LIPOSOMAL TARGETING OF GLUCOCORTICOIDS TO INHIBIT TUMOR ANGIOGENESIS

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Het doelgericht afleveren van liposomaal-ingekapselde glucocorticoïden aan tumoren om angiogenese te remmen
(met een samenvatting in het Nederlands)

Terapia la tinta cu glucocorticoizi incorporati in lipozomi pentru inhibarea angiogenezei tumorale
(cu un rezumat in limba romana)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 3 december 2007 des middags te 2.30 uur

door

Manuela Banciu

geboren op 26 mei 1974, te Sighisoara, Roemenië

Promotor: **Prof. dr. G. Storm**

Co-promotor: **Dr. R.M. Schiffelers**

"Would you tell me, please, which way I ought to go from here?" Alice speaks to Cheshire Cat
"That depends a good deal on where you want to get to," said the Cat.
"I don't much care where –" said Alice.
"Then it doesn't matter which way you go," said the Cat.
"– so long as I get somewhere," Alice added as an explanation.
"Oh, you're sure to do that," said the Cat, "if you only walk long enough."

Lewis Carroll "Alice's Adventures in Wonderland"

In memory of my father

To my mother and to my husband

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Chapter 1

General introduction

Partly based on a review published in Journal of Liposome Research 16: 185–194 (2006)

1. THERAPEUTIC APPLICATION OF GLUCOCORTICOIDS

Glucocorticoids (GC) can induce a variety of strong pharmacological effects [1]. These effects include the well-known immunosuppressive and anti-inflammatory actions. Additionally, GC can effectuate apoptosis, necrosis, stimulate or inhibit cell growth, and exert a variety of effects on bone, neurons, glial cells, lipid/carbohydrate/protein metabolism, and electrolyte and water balance. At the cellular level, these actions are primarily caused by the ability of GC to modulate DNA transcription in the nucleus, but can also be the result of direct actions on mRNA, proteins and membranes. The actions on DNA can be induced by very low concentrations of GC. These effects result from the binding of GC with their cytosolic receptors (cGCR) followed by receptor activation and translocation into the nucleus [5, 6]. Once inside the nucleus, the activated receptors modulate the activity of a number of transcription factors [5, 12-16]. This leads in turn to regulation of the expression of genes for many pathways, including immunoregulatory and inflammatory cytokines, apoptotic proteins, and angiogenic proteins [5, 13, 24, 25]. Higher concentrations of GC increase receptor occupation, which strengthens the GC effects at the DNA-level.

When the cytosolic receptors become saturated, GC can additionally induce a number of effects outside the nucleus. The following effects appear to be primarily the result of these non-genomic activities of GC such as: cGCR-mediated inhibition of arachidonic acid release; intercalation of GC molecules into cellular membranes altering cationic transport through the plasma membrane and increasing proton leakage out of the mitochondria; binding of GC to specific membrane-bound receptors; interference of the cGCR with phosphatidylinositol 3-kinase (PI3K)/ (protein kinase B) Akt signalling pathway [24, 33-36]. All these mechanisms, both at genomic and non-genomic level, could be involved in the various therapeutic effects of GC in numerous disease states (Table 1). The table illustrates that the desired potent effects of free GC on cellular function are primarily associated with suppression of the immune system, likely because these effects already occur at relatively low clinically applied GC doses.

Table 1. Therapeutic uses of GC

Inflammatory disorders/Auto-immune diseases	**Administration**	**Reference**
Allergic reactions (*general*)	Oral/parenteral	[2, 3]
Asthma (*anti-emetic*)	Inhaled/oral	[4]
Chronic obstructive pulmonary disorder	Oral/inhaled	[7, 8]
Crohn's disease/ulcerative colitis	Oral/rectal	[9-11]
Dermatitis (*atopic, allergic, seborrheic, exfoliative*)/pruritus (*and lichenification*) /eczema/ psoriasis/xerosis	Oral/topical	[17-21]
Drug hypersensitivity	Oral/parenteral	[22]
Graves' ophthalmopathy	Oral/parenteral	[23]
Hemorrhoids	Rectal	[19]
Idiopathic thrombocytopenia purpura	Parenteral	[26]
Multiple sclerosis	Oral/parenteral	[27]
Nasal polyps	Nasal	[28, 29]
Ocular inflammation (*conjunctivitis, cyclitis, uveitis, choroiditis, iritis, keratitis, neuritis*)	Ocular	[30-32]
Organ transplant/keratoplasty	Oral/Ocular	[32, 37]
Pemphigus (*vulgaris, foliaceus*)	Oral	[40]
Rheumatic disorders	Oral	[42]
Rhinitis	Nasal	[28, 45]
Transfusion reactions	Parenteral	[47]
Urticarial transfusion reaction	Parenteral	[47]
Injuries		
Acute spinal cord injury	Parenteral	[48]
Burns	Topical	[51]
Corneal injury	Ocular	[31]
Insect bites	Topical	[21]
Infections		
Bacterial meningitis	Parenteral	[52]
Cryptitis	Rectal	[53]
Hepatitis	Oral	[54]
Pneumocystis carinii-infection	Parenteral	[55]
Septic shock	Parenteral	[57]
Hormone insufficiency		
Addison's disease	Oral	[58]
Adrenal cortical insufficiency	Oral	[60]
Blood cell disorders		
Anemia (*hemolytic, congenital*)	Oral/parenteral	[61-63]
Leukemia	Oral	[64]
Lymphoma	Oral	[64]
Edema		
Acute mountain sickness	Oral	[65, 66]
Cerebral edema	Parenteral	[67]
Dystrophy		
Muscular dystrophy (*Duchenne*)	Oral	[68]

Apart from the desired effects, GC use is associated with undesired side effects. First of all, administration of GC may disrupt the hypothalamic-pituitary-adrenal axis leading to deregulation of physiological corticosteroid levels. In addition, musculoskeletal complications may occur, like osteoporosis, osteonecrosis and

myopathy [38, 39]. For inhaled GC, a growth suppressive effect in children has been documented [41]. Other complications include ocular subcapsular posterior cataracts and glaucoma, peptic ulcer disease, electrolyte imbalance, edema and hypertension [43, 44]. A variety of dermatological effects may occur including fat redistribution, thinning of the skin, purpura, striae and allergic reactions [46]. Wound healing may also be decelerated [49, 50]. Normal sugar and fat metabolism may be affected, featured by hyperglycemia, hypertriglyceridemia, and hypercholesterolemia [41, 43, 49, 50]. Steroid psychosis and steroid withdrawal syndrome are complications mediated by the central nervous system that may occur [43, 46, 56]. Finally, the immunosuppressive action may increase the risk for infections [44].

The powerful therapeutic effects of GC and multitude of GC side effects provide a rationale for targeted delivery of GC, to locally improve potency while minimizing toxicity. Additionally, the unfavorable pharmacokinetic profile of GC upon intravenous administration, characterized by rapid clearance in combination with a large volume of distribution, may be improved by means of drug targeting by employing liposomes as carrier systems for GC [59].

2. DRUG TARGETING

Drug targeting can be defined as the ability of a drug to accumulate at a target site in the body. Drug targeting can be realized by employing a drug carrier, which delivers the active compound to (pathological) target tissues and decreases side effects in healthy non-target tissues. Over the last decades, a broad variety of carriers has been designed for the purpose of drug targeting such as plasma proteins, antibodies, viruses, erythrocytes, blood platelets, polymeric nanoparticles, micelles, and liposomes [69-80].

The principal strategies of drug targeting include *direct application of a drug* into the affected zone, *passive drug targeting* (uptake of carrier by the cells of the mononuclear phagocyte system (MPS) or spontaneous drug accumulation in areas with leaky vasculature, as a result of the so-called “enhanced permeability and retention (EPR) effect”), *physical targeting* (based on abnormal pH value and/or temperature in the pathological zone), *magnetic targeting* (or targeting of a drug immobilized on paramagnetic materials under the action of an external magnetic field), and *active targeting* using specific ligands that have an increased affinity toward the area of interest (antibodies, antibody fragments, proteins, peptides, and polysaccharides) [69, 70, 81].

3. LIPOSOMES FOR LOCAL ADMINISTRATION OF GC

Encapsulation of GC in liposomes has been investigated early on in liposomal drug delivery research. Originally, the primary aim was to prolong local drug levels, e.g. after topical administration on the skin or cornea, or after intra-articular injection in the joint [82-89]. These studies showed extensive increases in the local area under the concentration-time curve (AUC) while minimizing systemic absorption, associated with less side effects. Generally, improved therapeutic effects were noted for the liposome-encapsulated GC, which was primarily attributed to the local AUC increase. Importantly, for liposome-encapsulated GC, the AUC has both a therapeutically available and liposome-encapsulated component, which makes the interpretation of AUC-data less straightforward, as usually only total AUC values are reported. In a clinical study, Korting et al. used a liposomal formulation of betamethasone for topical application in patients suffering from atopical eczema or psoriasis [86]. Whereas an improvement was noted on erythema and scaling for the eczema patients, liposomal betamethasone performed worse than control gel in psoriasis patients leading the authors to conclude that liposome-encapsulation improves the anti-inflammatory but not the antiproliferative action of the drug. This unfavorable observation may be explained by the reduction of therapeutically available peak drug concentrations and prolonged persistence of the liposomal betamethasone.

Taken together, the encapsulation of GC in liposomes for local administration may offer important benefits for controlled release of encapsulated agents, but application is, of course, limited to diseases that are accessible with a clearly defined pathological location.

For diseases that are not easily accessible, or cover multiple or unknown sites, intravenous administration is indicated. To maximize the degree of localization at the pathological site, long-circulating liposomes are preferred as they are better able to exploit the EPR effect that is the hallmark of the pathologies in which GC are effective [90].

4. LONG-CIRCULATING LIPOSOMES

In this thesis, we have used long-circulating liposomes as drug carriers for GC. These long-circulating liposomes are nanosized lipid bilayer vesicles coated with hydrophilic polymers to stabilize their structure and to prolong their circulation time. In our studies we have used poly(ethylene glycol) (PEG)-coated liposomes. PEG is a synthetic hydrophilic polymer that prolongs the circulation time of the liposomes when exposed on their surface.

These PEG-coated liposomes are also referred to as "sterically stabilized", or "stealth liposomes" due to the highly hydrated groups of PEG that inhibit both hydrophobic and electrostatic interactions of a variety of blood components at the liposome surface [91]. PEG coating reduces protein adsorption and opsonization of liposomes, thereby avoiding or retarding liposome recognition by the MPS. Therefore, PEG-liposomes have prolonged blood residence times (in humans with a half-life of up to 45 h) [92]. The long-circulation property of PEG-liposomes makes them especially suitable for the targeted delivery of drugs to tumors and sites of inflammation by exploiting the EPR-effect, as both pathological sites have an enhanced permeability of the vasculature compared to healthy endothelium [91-94].

5. THERAPEUTIC APPLICATIONS OF GC ENCAPSULATED IN LONG-CIRCULATING LIPOSOMES

Encapsulation of GC in long-circulating liposomes offers an increased therapeutic activity and an enhanced benefit-risk ratio. The increased local drug levels at sites of enhanced permeability may offer new treatment indications.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is still a major medical challenge. To this date, the exact cause of the disease is unknown. The difficulties in treatment are probably related to the multitude of processes that play a role in the disease. RA is characterized by joint pain and swelling as a result of inflammatory reactions against joint tissue. GC are able to inhibit the inflammatory processes but are also able to stop joint destruction through less obvious mechanisms [95].

In a series of studies, Metselaar et al. reported on the use of liposomal GC in experimental arthritis models [59, 96, 97]. To evaluate the targeting potential of long-circulating, PEG-coated liposomes in rheumatoid arthritis, whole body radioscintigraphy in rats with adjuvant arthritis was performed. GC-loaded liposomes were shown not to leak drug in the circulation and to preferentially localize in the inflamed joints. The beneficial effect of long-circulating behavior on inflamed tissue accumulation is in line with previous observations of Love et al., on rigid cholesterol-rich liposome compositions [98].

More importantly, both in rat adjuvant arthritis and in murine collagen-induced arthritis models, a strong anti-arthritic effect of intravenously administered liposomal prednisolone phosphate was observed. Fast and complete disappearance of joint inflammation was achieved with a single dose of 10 mg/kg, which lasted

up to a week post-injection. Unencapsulated prednisolone was totally ineffective at the same dose. When the dosing regimen was intensified and the free drug was administered daily, the activity of free drug was still poorer than the anti-arthritic activity of a single dose of liposomal prednisolone phosphate by far. The targeting effect of PEG-liposomes to the inflamed joints was proven to be crucial for achieving therapeutic efficacy. When PEG-liposomes with a large mean diameter (that primarily target the spleen) and liposomes without PEG-coating, (that primarily target liver and spleen) were used, a reduced circulatory half-life was observed leading to a reduced degree of localization in the inflamed joints. The reduced circulatory half-life was primarily accounted for by increased uptake of both liposomal formulations by liver and, most notably, spleen. These changes in tissue distribution profile were paralleled by a reduced anti-arthritic activity of the short-circulating formulations in the joints.
In an attempt to further increase specificity of the long-circulating liposomes, a peptide with an Arg-Gly-Asp motif was coupled to the distal end of the PEG-chain on the liposome surface [99]. Thereby, liposome interaction with the angiogenic endothelium during the inflammatory process is expected to be increased, through binding of the Arg-Gly-Asp containing peptide to alpha v-integrins overexpressed on the activated endothelial cell surface. The liposomes were shown to interact specifically with the vascular wall at sites of inflammation, and, when loaded with GC, demonstrated a strong and long-lasting anti-arthritic effect in the rat arthritis model. These observations support the notion that endothelial cells play a crucial role during inflammation progression and that targeting these cells with GC can contribute to therapeutic effects.

Multiple sclerosis

Multiple sclerosis is an auto-immune disease, propagated by a defect in immune system control [100]. Again, the cause for the disease is unknown, although it is speculated that infections may activate T cells through molecular mimicry and immune stimulation. Thereby myelin-reactive T cells are allowed to be activated, to proliferate, and to enter the central nervous system, where these cells instigate inflammation against the myelin sheath surrounding the axons. This activation favors a pro-inflammatory profile leading to the production of several pro-inflammatory cytokines including tumor necrosis factor alpha, interferon gamma, interleukin-1, interleukin-12, and the matrix metalloproteinases. High doses of GC are one of the cornerstones of treatment of relapses in multiple sclerosis [27].

Based on the previous studies in rheumatoid arthritis, the inflammatory lesion-targeting potential of liposomes was utilized in an animal model of multiple sclerosis: adoptive transfer-experimental auto-immune encephalomyelitis in rats [101]. Radioactively labeled PEG-liposomes selectively targeted inflamed nervous tissues, reaching up to 4.5-fold higher levels than in healthy controls. Targeting of experimental encephalomyelitis with radiolabeled liposomes for imaging purposes was previously demonstrated by Rousseau et al. [102]. This targeting effect led in turn to higher and prolonged levels of prednisolone in spinal cord as compared to levels observed after treatment with free GC. The disruptive effect of the inflammation on blood–brain barrier function was greatly reduced by liposomal prednisolone phosphate treatment, which was superior to a 5-fold higher dose of free methylprednisolone. Cellular markers of inflammation, featured by T cell and macrophage infiltration and tumor necrosis factor-staining were strongly decreased by liposomal treatment as compared to treatment with free drug. It is suggested that liposomal targeting of prednisolone phosphate achieves ultra-high concentrations at the site of inflammation, which are in range of the concentrations that can be responsible for the non-genomic effects of GC, adding non-genomic activities to the GC effects.

Cancer

Free GC have been shown to inhibit solid tumor growth [103-106]. The effects appear to be related to inhibition of angiogenesis but can only be achieved by extremely high and frequent dosing of GC up to 200 mg/kg per day. These doses resulted in death of part of the animals in the treatment groups in many experiments, due to opportunistic infections as a result of extensive immune suppression. Apparently, the minimum concentration required to observe the antitumor effects is far above the maximum concentrations below which genomic effects of GC occur. It was investigated whether the passive tumor targeting capacity of PEG-liposomal GC could create tumor concentrations of GC yielding antitumor activity in B16.F10 melanoma and C26 colon carcinoma tumor-bearing mice [107]. Liposomes were shown to accumulate at subcutaneous (s.c.) sites of malignancy. Between 5-10% of the injected dose localized in the tumor at 24h post injection. When looking at GC levels in the tumor after administration of 20 mg/kg liposome-encapsulated prednisolone phosphate, 10-20 μg GC was recovered per gram tumor tissue at 24h post injection. Although the respective contributions of therapeutically active and liposome-encapsulated prednisolone phosphate is unclear, levels were at least 100-fold higher than the levels induced by the administration of the free drug at this time point.

Liposomal prednisolone phosphate could inhibit tumor growth in a dose-dependent manner. Maximum levels of tumor growth inhibition were 80% to 90% after single dose of 20 mg/kg (Figure 1).

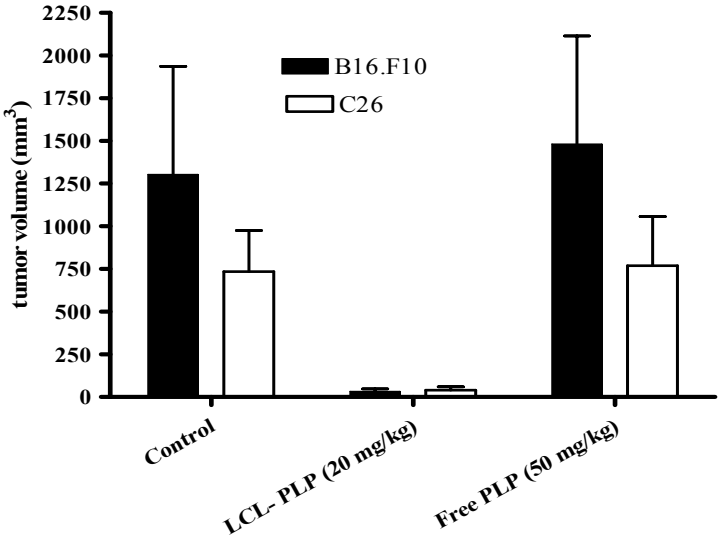

Figure 1. Effects of liposomal and free prednisolone phosphate on tumor growth in B16.F10 or C26 tumor-bearing mice. Mice received a single injection of the indicated dose and formulation of prednisolone phosphate on the day that the tumors became palpable. Tumor volume after 1 week is reported. Control= treatment with PBS, LCL-PLP = treatment with long-circulating liposome-encapsulated prednisolone phosphate, Free PLP= treatment with free prednisolone phosphate (adapted from [107])

Prednisolone phosphate in the free form was completely ineffective after single dose administration, even when administered at a dose of 50 mg/kg (Figure1). Again, the therapeutic effect could be attributed to local drug levels in the tumor as short-circulating liposomes failed to affect tumor growth rate.

6. AIM AND OUTLINE OF THE THESIS

The primary aim of this thesis was to investigate the mechanisms of tumor growth inhibition by glucocorticoids (GC) encapsulated in long-circulating liposomes (LCL) (LCL-GC). **Chapter 2** provides an overview of the current status of tumor-targeted delivery of GC and the possible mechanisms of GC action involved in their antitumor activity. The observation that the anti-angiogenic action of prednisolone phosphate-loaded poly(ethylene glycol) (PEG)-liposomes is responsible for tumor growth inhibition is reported in **Chapter 3**. To gain more insight into the antitumor mode of action of long-circulating liposome-encapsulated- prednisolone phosphate (LCL-PLP), **Chapter 4** aims to address the role of tumor-associated macrophages (TAM) in the antitumor effect of LCL-PLP. **Chapter 5** studies whether the differences in antitumor activity of LCL-GC other than LCL-PLP correlate with their efficacy to suppress tumor angiogenesis and inflammation. To investigate whether the liposomal GC tumor targeting concept can be exploited more efficiently by using other types of GC than PLP, the antitumor activity and side effects of LCL-GC were investigated in **Chapter 6**. **Chapter 7** deals with the tumor-targeted delivery of pravastatin. This therapeutic agent belongs to the class of statins. Statins have a primary activity not related to angiogenesis or tumor growth inhibition (i.e. cholesterol lowering in cardiovascular diseases) but also possess anti-angiogenic activity on tumor growth when they are delivered to tumor tissue. As an example, pravastatin was encapsulated into LCL. The capability of LCL to deliver cytostatic drugs to sites of malignancy is already successfully applied in the clinic. An example is Doxil^{TM} (PEG-liposomes with encapsulated doxorubicin). Based on the suppressive effects of Lip-PLP and LCL-encapsulated clodronate on TAM, we hypothesized that Doxil could also act in a similar way on these cells. **Chapter 8** focuses on this alternative mechanism of action of Doxil to its cytotoxicity towards tumor cells. **Chapter 9** provides a summarizing discussion on the results presented in this thesis.

