LIPOSOME-ENCAPSULATED PREDNISOLONE PHOSPHATE INHIBITS TUMOR GROWTH IN MICE

Raymond M. Schiffelers¹, Josbert M. Metselaar¹, Adriëne P.C.A. Janssen¹, Louis van Bloois¹, Julie Cornelis¹, Marcel H.A.M. Fens¹, Grietje Molema²,³, and Gert Storm¹

¹Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands
²Dept. Pathology and Laboratory Medicine, Groningen University Institute for Drug Exploration, Groningen University, The Netherlands
³Dept. Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration, Groningen University, The Netherlands
Glucocorticoids can inhibit solid tumor growth, which has been suggested to be caused by an inhibitory effect on angiogenesis. Several mechanisms have been proposed to explain their anti-angiogenic action, ranging from inhibition of endothelial cell proliferation and migration, modulation of basement membrane turnover, and/or inhibition of production of pro-angiogenic factors. The anti-tumor effects of the free drugs have only been observed using treatments schedules based on high and frequent dosing for prolonged periods of time. As long-circulating liposomes (LCL) accumulate at sites of malignancy, we investigated the tumor-inhibiting potential of LCL-encapsulated prednisolone phosphate. It appeared that liposomal prednisolone phosphate could inhibit tumor growth dose-dependently. 80-90% tumor growth inhibition was achieved in s.c. B16 melanoma and C26 colon carcinoma murine tumor models at a dose of 20 mg/kg by single or weekly doses. Prednisolone phosphate in the free form was completely ineffective at this low frequency treatment schedule, even at a dose of 50 mg/kg. Histological evaluation revealed that liposomal PLP-treated tumors were surrounded by a layer of connective tissue, whereas the tumor center contained areas of apoptotic cells. In addition, blood clots were observed in some of the larger blood vessels in these tumors. In conclusion, the present study shows the potent anti-tumor efficacy of a new, liposomal, formulation of glucocorticoids.
INTRODUCTION

Glucocorticoids (GC) have a wide spectrum of activities on cell trafficking, cell-cell interactions and cell communications, leading to pronounced anti-inflammatory and immunosuppressive effects. GC exert their effects by diffusion through the cell membrane and binding to their cytosolic receptors. Subsequently, these receptors become activated and translocate to the nucleus where they directly modulate DNA transcription of a variety of genes. In addition, GC-receptors may directly or indirectly antagonize the activity of several transcription factors, most notably nuclear factor kappa-B (1,2). GC also exert rapid non-genomic effects on cells by interacting non-specifically with cellular membranes, or specifically with membrane bound GC-receptors (3).

In tumor therapy, GC have been used for their anti-inflammatory and anti-emetic effects and for the treatment of hematological malignancies based on their efficient cytolytic activity on cells of lymphoid origin (4). Reports in the last two decades demonstrated that GC could also decelerate solid tumor growth in experimental animal models (5-8). However, one of the drawbacks of GC in tumor therapy, as shown in these pre-clinical studies, is the need for high or frequent dosing. In mice, doses of 100-200 mg/kg per day need to be administered for prolonged periods of time to obtain significant tumor growth inhibition (5-8). Moreover, these doses have been shown to cause considerable morbidity and mortality as a result of severe immune suppression in experimental animals (6,7).

Targeted delivery of GC to tumor tissue could be an attractive strategy to increase intratumoral drug concentrations, thereby reducing the overall dose decreasing the likelihood of side effects (9). In the present study, we investigated the use of long-circulating liposomes (LCL) to deliver GC selectively to tumor tissue. LCL have previously been shown to accumulate at sites of malignancy as a result of the enhanced permeability of tumor vasculature as compared to healthy endothelium (10). Furthermore, in previous studies in our group, the therapeutic activity of encapsulated in LCL was shown to be strongly increased in experimental models of arthritis (Chapter 3 and 4). In the present study, anti-tumor activity of liposomal prednisolone phosphate (PLP) was investigated in s.c. C26 colon carcinoma and B16F10 melanoma models and compared to the anti-tumor activity of free PLP in different dosing schemes.
MATERIALS AND METHODS

