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Solid lipid nanoparticles as drug carriers I. Incorporation and retention of the lipophilic prodrug 3′-azido-3′-deoxythymidine palmitate

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Abstract

Solid lipid nanoparticles (SLN) were prepared with trilaurin (TL) as the SLN solid core and dipalmitoylphosphatidylcholine (DPPC) or a mixture of DPPC and dimyristoylphosphatidylglycerol (DMPG) to produce neutral and negatively charged SLNs. The ester prodrug of 3′-azido-3′-deoxythimidine (Zidovudine®, AZT) with palmitic acid, AZT palmitate (AZT-P), was synthesized and its incorporation and retention in SLNs determined. The incorporation of hydrophilic AZT in SLNs was minimal; however the incorporation of AZT-P increased with increasing phospholipid (PL) content and was independent of the amount of TL used. The incorporation of AZT-P was greater in negatively charged SLNs than in neutral SLNs. The in vitro release of AZT-P from different SLNs formulation was studied at 37°C using a bulk-equilibrium reverse dialysis sac technique. Increased drug release was observed in SLNs formulated with PLs having a transition temperature below 37°C. The results obtained indicate that the highly packed TL core of the SLN is not compatible with lipophilic molecules such as AZT-P. The incorporation and subsequent retention of AZT-P appears to be dependent on the PL coating on the SLNs surface and is independent of the TL solid core.

Keywords: Azidothymidine; Drug incorporation in vitro drug release; Lipophilic prodrug; Solid lipid nanoparticles

1. Introduction

Azidothymidine (AZT) was the first drug reported as being capable of inhibiting human immunodeficiency virus (HIV) replication and its subsequent cytopathic effects (Mitsuya et al., 1985). The incorporation of amphiphilic AZT in liposomal carriers results in decrease bone marrow toxicity, increased bioavailibility and enhanced antiviral activity (Phillips et al., 1991; Phillips and Tsoukas, 1992), and such formula-

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tions have been evaluated clinically (Deutsch et al., 1993). Several investigators have converted this nucleoside analogue into a variety of lipophilic derivatives as a mean of increasing its antiviral activity (Kawaguchi et al., 1990; Steim et al., 1990; Hostetler et al., 1990; van Wijk et al., 1994; Sqalli-Houssaini et al., 1994). Liposomal incorporation and antiviral activity of a number of lipophilic derivatives of AZT (Schwendener et al., 1994) and other antiviral agents (Kamps et al., 1996) have also been evaluated.

The majority of drug delivery systems use a drug carrier which is a surfactant, a lipid, or an oil emulsified with surfactant. The amount of the drug that may be dissolved at equilibrium is limited by the solubility of the drug in the carrier (Müller, 1991). The administration of drugs in such systems is therefore limited by the large carrier to drug ratio that must be used. In some cases this may be a problem where large amounts of the drug need to be delivered. Although liposomes are biodegradable (Grit et al., 1989), may protect encapsulated drugs against degradative enzymes (Rowland and Woodley, 1980) and have been shown to reduce the toxicity of associated drugs such doxorubicin (Rahman et al., 1985) and amphotericin B (Lopez-Berestein et al., 1987), the limits for lipophilic drug incorporation in phospholipid bilayers (Shaw et al., 1976) has resulted in the investigation of alternative drug carriers such as fat emulsions. Phospholipid-triglyceride emulsions such as Interalipid® which have been widely used for parenteral nutrition over long periods of time (McNiff, 1977; Leibowitz et al., 1992) have been evaluated as carriers for a range of drugs (El Sayed and Repta, 1983; Caillot et al., 1993; Tibell et al., 1995; Yamaguchi et al., 1995; Higashi et al., 1995).

Solid lipid nanoparticles (SLN) were introduced as a new drug delivery system for lipophilic drugs combining the advantages of both liposomes and fat emulsions (Akbarieh et al., 1994; Schwarz et al., 1994; Heiati et al., 1996). SLNs are comprised of a high melting point triglyceride (TG) as the solid core and a phospholipid (PL) coating. Two of the potential advantages of SLNs over other drug carriers for lipophilic drugs are the use of natural lipids which are known from parenteral nutrition studies to be non-toxic (Davis et al., 1987), and the incorporation of lipophilic drugs in the TG core. Nanoparticles containing a polymeric solid core have been shown to prolong the release of the incorporated drugs (Koosha et al., 1989). Unlike fat emulsions, which have a fluid core, the solid core of SLNs may be applicable for this type of drug release.