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Chapter 2

Utility of targeted glucocorticoids in cancer therapy

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ABSTRACT

Glucocorticoids can inhibit solid tumor growth via downregulation of tumor-associated inflammation/ angiogenesis. In this minireview the possible mechanisms of glucocorticoid action involved in tumor growth inhibition are described. We also present an overview of the current status of tumor-targeted delivery of glucocorticoids. It appears that currently long-circulating liposomes are the only targeting system being explored for this purpose.

Keywords: glucocorticoids, tumor targeting, angiogenesis, inflammation

1. INTRODUCTION

More than 50 years after their introduction into clinical medicine, glucocorticoids (GC) are still among the most effective anti-inflammatory and immunosuppressive drugs. Unfortunately, these valuable drugs also harbor undesirable side effects though this has not prevented their widespread use. In clinical practice, high-dose intravenous GC therapy is being applied as first-line treatment in several situations, for example in the treatment of systemic lupus erythematosus [1]. In rheumatoid arthritis, GC can strongly reduce the generation and release of pro-inflammatory cytokines and cartilage degrading enzymes by macrophages in arthritic joints. However, serious adverse effects limit the systemic use of GC in arthritic patients. Moreover, high and frequent dosing is necessary to achieve sufficient activity in the joints, because target localization is usually poor as a result of efficient clearance [2].

To improve the anti-inflammatory effectiveness of GC, a long-circulating liposomal formulation of GC has recently been studied. Poly(ethylene glycol) (PEG)-coated liposomes encapsulating prednisolone phosphate (PLP) was used in the treatment of rats with experimental arthritis or autoimmune encephalomyelitis. In both models, much higher and more persistent levels of prednisolone at the site of inflammation were achieved after liposomal PLP treatment compared with an equal dose of free PLP [3]. As a result, a single injection of PLP liposomes at a dose of 10 mg/kg resulted in complete remission of the inflammatory response for almost a week in both experimental diseases. The same dose of unencapsulated PLP did not reduce inflammation, and only a slight effect was observed after repeated daily injections of high doses [3, 4].

In addition to anti-inflammatory activity, reports in the last two decades have indicated that GC can also exert antitumor activity in experimental animal models. However, these preclinical studies have also shown that high and frequent dosing of GC is a prerequisite for obtaining antitumor activity. GC are rapidly cleared from the circulation, and accumulate at tumor sites only to a very limited extent. Doses required to achieve antitumor effects are generally in the range of 100-200 mg/kg/day. Such high doses resulted in considerable morbidity and mortality as a result of severe immune suppression [5]. Therefore, it appears that the oncological application of GC would benefit greatly from the use of a tumor-targeted delivery system. The objective of this contribution is to offer an overview of the current status of tumor-targeted delivery of GC. In addition, we review the possible mechanisms of GC action involved in tumor growth inhibition via

downregulation of tumor-associated inflammation/angiogenesis. Perspectives on the oncological application of liposomally targeted glucocorticoids are given.

2. MECHANISMS OF ACTION OF GLUCOCORTICOIDS

GC exert a broad variety of effects on mammalian cells including anti-inflammatory, immunosuppressive, anti-angiogenic, apoptotic and necrotic effects. These actions are mediated by both genomic and non-genomic mechanisms. Below a condensed overview is given.

2.1. Genomic mechanisms

The genomic mechanisms are already induced by GC at low concentrations (≤7.5 mg prednisone equivalent per day in humans). These concentrations are much lower than those needed to induce non-genomic mechanisms [1]. The actions are determined by the interaction of GC with their cytosolic receptors (cGCR) (Figure 1) [6, 7]. In the absence of GC, cGCR is present in the cytoplasm in an inactive complex together with chaperones (such as heat shock proteins (hsp) 90, 70, 56, 40), and co-chaperones. When the GC bind, cGCR become activated by dissociating from the multimeric protein complex, leading to the exposure of two nuclear localization signals. This exposure leads to the rapid translocation of the GC-cGCR complex into the nucleus. Moreover, cGCR activation is followed by dimerization of cGCR. Within the nucleus, the GC-cGCR homodimer complex modulates transcriptional responses of the majority of inflammatory, angiogenic, immunomodulatory and apoptotic genes by binding directly to DNA (Figure 1, mechanisms A and B). Alternatively, it can interact with specific proteins, in particular transcription factors like nuclear factor κB (NF-κB), and activator protein 1 (AP-1), which are also involved in regulation of these genes (Figure 1, mechanisms C1 and C2) [6, 8-12]. Finally, GC can also interact at the post-transcriptional and translational level by regulation of transport, cytosolic localization, rate of decay and translation of mRNA (Figure 1, mechanisms D, E, F). Since all these genomic interactions of GC require interference with the transcription-translation machinery, their action becomes apparent only after a certain lag-time. The genomic mechanisms are discussed in more detail below.

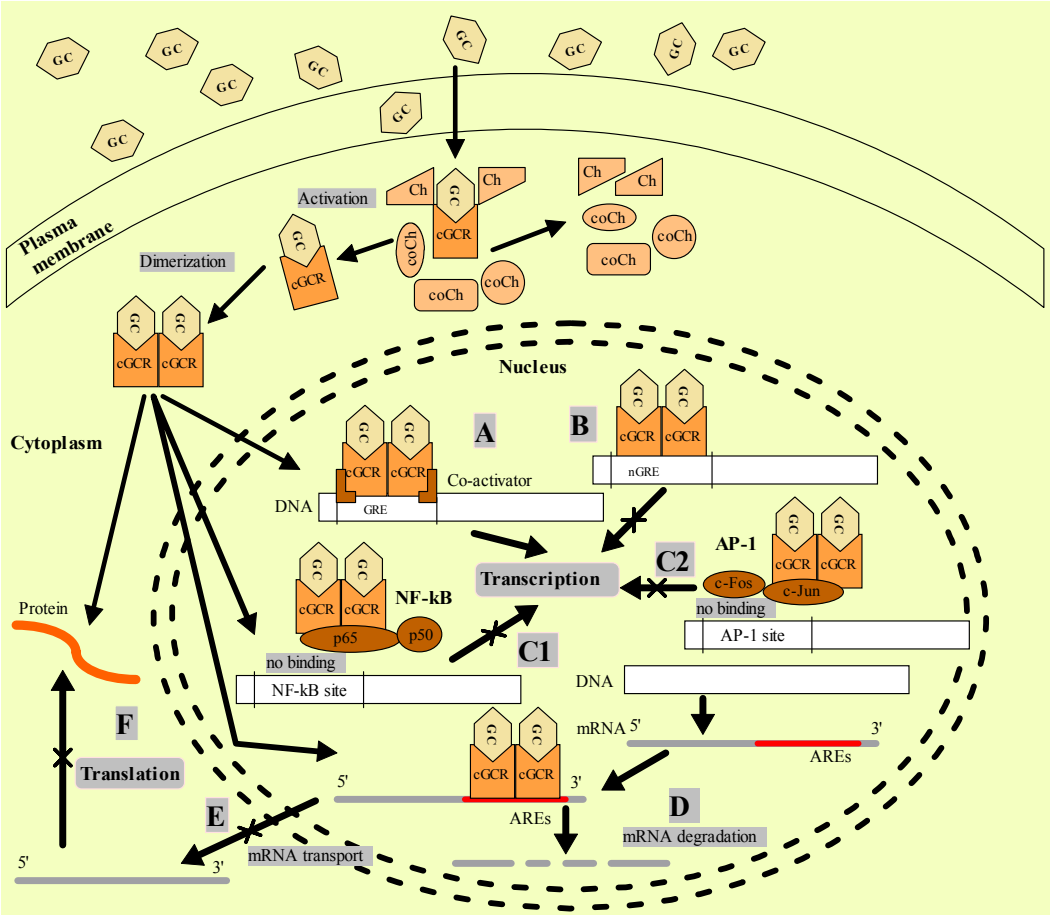

Figure 1 Main genomic mechanisms of glucocorticoids. The direct (A, B) and indirect (C1 and C2) regulation of transcription is induced by GC/cGCR complex in the nucleus. This complex also induces post-transcriptional (D, E) and translational (F) actions. GC= glucocorticoids, cGCR= cytosolic glucocorticoid receptor, GRE= glucocorticoid response elements, nGRE = negative GRE, NF-κB= nuclear factor κB, AP-1 = activator protein 1, AREs = adenylate/ uridylate-rich elements, Ch = chaperones, coCh = co-chaperones

2.1.1. Transcriptional mechanisms

After GC binding and dissociation of cGCR from the multimeric protein complex, the GC-cGCR homodimer is transported to the nucleus to bind to DNA at consensus sites that are termed glucocorticoid response elements (GREs) [6, 13]. Binding of the GC-cGCR complex to a GRE results in a conformational change of the cGCR that promotes the recruitment of several co-activators that also bind to the GC-cGCR-DNA complex,

such as the cAMP response element binding protein (CREB)-binding protein (CBP)/p300, and the steroid receptor co-activator 1 (Src-1) (Figure 1, mechanism A). These co-activators have histone acetylase activity necessary for nucleosomal rearrangement and DNA unwinding which allows the basal transcription machinery to access the promoter [6, 13]. GC-cGCR can also repress genes by directly binding to so called "negative GRE" (nGRE) which are responsible for gene repression (Figure 1, mechanism B). However, most genes that are represssed by GC do not have an nGRE, suggesting that other genomic mechanisms exist as well [7].

In addition to DNA binding, the GC–cGCR complex modulates transcriptional responses by interaction with transcription factors that are involved in regulation of genes encoding proteins involved in angiogenesis, inflammation, immunoregulation, and apoptosis, such as NF-κB, and AP-1 (Figure 1, mechanisms C1 and C2). Some studies suggest that the physical interactions between GC-cGCR and the p65 subunit of NF- κB (Figure 1, mechanism C1) and between GC-cGCR and c-Jun subunit of AP-1 (Figure 1, mechanism C2) sequester these factors, preventing their binding to DNA. Other studies suggest that the GC-cGCR complex and transcription factors (NF- κB, AP-1) compete for binding to certain co-factors such as CBP and Src-1 [6, 7]. In addition, it is known that the GC-cGCR complex can interact with a variety of other transcription factors such as the Nuclear Factor of activated T cells (NF-AT), and the Signal Transduction and Activator of Transcription (STAT) family transcription factors [6].

2.1.2. Post-transcriptional and translational mechanisms

The majority of post-transcriptional actions induced by GC appear to be mediated by binding of GC-cGCR complex to untranslated mRNA sequences, mostly flanking the coding regions at 3' ends of mRNAs. In particular, the adenylate/ uridylate-rich elements (AREs) are responsible for this interaction (Figure 1, mechanism D). GC-cGCR act as regulators of multiple mRNA functions, such as mRNA transport, mRNA subcellular localization, and rate of mRNA decay (Figure 1, mechanisms D, E). Post-transcriptional regulation of genes involved in inflammation, angiogenesis and apoptosis is also induced by the GC-cGCR complex through regulation of intracellular molecules that act as "general on/off switches". These molecules belong to serine/threonine kinase cascades, such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38 and kinase of NF-κB inhibitor (IKK) [11]. Finally, GC-cGCR can also inhibit translation of

certain inflammatory and angiogenic proteins by suppression of the translational initiation factors and ribosomal genes expression (Figure 1, mechanism F) [11].

2.2. Non-genomic mechanisms

Higher dosages (≥ 7.5 mg of prednisone equivalent per day in humans) of GC increases cGCR occupation, which intensifies the GC effects at the genomic level. At daily dosages ranging from 7.5 to 30 mg of prednisone weak non-genomic effects appear to occur but considered to be nonrelevant [14]. When the cGCR are saturated (which occurs at a dose of approximately 100 mg prednisone equivalent per day), GC additionally induce stronger non-genomic effects [1, 15]. Non-genomic mechanisms are characterized by a rapid onset of effects (generally in less than 15 minutes). This rapid onset is possible because gene transcription and translation are not necessary [16]. The non-genomic effects of GC are classified into direct effects when the GCs are acting on their own as an agonist and indirect effects when the GC needs an agonist partner to generate the response. These actions are also subdivided in nonspecific (Figure 2, mechanisms A1 and A2) and specific mechanisms (Figure 2, mechanisms B, C) [16]. The non-genomic mechanisms are detailed below.

2.2.1. Direct non-genomic mechanisms

Nonspecific non-genomic actions are induced at relatively high concentrations of GC without receptor involvement. It is mediated by GC-induced changes in membrane physicochemical properties (Figure 2, mechanisms A1 and A2) [16]. Nonspecific intercalation of GC molecules into the cell membranes can alter cell functions by influencing cation transport through the plasma membrane (Figure 2, mechanism A1) and/or by increasing proton leak out of mitochondria (Figure 2, mechanism A2) [1]. Recently, it was demonstrated that GC can alter the membrane potential, leading to specific changes in gene expression which are suggested to be involved in a newly hypothesized mechanism for the induction of apoptosis by GC [9].

Specific non-genomic mechanisms involve cytosolic glucocorticoid receptors (cGCR) or membrane-bound glucocorticoid receptors (mGCR) [16, 17]. An example of non-genomic actions induced by GC via cGCR is the inhibitory effect of GC-cGCR on the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway involved in tumor growth.

In a mouse skin tumor model it was found that cGCR appears to associate with the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), p85α, resulting in decreased Akt and IKK activity [18, 19].

In mouse lymphoma and human acute lymphoblastic leukemia cells, non-genomic actions via the mGCR have been functionally linked to GC-induced lysis of these cells. It has also been postulated to play a role in thymic regression and apoptosis [1, 11, 17, 20]. There are several signaling pathways with involvement of a steroid membrane receptor. More specifically, in human mononuclear leucocytes and vascular smooth muscle cells, fludrocortisone, a mineralocorticoid with glucocorticoid activity, binds to a membrane steroid receptor which leads to a rapid increase of intracellular Ca2+ concentration from intracellular inositol triphosphate (IP3)-sensitive stores (Figure 2, mechanism B). This is suggested to be mediated through a recently discovered G-protein coupled to a steroid membrane receptor [16, 21]. However, the causal connection between this receptor and these rapid effects is not clear yet.

2.2.2. Indirect non-genomic mechanisms

Specific indirect non-genomic actions occur after binding of GC molecules to the cytosolic glucocorticoid receptor (cGCR). Recent studies reported that dexamethasone can rapidly inhibit arachidonic acid release after binding to cGCR (Figure 2, mechanism C). The investigators considered that certain chaperones or co-chaperones (Src) of the multiprotein complex act as mediators of this inhibitory effect of dexamethasone after cGCR release from the multiprotein complex [1, 17].

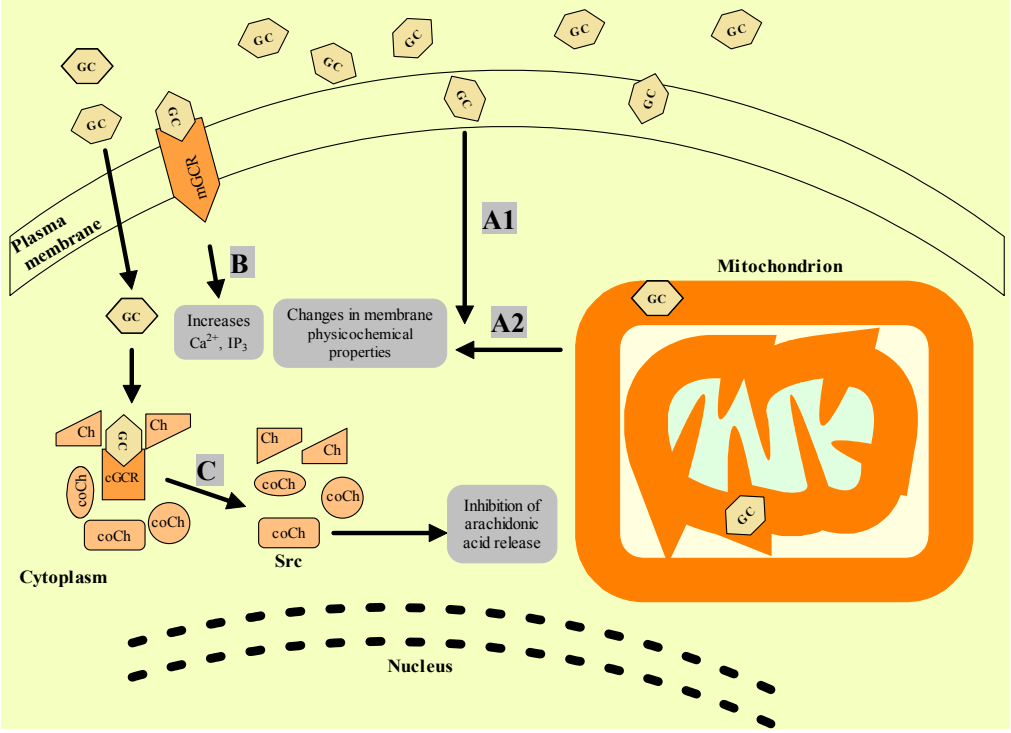

Figure 2 Main non-genomic mechanisms of glucocorticoids. Direct nonspecific non-genomic mechanisms (A1, A2) of GC change physicochemical properties of the cellular membranes. Direct specific non-genomic mechanisms are mediated by GC interaction with mGCR (B). Indirect mechanisms are exerted by Src released from multiprotein complex of inactive cGCR (C). GC= glucocorticoids, cGCR= cytosolic glucocorticoid receptor, mGCR= membrane glucocorticoid receptor, Src= co-chaperone released from multiprotein complex Ch = chaperones, coCh = co-chaperones

3. TARGETED DELIVERY OF GC

In the clinical setting, GC have been used for their anti-inflammatory and anti-emetic effects. In addition, GC have been applied clinically in the treatment of hematological malignancies as they have efficient cytolytic activity on cells of lymphoid origin [5]. There are indications that GC have inhibitory actions on solid tumor growth due to suppressive effects on tumor angiogenesis and inflammation [22, 23]. When evaluating the preclinical studies on solid tumor growth inhibition, it appears that GC-induced antitumor effects are achieved either by using substantially higher doses than the minimal doses needed to achieve inhibition of inflammatory disease processes or by employing tumor-targeted delivery systems. For example, to obtain antitumor effects in tumor-bearing mice, frequent and high dosing of GC is required: cumulative weekly doses of cortisone are in the range of 500-700 mg/kg [22, 24]. The high doses of GC used in these antitumor studies resulted in death of some of the animals due to opportunistic infections, indicating that severe systemic side effects can occur [24].

As GC are rapidly cleared from the circulation, and localize at tumor sites only to a very limited extent, targeted delivery of GC to tumor tissue is an attractive strategy to increase intratumoral drug concentrations. As described in the previous section, GC regulate the production and activity of a broad variety of inflammatory and angiogenic proteins, such as enzymes responsible for the synthesis of key mediators of inflammation, for the degradation of basal membranes and for the reorganization of extracellular matrix of blood vessels, peptide growth factors, mediators of inflammatory reactions, and cell adhesion molecules [7, 12, 25-28]. These actions are exerted by GC at both the genomic and non-genomic level (Figure 3). One of the main inhibitory actions of GC on tumor inflammation is downregulation of expression of genes encoding for enzymes involved in synthesis of prostaglandin E_2 (PGE_2) such as cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase 2 (COX-2) [29, 30]. Inhibition of the production of PGE_2, a key mediator of inflammation, leads to a supplementary reduction of growth factors, cell adhesion molecules, and metalloproteinases involved in different steps of tumor angiogenesis [31, 32]. Remarkably, GC appear to have no reducing effects on the levels of most of the *anti*-angiogenic and *anti*-inflammatory proteins allowing these proteins to mediate downregulation of genes encoding for *pro*-angiogenic factors (VEGF, bFGF, MMPs) [23, 33, 34]. These effects of GC on the production of angiogenic/ inflammatory factors in tumor tissue can cause a shift in the balance between *pro*- and *anti*-angiogenic factors in favor of inhibition of inflammation–associated angiogenesis, thus diminishing tumor

growth (Figure 3). Therefore, antitumor therapy with targeted GC appears to be an useful tool for inhibition of most of *pro*-angiogenic/ *pro*-inflammatory factors production in tumor tissue leading to blockage of tumor growth and metastasis (Figure 3). Due to the targeting effect, the overall dose needed is relatively small and hence the probability for the occurrence of side effects is low.