Liposome preparation

LCL were prepared as described previously (11). In brief, appropriate amounts of dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) 2000-distearoyl phosphatidylethanolamine (PEG-DSPE) (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in chloroform:methanol (2:1 vol:vol) in a round-bottom flask. A lipid film was made under reduced pressure on a rotary evaporator and dried under a stream of nitrogen. Liposomes were formed by addition of an aqueous solution of 100 mg/ml PLP. A water-soluble phosphate ester of prednisolone was used to ensure stable encapsulation in the liposomes. For labeling of the liposomes with 0.5 mCi $^{111}$In-oxine (Mallinckrodt Medical, Petten, The Netherlands), the lipid film was hydrated in 5 mM diethylene-triamine penta-acetic acid (DTPA) in 10 mM N-[(2-hydroxyethyl) piperazine-N'-ethane sulfonic acid (HEPES)/135 mM NaCl-buffer pH 7 to a final lipid concentration of 10 µmol/ml, according to a procedure described by Boerman et al. (12). The liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm.

Short-circulating liposomes (SCL) were prepared similarly, only PEG-DSPE was replaced by egg phosphatidylglycerol (EPG). SCL were used to determine the dependency of anti-tumor effects on the degree of liposome localization in the tumor, which is positively correlated to liposomal circulation time (10). Therefore, PEG-DSPE was exchanged, as it is responsible for the prolonged circulation of the LCL by providing a layer of steric stabilization around the liposome surface, decelerating MPS uptake. Moreover, exchanging it for EPG introduced a negative charge on the liposome surface, which promotes MPS-uptake and thereby reduces circulation time of the SCL even further (13). The SCL were formed by addition of 10 mg/ml PLP in 10 mM HEPES/135 mM NaCl-buffer pH 7.4 to the lipid film, and extrusion took place through polycarbonate membranes of 400 nm. Unencapsulated material was removed by dialysis with repeated changes of buffer against 10 mM HEPES/135 mM NaCl-buffer pH 7.4 at 4 °C.

Mean particle size of the LCL was determined by dynamic light scattering and found to be 0.1 µm with a polydispersity value of around 0.1, whereas the SCL had a mean particle size of 0.5 µm with a polydispersity value of around 0.3. The low polydispersity values indicate limited variation in particle size. The large size of the SCL also reduces circulation time as compared to the LCL and also prevents efficient extravasation at the target site (13,14). Taken together, the changes in lipid composition and liposome size strongly reduce the degree of liposome localization in the tumor for SCL as compared to LCL.

Phospholipid content was determined with a phosphate assay, performed according to Rouser (15), on the organic phase after extraction of liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content by high performance liquid chromatography using a mobile phase of acetonitril-water with
pH of 2 and monitoring the eluens with a UV-detector, which was set at 254 nm. The liposomal preparation contained around 2 mg PLP/ml and 60 µmol/ml phospholipid. Using this setup it was established that liposomes contained 25-35 µg PLP/µmol lipid.

Cells
B16 murine melanoma and C26 murine colon carcinoma cells were cultured at 37 °C in a 5% CO₂-containing humidified atmosphere in culture medium (DMEM) (Gibco, Breda, The Netherlands) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Gibco). To determine whether prednisolone (phosphate) had a direct cytotoxic effect on tumor cells, 10⁴ cells/well were plated in a 96-well plate. Prednisolone (PL) was added dissolved in ethanol, using corresponding concentrations of ethanol as controls, whereas PLP was added in HEPES/NaCl-buffer. Cell viability was determined after 24 h and 48 h of incubation by XTT-assay (Sigma, St. Louis, USA) according to manufacturer’s instructions.

Murine tumor models
Male Balb/c and C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (the Netherlands) kept in standard housing with standard rodent chow and water available ad libitum, and a 12 h light/dark cycle. Experiments were performed according to national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10⁶ B16 melanoma or C26 colon carcinoma cells were inoculated subcutaneously in the flank of syngeneic C57Bl/6 or Balb/c mice, respectively.