Although it is desirable to incorporate the drug into the innermost phase of the emulsion in order to achieve the potential advantage of the SLN as a slow and controlled release drug carrier, drug solubility in solid phase TG may be a major problem for incorporation in the SLNs solid core. The objective of this study therefore was to investigate the effect of PL and TG content on the incorporation and subsequent in vitro release of AZT palmitate (AZT-P) using different SLN formulations.

2. Materials and methods

2.1. Materials

Trilaurin (TL), palmitoyl chloride and 3′-azido-3′-deoxythimidine-methyl-^{3}H (8.18×10^{10} Bq/mmol) were purchased from Sigma Chemical Company (St. Louis, MO). Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and dimyristoylphosphatidylglycerol, sodium salt (DMPG) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Sephadex G-25M PD-10 columns were purchased from Pharmacia (Uppsala, Sweden). TLC Silica gel 60 F_{254} plates were obtained from Merck (Darmstadt, Germany). Silica gel (60–200 mesh) was from Baker Chemical (Phillipsburg, NJ). 3′-azido-3′-deoxythimidine (Zidovudine®, azidovudine, AZT) was supplied by GlaxoWellcome (Research Triangle Park, NC). HEPES was purchased from Boehringer Mannheim (Germany). HEPES buffer used in the experiments was 10 mM HEPES buffer at pH 7.4. All materials were used without further purification, and all other chemicals were of analytical or

USP grade. Cellulose dialysis tubing (MW cut-off range: 12 000–14 000 Da) was obtained from Spectra/por®, Spectrum Medical Industries, (Los Angeles, CA). Radioactivity was counted in a liquid scintillation cocktail (CytoScint™ ES, ICN Biomedicals, Costa Mesa, CA) using a LKB-Wallac Rackbeta (Turku, Finland) at an efficiency of 40%.

2.2. Methods

2.2.1. Synthesis of azidothymidine palmitate (AZT-P)

AZT-P was synthesized according to the procedure of Kawaguchi et al. (1990) with a slight modification. In order to increase the specific radioactivity of the end product, [^{3}H]AZT (3.7×10^6 Bq) was added to a solution of palmitoyl chloride (2 mmol) in pyridine (10 ml) 1 h prior to AZT (1 mmol). The mixture was subsequently stirred at room temperature for 24 h. The solution was evaporated to dryness in vacuo at 45°C and the residue was dissolved in 2 ml chloroform:ethanol (95:5). This solution was applied to a silica gel column (2.5 × 15 cm) and eluted with chloroform:ethanol (95:5), (1 ml/min). Fractions of 2 ml were collected and an aliquot (10 μl) of each was taken for TLC using chloroform:ethanol (95:5) as the mobile phase. Fractions containing AZT-P were evaporated to dryness in vacuo in a round bottom flask at 45°C. The dry residue on the flask wall was dissolved in a minimum amount of *t*-butanol, frozen at −40°C and lyophilized at the same temperature in vacuo (0.07 mbar) for 16 h, using a Lyph lock 6 lyophilizer (Labconco Inc., Kansas City, MO). The chemical structure of the resulting product, AZT-P, was supported by IR and ^{1}H-NMR spectroscopy, mass spectroscopy (calculated molecular mass: 506.5, observed: 506.5) and chemical analysis (H, calculated: 8.5%, observed: 9 ± 0.3% C, calculated: 61.8%, observed: 63 ± 0.3% N, calculated: 13.9%, observed: 12.4 ± 0.4%). The specific radioactivity of the AZT-P was 4.1×10^3 Bq/mg, giving a reaction yield of 59% and a radioisotope recovery of 92%. The product gave only one spot on silica gel TLC (Rf = 0.61).

2.2.2. Preparation of SLN

To prepare SLNs, different weight ratios of trilaurin (TL) to phospholipid (PL) were dissolved in chloroform (20 ml) in a round bottom flask at room temperature. To produce SLNs containing drug, AZT-P (17 mg, 6.9×10^7 Bq) was added to the preparations. Neutral SLNs were prepared using DPPC as the PL, and negatively charged SLNs were prepared using various mol ratios of DPPC:DMPG as the PL. After removal of the organic phase by rotary evaporation in vacuo at 50°C, an oily lipid film was obtained on the flask wall. The lipid film was hydrated with 20 ml HEPES buffer and rotated for 5 min at 50°C. The emulsion obtained (mean diameter 1–2 μm) was homogenized for ten cycles at 60–70°C and 15 000 psi, using a high pressure homogenizer (Emulsiflex®-30, Avestin Inc., Ottawa) to produce SLNs. The SLNs were allowed to cool to 20°C over a period of 1 h.