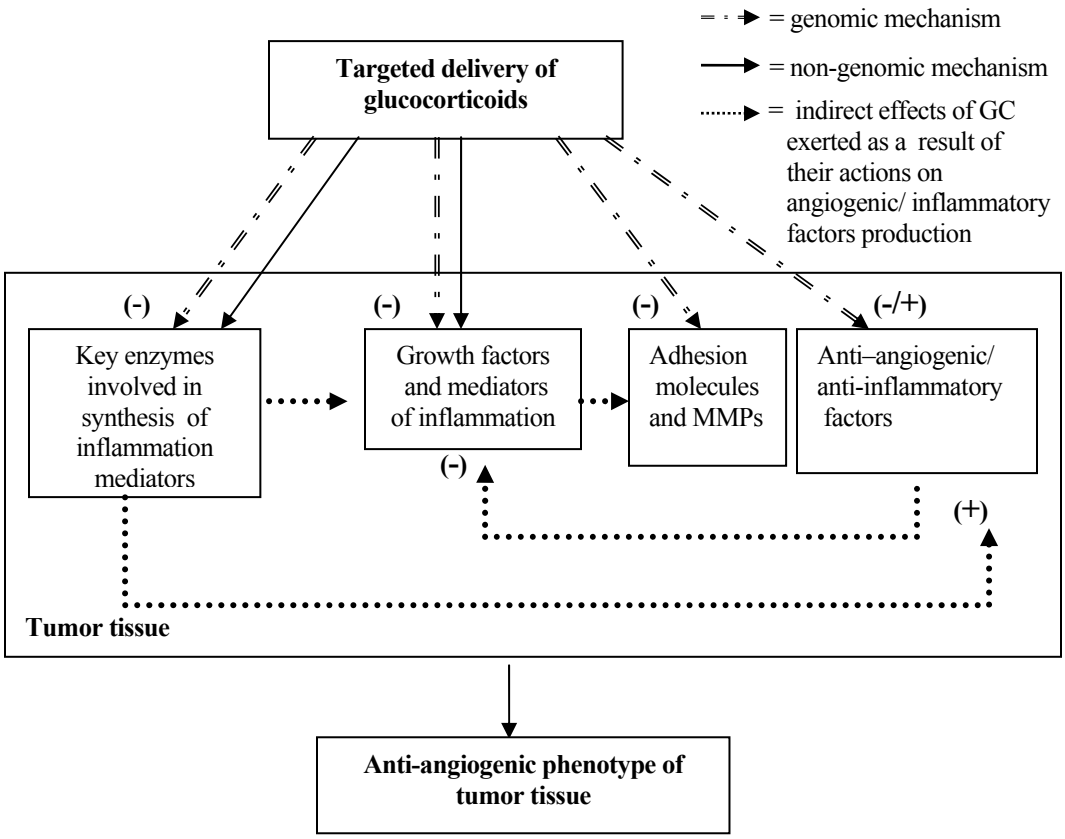

Figure 3 Inhibition of tumor angiogenesis/ inflammation by targeted delivery of glucocorticoids. Glucocorticoids exert their genomic and non-genomic effects on angiogenic/inflammatory factors in tumor tissue by shifting the balance between pro- and anti-angiogenic/inflammatory factors in favor of inhibition of inflammation-associated angiogenesis. MMPs= metalloproteinases, (-)= reduction of production of angiogenic/inflammatory factors, (+)= stimulation of production of angiogenic/ inflammatory factors, (-/+)= differentiated reduction/stimulation of production of angiogenic/inflammatory factors.

Thorpe et al. developed a strategy by employing a nonanticoagulating derivative of heparin as tumor vasculature-targeting motif for the corticosteroid [35]. An inhibitor of angiogenesis was obtained by covalently linking heparin adipic hydrazide (HAH) to the antiangiogenic steroid cortisol (HAH-cortisol), as evidenced by the inhibition of the proliferation and migration of vascular endothelial cells *in vitro* [35]. In mice bearing subcutaneous Lewis lung carcinoma, HAH-cortisol administered i.v. daily was shown to inhibit tumor growth up to 40-60 % as compared to untreated tumors. The doses of cortisol that were used in this study were low (7.5 mg/kg) and were injected daily for 10 days [35]. The mechanism behind the inhibitory effect of HAH-cortisol on tumor growth was hypothesized to be related to the accumulation of HAH-cortisol on the surface of tumor vascular endothelial cells. The accumulation was suggested to be mediated by heparin binding to sulfated polyanion receptors on endothelial cells. Although these receptors are present on other cell types as well, like hepatocytes and fibroblasts, the majority of systemically administered heparin is taken up by vascular endothelial cells, presumably due to the vast surface area of these cells which is in contact with the blood [35]. In addition, the preference for the tumor endothelium might be explained by the observation that angiogenic endothelial cells divide and endocytose about 10–fold more heparin than nondividing endothelial cells. Therefore, tumor endothelial cells are selectively targeted and experience larger drug exposure. Following uptake, the active free cortisol is released within the vascular endothelial cells resulting in changes in gene transcription and inhibition of endothelial cell proliferation [36]. The antiproliferative effect of HAH-cortisol on endothelial cells appeared independent of the glucocorticoid activity of the steroid since HAH conjugated to 5β-pregnane-3α, 17α, 21-triol-20-one (Tetrahydro S), a steroid completely lacking glucocorticoid or mineralocorticoid activity, was more effective at inhibiting DNA synthesis by murine pulmonary capillary endothelial (MPCE) cells than was HAH-cortisol [35]. A possible explanation is that cells have intracellular receptors other than GC receptors for tetrahydro steroids or their metabolites which mediate the angiostatic action of the conjugates [37, 38].

Another strategy investigated for GC targeting to tumors is the incorporation of GC into long-circulating liposomes. A formulation of PEG-coated liposomes encapsulating prednisolone phosphate (PLP) had an antitumor effect as a result of the passive tumor-targeting property of the liposomal formulation.

These liposomes were sized to a diameter between 90 and 110 nm. It was shown that liposome-encapsulated GC exerted a strong tumor growth-inhibiting effect *in vivo* in two subcutaneous murine tumor models: B16.F10 melanoma and C26 colon carcinoma. After administration of 20 mg/kg liposome-encapsulated GC, 10-20 μg (about 5% of the injected dose) of drug was recovered per gram of tumor tissue at 24 h post injection. When PLP was administered in free form, it was rapidly cleared from the circulation and resulting tumor levels were at least 100-fold lower than the levels induced by administration of liposomal drug at this time point. Liposomal PLP could inhibit tumor growth in a dose-dependent manner up to 80-90% after a single dose of 20 mg/kg whereas PLP in the free form was completely ineffective at this single dose treatment schedule [5].

The antitumor effects of the PEG-liposomal PLP formulation is enabled by the high degree of tumor accumulation of these liposomes, which in turn is favoured by the structural and functional abnormalities of tumor neovessels. Tumor vessels are often immature and tortuous, they may contain blind ends and they show increased permeability [39]. PEG-liposomes can extravasate through the permeable pathological vasculature and thereby accumulate into the malignant tissue (referred to as the "enhanced permeability and retention (EPR) effect"). Once extravasated into the tumor, liposomes appear to accumulate in the vicinity of capillaries within macrophages, further increasing effects on a major cell type driving angiogenesis: the tumor-associated macrophages (TAM). The preferential localization of liposomes in the immediate vicinity of tumor neovasculature may cause extraordinarily high local drug levels, which can mediate genomic effects and non-genomic effects. In addition, the hypothesis that inflammatory processes in and around the tumor are important in the angiogenic cascade would suggest that, apart from the anti-angiogenic properties, and anti-inflammatory actions of GC also have a pivotal importance in the mode of antitumor action. The observations of macrophage uptake of GC-loaded PEG-liposomes in the tumor tissue support the theory of liposomal GC-induced suppression of macrophage activity as being critical for achieving antitumor action [5]. It has also been shown that tumor-associated macrophages secrete mitogens, growth factors, and enzymes that stimulate both tumor cell survival and growth as well as angiogenesis. Accumulation of GC-containing liposomes within macrophages may lead to the suppressive effects on the production of these factors. In this view, liposomal GC may disturb the symbiotic relationship between tumor cells, macrophages and endothelial cells of tumor blood vessels [5].

To gain more insight into the mechanism of liposomal GC involved in tumor growth inhibition, *in vitro* toxicity and proliferation of tumor and endothelial cells were investigated together with *in vivo* tumor angiogenic protein levels [23, 40]. *In vitro* studies showed that the viability and proliferation of tumor cells was only slightly affected by liposomal GC treatment. However, liposomal PLP had strong antiproliferative effects on human umbilical vein endothelial cells (HUVEC), whereas free PLP had hardly any effect. *In vivo*, liposomal PLP had a strong reducing effect on pro-angiogenic protein levels, whereas levels of anti-angiogenic proteins were hardly affected. Free PLP, however, did not show strong effects on angiogenic protein levels.

Taken together, these studies point to a strong inhibitory effect of liposomal GC on tumor angiogenesis by reduction of the intratumoral production of pro-angiogenic factors combined with a direct inhibition of endothelial cell proliferation, as a result of high prolonged levels of prednisolone in the tumor by liposomal delivery [23]. All these actions are exerted by GC at both the genomic and non-genomic level, and are enabled by liposomal targeting of GC to tumor tissue. Overall, the non-genomic mechanisms occurring at the relatively high tumor concentrations delivered by targeted liposomal GC complete the genomic actions, promoting successful cancer treatment.

4. CONCLUSION AND PERSPECTIVES

The present minireview deals with GC targeting to tumors. Long-circulating liposomes are currently the only targeting system being explored for this purpose. Encapsulation of glucocorticoids in long-circulating liposomes provides effective means to achieve tumor-targeted delivery of GC producing antitumor effects based on actions at the non-genomic level that strengthen and complete their genomic actions.

One of the future issues is to identify the types of GC which show the strongest antitumor activity when they are incorporated in liposomes. Rapid clinical application may be feasible as both drug and carrier system are already on the market for many years.

Liposomal GC may be used in combination with other agents like conventional cytostatic drugs, and antitumor cytokines (IFN, TNF, IL-12p70) to improve cancer treatment. Moreover, suppression of tumor-associated inflammation is a promising concept that should be translated into new anticancer therapeutics not only based on GC but also on other inflammation inhibitors such as non steroidal anti-inflammatory drugs.

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Chapter 3

Anti-angiogenic effects of liposomal prednisolone phosphate on B16 melanoma in mice

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ABSTRACT

Prednisolone phosphate (PLP) encapsulated in long-circulating liposomes (LCL) (LCL-PLP) can inhibit tumor growth after intravenous administration (i.v.). The antitumor effects of LCL-PLP are the result of the tumor-targeting property of the liposome formulation. The mechanism by which LCL-PLP inhibits tumor growth is unclear. We investigated the effects of liposome-encapsulated PLP versus free PLP on angiogenic protein production in tumor tissue *in vivo* and on viability and proliferation of tumor and endothelial cells *in vitro*. *In vivo*, LCL-PLP had a stronger reducing effect on *pro*-angiogenic protein levels than free PLP, whereas levels of *anti*-angiogenic proteins were hardly affected. Cell viability was only slightly affected with either treatment. LCL-PLP had strong anti-proliferative effects on human umbilical vein endothelial cells whereas free PLP had hardly any effect. Taken together, the present study points to a strong inhibitory effect of LCL-PLP on tumor angiogenesis by reduction of the intratumoral production of the majority of *pro*-angiogenic factors studied and direct inhibition of endothelial cell proliferation, which is the result of high prolonged levels of prednisolone in the tumor by liposomal delivery.

Keywords: liposomes, drug targeting, angiogenesis, cancer, glucocorticoids

INTRODUCTION

Our previous studies indicate that PLP encapsulated in long-circulating liposomes (LCL) (LCL-PLP) exerts strong inhibitory effects on tumor growth in a low dose and low frequency schedule after intravenous administration. LCL-PLP inhibits tumor growth by 80-90% in subcutaneous B16.F10 melanoma and C26 colon carcinoma murine tumor models at a dose of 20 mg/kg [1]. In the case of free (i.e. non-encapsulated) PLP, the antitumor effects have only been observed using treatment schedules based on high and frequent dosing for prolonged periods of time. These doses resulted in a considerable morbidity and mortality as a result of severe immune suppression [1]. Therefore, targeted delivery of glucocorticoids (GC) to tumor tissue is an attractive strategy to increase intratumoral drug concentration and to prolong the antitumor effects of GC. By virtue of the enhanced permeability of tumor vasculature, as compared to healthy endothelium, LCL are able to extravasate into subcutaneous tumor tissue thereby increasing and prolonging intratumoral glucocorticoid (GC) concentrations. The mechanism by which extravasated LCL-PLP inhibits tumor growth is, however, unclear.

It is known that GC can exert a broad variety of activities on mammalian cells including immunosuppressive, anti-inflammatory, apoptotic, necrotic and anti-angiogenic effects. In principle, all these effects, both at the genomic and non-genomic level, could play a role in the antitumor activity exerted by LCL-PLP [1]. The genomic mechanisms can be induced by very low concentrations of GC. These mechanisms are determined by the interaction of GC with their cytosolic receptors (cGCR) followed by cGCR activation and translocation into the nucleus. Once in the nucleus, GC/cGCR complexes modulate the activity of transcription factors, such as activator protein-1(AP-1), nuclear factor kB (NF-kB) and nuclear factor of activated T cells (NF-AT). This leads to regulation of the expression of genes for many immunoregulatory and inflammatory cytokines (TNF-α, GM-CSF, IL-1β, IL-2, IL-3, IL-6, IL-8, IL-11), for apoptotic proteins (members of the Bcl-2 family such as Bcl-xs, Bad, Bax, Bid, FasL) as well as for *pro*-angiogenic proteins (like bFGF and VEGF) [2-4]. Higher dosages increase cGCR occupation, which intensifies the GC effects at the genomic level. If cGCR are saturated, GC can additionally induce non-genomic effects. Non-genomic actions comprise three different mechanisms: 1) cGCR-mediated inhibition of arachidonic acid release, 2) intercalation of GC molecules into cellular membranes altering cationic transport through the plasma membrane and increasing proton leak out of the mitochondria, and 3) binding of GC to specific membrane-bound receptors [5]. The responses induced by non-genomic

mechanisms of GC include immunosuppressive and anti-inflammatory effects and induction of necrosis.

To gain further insight into the mechanism of action of liposomal GC, we investigated the effects of LCL-PLP and free PLP on angiogenic protein levels *in vivo,* as well as on tumor cell and endothelial cell viability and proliferation *in vitro*. PLP encapsulated in long-circulating liposomes induced a strong inhibition of tumor angiogenesis by reduction of the intratumoral production of the majority of *pro*-angiogenic factors studied and by direct inhibition of endothelial cell proliferation.

MATERIALS AND METHODS

LCL-PLP preparation

LCL were prepared as described previously [1]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly (ethylene glycol) 2000-distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol 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 of prednisolone disodium phosphate (PLP) (Bufa, Uitgeest, The Netherlands). A water-soluble phosphate derivative of prednisolone was used to ensure stable encapsulation in the liposomes. Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering and found to be 0.1 μm with a polydispersity value lower than 0.1. The polydispersity values obtained indicate limited variation in particle size. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of liposomal preparations with chloroform, according to Rouser [6]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. The aqueous phase after extraction was used for determining the prednisolone phosphate content by high performance liquid chromatography as described previously [7]. The type of column was RP18 (5 μm) (Merck) and the mobile phase consisted of acetonitril and water (1:3 v/v), pH 2. The eluent was monitored with an ultraviolet detector set at 254 nm.

The detection limit for the high performance liquid chromatography setup was 20 ng/ml. The liposomal preparation contained ~5 mg prednisolone phosphate/ml and ~70 μmol phospholipid/ml. As control, empty liposomes were prepared with the same lipid composition but without incorporated drug.

Cells

B16.F10 murine melanoma and C26 murine colon carcinoma cells were cultured as monolayers at 37°C in a 5% CO_2-containing humidified atmosphere in DMEM medium (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). Human umbilical vein endothelial cells (HUVEC) (Glycotech, Rockville, USA) were cultured as a monolayer at 37°C in a 5% CO_2-containing humidified atmosphere in complete EGM endothelial cell growth medium (Cambrex, East Rutherford, NJ, USA).

For *in vitro* studies, the following protocol was established. All three cells types were trypsinized off the substratum and counted in Bürker counting chamber under microscope in the presence of trypan blue. Only cells excluding the dye were counted as viable cells. Cells were plated in 96-well plates at the appropriate concentrations according to the assay performed.

Murine tumor model

Male C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (The Netherlands) and 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 the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the flank of syngeneic C57Bl/6 mice. B16.F10 tumors became palpable around 7 days after tumor cell inoculation.

Effects of LCL-PLP versus free PLP on cell viability *in vitro*

To determine whether LCL-PLP and free PLP (i.e. not encapsulated in liposomes) had a direct cytotoxic effect on cells, 5 x 10^3 HUVEC, C26 and B16.F10 cells/well were plated in a 96-well plate for 24h. Then, LCL-PLP and free PLP (i.e. dissolved in aqueous solution) were added in PBS and incubated for 24h, 48h, and 72h. After exposure time, cell viability was determined by XTT-assay (Sigma, St. Louis, USA) according to the manufacturer's instructions [8]. All three cell types were incubated with tetrazolium salt XTT and electron-coupling reagent (N-methyl dibenzopyrazine methylsulfate) for 1 hour at 37°C in the CO_2-incubator. Using an ELISA microplate reader, the absorbance was measured at 490 nm with a reference wavelength of 655 nm.

Effects of LCL-PLP versus free PLP on cell proliferation *in vitro*

To determine the effect of PLP (liposomal and free) on cell proliferation, 1 x 10^3 HUVEC, C26 and B16.F10 cells/well were plated in a 96-well plate for 24 h. After that, LCL-PLP and free PLP, were added in PBS. The anti-proliferative effect of (liposomal

and free) PLP was determined after 24h, 48h, and 72h of incubation by ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions [9, 10]. This technique is based on the incorporation of the pyridine analogue bromodeoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. Cells were incubated with BrdU solution for 24h and then media were completely removed from the wells. Then, cells were fixed and DNA was denatured. To detect BrdU incorporated in newly synthesized cellular DNA, a monoclonal antibody conjugated with peroxidase, anti-BrdU-POD, was added. After 90 minutes of incubation, antibody was removed and cell lysates were washed three times with PBS. The immune complexes were detected by the subsequent substrate of peroxidase (tetramethyl-benzidine) reaction. The reaction product was quantified by measuring the absorbance at 450 nm with a reference wavelength of 655 nm.

Effects of LCL-PLP versus free PLP on the production of angiogenic factors *in vivo*

At 7 days after tumor cell inoculation, tumor size was measured and tumor volume calculated according to the formula $V = 0.52 \times a^2 \times b$, in which a is the smallest and b, the largest superficial diameter.

3-4 animals were used per experimental group. The first group was used as control and treated with PBS. The second group was treated with empty liposomes. The third group was treated with free PLP. The fourth group was treated with LCL-PLP. Free PLP and LCL-PLP were administered i.v. at a dose of 20 mg/kg at day 7, 10 and 13 after tumor cell inoculation. On day 14, the mice were sacrificed and tumors were isolated. Empty liposomes were administered i.v. using the same lipid concentration as for PLP-loaded liposomes.

To evaluate the effect of free PLP and LCL-PLP at a molecular scale, a screening of angiogenic proteins present in tumor tissues was performed using an angiogenic protein array of RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1 (RayBiotech Inc., Norcross, GA) [11]. Each membrane contains 24 types of primary antibodies against certain angiogenic proteins. To detect the levels of angiogenic factors, the tumor tissue for each group was lysed with Cell Lysis Buffer, provided by manufacturer, after 30 minutes of incubation. Protease Inhibitor Cocktail (Sigma) was added to the lysis buffer. After obtaining the pooled tumor tissue lysates for each group, the protein content of the lysates was determined by protein determination according to Peterson [12]. One array membrane was used per tumor tissue lysate.

The array membrane was incubated with 250 μg of protein from tissue lysate for 2h, at room temperature. Each membrane was incubated with a mixture of secondary Biotin-Conjugated Antibodies against the same angiogenic factors as those for primary antibodies bound onto the membranes, for 2h, at room temperature. Then, membranes were incubated with HRP-conjugated streptavidin for 2h, at room temperature. After that, the membranes were incubated with a mixture of two detection buffers, provided by manufacturer, for 1 minute, at room temperature. Each step of membrane incubation was followed by five washing steps. The membranes were exposed to x-ray film for 4 minutes and signal detected using film developer. Each protein for each experimental group was determined in duplicate. The tumor protein levels were obtained by quantification of the color intensity of each spot. Using GelPro Analyzer software, version 3.1, the color intensity was determined for each spot in comparison to positive control spots already bound to the membrane. Then the angiogenic protein levels in tumors from mice treated with empty liposomes, free PLP, and LCL-PLP were expressed as percentage of inhibition by comparison to tumor angiogenic protein levels in tumors from mice treated with PBS. The final results represent mean±SD of three independent experiments.

Statistical Analysis

Data from different experiments were reported as mean ± SD. For statistical analysis, Student's *t*- test for independent means was used. A value of $P<0.05$ was considered significant. The differences between the effects of different treatments on angiogenic factors were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Effects on cell viability *in vitro*

To evaluate the cytotoxic effects of LCL-PLP versus free PLP, HUVEC, B16.F10, and C26 cells were incubated in vitro for 24, 48, and 72 hours with increasing concentrations ranging from 5 to 200 μg/ml. The same cell types incubated with PBS were used as control. The differences in viability of cells incubated with PBS and cells incubated with culture media were not significant. Cell viability was not reduced for any cell type at any concentration at the earliest time point (24h). Also, at 48 and 72h, lower drug concentrations tested (5-100 μg/ml) did not affect cell viability. Therefore, Figure 1 presents only the relative cytotoxic effects of LCL-PLP and free PLP for the highest concentration tested (200 μg/ml) at 48 and 72h.