Tissue distribution of ¹¹¹In-labeled LCL in tumor bearing mice
At a tumor volume of approximately 1 cm³, mice were injected i.v. with 25 µmol lipid/kg (corresponding to 30 x 10⁶ cpm/mouse) of ¹¹¹In-labeled LCL. At 6 h and 24 h after injection animals were sacrificed, a blood sample was taken and tumor, lungs, liver, spleen and kidneys were dissected, the tissues were weighed and radioactivity was counted with a Philips PW 4700 liquid scintillation counter.

Tumor growth inhibition
Effect of dose. Mice received a single intravenous injection of an indicated dose of free PLP or liposomal PLP at the time when the tumor became palpable. At 7 days after treatment tumor size was measured and tumor volume calculated according to the formula V = 0.5 * a² * b, in which a is the smallest and b the largest superficial diameter. Effect of tumor size. Free PLP or liposomal PLP were i.v. administered at a dose of 20 mg/kg at day 1, 7, and 14 or by single injection at day 7 or day 14 after tumor cell inoculation. As a reference, B16F10 tumors became palpable around 7 days and C26 tumors around 11 days after tumor cell inoculation. Tumor size was measured regularly, and tumor volume was calculated as described above.
Analysis of amount of PLP or prednisolone in tissues
At a tumor volume of approximately 1 cm³, mice were injected i.v. with 20 mg/kg liposomal PLP or free PLP. At 24 h after injection animals were sacrificed and tumor, liver, and spleen were dissected. The tissues were weighed and homogenized. 2 µg methylprednisolone was added as an internal standard. PLP and free prednisolone were extracted from the tissue with ethylacetate at pH 2, and concentrated under a nitrogen flow. Samples were diluted in ethanol:water 1:1 vol/vol and analyzed by HPLC as described above. Standard lines were prepared by extracting known amounts of PLP and prednisolone from control organs from untreated mice. The detection limit for the HPLC setup was 20 ng/ml.

Histology
Tumors were dissected at 72 h after a single i.v. injection of either free PLP or liposomal PLP 20 mg/kg. Tumors were fixed in 10% PBS-buffered formaldehyde and embedded in paraffin. 5 µm slides were cut and stained with hematoxilin/eosin and evaluated by light microscopy.

Statistical analysis
Data were analyzed by one-way ANOVA with Dunnett's post-test using GraphPad InStat version 3.05 for Windows, GraphPad Software (San Diego, USA). Data were logarithmically transformed to correct for significant differences between SD of groups, when appropriate according to Bartlett’s test. Spearman rank correlation coefficient was calculated to identify dose-response.
RESULTS

Tissue distribution of LCL

Figure 1 presents tissue distribution data of the LCL at 6 h and 24 h after intravenous injection in C26 or B16-tumor bearing mice. Approximately 60% of the injected dose (ID) was still present in the circulation at 6 h after administration in both mouse models, whereas 15% ID was still circulating at 24 h post-injection. These values correspond to previous data on circulation kinetics of LCL (10). Approximately 7-10% ID could be recovered from tumor tissue in both the C26 and B16F10 model at 24 h after injection, which was approximately two-fold higher than the levels at 6 h post-injection. At both time-points approximately the same amount was present in the livers of both strains of tumor-bearing mice. Relatively low amounts of LCL were recovered from spleen, kidney and lung in the two mouse models both at 6 h and 24 h after injection.