2.2.3. Particle size measurement and ζ potential

The mean particle size of the samples was determined using photon correlation spectroscopy (N4 Plus, Coulter Electronics Inc., Hialeah, FL). SLNs were diluted with HEPES buffer to give a particle count rate between 5×10^4 and 1×10^6 counts per second. Mean particle diameter was calculated in size distribution processor mode (SDP) using the following conditions: fluid refractive index 1.33; temperature 20°C; viscosity 0.93 centipoise; angle of measurement 90.0°; sample time 10.5 μs, and sample run time 90 s.

The zeta (ζ) potential was measured using a Delsa 440SX (Coulter Electronics Inc., Hialeah, FL) using the following conditions: current 0.7 mA; frequency range 500 Hz; temperature 20°C; fluid refractive index 1.33; viscosity 0.93 centipoise; dielectric constant 78.3; conductivity 16.7 ms/cm; on time 2.5 s, off time 0.5 s, and sample run time 60 s.

2.2.4. Determination of incorporated AZT-P

Free AZT-P was removed from the SLN preparations by gel permeation chromatography using Sephadex G-25 (PD-10 column). Preliminary calibration of PD-10 columns showed that SLNs eluted between 2.0 and 3.5 ml, AZT-P eluted

between 4.5 and 6 ml and AZT eluted between 7 and 10 ml. SLN preparations (500 μl) were applied to the PD-10 column and eluted with HEPES buffer (0.5 ml/min). Fractions of 0.5 ml containing SLNs were collected to which 10 ml scintillation cocktail was added prior to β-counting. The percentage of AZT-P incorporated in the SLNs was calculated relative to the radioactivity of 0.5 ml of SLN suspension before gel permeation chromatography.

2.2.5. Calculation of incorporation density of AZT-P in SLN PL membrane

The number of mol PL able to incorporate 1 mol AZT-P in a saturated PL membrane was calculated. The calculation was performed using the SLN formulation containing 20 mg PL. This formulation was considered to be saturated with AZT-P as only 30% (negatively charged SLNs) and 16% (neutral SLNs) of the initial input of AZT-P was incorporated.

2.2.6. Assessment of AZT-P retention by bulk-equilibrium reverse dialysis

Reverse dialysis was carried out according to the procedure described by Levy and Benita (1990). AZT-P suspended in HEPES buffer or SLNs containing AZT-P (5 ml, 0.85 mg/ml, 0.35×10^7 Bq/ml) were placed directly into 500 ml of a stirred HEPES sink solution where ten dialysis sacs containing 2 ml of the same sink solution were immersed. The dialysis sacs were equilibrated with the sink solution for 5 h prior to the experiments. The experiments were performed at 37°C under constant magnetic stirring, and at predetermined intervals the contents of the dialysis sacs were assayed for AZT-P.

2.2.7. Freeze-fracture electron microscopy

A drop of SLN samples was placed on the surface of a standard 3 mm diameter gold specimen disc (Balzers, Liechtenstein) and frozen and stored in a slurry of liquid nitrogen-cooled isopentane. The frozen samples were fractured at −112°C in a Balzers BAF 301 Freeze-Etch Unit and replicated with a thin film of platinum followed by a thin film of carbon according to the procedure of Shivers and Brightman (1976). Platinum replicas were placed in absolute methanol overnight and cleaned for a minimum of 3 h in household bleach. The cleaned replicas were picked up on bare 200-mesh copper grids and imaged in a Philips 201 transmission electron microscope operating at an accelerating voltage of 60 kV. Selected SLN images were further magnified optically.