For all cell types tested, the viability reduction induced by free drug (ranging from 25% to 45%) was higher as compared to the liposomal drug reduction (ranging from 10% to 25%). The minor cytotoxic effects induced by LCL-PLP, are probably due to liposomal lipids rather than the encapsulated drug, as empty liposomes (i.e. devoid of drug) induced the same degree of viability reduction.

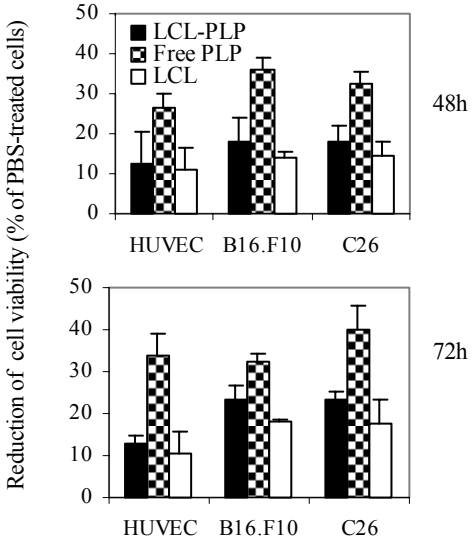

Figure 1. Effect of LCL-PLP and free PLP on cell viability. HUVEC, B16.F10, and C26 cells were incubated for 24, 48, and 72h with increasing concentrations of PLP ranging from 5 to 200 μg PLP/ml. Cell viability was not reduced for any cell type at any concentration at the earliest time point (24h). Also, at 48 and 72h, lower drug concentrations tested (5-100 μg/ml) did not affect cell viability. Only data obtained at the concentration of 200 μg prednisolone phosphate/ml are shown. Mean±SD; n= 3 measurements; LCL-PLP= Long-circulating liposome-encapsulated prednisolone phosphate; Free PLP= free prednisolone phosphate; LCL= empty long-circulating liposomes

Effects on cell proliferation *in vitro*

To study the anti-proliferative effects of LCL-PLP versus free PLP, HUVEC, B16.F10, and C26 cells were incubated *in vitro* for 24, 48 and 72 hours with increasing concentrations of PLP ranging from 5 to 200 μg/ml. Results are shown in Figure 2.

Inhibition of cell proliferation was moderate (up to 35%) for all three cell types incubated with free PLP. Slightly stronger inhibitory effects were observed when the B16.F10 and C26 tumor cells are incubated with LCL-PLP. This effect is due to the liposomal lipids rather than the encapsulated drug, as empty liposomes (i.e. devoid of drug) induced a similar degree of proliferation inhibition. Remarkably, only in case of the HUVEC, LCL-PLP yielded a strong inhibitory effect on cell proliferation (up to 75% at the highest concentration tested after 72 hours of incubation) which was clearly mediated by the encapsulated PLP.

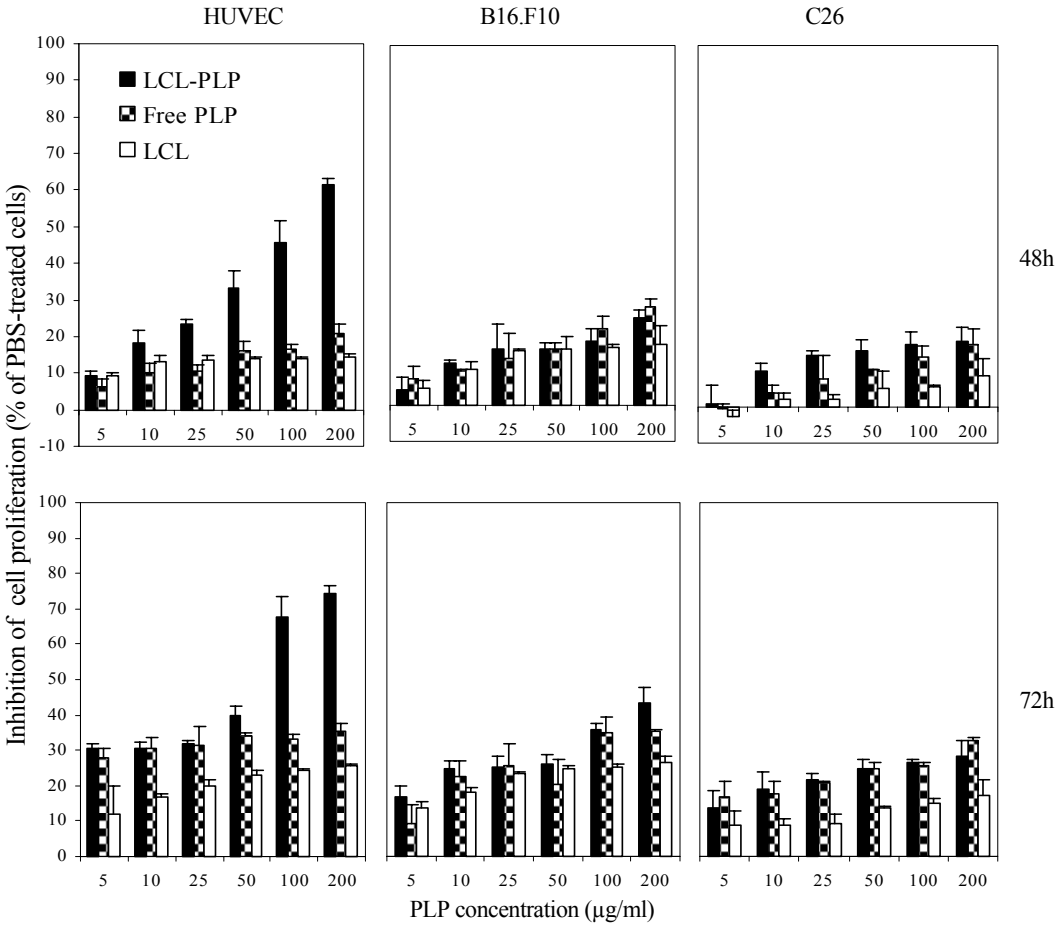

Figure 2. Effects of LCL-PLP and free PLP on cell proliferation. HUVEC, B16.F10, and C26 cells were incubated for 24, 48 and 72 hours with increasing concentrations of PLP ranging from 5 to 200 µg PLP/ml. Cell proliferation was unaffected after 24h incubation. Therefore, results are only shown for 48h and 72h incubation. Mean±SD; n= 3 measurements; LCL-PLP= Long-circulating liposome-encapsulated prednisolone phosphate; Free PLP= free prednisolone phosphate; LCL= empty long-circulating liposomes

Effects on production of angiogenic factors *in vivo*

To study the effects of LCL-PLP and free PLP on levels of angiogenic proteins in tumor tissue, we used the subcutaneous B16.F10 murine melanoma model. In accordance with our previous data [1], growth of tumors from mice treated by LCL-PLP was inhibited by 85% compared to controls, whereas free PLP did not affect tumor growth. A screening of angiogenic proteins present in tumor tissue was performed using an angiogenic protein array. With this array, levels of 24 proteins involved in angiogenesis, inflammation and apoptosis can be determined. The differences in protein levels in tumors from PBS-treated mice and those in tumors from mice treated with empty liposomes were not statistically significant (P=0.795). The effects of LCL-PLP and free PLP on the intratumoral production of *pro*-angiogenic proteins are shown in Table 1 and Figure 3.

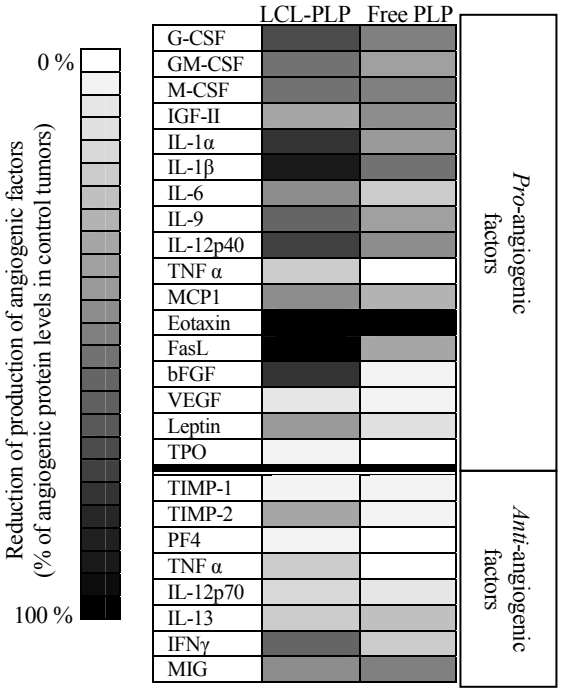

Figure 3. Effects of LCL-PLP and free PLP on levels of angiogenic factors *in vivo*. Free PLP and LCL-PLP were administered i.v. at a dose of 20 mg/kg at day 7, 10 and 13 after tumor cell inoculation. Only LCL-PLP reduced tumor growth over 85% whereas free PLP did not affect tumor growth rate. On day 14, the mice were sacrificed and tumors were isolated. Tumors were lysed and levels of angiogenic proteins in the lysates were analyzed by an angiogenic protein array. Degree of reduction of levels of tumor angiogenic factors ranged from 0% (white) to 100% (black) compared to angiogenic factors in control tumors (tumors in mice treated with PBS); LCL-PLP= Long-circulating liposome-encapsulated prednisolone phosphate treatment; Free PLP= free prednisolone phosphate treatment.

The levels of the majority of *pro*-angiogenic factors determined were reduced in case of liposomal and free PLP compared to PBS treatment. Remarkably, for 11 out of 17 *pro*-angiogenic proteins studied, reduction was stronger and statistically significant after treatment with LCL-PLP than with free PLP. On average, the effect of LCL-PLP on *pro*-angiogenic protein levels is 25% higher than the effect of free PLP (P =0.0191). More

specifically, LCL-PLP treatment inhibited expression of the *pro*-angiogenic factors G-CSF, GM-CSF, M-CSF, IL-9 (by 50-75%) and IL-1α, IL-1β, IL-12p40, bFGF (by 75-100%). Interestingly, expression of one of the most important *pro*-angiogenic factors, bFGF, was only strongly inhibited (by 78%) after LCL-PLP treatment whereas free PLP did not induce any reduction. Also the production of eotaxin in tumors was very strongly inhibited (75-100%) by LCL-PLP as well as free PLP treatment. FasL was no longer detectable in tumors from mice treated by LCL-PLP but still detectable in case of free drug treatment. Tumor amounts of IGF-II, TNF-α, thrombopoietin, VEGF were not or only slightly influenced by free and LCL-PLP treatment (Table 1).

Table 1. **Effects of i.v. administered LCL-PLP and free PLP on *pro*-angiogenic protein levels in subcutaneous B16.F10 tumor tissue.**

***Pro*-angiogenic factors**	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Reduction induced by free PLP (% of reduction as mean±SD)	Statistical differences
Granulocyte-colony stimulating factor (G-CSF)	67.16 ± 5.96	45.33 ± 2.51	**
Granulocyte-macrophage- colony stimulating factor (GM-CSF)	52.33 ± 3.51	35 ± 5.56	*
Monocyte-colony stimulating factor (M-CSF)	52 ± 7.21	48.83 ± 8.03	ns
Insulin growth factor II (IGF-II)	33.66 ± 6.5	42.33 ± 3.05	ns
Interleukin 1α (IL-1α)	79.5 ± 8.41	38.83 ± 10.91	***
Interleukin 1β (IL-1β)	88 ± 2.64	54.83 ± 9.38	***
Interleukin 6 (IL-6)	43.16 ± 5.48	18.33 ± 6.02	***
Interleukin 9 (IL-9)	57.66 ± 6.11	35.66 ± 3.51	**
Interleukin 12 p40 (IL-12 p40)	74.83 ± 9.11	41.5 ± 1.8	***
Tumor necrosis factor α (TNF α)	17.16 ± 4.64	-3.8 ± 3.88	**
Monocyte chemoattractant protein-1 (MCP1)	42.33 ± 4.72	27 ± 3	ns
Eotaxin	99.33 ± 1.15	98 ± 3.46	ns
Fas ligand (FasL)	97.33 ± 3.78	32.83 ± 10.29	***
Basic fibroblast growth factor (bFGF)	78.33 ± 5.85	2.83 ± 6	***
Vascular endothelial growth factor (VEGF)	5.16 ± 3.54	1.5 ± 1.8	ns
Leptin	39 ± 6.24	12.5 ± 5.5	***
Thrombopoietin (TPO)	2.5 ± 3.12	-1 ± 3.6	ns

Pro-angiogenic factors are defined as proteins reported in literature to favor angiogenesis and tumor-associated inflammation. The protein levels are compared to control protein levels in tumors in PBS-treated animals. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05);* *******, P<0.05;* ********, P<0.01; and* *********, P<0.001. The results represent mean±SD of three independent experiments.*

The level of the majority of *anti*-angiogenic proteins was not or only slightly suppressed by liposomal and free PLP treatment, except for the IFN-γ and MIG factors which showed a strong decrease in tumor level after treatment (by 45-60%). The decrease of IFN-γ level was considerably stronger in the case of LCL-PLP treatment (Table 2 and Figure 3).

Table 2. Effects of i.v. administered LCL-PLP and free PLP on *anti*-angiogenic protein levels in subcutaneous B16.F10 tumor tissue.

***Anti*-angiogenic factors**	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Reduction induced by free PLP (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	2.66 ± 1.15	1.66 ± 6.5	ns
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	31 ± 10.58	4.66 ± 8.14	***
Platelet factor 4 (PF4)	4 ± 5.29	-1.33 ± 2.88	ns
Tumor necrosis factor α (TNF α)	17.16 ± 4.64	-3.8 ± 3.88	**
Interleukin 12 p70 (IL-12 p70)	14.66 ± 8.02	9.33 ± 1.52	ns
Interleukin 13 (IL-13)	16.66 ± 9.71	21 ± 12.76	ns
Interferon γ (IFN-γ)	59 ± 12.28	17.33 ± 6.11	***
Monokine induced by IFN-γ (MIG)	44 ± 4.58	48.33 ± 4.72	ns

The anti-angiogenic factors are defined as proteins reported in literature to impede angiogenesis and tumor-associated inflammation. The protein levels are compared to control protein levels in tumors fromPBS-treated animals. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05);* ********, *P<0.01; and* *********, *P<0.001. The results represent mean±SD of three independent experiments.*

DISCUSSION

The present study provides insight into the mechanism of antitumor action of LCL-PLP [1]. The *in vitro* and *in vivo* studies indicate that the underlying mechanism of LCL-PLP responsible for inhibition of tumor growth is based on inhibition of angiogenesis. Inhibition of tumor angiogenesis appears to be due to a strong reduction of intratumoral levels of *pro*-angiogenic factors as well as to a direct inhibition of endothelial cell proliferation.

To demonstrate *in vivo* effects of liposomal and free PLP on tumor angiogenesis, we have measured the tumor levels of angiogenic proteins in a subcutaneous B16.F10 melanoma model. The angiogenic protein array monitored both *pro*- and *anti*-angiogenic proteins. 17 out of 24 proteins are *pro*-angiogenic factors. The majority of them (G-CSF, GM-CSF, IGF-II, IL-1α, IL-1β, IL-6, IL-9, TNF α, MCP1, eotaxin, bFGF, VEGF, leptin, and thrombopoietin) are involved in all tumor angiogenesis steps [13-26]. Most of these factors such as G-CSF, GM-CSF, M-CSF, IL-1α, IL-1β, IL-6, IL-9, TNF α, MCP1, have pro-inflammatory effects that strengthen their pro-angiogenic effects and support tumor growth [13, 14, 16-20, 27, 28]. Moreover, IL-6 has an anti-apoptotic effect on cancer cells by inhibition of p53 induced-apoptosis [29]. On the other hand, FasL helps tumor cells to escape immune surveillance by inducing apoptosis of T cells [30].

The most important effect of LCL-PLP and free PLP on tumor angiogenesis was a strong reduction of most *pro*-angiogenic protein levels, whereas the levels of the majority of *anti*-angiogenic proteins were not affected. As shown in Table 1 and Figure 3, the reduction of *pro*-angiogenic protein levels from tumors in mice treated with LCL-PLP was much stronger than that from tumors in mice treated with free PLP. This strong effect is related to the tumor-targeting property of the liposome formulation. The enhanced permeability of blood vessels in solid tumor tissue enables LCL, like those used in this study, to extravasate into the malignant tissue, leading to preferential intratumoral localization of PLP. Once extravasated into the tumor, liposomes appear to accumulate in the surrounding area of capillaries and in macrophages, further increasing effects on two major cell types driving angiogenesis, the endothelial cells and macrophages [31-34]. In contrast, when PLP is administered in free form, it is rapidly cleared from the circulation and therefore it is not able to localize in the tumor to a substantial degree, with consequently lower antitumor activity as a result [1]. These findings are supported by our previous observations of intratumoral accumulation of LCL in the immediate vicinity of tumor blood vessels and strong uptake of liposomes by intratumoral macrophages [1].

Reduction of *pro*-angiogenic factors produced principally by macrophages and endothelial cells may shift the balance between *pro*- and *anti*-angiogenic proteins in favor of inhibition of angiogenesis (Figure 3, Tables 1 and 2).

Remarkably, expression of bFGF, one of the key *pro*-angiogenic factors, was strongly reduced in tumors treated with LCL-PLP whereas its tumor expression was not affected by free PLP treatment (Table 1 and Figure 3). bFGF is very important for almost all steps in the angiogenesis process, like degradation of basement membrane, migration of endothelial cells into interstitial space and sprouting and endothelial cell proliferation [17, 26, 35]. A reduction of bFGF level will also inhibit anti-apoptotic effects of bFGF on tumor cells [17, 26, 29, 36].

Production of VEGF, the other key *pro*-angiogenic factor, was not affected by free or LCL-PLP treatment, indicating that not all pro-angiogenic pathways are equally affected. The similar levels of VEGF in tumors treated with free or LCL-PLP as well as control tumors used could be maintained by stimulating factors of VEGF production such as thrombopoietin and TNF-α [18, 19, 37-41]. The levels of these factors were not or only slightly inhibited by free or LCL-PLP treatment (Table 1). Altogether, these results indicate that LCL-PLP treatment does lead to a broad inhibition of *pro*-angiogenic proteins, but with differential effects on different pathways within the angiogenic process.

In contrast to the *pro*-angiogenic protein production, the levels of the majority of *anti*-angiogenic proteins were not affected by LCL-PLP or free PLP treatment (Table 2). The continuing presence of these factors produced mainly by tumor macrophages and T cells strengthens the inhibitory effects resulting from reduction of *pro*-angiogenic proteins by liposomal and free PLP, through their anti-angiogenic effects (by TNF-α, PF4, TIMP-1, TIMP-2, IL-12p70), anti-inflammatory effects (by IL-13) and cytotoxic effects on cancer cells (by TNF-α, IL-12p70 and IL-13) [21, 42-47]. In our study, IFN-γ was the only *anti*-angiogenic factor that was strongly inhibited by LCL-PLP treatment. This may relate to suppresive effects of LCL-PLP on immune cells like T cells.

The *in vitro* cytotoxicity studies show that cell viability was only modestly affected for all cell types and both LCL-PLP and free PLP (Figure 1). Only incubation at the highest concentration of 200 μg/ml over 48h induced cytotoxic effects. The *in vivo* relevance of these observations at extremely high and static drug concentrations for several days is unclear. Average levels of LCL-PLP were 10-20 μg/g tumor tissue at 24h after injection of a 20 mg/kg dose [1]. Intratumoral drug levels induced by LCL-PLP may exceed these values at other time points, but is unlikely to be maintained for days.

Therefore, a direct tumor cell killing effect seems to play a relatively minor role, if at all, and certainly does not explain the magnitude of the *in vivo* antitumor effects of LCL-PLP. Effects of LCL-PLP on *in vitro* cell proliferation occurred at lower concentrations, more relevant for the *in vivo* situation. In the case of HUVEC, the anti-proliferative effect of LCL-PLP was approximately 2- to 3-fold stronger as compared to the two tumor cell types. Interestingly, LCL-PLP inhibited HUVEC proliferation to a 2-fold higher degree than free PLP. This strong effect might be due to a higher intracellular drug concentration induced by liposomal encapsulation possibly as a result of endocytosis of the lipid particles by the endothelial cells.