![Figure 1. Tissue distribution of 111In-labeled liposomes at 6 h and 24 h after intravenous administration in B16F10-tumor bearing C57Bl/6 mice or C26-tumor bearing Balb/c mice. Tumors weighed approximately 1 g. Mean ± SD, n=5 animals/experimental group.](image)

Anti-tumor activity of liposomal PLP versus free PLP: dose–response relationship

To compare the effect of different doses of liposomal PLP to free PLP on tumor growth, B16 or C26-tumor bearing mice received a single injection of either formulation at the moment that the tumor became palpable. At 1 week after injection the tumor volume was smaller with an inverse relationship to the dose of injected liposomal PLP in both mouse models as shown in Figure 2 (B16: Spearman correlation coefficient r=-0.92 (p<0.001); C26 Spearman correlation coefficient r=-0.82 (p<0.01)). 20 mg/kg PLP was the maximum dose that could be administered for the liposomal formulation in view of the maximal injection volume. Treatment of B16 or C26 tumor bearing mice with 20 mg/kg or 50 mg/kg free PLP did not result in significantly different tumor volumes compared to buffer treated control animals (Figure 2).

To evaluate whether the anti-tumor effect was due to a direct cytotoxic effect of prednisolone or PLP on the tumor cells, B16 and C26 cells were incubated in vitro with increasing concentrations of prednisolone and PLP. No decrease in cell viability was noted up to the maximum concentration tested of 10 μg/ml (data not shown).
Figure 2. Effect of dose of liposomal (left) or free PLP on tumor growth in B16F10 or C26 bearing mice. Mice received a single injection with the indicated dose and formulation of PLP on the day tumors became palpable (= day 7 after inoculation). Tumor volume after 1 week is reported. Mean ± S.D, n=5 animals/experimental group.

Analysis of level of PLP or prednisolone in tissues
PLP and prednisolone levels at 24 h after i.v. injection of liposomal PLP in liver, spleen and tumor tissue were determined by HPLC analysis. Figure 3 shows that the highest amount of PLP (± 5 µg) was present in the tumor and a similar amount was present in the form of PL. The level of PLP in the spleen was relatively low, whereas the prednisolone level was similar to that in the tumor. Hardly any PLP could be detected in liver tissue, but approximately 20 µg was present in the form of PL. Neither PLP nor prednisolone was detected in any of these tissues at 24 h after injection of free PLP (data not shown).

Figure 3. Amount of PLP and PL recovered from mouse tissues. Mice received a single injection of 20 mg/kg liposomal PLP and tissues were excised 24 h later. Mean ± SD, n=5 animals/experimental group.

Dependence of anti-tumor effect on tumor size
To determine if, and to what extent the anti-tumor effect depends on the tumor size at day of treatment, liposomal and free PLP were injected at a dose of 20 mg/kg at day 1, 7, and 14 or a single dose at day 7 or day 14. The results are shown in Figure 4.
liposome-encapsulated prednisolone phosphate inhibits tumor growth in mice

Figure 4. Effect of tumor size on the antitumor effect of free and liposomal PLP. The formulations were injected at a dose of 20 mg/kg at day 1, 7, and 14 (A and B) or single injection at day 7 (C and D) or day 14 (E and F) in B16F10-bearing C57Bl/6 mice (A, C, and E) or C26-bearing Balb/c mice (B, D, and F). Mean ± SD, n=5 animals/experimental group.
**B16-model.** The tumor volumes of B16-tumor bearing mice that received either no treatment, or treatment with free PLP or liposomal PLP at day 1, 7, and 14 are shown in Figure 4 A. Tumors became palpable at day 7 in all treatment groups indicating that neither of the treatments delayed tumor growth between day 1 and day 7. A second dose of liposomal PLP at day 7 resulted in 92% tumor growth inhibition between day 7 and day 14 as compared to controls (p<0.05), whereas free PLP did not affect tumor volume. On day 14, mice received a third injection. At day 17, some of the mice in the free PLP and control group had to be euthanized because of large tumor sizes (>2 cm$^3$), whereas average tumor volume in the liposomal PLP group was approx. 79% smaller (p<0.01).

After a single injection of liposomal or free PLP at day 7, a significantly smaller tumor volume was only seen after treatment with liposomal PLP with average inhibition of tumor growth of 89% at day 14 and 67% at day 17 as compared to controls (p<0.05, both time-points) (Figure 4 C). A single injection of liposomal PLP at day 14 produced 58% tumor growth inhibition at day 17 compared to controls (p<0.05) (Figure 4 E).