3. Results and discussion

3.1. Incorporation of AZT and AZT-P in SLN

Despite the lipophilicity conferred by the azido function of the AZT molecule (Robins et al., 1989), the incorporation of AZT in the neutral and the negatively charged SLNs was minimal (Table 1). Linkage of a palmitic acid group to AZT to give the prodrug AZT-P increased the incorporation of this drug up to 90% using a high PL/TL ratio (Fig. 1), with negatively charged SLNs incorporating more AZT-P than neutral SLNs. Calculation of the incorporation density gives 1 molecule of AZT-P per 4 molecules of DPPC/DMPG in the case of the negatively charged SLNs and 1 molecule of AZT-P per 7 molecules of DPPC in the case of neutral SLNs. Neutral SLNs are smaller in size (203 ± 31 nm) than negatively charged SLNs (294 ± 32 nm). The larger surface curvature in the negatively charged SLNs, resulting from the repulsion of the negative charges between the PL molecules, may facilitate the packing of AZT-P within the PL bilayers. Conversely in neutral SLNs the small curvature of the PL membrane may impair the close packing

Table 1
Incorporation of AZT in SLNs

PL:TL (mg)	Neutral SLN (%)	Negatively charged SLN (%)
20:200	0	0
50:200	0.4 ± 0.3	0.9 ± 0.4
70:200	0.6 ± 0.5	1.0 ± 0.3

DPPC was used as PL for the neutral SLNs and a mixture of DPPC:DMPG, 95:5 mol%, was used as PL for the negatively charged SLNs. Mean ± S.D. of three experiments.

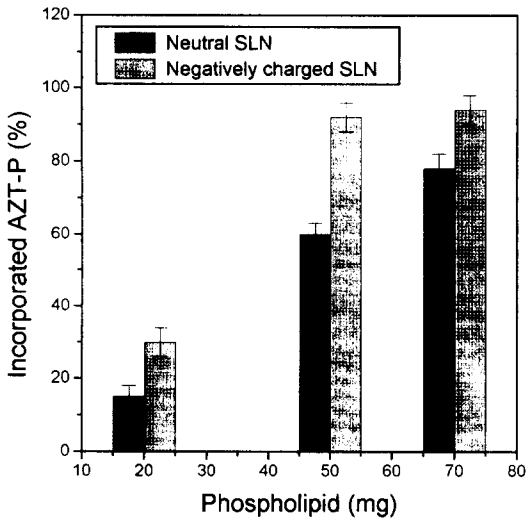

Fig. 1. Incorporation of AZT-P in SLNs as a function of PL content. 200 mg TL was used as SLNs solid core. DPPC was used as PL for the neutral SLNs and a mixture of DPPC: DMPG, 95: 5 mol%, was used as PL for the negatively charged SLNs. Mean ± S.D. of three experiments.

and incorporation of AZT-P within PL bilayers. Increased incorporation was not directly related to PL charge density. Increasing the DMPG content from 5 to 30 mol% in negatively charged SLNs containing AZT-P, which resulted in a significant increase in ζ potential but not in diameter, did not significantly increase the amount of AZT-P incorporated (Table 2).

Benameur et al. (1993) have shown that palmitic acid ester prodrugs are amphiphilic, and several investigators have studied the interaction of ester prodrugs with PL membranes (Shaw et al., 1976; Taniguchi et al., 1987). Other amphiphilic prodrugs based on muramyl dipetides (Phillips, 1992) or 5-fluorouracil (Elorza et al., 1993) have been investigated in liposomal carriers. The fatty acid chain and the AZT head group of AZT-P give an amphiphilic characteristic to the AZT-P molecule which may facilitate its incorporation within PL membranes on the surface of SLNs. The formation of PL bilayer structure on the SLNs core resulting from an excess of PL (Heiati et al., 1996) and the ability of amphiphilic prodrug molecules such as AZT-P to integrate within PL bilayers appears to be responsible for the increased incorporation of AZT-P in SLNs produced with a high PL/TL ratio.

Increasing the TL content of the SLNs did not affect the incorporation of AZT-P (Fig. 2), indicating that the close packing of the triglyceride core in the β-phase (Larsson, 1964) is not compatible with the incorporation of AZT-P. Considering the very low or non-mutual solubility of high melting point TGs which has been reported by Knoëster et al. (1972), our results were not unexpected. Although they have been a number of communications (Akbarieh et al., 1994; Seki et al., 1994; Müller et al., 1994, 1996) regarding drug incorporation in SLNs, it is clear from this study that the actual location of drugs may not be within the solid core. Whether other drugs are capable of dissolving in the TGs remain to be determined. The increased incorporation of AZT-P in SLNs prepared with high PL/TL ratios, and the lack of correlation between the incorporation of AZT-P and the amount of TL used, strongly indicates that AZT-P is incorporated within the PL bilayer structures surrounding the SLN core. Modification of PL bilayers, rather than TG core, would therefore appear to be crucial factor with respect to amphiphilic prodrug incorporation in SLNs.