In conclusion, the screening of proteins known to be involved in the angiogenesis process reveals that the main action of LCL-PLP treatment is inhibition of the production of *pro*-angiogenic proteins. The reduction of *pro*-angiogenic factors shifts the balance between *pro*- and *anti*-angiogenic proteins in the tumor to a distinct anti-angiogenic phenotype. In addition, LCL-PLP had a strong inhibitory effect on endothelial cell proliferation *in vitro.* Although this inhibition was only seen after exposure of HUVEC to high drug concentrations that remained static for prolonged periods of time, the local accumulation of liposomes near tumor capillaries may achieve drug concentrations in this range resulting in direct inhibition of endothelial cell proliferation. Taken together, the present results point to a strong inhibition of tumor angiogenesis as the principal cause for the antitumor activity of LCL-PLP *in vivo*. One of the future issues is to identify the types of glucocorticoids which show the strongest antitumor activity when they are incorporated in liposomes. Liposomal glucocorticoids could be used in combination with conventional cytostatic agents or antitumor cytokines (IFN, TNF, IL-12p70) to improve cancer treatment. Rapid clinical application may be feasible as both drug and carrier system have been extensively used in the clinic.

CONCLUSION

The present study demonstrates that the principal cause of antitumor activity of liposomal glucocorticoids is the strong inhibition of tumor angiogenesis. This beneficial effect is a result of tumor-targeting property of the liposome formulation that increases intratumoral drug concentration and prolongs the inhibitory effects of GC on tumor growth. Consequently, LCL-PLP offers promise for liposomal glucocorticoids as novel antitumor agents.

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Chapter 4

Antitumor activity of liposomal prednisolone phosphate depends on the presence of functional tumor-associated macrophages in tumor tissue

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ABSTRACT

Prednisolone phosphate (PLP) encapsulated in long-circulating liposomes (LCL) (LCL-PLP) exerts antitumor activity via inhibition of tumor angiogenesis. It is known that tumor-associated macrophages (TAM) play a crucial role in tumor growth as they are actively involved in promoting and maintaining tumor angiogenesis. To gain more insight into the anti-angiogenic mechanisms of LCL-PLP, this study aimed to investigate the role of TAM in the antitumor mode of action of LCL-PLP in B16.F10 melanoma-bearing mice. Our results show that TAM have a pivotal function in the growth of B16.F10 melanoma via production of *pro*-angiogenic/*pro*-inflammatory factors. One of the major inhibitory actions of LCL-PLP on tumor growth is the reduction of the TAM-mediated production of *pro*-angiogenic factors, whereas production of *anti*-angiogenic factors by these cells is hardly affected.

Keywords: prednisolone phosphate, long-circulating liposomes, tumor-associated macrophages, angiogenesis, inflammation

INTRODUCTION

Prednisolone phosphate (PLP) encapsulated in long-circulating liposomes (LCL) (LCL-PLP) has been shown to exert strong inhibitory effects on tumor growth in subcutaneous (s.c.) B16.F10 melanoma and C26 colon carcinoma murine tumor models [1, 2]. The antitumor activity of the LCL-PLP formulation mediated by anti-angiogenic effects is enabled by the tumor-targeting property of the liposomes. Site-specific delivery increases the intratumoral drug concentration and thereby intensifies the inhibitory effects of PLP [3]. The tumor-targeting capability of LCL is the combined result of their long circulation time and an enhanced permeability of tumor vasculature, as compared to healthy endothelium [1, 4]. LCL can extravasate through the hyperpermeable pathological vasculature and thereby accumulate in malignant tissue. This effect is referred to as the "enhanced permeability and retention (EPR) effect" [3]. Interestingly, LCL localize in the immediate vicinity of tumor blood vessels and can be visualized in the endosomal/lysosomal compartment of tumor–associated macrophages (TAM) [2]. Among the immune cell populations present in tumor tissue, TAM seem most important in promoting and coordinating tumor growth [5]. TAM are known to be an important source of inflammatory and angiogenic factors involved in all steps in tumor angiogenesis [4]. Therefore, to gain more insight into the antitumor mode of action of LCL-PLP, this study aims to address the role of TAM in the antitumor effect of LCL-PLP in the murine B16.F10 melanoma model. Firstly, the ability of clodronate-containing liposomes to deplete macrophages was used as a tool to evaluate whether TAM play a pivotal role in the growth of B16.F10 melanoma. Secondly, tumor-bearing animals were pretreated with clodronate-liposomes before the actual treatment with LCL-PLP in order to study the antitumor activity of LCL-PLP towards tumors with suppressed TAM function. The effect of LCL-PLP treatment on the levels of *pro*-angiogenic and *anti*-angiogenic factors was determined in B16.F10 melanoma-bearing mice with and without pretreatment with liposomal clodronate (Lip-CLOD). To suppress TAM functions in tumors, a mixture of two types of clodronate liposomes was used: LCL-encapsulated clodronate to deplete TAM and large negatively charged clodronate-liposomes to prevent chemoattraction of new monocytes from the bloodstream in the tumor tissue. Our results show that LCL-PLP exert a strong suppressive effect on TAM as reflected by a reduced production of *pro*-angiogenic factors by these cells.

MATERIALS AND METHODS

Preparation of LCL-PLP

LCL were prepared as described previously [2]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) 2000-distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol in a round-bottom flask. After lipid film formation, the film was hydrated with a solution of 100 mg/ml PLP, (obtained from Bufa, Uitgeest, The Netherlands). Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering and found to be 100 nm with a polydispersity value lower than 0.1. The polydispersity values obtained indicate limited variation in particle size. Phospholipid content was determined with a phosphate assay according to Rouser [6]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. Glucocorticoid phosphate content was assessed by high performance liquid chromatography as described previously [7]. The type of column was RP18 (5 μm) (Merck) and the mobile phase consisted of acetonitril and water (1:3 v/v), pH 2. The eluent was monitored with an ultraviolet detector set at 254 nm. The detection limit for the high performance liquid chromatography setup was 20 ng/ml. The liposomal preparation contained about 5 mg PLP /ml and ~60 μmol phospholipid/ml.

Preparation of Lip-CLOD

Clodronate-containing liposomes as macrophage-suppressive agents have already been used in inflammatory and auto-immune diseases, where macrophages have been suggested to be involved in pathological processes [8]. Previous studies demonstrated that macrophages were efficiently eliminated at 24h after intravenous (i.v.) administration of a dose of 25 mg/kg of liposomal clodronate [9]. To deplete TAM, clodronate-containing LCL (mean size about 100 nm) were essentially prepared as described above for LCL-PLP. After lipid film formation, the film was hydrated with a solution of 60 mg/ml dichloromethylene bisphosphonate, disodium clodronate (Bonefos™ infusion, Schering, Weesp, The Netherlands). To reduce chemoattraction of new monocytes in tumors, large negatively charged liposomes (mean size around 1 μm) were used as delivery systems for

clodronate. For this reason, appropriate amounts of egg phosphatidylcholine and egg phosphatidylglycerol (both obtained from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma, St. Louis, USA) in a ratio of 1.85:0.3:1 were dissolved in ethanol. The hydration of lipid film was performed with 10 ml Bonefos infusion. Liposomes were extruded twice through a filter with a pore size of 8 μm. Phospholipid content was determined with a phosphate assay according to Rouser [6]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. The aqueous phase after extraction was used for determining clodronate content by UV spectrophotometry at 238 nm after formation of clodronate complex with $CuSO_4$ solution [10]. Both types of liposomes contained about 5 mg clodronate /ml and ~70 μmol phospholipid/ml.

Cells

B16.F10 murine melanoma cells were cultured as monolayers at 37°C in a 5% CO_2-containing humidified atmosphere in DMEM medium (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).

Murine tumor model

To exclude antitumoral effect of T cells [11], male Balb/c athymic nude Foxn1 $^{nu-/nu-}$ mice (6 – 8 weeks of age) were used. They were obtained from Harlan (The Netherlands) and kept in standard housing under filter tops with standard rodent chow and water available *ad libitum*, and a 12 h light/dark cycle. Experiments were performed according to the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the right flank of mice. B16.F10 tumors became palpable at day 7 after tumor cell inoculation.

Effects of Lip-CLOD on tumor growth

To determine whether TAM play an important role in tumor growth, B16.F10 melanoma-bearing athymic mice were injected i.v. with a mixture of both types of clodronate liposomes (ratio 1:1 (w/w)) (Lip-CLOD) at a dose of 25 mg/kg at day 7 (when tumors became palpable). As control tumors, tumors from mice treated with PBS which did not receive Lip-CLOD treatment were used. 5 animals were used per experimental group.

Effects of pretreatment with Lip-CLOD on antitumor activity of LCL-PLP

To compare the effects of LCL-PLP and free PLP on the growth of the tumors in mice pretreated with Lip-CLOD and in mice when Lip-CLOD pretreatment was not administered, LCL-PLP and free PLP were injected i.v. at a dose of 20 mg/kg at day 8 after tumor cell inoculation. To eliminate TAM functions in tumors, mice received i.v. a dose of 25 mg/kg of Lip-CLOD at day 7 after tumor cell inoculation. Controls received PBS or empty liposomes at day 8 after tumor cell inoculation. 5 animals were used per experimental group. Since day 7, tumor volume was measured regularly and calculated according to the formula $V = 0.52 \times a^2 \times b$, in which *a* is the smallest and *b* the largest superficial diameter (in mm). Mice were sacrificed when the tumor volumes were larger than 2 cm^3.

Effect of Lip-CLOD pretreatment on TAM-mediated production of angiogenic factors

To evaluate the role of TAM in intratumoral production of angiogenic factors, mice received Lip-CLOD at a dose of 25 mg/kg, at day 7 after tumor cell inoculation. Controls received PBS. 4-5 animals were used per experimental group. On day 12, mice were sacrificed and tumors were isolated. A screening of angiogenic proteins in tumor tissue was performed using an angiogenic protein array (RayBiotech Inc. Norcross, GA) [12] for 24 proteins involved in angiogenesis, inflammation and apoptosis as described previously [1].

Effect of pretreatment with Lip-CLOD on anti-angiogenic activity of LCL-PLP

To compare the effects of LCL-PLP and free PLP on angiogenic protein production in tumors from mice pretreated with Lip-CLOD and from mice when Lip-CLOD pretreatment was not administered, LCL- PLP and free PLP were injected i.v. at a dose of 20 mg/kg at day 8 and 11 after tumor cell inoculation. To deplete TAM, mice received i.v. Lip-CLOD at a dose of 25 mg/kg, at day 7 after tumor cell inoculation. Controls received PBS or empty liposomes at days 8 and 11 after tumor cell inoculation. 4-5 animals were used per experimental group. On day 12, mice were sacrificed and tumors were isolated. A screening of angiogenic proteins in tumor tissue was performed as described previously [1].

Immunohistochemical examination of tumor tissue after LCL- PLP treatment

To evaluate the effects of (LCL-) PLP and Lip-CLOD on TAM infiltration in tumor tissue, we compared F4/80-stained sections. LCL- PLP and free PLP were injected i.v. at a dose of 20 mg/kg at day 8 after tumor cell inoculation. Tumors-bearing mice in which TAM were depleted received i.v. a dose of 25 mg/kg of Lip-CLOD, at day 7 after tumor cell inoculation. 2-3 animals were used per experimental group.

Tumors were dissected 48h and 96h after Lip-CLOD injection. Tumors were snap frozen in liquid nitrogen for immunohistochemical staining. Rat anti-mouse F4/80 antibody (Serotec Oxford, United Kingdom) was used as a primary antibody. As a secondary antibody biotinylated Rabbit anti rat IgG (Vector laboratories Burlingame, USA) was used. After incubation with HRP-Streptavidin (Vector laboratories) and peroxidase-substrate, slides were counterstained in hematoxylin (Sigma-Aldrich Co., Zwijndrecht, The Netherlands) and mounted in Kaiser's glycerol-gelatin (Merck, Darmstadt, Germany). All slides were examined by light microscopy regarding TAM distribution in tumor tissue.

Statistical Analysis

Data from different experiments were reported as mean ± SD. For statistical analysis, Student's *t*- test for independent means was used. A value of $P<0.05$ was considered significant. To compare the effects of different treatments on tumor growth *in vivo*, one-way ANOVA with Dunnett's Multiple Comparison Test was used. The differences between the effects of different treatments on angiogenic factor production were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Effect of Lip-CLOD on tumor growth

To determine whether TAM play a critical role in supporting tumor growth, a mixture of two types of clodronate-containing liposomes in a ratio 1:1 (w/w) (Lip-CLOD) was injected i.v. at day 7 (when tumors became palpable), at a dose of 25 mg/kg. To deliver clodronate to TAM, LCL (mean size about 100 nm) were used [2, 13]. In addition, to reduce chemoattraction of new monocytes in tumors, clodronate-containing large negatively charged liposomes (mean size about 1 μm) were co-injected [14]. Depletion of TAM in tumor tissue was verified by immunohistochemical examination of tumor tissue for the macrophage antigen F4/80, at 48h and 96h after Lip-CLOD administration (Figure 1, Table 5). At 48h, without Lip-CLOD treatment, TAM were observed at the rim of the control tumors (i.e. tumors in mice treated only with PBS) (Figure 1, panel B). At the same time point, TAM were not noted in tumors from mice that received treatment with Lip-CLOD (Figure 1, panel A). At the 96h observation time point, TAM were observed spread over the tumor tissue in large areas in control tumors (Figure 1, panel D). At the same time point, after treatment with Lip-CLOD, tumors also contained TAM in large areas but at a lower density as compared to TAM in control tumors (Figure 1, panel C, and Table 5).

Interestingly, at day 14 after tumor cell inoculation (the day when the first tumors from the control group reached a volume of 2 cm^3), tumor volume after Lip-CLOD was 55% smaller ($P= 0.02$) compared to control tumors (Figure 2). This strong inhibitory effect on tumor growth induced by Lip-CLOD demonstrates the pivotal role of TAM in B16.F10 melanoma growth.

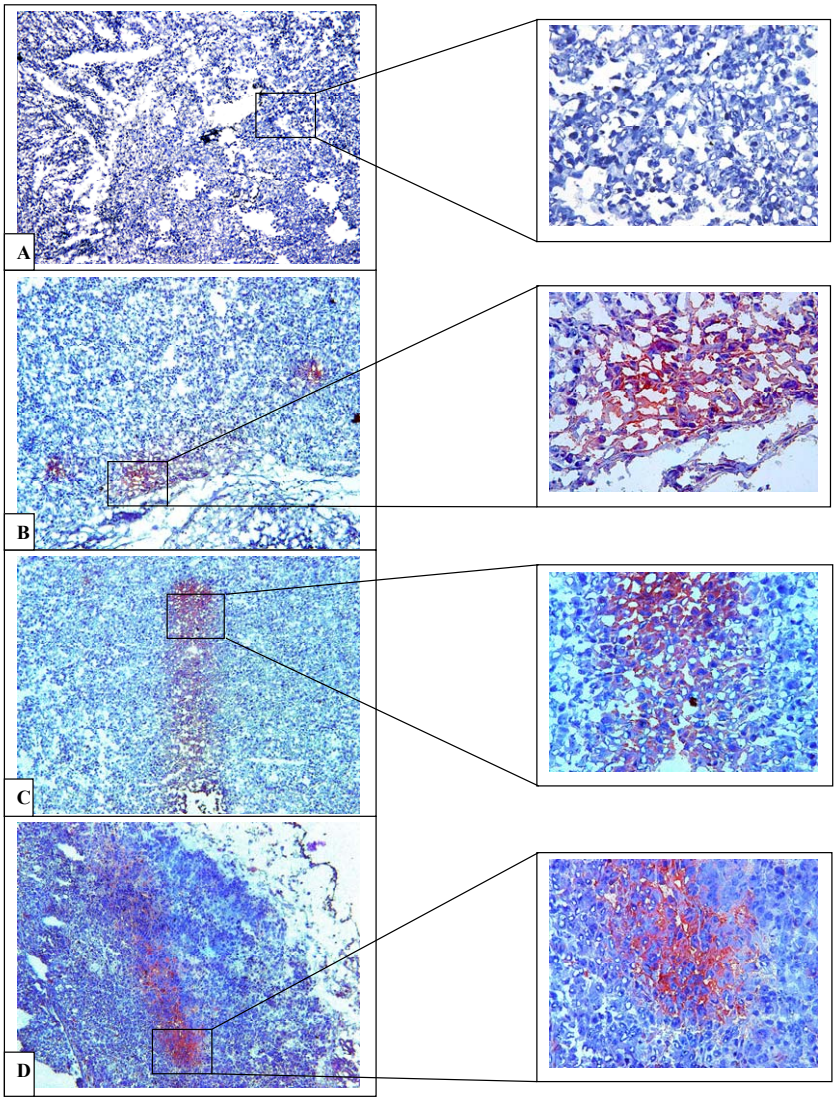

Figure 1. Immunohistochemical analysis of the macrophage antigen F4/80 in B16.F10 melanoma tumor sections from mice treated with Lip-CLOD. Red staining indicates areas with infiltrated TAM in tumor tissue. Sections were counterstained with hematoxylin. TAM distribution in tumor tissue was verified at 48h and 96h after Lip-CLOD administration. Panel A. Lip-CLOD treatment (48h): tumors do not contain TAM. Panel B. PBS treatment at the same time point: TAM at the rim of the tumors. Panel C. Lip-CLOD treatment (96h): tumors show similar dispersion of TAM in large areas but containing TAM at a lower density as compared to TAM in control tumors. Panel D. PBS treatment at the same time point: tumors show large areas with TAM infiltrated in tumor tissue. Control tumors are tumors from mice not treated with Lip-CLOD but treated with PBS (panels B and D). Left panels were magnified 10x and right panels were magnified 40x.

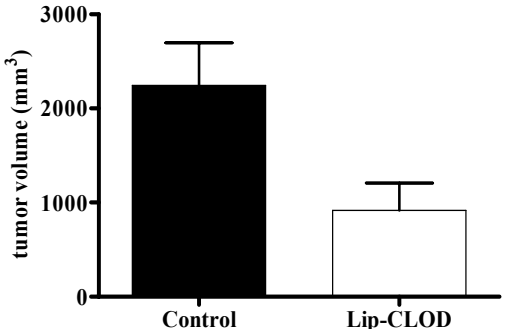

Figure 2. **Effect of Lip-CLOD treatment on growth of s.c. B16.F10 melanoma.** Tumor volumes at day 14 (the day when the first tumors from the control group reached a volume of 2 cm3) were compared to volumes of control tumors at the same time point. Control tumors are tumors from mice that received only PBS. Student's *t*- test for comparison of tumor volumes was used. *P* value = 0.02. The results represent mean±SD of 5 mice. Control= treatment with PBS, Lip-CLOD= treatment with Lip-CLOD.

Effect of pretreatment with Lip-CLOD on antitumor activity of LCL-PLP

To determine whether the antitumor activity of LCL-PLP in the B16.F10 melanoma model is dependent on the TAM functions in tumor tissue, tumor-bearing mice were injected i.v. with LCL-PLP at a dose of 20 mg/kg, 24h after Lip-CLOD administration. The inhibition of tumor growth induced by LCL-PLP and free PLP was analyzed using area under tumor growth curves (AUTC) until day 14 after tumor cell inoculation. The results are shown in Figure 3, panels A and B. Tumor volume results at day 14 are also shown in Figure 3, panels C and D.

Both with and without Lip-CLOD pretreatment, empty liposomes as well as free PLP did not have a statistically significant antitumor effect compared to PBS. In line with the results shown in Figure 2, Lip-CLOD pretreatment inhibited tumor growth in both PBS- and empty liposomes-treated groups (Figure 3, panels B and D compared with panels A and C).When the Lip-CLOD pretreatment was not given, LCL-PLP inhibited tumor growth by 83% ($P<0.05$) compared to PBS treatment (Figure 3, panels A, and C). In the case of LCL-PLP treatment, AUTC values and tumor volumes after Lip-CLOD pretreatment were not significantly different than those without Lip-CLOD pretreatment (Figure 3, panels B and D compared with panels A and C). It is likely, however, that in the Lip-CLOD pretreated-groups the antitumor effect of LCL-PLP treatment is overshadowed by the pretreatment effect.