**C26 model.** C26-bearing mice received the first of the three doses of liposomal PLP or free PLP on day 1 and a second on day 7 after tumor cell inoculation. As tumors in all treatment groups became palpable around day 10, the effect on tumor growth of the first injections appeared to be minimal, although tumor volume was 89% smaller in liposomal PLP-treated animals than in controls at day 14. At day 21, 1 week after the third dose at day 14, average tumor volume in liposomal PLP-treated animals was 89% smaller than that in controls (p<0.01) (Figure 4 B).

Although a single dose of liposomal PLP on day 7 resulted in 66% tumor growth inhibition at day 14 and 67% inhibition at day 21, these differences were not statistically significant from the control and free drug-treated groups (Figure 4 D). A single injection of liposomal PLP at day 14 resulted in 78% tumor growth inhibition (p<0.05) (Figure 4 F).

*Figure 5.* Effect of short-circulating or long-circulating PLP-liposomes on tumor growth. Mice received a single injection of 20 mg/kg of the indicated formulation of PLP on the day tumors became palpable. Mean ± SD, n=5 animals/experimental group.
Importance of the long-circulation property of liposomes for tumor growth inhibition

To determine whether liposomal circulation time is critical for achieving anti-tumor efficacy we tested a SCL and LCL formulation of PLP for anti-tumor activity in C26 tumor-bearing mice. Both formulations were injected at day 14 after tumor cell inoculation in C26-tumor bearing mice. Tumor volume of SCL-encapsulated PLP-treated animals was not significantly different from saline-treated animals, whereas animals treated with LCL-encapsulated PLP experienced a significantly reduced tumor growth rate (Figure 5).

Histological examination of tumor tissue

Histological examination of tumor tissue at 3 days after treatment with a single dose of 20 mg/kg liposomal PLP or free PLP revealed 3 prominent differences between treatment groups as is illustrated in Figure 6 A. Firstly, tumors treated with liposomal PLP were smaller and surrounded by a layer of connective tissue, which was absent in the tumors treated with free PLP. Secondly, liposomal PLP-treated tumors showed areas of apoptotic tumor cells, which were not noted in the free PLP-treated animals. And finally, large fibrin clots were present in some of the larger blood vessels in liposomal PLP-treated tumors, which were not observed in free PLP-treated tumor tissue. A large, leukocyte infiltrated clot is shown in Figure 6 B.

Figure 6. Micrographs of C26 tumor tissue dissected at 3 days after treatment with a single dose of 20 mg/kg of liposomal PLP. Pictures show an overview (A) and an occluded blood vessel (B). Tissues were stained with hematoxilin/eosin. Legend of figure A: x layer of connective tissue, + apoptotic region, arrows point to occluded blood vessels.
DISCUSSION

The present study demonstrates for the first time anti-tumor effects of LCL-encapsulated GC. Strong anti-tumor effects were observed when liposomal PLP was administered in a low frequency (single dose or weekly) dosing schedule and at substantially lower doses than reported for free GC (conjugates) (5-7).

LCL have been previously used to increase the delivery of a variety of drugs to tumor tissue (16-18). Probably, the best-known formulation in this respect is LCL-encapsulated doxorubicin, marketed as Doxil® or Caelyx® (17). The long-circulating property allows liposomes to extravasate as a result of the enhanced vascular permeability in solid tumor tissue leading to their accumulation at the malignant site (16-18). In both the B16 and C26 tumor models used in this study, 7 to 10% of the injected dose of LCL had localized in the tumor at 24 h after injection, which is similar to previously reported data (10,16-18). At this time-point approximately 15% of the injected dose was still circulating in the blood stream, which is consistent with a long-circulatory behavior.