3.2. The release of AZT-P from SLN

The stability of ester prodrugs of AZT at different pHs and temperatures has been studied by Kawaguchi et al. (1990), with the palmitic acid ester of AZT, AZT-P, being quite stable under moderate pH conditions at 40°C (chemical hydrolysis rate, $k_{obs} < 0.0005\ h^{-1}$). The very low hydrolysis rate of AZT-P make this molecule suitable for prolonged retention and stability studies. Many investigators have studied drug release using the bulk-equilibrium reverse dialysis sac technique (Levy and Benita, 1990; Santos Magalhaes et al., 1995). This method has been suggested as being appropriate for colloidal carriers with a slow drug release rate (Levy and Benita, 1990). Drug hydrolysis during the release studies was negligible, as shown by an absence of free AZT as determined by TLC. Fig. 3 shows the release of AZT-P from SLNs prepared with differ-

Table 2
SLN ζ potential and incorporation of AZT-P as a function of DPMG content

Phospholipid mixture DPPC/DMPG (mol%)	Diameter (nm)	ζ potential (mV)	Incorporated AZT-P (%)
95/5	294 ± 32	−15 ± 4	31 ± 7
90/10	301 ± 29	−27 ± 4	31 ± 8
80/20	281 ± 37	−39 ± 6	37 ± 6
70/30	315 ± 36	−50 ± 6	36 ± 7

SLNs were prepared using 200 mg TL and 20 mg PL. Mean ± S.D. of three experiments.

ent PLs at 37°C. The initial loss of a small amount of AZT-P from the SLNs formulated with DPPC and DSPC may be related to a low level of AZT-P micelle contamination (Heiati et al., 1996). The sustained loss of AZT-P from SLNs formulated with DMPC may be related to the fluid state of DMPC at 37°C. The effect of phospholipid T_t on the retention of lipophilic drugs in liposomes has been studied by Shaw et al. (1976). Differences in release profile of AZT-P from different PLs can be attributed to the phase transition of the PL used. DMPC has a T_t of 23°C (Chen and Sturtevant, 1981) and is therefore present in a liquid-like conformation at 37°C. The fluid PL coating of SLNs produced with DMPC may result in the rapid release of AZT-P at 37°C. On the other hand, DPPC, with a T_t of 41°C and DSPC, with a T_t of 54.1°C, are present in a gel conformation at 37°C (Chen and Sturtevant, 1981). Enhanced membrane rigidity may prevent the release of incorporated AZT-P at 37°C from the SLNs produced with DPPC or DSPC. Although the use of different PLs did not affect the initial incorporation efficiency of AZT-P in SLNs, the use of a defined PL with defined T_t would appear to allow some control of the release of AZT-P from SLNs. Other studies have not evaluate the effect of different PL coat in the retention of the incorporated drug (Seki et al., 1994; Müller et al., 1994, 1996). This may be an important factor for the prolonged release of AZT-P from SLNs following the parentral administration as well as targeting SLNs to defined cellular targets, as has been demonstrated with liposomal drug carriers.

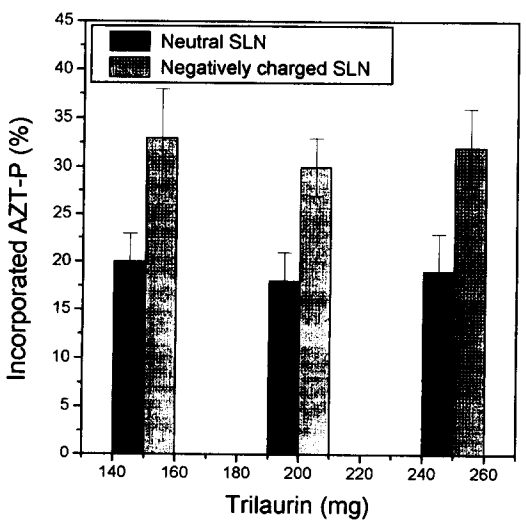

Fig. 2. Incorporation of AZT-P in SLNs as a function of TL content. Approximately 20 mg PL was used as SLNs PL coating. Mean ± S.D. of three experiments.