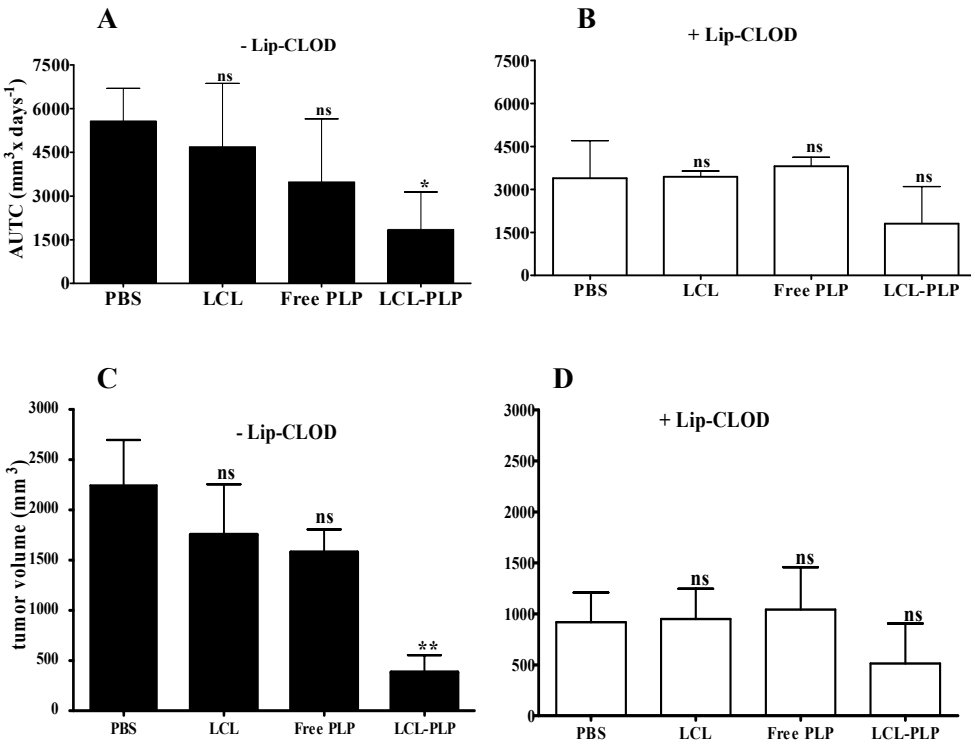

Figure 3. Effect of Lip-CLOD pretreatment on the antitumor activity of LCL-PLP. Tumor growth for each experimental group is analyzed using the "area under the tumor growth curve (AUTC)" until day 14 (the day when the first tumors from the control group reached a volume of 2 cm3). Panels A and B show AUTCs by day 14. Panel A. Only LCL-PLP treatment. Panel B. Lip-CLOD pretreatment given before LCL-PLP treatment. Panels C and D show tumor volume at day 14. Panel C. Only LCL-PLP treatment. Panel D. Lip-CLOD pretreatment given before LCL-PLP treatment. The results are compared to PBS-treated groups. One-way ANOVA with Dunnett's Multiple Comparison Test was used; ***ns***, not significant ($P>0.05$); *, $P<0.05$; **, $P<0.01$. The results represent mean±SD of 5 mice. AUTC= area under the tumor growth curve, -Lip-CLOD = no pretreatment with Lip-CLOD, +Lip-CLOD = pretreatment with Lip-CLOD, PBS= treatment with PBS, LCL= treatment with empty LCL, Free PLP= treatment with free PLP, LCL-PLP= treatment with LCL-PLP.

Effect of Lip-CLOD treatment on the production of angiogenic proteins *in vivo*

To investigate whether TAM are an important source of angiogenic factors in tumors, B16.F10 melanoma-bearing mice were injected i.v. with Lip-CLOD at a dose of 25 mg/kg at day 7 after tumor cell inoculation. On day 12, the mice were sacrificed, tumors were isolated, and angiogenic protein levels in tumor tissue were determined by using an

angiogenic protein array (RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1; RayBiotech Inc.Norcross, GA) [12]. Lip-CLOD treatment reduced the level of most of the *pro*-angiogenic factors by 35% (P=0.0001) compared to the levels in control tumors (i.e. in mice not treated with Lip-CLOD) (Table 3, and Figure 4, column A). More specifically, Lip-CLOD reduced the level of GM-CSF, IL-6, IL-9, FasL, bFGF, and thrombopoietin by 25-50%, G-CSF, IL-1α, IL-1β, TNF α, MCP1 by 50-75%, and leptin by 95%. Interestingly, Lip-CLOD treatment also strongly reduced the production of two *anti*-angiogenic factors (TIMP-1 and 2) (Table 4, and Figure 4, column A). This inhibitory effect on angiogenic factor production induced by Lip-CLOD demonstrates the important role of TAM in production of these factors in B16.F10 melanoma.

Effect of pretreatment with Lip-CLOD on the inhibitory action of LCL-PLP on tumor angiogenesis

To assess the effects of the treatment with LCL-PLP and free PLP on the production of angiogenic factors by TAM in s.c. B16.F10 melanoma tumors, mice were pretreated with Lip-CLOD. Subsequently, LCL-PLP and free PLP were administered i.v. 20 mg/kg at days 8 and 11 after tumor cell inoculation. On day 12, mice were sacrificed, tumors were isolated, and angiogenic protein levels in tumor tissue were screened. No changes were observed between angiogenic protein levels in tumors from mice treated with PBS and empty liposomes (data not shown). In case of treatment with LCL-PLP and free PLP, however, changes were observed.

In the absence of Lip-CLOD pretreatment, both LCL-PLP and free PLP reduced the level of the majority of *pro*-angiogenic factors compared to control treatment (Table 1). For 10 out of 17 *pro*-angiogenic proteins studied, reduction was significantly stronger after treatment with LCL-PLP than after free PLP (Table 1). LCL-PLP treatment suppressed expression of the *pro*-angiogenic factors GM-CSF, M-CSF, IL-1α, IL-1β, IL-6, IL-9, IL-12p40, TNF α, MCP1, FasL (by 50-75%) and G-CSF, bFGF, leptin, eotaxin (by 75-100%).

Table 1. Effects of i.v. administered LCL-PLP and free PLP on *pro*-angiogenic protein levels in s.c. B16.F10 tumors when Lip-CLOD pretreatment was not given.

***Pro*-angiogenic factors**	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Reduction induced by free PLP (% of reduction as mean±SD)	Statistical differences
Granulocyte- colony stimulating factor (G-CSF)	82.5 ± 3.6	42.6 ± 11.4	*
Granulocyte-macrophage-colony stimulating factor (GM-CSF)	59.4 ± 5.2	23.3 ± 16.0	*
Monocyte-colony stimulating factor (M-CSF)	60.0 ± 3.0	19.3 ± 12.6	*
Insulin growth factor II (IGF-II)	41.3 ± 5.3	21.0 ± 29.8	ns
Interleukin 1α (IL-1α)	63.3 ± 8.5	29.5 ± 13.2	**
Interleukin 1β (IL-1β)	63.5 ± 10.0	15.0 ± 11.0	**
Interleukin 6 (IL-6)	74.5 ± 9.6	31.0 ± 2.7	*
Interleukin 9 (IL-9)	61.5 ± 24.0	19.2 ± 5.4	***
Interleukin 12 p40(IL-12 p40)	58.5 ± 7.3	29.5 ± 3.5	ns
Tumor necrosis factor α (TNF α)	65.2 ± 0.3	25.0 ± 22.0	***
Monocyte chemoattractant protein-1 (MCP1)	70.5 ± 18.7	4.0 ± 5.0	ns
Eotaxin	100.00 ± 0.00	97.4 ± 3.7	ns
Fas ligand (FasL)	61.4 ± 3.5	26.7 ± 2.7	ns
Basic fibroblast growth factor (bFGF)	79.6 ± 10.5	24.2 ± 10.2	***
Vascular endothelial growth factor (VEGF)	0.00 ± 0.00	0.00 ± 0.00	ns
Leptin	78.4 ± 23.4	13.0 ± 3.0	***
Thrombopoietin (TPO)	22.4 ± 9.7	10.5 ± 8.3	ns

Pro-angiogenic factors are defined as proteins reported in literature to favor angiogenesis and tumor-associated inflammation. The protein levels are compared to protein levels in control tumors. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05);* *******, P<0.05;* ********, P<0.01; and* *********, P<0.001. The results represent mean±SD of 4 measurements.*

The level of the majority of *anti*-angiogenic proteins was not or only slightly suppressed by LCL-PLP and free PLP treatments (Table 2) except for the levels of the *anti*-angiogenic factors IFN-γ and MIG which dropped strongly after LCL-PLP treatment (by 55-65%) (Table 2).

Table 2. **Effects of i.v. administered LCL-PLP and free PLP on *anti*-angiogenic protein levels in s.c. B16.F10 tumors when Lip-CLOD pretreatment was not given. .**

***Anti*-angiogenic factors**	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Reduction induced by free PLP (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	0.2 ± 0.3	0.0 ± 0.0	ns
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	12.4 ± 3.2	16.3 ± 7.0	ns
Platelet factor 4 (PF4)	22.6 ± 3.2	13.6 ± 13.3	ns
Interleukin 12 p70 (IL-12 p70)	11.6 ± 11.3	13.5 ± 12.3	ns
Interleukin 13 (IL-13)	38.3 ± 8.7	17.3 ± 0.6	ns
Interferon γ (IFN-γ)	65.2 ± 12.5	10.1 ± 0.2	***
Monokine induced by IFN-γ (MIG)	54.2 ± 8.7	37.0 ± 11.2	ns

The anti-angiogenic factors are defined as proteins reported in literature to impede angiogenesis and tumor-associated inflammation. The protein levels are compared to protein levels in control tumors. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05); and* *********, *P<0.001. The results represent mean±SD of 4 measurements.*

In case of Lip-CLOD pretreatment, LCL-PLP only strengthened the reducing effect of Lip-CLOD on the *pro*-angiogenic protein levels, by about 20% (P=0.0001) (Table 3, and Figure 4, column A compared to column C). The reducing effects of LCL-PLP treatment on the production *anti*-angiogenic factors in tumors was dominated by the effect of Lip-CLOD administration (Table 4, Figure 4, column A compared to column C).

Table 3. Effects of i.v. administered Lip-CLOD and LCL-PLP on *pro*-angiogenic protein levels in s.c. B16.F10 tumors

***Pro*-angiogenic factors**	Reduction induced by Lip-CLOD + LCL-PLP (% of reduction as mean±SD)	Reduction induced by Lip-CLOD (% of reduction as mean±SD)	Statistical differences
Granulocyte- colony stimulating factor (G-CSF)	73.8 ± 13.3	52.3 ± 20.7	ns
Granulocyte- macrophage-colony stimulating factor (GM-CSF)	56.5 ± 16.2	36.4 ± 7.0	ns
Monocyte-colony stimulating factor (M-CSF)	52.8 ± 23.6	19.7 ± 13.3	ns
Insulin growth factor II (IGF-II)	41.8 ± 16.1	6.0 ± 8.4	ns
Interleukin 1α (IL-1α)	64.0 ± 17.6	53.0 ± 13.8	ns
Interleukin 1β (IL-1β)	60.1 ± 6.3	59.4 ± 20.4	ns
Interleukin 6 (IL-6)	60.8 ± 19.6	49.7 ± 8.0	ns
Interleukin 9 (IL-9)	59.5 ± 16.5	48.4 ± 3.5	ns
Interleukin 12 p40 (IL-12 p40)	37.0 ± 48.2	19.1 ± 25.0	ns
Tumor necrosis factor α (TNF α)	59.4 ± 3.0	53.0 ± 6.6	ns
Monocyte chemoattractant protein-1 (MCP1)	73.1 ± 11.0	59.4 ± 3.6	ns
Eotaxin	98.4 ± 0.5	62.4 ± 2.8	ns
Fas ligand (FasL)	61.1 ± 6.5	30.6 ± 8.2	ns
Basic fibroblast growth factor (bFGF)	92.1 ± 3.9	36.3 ± 24.0	**
Vascular endothelial growth factor (VEGF)	0.0 ± 0.0	0.0 ± 0.0	ns
Leptin	87.7 ± 17.5	94.5 ± 3.6	ns
Thrombopoietin (TPO)	43.5 ± 2.2	37.7 ± 2.7	ns

The protein levels are compared to protein levels in control tumors which were not treated with Lip-CLOD. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05); and* ********, *P<0.01. The results represent mean±SD of 4 measurements.*

Table 4. Effects of i.v. administered lip-CLOD and LCL-PLP on *anti*-angiogenic protein levels in s.c. B16.F10 tumors

***Anti*-angiogenic factors**	Reduction induced by Lip-CLOD + LCL-PLP (% of reduction as mean±SD)	Reduction induced by Lip-CLOD (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	68.0 ± 12.0	62.0 ± 12.0	ns
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	100.0 ± 0.0	100.0 ± 0.0	ns
Platelet factor 4 (PF4)	9.4 ± 11.1	9.7 ± 13.7	ns
Interleukin 12 p70 (IL-12 p70)	20.8 ± 0.8	19.7 ± 10.8	ns
Interleukin 13 (IL-13)	43.7 ± 24.5	23.1 ± 32.7	ns
Interferon γ (IFN-γ)	77.8 ± 2.6	3.0 ± 4.1	***
Monokine induced by IFN-γ (MIG)	52.4 ± 0.3	27.0 ± 3.0	ns

The protein levels are compared to protein levels in control tumors which were not treated with Lip-CLOD. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05); and* ***, *P<0.001. The results represent mean±SD of 4 measurements.*

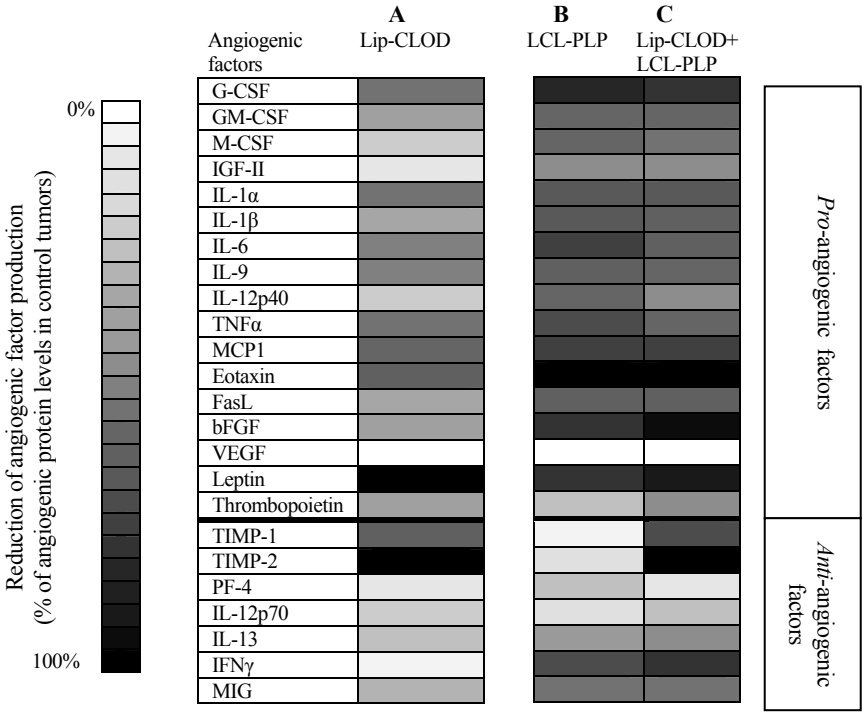

Figure 4. Effect of Lip-CLOD pretreatment on anti-angiogenic activity of LCL-PLP. Results presented as %reduction of tumor angiogenic factors ranging from 0% (white) to 100% (black) compared to levels of angiogenic factors in control tumors (tumors in mice treated with PBS and not treated with Lip-CLOD). Column A. Lip-CLOD = treatment with Lip-CLOD. Column B. LCL-PLP= treatment with LCL-PLP. Column C. Lip-CLOD+LCL-PLP = pretreatment with Lip-CLOD followed by LCL-PLP treatment.

Immunohistochemical examination of tumor tissue

To compare the effects of LCL-PLP treatment with and without Lip-CLOD pretreatment on TAM infiltration, we evaluated macrophage antigen F4/80-stained sections of B16.F10 melanoma by light microscopy (Table 5). LCL-PLP were injected i.v. at a dose of 20 mg/kg 24h after Lip-CLOD pretreatment. Tumors were dissected 48h and 96h after Lip-CLOD administration. Immunohistochemical analysis for the macrophage antigen F4/80 showed different patterns of TAM distribution in tumor sections (Figures 1 and 5, Table 5). At 48h, in all groups receiving treatment with Lip-CLOD, TAM were hardly present in tumor tissue (Figure 1, panel A, and Table 5) whereas in control tumors large areas with TAM at the rim of the tumors were observed (Figure 1, panel B).

Interestingly, at this time point, small groups of TAM were seen in tumors from animals treated only with LCL-PLP (Figure 5, panel A, and Table 5). At 96h, TAM were present in large areas spread over the tumor tissue in control tumors (Figure 1, panel D, and Table 5). After treatment with Lip-CLOD, tumors show similar dispersion of TAM in large areas but containing TAM at a lower density as compared to TAM in control tumors. (Figure 1, panel C, and Table 5). At 96h after Lip-CLOD pretreatment, TAM were grouped in small areas in tumor tissue from LCL-PLP-treated mice (Table 5). Interestingly, after treatment with LCL-PLP only, tumors showed the same distribution of TAM in small clusters in tumor tissue as observed at the 48h observation time point (Figure 5, panel B, and Table 5).

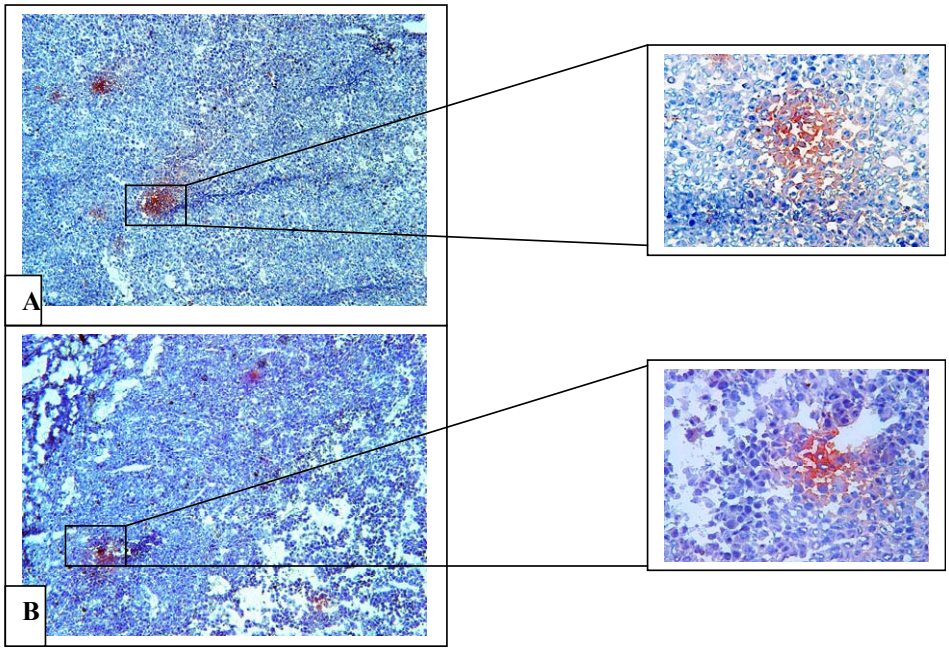

Figure 5. Immunohistochemical analysis of the macrophage antigen F4/80 in B16.F10 melanoma tumor sections from mice treated with LCL-PLP. Red staining indicates areas with infiltrated macrophages in tumor tissue. Sections were counterstained with hematoxylin. Macrophage distribution in tumor tissue was verified at 48h and 96h after Lip-CLOD administration. Panel A (48h): LCL-PLP treatment without Lip-CLOD pretreatment: small areas with macrophages. At this time point, tumor sections from mice pretreated with Lip-CLOD followed by LCL-PLP treatment do not show macrophages (data not shown). Panel B (96h). LCL-PLP without Lip-CLOD pretreatment: small areas with macrophages. The same pattern of macrophage distribution was noted in tumors from mice pretreated with Lip-CLOD followed by LCL-PLP treatment (data not shown). Left panels were magnified 10x and right panels were magnified 40x.

Table 5. Effects of LCL-PLP treatment on TAM dispersion in B16.F10 melanoma tumors visualized after F4/80 immunostaining

Time point	TAM distribution	Without Lip-CLOD			Lip-CLOD	Lip-CLOD + Free PLP	Lip-CLOD + LCL-PLP
		PBS	Free PLP	LCL-PLP			
48h	Density (+ few;++ normal, +++ many TAM)	++	+	++	+	+	+
	Mainly in rim	*					
	Spread over the tumor tissue in large areas						
	Grouped in tumor tissue in small areas		*	*			
96h	Density (+ few;++ normal,+++ many TAM)	+++	+++	+	++	++	+
	Mainly in rim						
	Spread in tumor tissue in large areas	*	*		*	*	
	Grouped in tumor tissue in small areas			*			*

DISCUSSION

The present study provides confirmatory evidence for an anti-angiogenic/anti-inflammatory mode of antitumor action of LCL-PLP via suppressive effects on TAM functions. Our previous observations on intratumoral accumulation of LCL in the endosomal/lysosomal compartment of TAM pointed to a route for therapeutic intervention using LCL-PLP [2]. TAM play a crucial role in tumor growth being actively involved in promoting the angiogenic switch as well as in the maintenance of tumor angiogenesis [15]. TAM are an important source of inflammatory and angiogenic factors such as TNF α, IL-8, IL-1β, IL-6, VEGF, bFGF, proteases present in tumors such as matrix metalloproteinases (MMP), urokinase plasminogen activator, and plasmin [4, 16-20]. To evaluate whether TAM play a crucial role in B16.F10 melanoma growth, tumor-bearing mice were treated with Lip-CLOD due to their ability to deplete macrophages [13]. Previous studies already showed the feasibility of clodronate encapsulated in liposomes for elimination of TAM from s.c. tumor tissue [21]. Our results show that i.v. administered Lip-CLOD inhibited tumor growth by approximately 55% compared to tumor growth in control animals (Figure 2). Furthermore, Lip-CLOD induced a moderate to strong reduction of the intratumoral production of the majority of the *pro*-angiogenic factors (Figure 4, column A, and Table 3). These antitumor effects induced by Lip-CLOD demonstrate the role of TAM in supporting the growth of B16.F10 melanoma via the production of the *pro*-angiogenic factors. Most of the *pro*-angiogenic factors produced by TAM, (e.g. G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-9, TNF α, MCP1, have also *pro*-inflammatory effects involved in tumor growth [22-31]. Tumor angiogenesis and tumor inflammation are interconnected and TAM are able to drive both processes making them central forces in tumor growth and expansion [32].