Furthermore, LCL encapsulation increased levels of the drug in the tumor, liver and spleen at 24 h after injection compared to administration of the free drug. Approximately, 2% of the injected dose of PLP was recovered from the tumor tissue as PLP or prednisolone. This percentage is substantially lower than the 7-10% of the ID of LCL that accumulate at the site of the malignancy. Probably, intratumoral conversion of PLP to prednisolone leads to a redistribution of the drug over the body as prednisolone can easily pass membranes. Conversion of PLP to prednisolone likely forms also the explanation for the virtual absence of PLP in liver tissue.

Administration of LCL-encapsulated PLP resulted in a dose-dependent anti-tumor effect in both the B16 and C26 s.c. tumor model. The maximum dose of 20 mg/kg (determined by the maximal injection volume) resulted in approximately 90% tumor inhibition over a 1 week period, when administered as a single dose at the moment that the tumor became palpable, in both tumor models. Free PLP did not inhibit tumor growth even at a dose of 50 mg/kg in this treatment schedule.

The underlying mechanism of tumor inhibition is at present unclear. Several studies showed that an anti-tumor effect of GC was not directly aimed at the tumor cells, but rather mediated by interference with the tumor vascularization (19-23). Also in the present study PLP or prednisolone did not inhibit the proliferation of the murine tumor cells in vitro. It has been suggested that GC’s anti-angiogenic effect is mediated by inhibition of endothelial cell proliferation and migration (20) or effects on basement membrane turnover (21). Furthermore, inhibition of production and/or release of pro-angiogenic factors (like plasminogen activator and vascular endothelial growth factor) may play a role (22,23). In addition, the hypothesis that inflammatory processes in and around the tumor are important in the angiogenic cascade suggests that GC’s immunosuppressive action may also be of relevance in this respect (24,25). Likely, these mechanisms act together leading to the observed anti-tumor effects.
Regardless of the proposed underlying mechanism, these studies report a concentration-dependent inhibition of the angiogenic process \textit{in vitro} or \textit{in vivo} by GC (19-23). The dose-dependent inhibition of angiogenesis is further illustrated by a study in rats demonstrating that local administration of GC in sponge implants was more effective in inhibiting angiogenesis than systemic treatment (26). Also in the clinical treatment of hemangiomas, intrallesional injection of GC is often used (27). The importance of prolonged high local drug levels is further supported by the observation in this study that the same dose of liposomal PLP in SCL inhibited tumor growth to a much lower extent. SCL are rapidly taken up by macrophages mainly in liver and spleen and are therefore unable to accumulate at the tumor. Consequently, the limited localization of SCL in the tumor is paralleled by a decrease in activity.

The lack of an inhibitory effect of liposomal PLP against small, not-yet palpable, tumors as observed in this study, is indicative of the importance of tumor localization of the liposomes for anti-tumor efficacy. Both in the B16 and C26 model, liposomal PLP injection at 1 day after tumor cell inoculation did not delay the time-point at which the tumor became palpable. At 24 h after tumor cell inoculation, the tumor mass is still minimal and vascular integrity is hardly affected yet. Therefore the circulating liposome particles are not able to extravasate at this time point. Once a tumor mass has formed, however, administration of liposomal PLP reduced tumor growth rate significantly, irrespective of the size of the tumor at the time of liposome injection.

Histological evaluation indicates that apoptosis of tumor cells in the core of the tumor occurs, which could be the result of inhibition of angiogenesis as the cells at this location are most dependent on intratumoral blood supply. Another observation, which was made solely in some of the liposomal PLP treated tumors, was the presence of blood clots inside the larger blood vessels in the tumor tissue. As thrombosis has been suggested to precede angiogenesis, we may be dealing with tissue that is again in or converting to a pro-angiogenic state (28,29).

In conclusion, the present study shows for the first time potent anti-tumor efficacy of LCL-encapsulated PLP. This formulation yields high intratumoral levels of PLP for a prolonged period of time. The advantage that the current system may offer for clinical use is the relatively low dose and low frequency schedule with which PLP needs to be administered to produce anti-tumor efficacy.

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