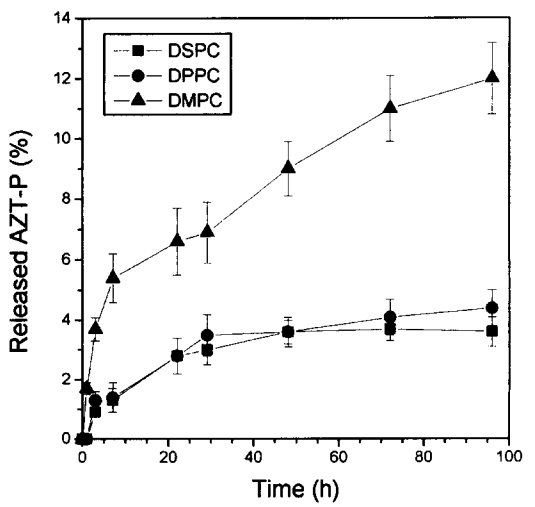

Fig. 3. Release profile of the negatively charged SLNs at 37°C using PLs with different T_t. SLN's were produced with 50 mg PL and 200 mg TL. Mean ± S.D. of three experiments.

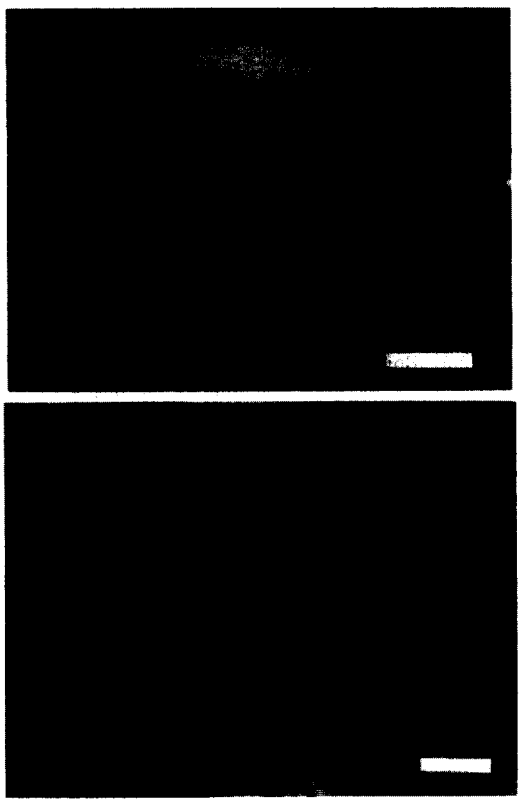

Fig. 4. Freeze fracture and electron micrograph of negatively charged SLNs containing AZT-P (a) and without AZT-P (b). SLNs were produced with 60 mg PL and 200 mg TL. Bar represents 50 nm.

3.3. Freeze-fracture electron microscopy

Multiple PL bilayers that appear to surround a TL core are shown in the freeze-fracture electron micrographs of SLN (Fig. 4(a)) and SLN containing AZT-P (Fig. 4(b)). Incorporation of AZT-P in SLNs did not change the physical structure of the SLN. Assuming a bilayer distance of 6.5 nm for DPPC (Janiak et al., 1979), measurement of the outer coat of the SLN (Fig. 4(a,b), approximately 25 nm) indicates that there are three to four PL bilayers surrounding the TL core. These results are in agreement with our previous report on the theoretical amount of PL needed to form a bilayer on the TL core (Heiati et al., 1996). Changes in PL/TL ratio will therefore allow the construction of defined number of PL bilayers available for amphiphilic drug incorporation.

4. Conclusions

The incorporation and the release kinetics of AZT-P are related to the nature and amount of PL surrounding the SLNs core. Our results clearly demonstrate that AZT-P incorporation is dependent on PL content and independent of TG content. AZT-P as an amphiphilic molecule would appear to preferentially integrate within the PL bilayers. Amphiphilic drugs such as AZT-P (this study) and dexamethasone and dexamethasone palmitate (manuscript in preparation) do not appear to be incorporated within the TG core of SLNs. The close molecular packing of TG molecules in the solid phase prevents the incorporation of these drugs in the SLNs solid core. The amount of SLN associated drug can however be controlled by changing the PL content. This can be used in a precise manner to control the incorporation of drug by changing the PL/TL ratio. The controlled release of incorporated drug would appear to depend on the nature of the PL and its T_t relative to physiological temperature. The principal function of the TG in SLNs may therefore be to act as a matrix for the construction of a defined number of PL bilayers capable of incorporating amphiphilic drugs. The incorporation of lipophilic drugs in the solid core of SLNs may be limited to those molecules that are physically and chemically compatible with TG. The effect of PL composition on the incorporation and retention of amphiphilic prodrugs in SLNs is currently being investigated.

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