A particular finding was that Lip-CLOD treatment strongly reduced the tumor levels of two *anti*-angiogenic factors TIMP-1 and TIMP-2 (Figure 4, column A, and Table 4). Besides tumor growth promoting effects, TAM exert antitumor effects via the production of these *anti*-angiogenic factors. It is known that TIMP's produced by TAM and fibroblasts can inhibit the tumorigenic and metastatic phenotype of cancer cells [33-36]. Several studies support the hypothesis of a dual role of TAM in tumor growh [5, 16, 37].

Taken together, the results obtained with Lip-CLOD treatment suggest that TAM are an important source of *pro*-angiogenic factors as well as of certain *anti*-angiogenic factors (Figure 4, column A, and Tables 3 and 4).

To further study the earlier suggested role of TAM in the mode of antitumor action of LCL-PLP [1], we investigated the effects of pretreatment with Lip-CLOD on the antitumor activity of LCL-PLP. In line with previous results [1, 2], when Lip-CLOD pretreatment was not given, LCL-PLP strongly inhibited tumor growth compared to the growth of control tumors (Figure 3, panels A and C). However, when B16.F10 melanoma-bearing mice were pretreated with Lip-CLOD, no additional inhibitory effect of LCL-PLP on tumor growth was noted. (Figure 3, panels B and D). This is obviously due to the overshadowing effect of Lip-CLOD pretreatment, indicating that the antitumor activity of LCL-PLP depends on the presence of functional TAM in the tumor.

The antitumor effects of LCL-PLP are likely primarily caused by their suppressive effects on the TAM-mediated production of *pro*-angiogenic factors in tumors. Without Lip-CLOD pretreatment, LCL-PLP exerts a strong reducing effect on the level of most of the *pro*-angiogenic factors. Notably, LCL-PLP reduced strongly the production of following key factors responsible for the regulation of TAM functions, such as GM-CSF, M-CSF, G-CSF, and MCP1. These factors are involved in attracting new monocytes from the bloodstream into the tumor tissue and stimulating these newly recruited tumor macrophages to produce *pro*-angiogenic factors [16, 38, 39]. The suppressive effect of LCL-PLP on TAM-mediated production of *pro*-angiogenic factors in tumors is supported by the immunohistochemical observations. The microscopic images of F4/80-stained tumor sections show that in tumors from mice treated with LCL-PLP, TAM are inactivated or unable to infiltrate the tumor tissue, leading to their clustering in small spots (Figure 5, panels A and B, and Table 5). Likely, the impaired macrophage infiltration in tumor tissue is a reflection of the reduced capability of TAM to produce *pro*-angiogenic proteins, due to the treatment with LCL-PLP, as these factors are also responsible for chemoattraction and spreading of macrophages in tumor tissue [16, 38, 39].

In tumors from mice which were not pretreated with Lip-CLOD, the production of *anti*-angiogenic/*anti*-inflammatory factors was only slightly affected by LCL-PLP. In our study, only the *anti*-angiogenic factors IFN-γ and MIG showed a strong decrease in tumor level after LCL-PLP treatment (Figure 4 column B, and Table 2). The expression of these two factors may suffer from suppressive effects of LCL-PLP on cells other than TAM such as NK cells (for IFN-γ), fibroblasts and endothelial cells (for MIG) [40-42]. Interestingly, LCL-PLP treatment did not affect the TAM-mediated production of TIMP-1 and TIMP-2 whereas the production of these two *anti*-angiogenic factors was

drastically reduced after TAM suppression induced by Lip-CLOD pretreatment (Figure 4 column B compared to column A, Tables 2 and 4). This remarkable observation may relate to studies demonstrating a stimulatory effect of prednisolone on TIMP production in patients with chronic bronchitis [43].

When Lip-CLOD pretreatment was administered, LCL-PLP only slightly strengthened the reducing effect of Lip-CLOD pretreatment on the tumor levels of *pro*-angiogenic proteins, as the inhibitory effect of LCL-PLP on tumor angiogenesis depends on the presence of functional TAM in the tumor. These results are in line with the critical role of functional TAM in supporting tumor angiogenesis and inflammation via production of *pro*-angiogenic/ *pro*-inflammatory factors [4, 32]. Apparently, LCL-PLP have little effects on other cell types in tumor than TAM like fibroblasts, endothelial cells and NK cells [22, 25, 42, 44].

Taken together, our studies indicate that TAM play a vital role in coordinating tumor growth being an important source of *pro*-angiogenic/ *pro*-inflammatory factors involved in all steps in tumor angiogenesis. One of the major inhibitory actions of LCL-PLP on tumors is based on the reduction of the TAM-mediated production of *pro*-angiogenic factors, whereas production of *anti*-angiogenic factors by these cells is hardly affected. LCL-PLP are likely not to induce strong suppressive effects on other cell types present in tumor tissue than TAM, such as fibroblasts, endothelial cells and NK cells.

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Chapter 5

Antitumor activity of long-circulating liposomes containing glucocorticoids in B16 melanoma-bearing mice. Effect of encapsulated glucocorticoid type

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ABSTRACT

This study evaluates whether the inhibitory effects of prednisolone phosphate (PLP) encapsulated in long-circulating liposomes (LCL-PLP) on tumor growth and tumor angiogenesis described previously can be generalized to other types of glucocorticoids (GC) encapsulated in LCL (LCL-GC). Four types of synthetic GC (as disodium salts of the phosphate derivatives), i.e. budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), methylprednisolone disodium phosphate (MPLP), and PLP, were selected based on the difference in their potency to activate the human glucocorticoid receptor. The effects of all LCL-GC on the production of angiogenic/inflammatory factors *in vivo* in the B16.F10 murine melanoma model as well as on the viability and proliferation of tumor cells and endothelial cells *in vitro* were investigated.

Our results show that all four selected LCL-GC formulations inhibit tumor growth, albeit to different degrees. The differences in antitumor activity of LCL-GC correlate with their efficacy to suppress tumor angiogenesis and inflammation. The strongest antitumor effect is achieved by LCL-encapsulated BUP (LCL-BUP), due to the highest potency of BUP versus the other three GC types. In addition, the *in vitro* results presented herein suggest that LCL-BUP has strong cytotoxic effects on B16.F10 melanoma cells. The *in vitro* data also suggest that anti-proliferative effects of LCL-GC towards angiogenic endothelial cells may play a role in their antitumor activity.

Keywords: liposomes, glucocorticoids, angiogenesis, inflammation, cancer

INTRODUCTION

Our previous studies showed that prednisolone phosphate encapsulated in long-circulating liposomes (LCL-PLP) exerts strong inhibitory effects on tumor growth via inhibition of tumor angiogenesis [1, 2]. For efficient delivery of PLP into tumors by intravenous (i.v.) treatment, small-sized liposomes coated with poly(ethylene glycol) (PEG) were used. PEG has been shown to be very effective in reducing recognition and rapid removal of liposomes from the circulation by the mononuclear phagocyte system (MPS), enabling liposomes to stay in the circulation for a prolonged period of time. The long-circulation property provides the liposomes the opportunity to substantially extravasate and accumulate in tumors [3]. Tumor accumulation of PEG-liposomes is favored by the structural and functional abnormalities of tumor neovessels. Tumor vessels are often immature and tortuous, may contain blind ends and show increased permeability [4]. PEG-liposomes can extravasate through the permeable pathological vasculature and thereby accumulate into the malignant tissue (referred to as the "enhanced permeability and retention (EPR) effect") [5]. The EPR effect enables the antitumor effects of the LCL-PLP formulation.

The underlying actions of LCL-PLP on tumor angiogenesis involve a reduction of pro-angiogenic protein levels in tumor tissue. In principle, these anti-angiogenic actions induced by LCL-PLP localized in tumor tissue can be mediated at the genomic as well as non-genomic level. Genomic effects are already induced at very low concentrations of GC. They are effectuated by the interaction of GC with the cytosolic GC receptors (cGCR) followed by cGCR activation and translocation into the nucleus. Once in the nucleus, GC/cGCR complexes modulate transcriptional responses of the majority of inflammatory, angiogenic, immunomodulatory and apoptotic genes, by binding directly to DNA, or by binding to proteins, e.g. to transcription factors involved in regulation of these genes [6-12]. GC can also interact at the posttranscriptional and translational level leading to suppression of a broad variety of factors responsible for angiogenesis, inflammation, apoptosis, and immune function [10]. Higher dosages of GC increase cGCR occupation, which intensifies the GC effects at the genomic level. If cGCR are saturated, GC can additionally induce non-genomic effects. Non-genomic actions of GC strengthen the genomic effects. The non-genomic actions of GC are not simply concentration-dependent. They are mediated via distinct cellular pathways: cGCR-mediated inhibition of arachidonic acid release, interference of the cGCR with phosphatidylinositol 3-kinase/Akt signalling pathway, intercalation of GC molecules into

cellular membranes altering cationic transport through the plasma membrane and increasing proton leakage from the mitochondria, and binding of GC to specific membrane-bound receptors [13, 14].

To gain more insight into the inhibitory mechanisms of LCL-PLP on tumor angiogenesis, the type of GC encapsulated in the LCL was varied. We have investigated the effects of four GC types, each encapsulated in LCL (LCL-GC), on the production of angiogenic/inflammatory factors *in vivo* in the B16.F10 murine melanoma model as well as on the viability and proliferation of tumor cells and endothelial cells *in vitro*. Attempts were made to correlate the results with the antitumor activity of the four LCL-GC formulations *in vivo*. The four synthetic GC were used as disodium salt of the phosphate derivatives, as this ensures stable encapsulation in the aqueous interior of the liposomes. The following GC types were encapsulated into LCL: budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), methylprednisolone disodium phosphate (MPLP), and prednisolone disodium phosphate (PLP). They were selected due to their difference in the ranking order of their potency in terms of activation of the human glucocorticoid receptor: budesonide > dexamethasone > methylprednisolone ~ prednisolone [15-19].

MATERIALS AND METHODS

LCL-GC preparation

LCL were prepared as described previously [1]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) (PEG)2000-distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol in a round-bottom flask. A lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone disodium phosphate (PLP), dexamethasone disodium phosphate (DXP) (both obtained from Bufa, Uitgeest, The Netherlands), budesonide disodium phosphate (BUP) or methylprednisolone disodium phosphate (MPLP) (synthesized by Syncom, Groningen, The Netherlands). Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of liposomal preparations with chloroform, according to Rouser [20]. Unencapsulated GC were removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. The aqueous phase after extraction was used for determining the glucocorticoid phosphate content by high performance liquid chromatography as described previously [21]. The type of column was RP18 (5 μm) (Merck) and the mobile phase consisted of acetonitril and water (1:3 v/v), pH 2. The eluent was monitored with an ultraviolet detector set at 254 nm. The detection limit for the high performance liquid chromatography setup was 20 ng/ml.

Cells

B16.F10 murine melanoma and C26 murine colon carcinoma cells were cultured as monolayers at 37 °C in a 5% CO_2-containing humidified atmosphere in DMEM medium (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). Human umbilical vein endothelial cells (HUVEC) (Glycotech, Rockville, USA) were cultured as a monolayer at 37 °C in a 5% CO_2-containing humidified atmosphere in complete EGM endothelial cell growth medium (Cambrex, East Rutherford, NJ, USA).

For *in vitro* studies, the following protocol was established. All three cells types were trypsinized off the substratum and counted in a Bürker counting chamber under microscope in the presence of trypan blue. Only cells excluding the dye were counted as viable cells. Cells were plated in 96-well plates at the appropriate concentrations according to the assay performed.

Murine tumor model

Male C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (The Netherlands) and 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 the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the right flank of syngeneic C57Bl/6 mice. B16.F10 tumors became palpable around 7 days after tumor cell inoculation.

Effects of LCL-GC versus free GC on cell viability *in vitro*

To determine whether LCL-GC and free GC (i.e. not encapsulated in liposomes) had a direct cytotoxic effect on cells, 5 x 10^3 HUVEC, C26 and B16.F10 cells/well were plated in a 96-well plate for 24h. Then, LCL-GC and free GC (i.e. dissolved in aqueous solution) were added in PBS and incubated for 24h, 48h, and 72h. After exposure time, cell viability was determined by XTT-assay (Sigma, St. Louis, USA) according to the manufacturer's instructions [22]. All three cell types were incubated with tetrazolium salt XTT and electron-coupling reagent (N-methyl dibenzopyrazine methylsulfate) for 1 hour at 37°C in the CO_2-incubator. Using an ELISA microplate reader, the absorbance was measured at 490 nm with a reference wavelength of 655 nm.

Effects of LCL-GC versus free GC on cell proliferation *in vitro*

To determine the effect of GC (liposomal and free) on cell proliferation, 1 x 10^3 HUVEC, C26 and B16.F10 cells/well were plated in a 96-well plate for 24h. After that, LCL-GC and free GC were added in PBS. The anti-proliferative effect of LCL-GC and free GC was determined after 24h, 48h, and 72h of incubation by ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions [23, 24]. This technique is based on the incorporation of the pyridine analogue bromodeoxyuridine (BrdU) instead of thymidine into the DNA of

proliferating cells. Cells were incubated with BrdU solution for 24h and then media were completely removed from the wells. Then, cells were fixed and DNA was denatured. To detect BrdU incorporated in newly synthesized cellular DNA, a monoclonal antibody conjugated with peroxidase, anti-BrdU-POD, was added. After 90 minutes of incubation, antibody was removed and cell lysates were washed three times with PBS. The immune complexes were detected by the subsequent substrate of peroxidase (tetramethyl-benzidine) reaction. The reaction product was quantified by measuring the absorbance at 450 nm with a reference wavelength of 655 nm.

Effects of LCL-GC versus free GC on the production of angiogenic factors *in vivo*

At 7 days after tumor cell inoculation, tumor size was measured and tumor volume calculated according to the formula $V = 0.52 \times a^2 \times b$, in which a is the smallest and b, the largest superficial diameter (in mm).

4 animals were used per experimental group. The groups were treated as following: with PBS (group 1), with empty liposomes (group 2), free PLP (group 3), LCL-PLP (group 4), free MPLP (group 5), LCL-MPLP (group 6), free DXP (group 7), LCL-DXP (group 8), free BUP (group 9), LCL-BUP (group 10). Free GC and LCL-GC were administered intravenously (i.v.) at a dose of 10 mg/kg at day 7, 11 after tumor cell inoculation. On day 14, the mice were sacrificed and tumors were isolated. Empty liposomes were administered i.v. at the same lipid concentration as that used for LCL-GC.

To evaluate the effect of LCL-GC and free GC at a molecular scale, a screening of angiogenic proteins present in tumor tissues was performed using an angiogenic protein array of RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1 (RayBiotech Inc. Norcross, GA) [25]. Each membrane contains 24 types of primary antibodies against certain angiogenic proteins. To detect the levels of angiogenic factors, the tumor tissue for each group was lysed with Cell Lysis Buffer, provided by manufacturer, after 30 minutes of incubation. Protease Inhibitor Cocktail (Sigma) was added to the lysis buffer. After obtaining the pooled tumor tissue lysates for each group, the protein content of the lysates was measured by protein determination according to Peterson [26]. One array membrane was used per tumor tissue lysate. The array membrane was incubated with 250 μg of protein from tissue lysate, followed by a mixture of secondary Biotin-Conjugated Antibodies against the same angiogenic factors as those for primary antibodies, and finally HRP-conjugated streptavidin. All incubations steps were for 2h, at room temperature and each incubation was followed by five washing

steps. After that, the membranes were incubated with a mixture of two detection buffers, provided by manufacturer, for 1 minute, at room temperature. The membranes were exposed to x-ray film for 4 minutes and signal detected using film developer. Each protein for each experimental group was determined in duplicate. The tumor protein levels were obtained by quantification of the color intensity of each spot. Using GelPro Analyzer software, version 3.1, the color intensity was determined for each spot in comparison to positive control spots already bound to the membrane. Then the angiogenic protein levels in tumors from mice treated with empty liposomes, free GC, and LCL-GC were expressed as percentage of inhibition by comparison to tumor angiogenic protein levels in control tumors (tumors from mice treated with PBS). The final results represent mean±SD of four independent measurements.

Effects of LCL-GC on the production of COX-2 *in vivo*

The effects of LCL-GC on COX-2 production in tumor tissue were assessed by western blot. Tumor tissue lysate was obtained as described above for angiogenic protein screening. The total protein content was measured using protein determination according to Peterson [26]. 50 μg of total protein was loaded per lane onto a 7.5% polyacrylamide gel. Electrophoresis was performed at 20–25 mA and subsequently proteins were electrotransferred onto a nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK), for 1 h at 100 mA, with a Scie-Plas semidry blotter (Scie-Plas, Warwickshire, UK). The non-specific binding to the membrane was blocked using 5% BSA in PBS with 0.05% Tween-20 (PBS-T) buffer for 1 h at room temperature, with constant shaking. Thereafter, the membrane was incubated with the primary antibody, rabbit polyclonal anti-mouse COX-2 (Abcam, Cambridge, UK) at a dilution of 1:200 in PBS-T buffer with 0.1% BSA, followed by incubation with the secondary antibody, goat anti-rabbit antibody labeled with Cy5 dye (Amersham Pharmacia Biotech, Little Chalfont, UK) at a dilution of 1:1250 in PBS-T buffer with 0.1% BSA. For fluorescence visualization, a Typhoon 9400 scanner (Amersham Biosciences, Buckinghamshire, UK) was used. The intensity of the bands was quantified by GelPro Analyzer software, version 3.1.

Statistical Analysis

Data from different experiments were reported as mean ± SD. For statistical analysis, Student's t- test for independent means was used. A value of $P<0.05$ was considered significant. To compare the effects of different treatments on tumors *in vivo* with control tumors, one-way ANOVA with Dunnett's test for multiple comparisons was used. The differences between the effects of different treatments on angiogenic factors were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism version 4.02 for Windows software, GraphPad Software (San Diego, CA).

RESULTS

Characterization of LCL-GC

LCL, used in this study, contained 5 mol% of PEG2000-conjugated distearoylphosphatidylethanolamine. LCL-GC were characterized with respect to mean particle size, phospholipid concentration, and GC content. Mean particle size was found to be 100 nm with a polydispersity value lower than 0.1. The polydispersity values obtained indicate limited variation in particle size. The liposomal preparations contained about 4.25 mg GC/ml and ~65 μmol phospholipid/ml. Degree of loading (on average 6.5 μg GC/μmol phospholipid) was independent from the type of GC encapsulated.

In vitro cytotoxicity

In vitro cytotoxicity of LCL-GC and free GC was investigated with regard to cell viability and cell proliferation using human umbilical vein endothelial cells (HUVEC), B16.F10 murine melanoma and C26 murine colon carcinoma cells. Cells were incubated *in vitro* for 24, 48, and 72 hours with increasing (LCL-)GC concentrations ranging from 5 to 200 μg/ml. PBS-treated cells were used as controls. There were no changes in viability and proliferation of cells incubated with PBS and cells incubated with culture media.

Effects on cell viability

The effects of different formulations on cell viability were expressed as % of reduction compared to the viability of the controls.

At 24h, viability of all cell types was slightly (<25%) reduced at the two highest concentrations tested (100 and 200 μg/ml) in the case of DXP and BUP, both for the free and LCL forms. These cytotoxic effects are partly caused by the liposomal lipids, as empty liposomes (i.e. devoid of drug) also induced a slight reduction of cell viability for all cell types at equivalent phospholipid concentrations (data not shown).

At 48 and 72h, viability of HUVEC and C26 cells was moderately affected (25 - 50%) at the two highest concentrations (100 μg/ml and 200 μg/ml) of DXP and BUP in free and LCL form (data not shown). Remarkably, only in the case of B16.F10 cells, major cytotoxic effects were observed at these time points for LCL-BUP at concentrations starting at 25 μg/ml (Figure 1). The cytotoxicity induced by LCL-BUP is due to the drug component, as empty liposomes caused only a slight reduction in cell viability.

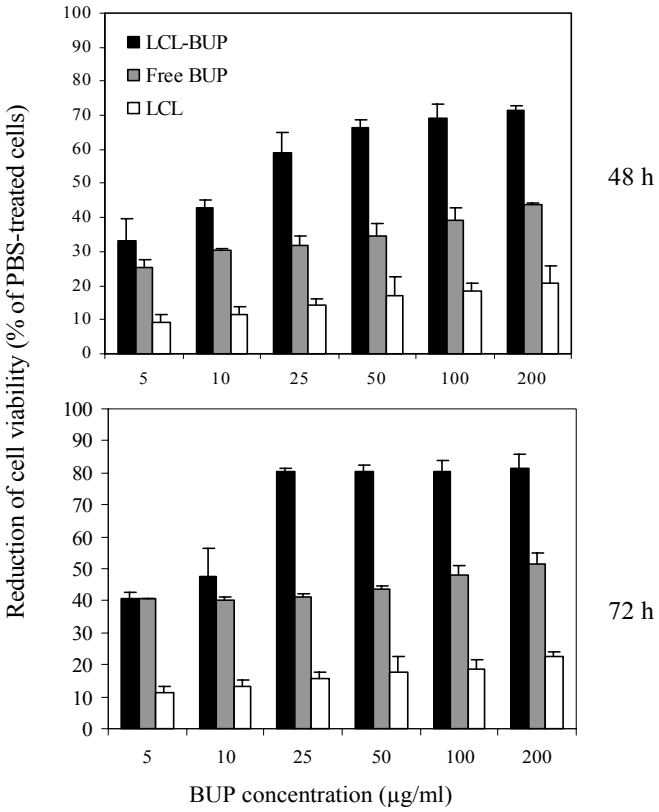

Figure 1. Effect of LCL-BUP and free BUP on the viability of B16.F10 cells. Only data obtained at 48h and 72h of cell incubation with LCL-BUP and free BUP treatments are shown. Mean±SD; n= 3 measurements; LCL-BUP= treatment with LCL-BUP; Free BUP= treatment with free BUP; LCL= treatment with empty LCL (i.e. devoid of drug)

Effects on cell proliferation

The effects of different formulations on cell proliferation were expressed as % of inhibition compared to the proliferation of the controls.

C26: At 24h, proliferation of C26 cells was inhibited by 25 - 40% only after treatment with BUP and DXP, added as liposomal or free drug, at the highest concentrations tested (100 and 200 µg/ml). At 48h and 72h, inhibition of C26 cell proliferation was observed in case of all GC formulations but remained below 50% even at the highest concentrations (data not shown).

B16.F10: At 24h of incubation, all formulations of GC, except for BUP, showed moderate inhibition of B16.F10 cell proliferation but only at the two highest concentrations tested (100 and 200 μg/ml). Free and LCL-BUP induced a moderate inhibition of cell proliferation at lower concentrations (between 10 - 50 μg/ml BUP). The inhibitory effects became more pronounced at the 100 μg/ml and 200 μg/ml BUP concentrations (data not shown). At 48h and 72h, only LCL-BUP exerted a strong inhibitory effect on the proliferation of B16.F10 cells (Figure 2). At 72h, LCL-BUP-mediated inhibition of cell proliferation was at the level of 60-90% at concentrations ranging from 25 μg/ml to 200 μg/ml (Figure 2).

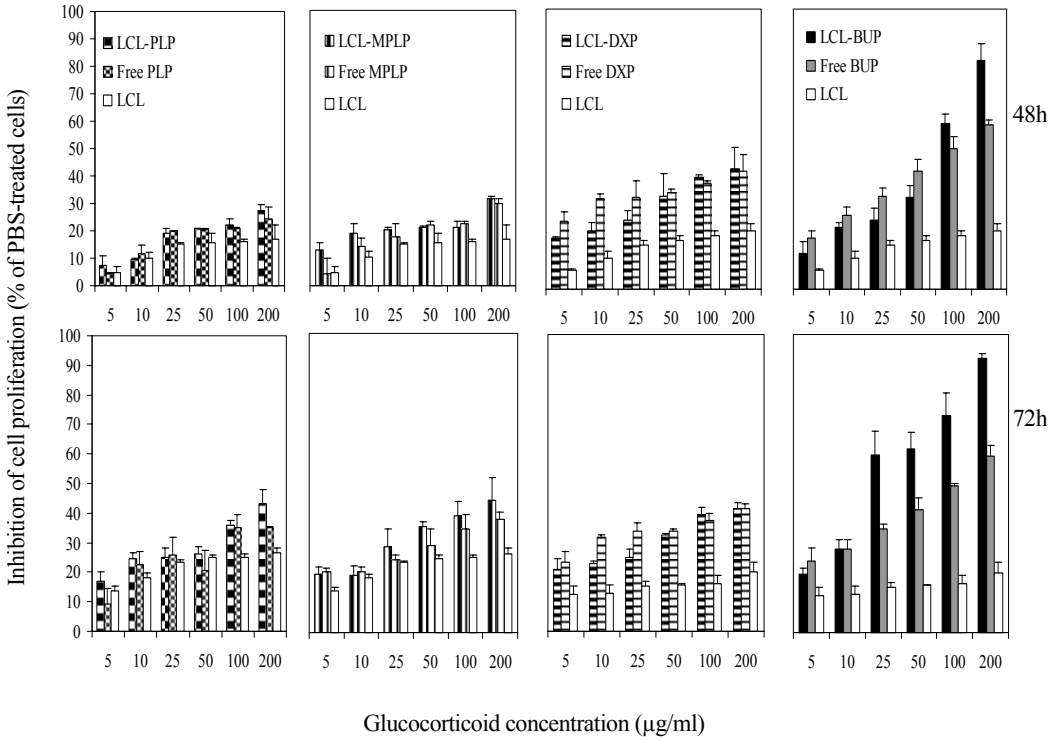

Figure 2. Effect of LCL-GC and free GC on the proliferation of B16.F10 cells. Mean±SD; n= 3 measurements; LCL-PLP= treatment with LCL-PLP; free PLP= treatment with free PLP; LCL-MPLP= treatment with LCL-MPLP; free MPLP= treatment with free MPLP; LCL- DXP= treatment with LCL-DXP; free DXP= treatment with free DXP; lip BUP= treatment with LCL-BUP; free BUP= treatment with free BUP; LCL= treatment with empty LCL (i.e. devoid of drug)

HUVEC: At 24h, inhibition of HUVEC proliferation was moderate (25-50%) only at the highest GC concentrations tested in case of all formulations except for LCL-BUP which was again an exception. LCL-BUP caused a much stronger inhibition up to 60% at the highest concentrations of 100 μg/ml and 200 μg/ml (data not shown). After 48h and 72h, proliferation of HUVEC cells was moderately inhibited (up to 45%) in case of treatment with all free GC types (Figure 3). Remarkably, all four LCL-GC formulations had strong inhibitory effects on HUVEC proliferation (ranging from 75-90%, at the highest concentrations of 100 μg/ml and 200 μg/ml after 72h of incubation) which was clearly mediated by the encapsulated GC (Figure 3). Notably, the anti-proliferative effects of LCL-BUP started at lower concentrations (25-100 μg/ml).

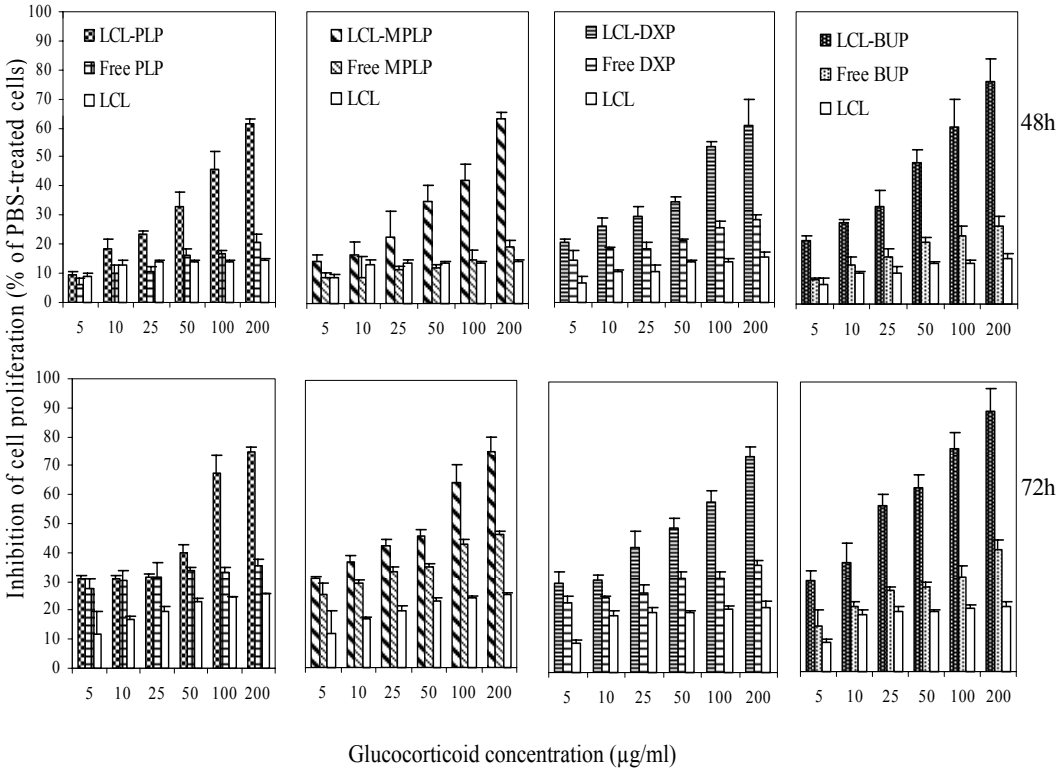

Figure 3. Effect of liposomal GC and free GC on the proliferation of HUVEC. Mean±SD; n= 3 measurements; LCL-PLP= treatment with LCL-PLP; free PLP= treatment with free PLP; LCL-MPLP= treatment with LCL-MPLP; free MPLP= treatment with free MPLP; LCL-DXP= treatment with LCL-DXP; free DXP= treatment with free DXP; LCL-BUP= treatment with LCL-BUP; free BUP= treatment with free BUP; LCL= treatment with empty LCL (i.e. devoid of drug)

In vivo production of angiogenic factors

Tumors from mice treated with LCL-GC showed statistically significant growth inhibition compared with control tumors (i.e. tumors in mice treated with PBS) at a dose of 10 mg/kg (Figure 4). For each GC, LCL-GC formulation induced a stronger antitumor activity than free GC. For the free drugs, only DXP and BUP inhibited tumor growth significantly as compared with controls (Figure 4).

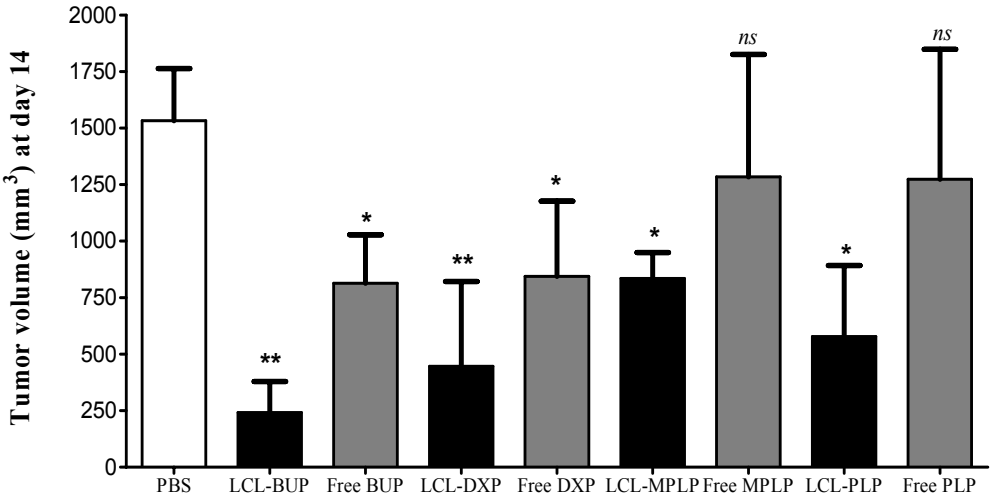

Figure 4. Effect of LCL-GC and free GC on s.c. B16.F10 tumor growth. The formulations were injected i.v. at a dose of 10 mg/kg on days 7, and 11 in B16.F10 tumor bearing mice. Mean±SD; n= 3-4 animals per experimental group; PBS= treatment with PBS; LCL-BUP= treatment with LCL-BUP; free BUP= treatment with free BUP; LCL-DXP= treatment with LCL-DXP; free DXP= treatment with free DXP; LCL-MPLP= treatment with LCL-MPLP; free MPLP= treatment with free MPLP; LCL-PLP= treatment with LCL-PLP; free PLP= treatment with free PLP. To compare the effects of different treatments on tumor growth with control tumors (tumors from mice treated with PBS), one-way ANOVA with Dunnett Test for multiple comparisons was used and the P values are indicated as follows*:* ***ns,*** not significant (P>0.05); *, P<0.05; **, P<0.01.

In addition, the effects of LCL-GC and free GC on the level of angiogenic proteins in tumor tissue were studied in the s.c. B16.F10 murine melanoma model. As shown previously, there were no significant differences between the effects of PBS and empty liposomes on angiogenic protein levels (P=0.795) [2]. All free GC types had no or only a

slight reducing effect on angiogenic protein levels. LCL-GC exerted moderate to strong reducing effects on the intratumoral production of *pro*-angiogenic proteins except for LCL-MPLP (Figure 5). Average reductions on total *pro*-angiogenic protein level compared to PBS treatment were 68% for LCL-BUP (P <0.0001), 44% for LCL-DXP (P <0.0001), and 26% for LCL- PLP (P <0.0009). The ranking order of the reducing effects of LCL-GC on *pro*-angiogenic protein production was: LCL-BUP > LCL-DXP > LCL-PLP > LCL-MPLP (Table 3).

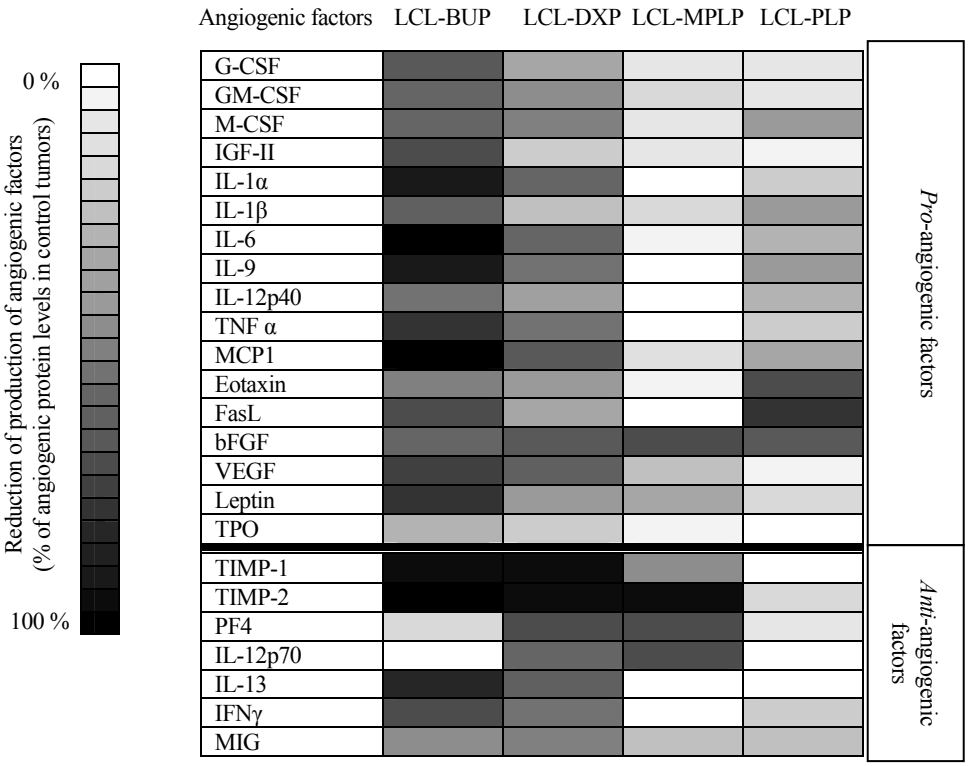

Figure 5. Effect of LCL-GC on the production of angiogenic factors *in vivo*. Results presented as % reduction of tumor angiogenic factors ranging from 0% (white) to 100% (black) compared to levels of angiogenic factors in control tumors (tumors from mice treated with PBS); LCL-BUP= treatment with LCL-BUP; LCL-DXP= treatment with LCL-DXP; LCL-MPLP= treatment with LCL-MPLP; LCL-PLP= treatment with LCL-PLP.

Among all GC tested, BUP exerted the strongest inhibitory effects on angiogenic protein levels in tumors (Figure 5). Therefore, the effects of LCL-BUP and free BUP on the intratumoral production of *pro*-angiogenic proteins and *anti*-angiogenic proteins are presented in more detail in Tables 1 and 2. For 12 out of 17 *pro*-angiogenic proteins studied, reduction was stronger after treatment with LCL-BUP as compared to free BUP.

Table 1. Effects of i.v. administered LCL-BUP and free BUP on *pro*-angiogenic protein levels in s.c. B16.F10 tumor tissue.

***Pro*-angiogenic factors**	Reduction induced by LCL-BUP (% of reduction as mean±SD)	Reduction induced by free BUP (% of reduction as mean±SD)	Statistical differences
Granulocyte-colony stimulating factor (G-CSF)	64.0±0.8	1.5±3.4	**
Granulocyte-macrophage-colony stimulating factor (GM-CSF)	58.5±4.0	-3.5±28.0	**
Monocyte-colony stimulating factor (M-CSF)	59.0±13.0	20.0±0.5	ns
Insulin growth factor II (IGF-II)	66.0±8.6	9.5±2.0	**
Interleukin 1α (IL-1α)	89.0±0.6	29.4±6.2	**
Interleukin 1β (IL-1β)	62.5±35.5	15.0±18.5	ns
Interleukin 6 (IL-6)	97.5±1.3	10.0±4.1	***
Interleukin 9 (IL-9)	88.0±4.5	23±0.8	**
Interleukin 12p40 (IL-12 p40)	53.0±31.2	5.5±5.0	*
Tumor necrosis factor α (TNF α)	80.0±15.0	3.2±8.3	***
Monocyte chemoattractant protein-1 (MCP1)	98.0±1.2	6.0±8.4	***
Eotaxin	46.5±8.0	8.5 ± 20.1	ns
Fas ligand (FasL)	65.3±20.0	23.6±12.0	ns
Basic fibroblast growth factor (bFGF)	59.3±39.2	3.4±11.1	**
Vascular endothelial growth factor (VEGF)	73.6±3.0	7.3±8.0	***
Leptin	79.0±27.0	28.0±22.0	*
Thrombopoietin (TPO)	25.8±12.6	2.7±21.1	ns

Pro-angiogenic factors are defined as proteins reported in literature to favor angiogenesis and tumor-associated inflammation. The protein levels are compared to protein levels in control tumors (tumor from mice treated with PBS). The results were analyzed for statistically significant differences between the effects of LCL-BUP versus freeBUP at the level of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05);* *******, P<0.05;* ********, P<0.01; and* ***********, P<0.001. The results represent mean±SD of four independent measurements.*

More specifically, LCL-BUP treatment reduced expression of the *pro*-angiogenic factors G-CSF, GM-CSF, M-CSF, IGF-II, IL-1β, IL-12p40, FasL, bFGF, VEGF (by 50-75%) and IL-1α, IL-6, IL-9, TNF α, MCP1, leptin (by 75-100%). Remarkably, expression of one of the most important *pro*-angiogenic factors, VEGF, was strongly inhibited (by 73%) only after LCL-BUP treatment with free BUP inducing only a slight reduction (by 7%). Tumor levels of eotaxin, and thrombopoietin were moderately (by 25-50%) reduced by LCL-BUP treatment (Table 1, Figure 5). Notably, in the case of LCL-MPLP, only the level of bFGF was reduced strongly (by 65%) (Figure 5).

Interestingly, although LCL-PLP hardly showed any suppressive effect on the level of the *anti*-angiogenic proteins, all LCL-GC other than LCL-PLP suppressed strongly (by 40-100%) the production of these proteins (Figure 5). Remarkably, the levels of IL-12p70 and PF4 were not or only slightly reduced after LCL-BUP treatment (Figure 5, Table 2). The ranking order of the reducing effects of LCL-GC on *anti*-angiogenic protein production was: LCL-DXP > LCL-BUP > LCL-MPLP > LCL-PLP (Table 3).

Table 2. Effects of i.v. administered LCL-BUP and free BUP on *anti*-angiogenic protein levels in s.c. B16.F10 tumor tissue.

***Anti*-angiogenic factors**	Reduction induced by LCL-BUP (% of reduction as mean±SD)	Reduction induced by free BUP (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	92.1±1.6	44.5±4.2	ns
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	100.0±0.0	43.5±13.0	**
Platelet factor 4 (PF4)	13.2±12.5	-4.3±5.3	ns
Interleukin 12 p70 (IL-12 p70)	7.0±3.6	-1.6±3.0	ns
Interleukin 13 (IL-13)	82.0±7.5	46.0±12.3	ns
Interferon γ (IFN-γ)	69.6±17.0	-6.6±14.0	***
Monokine induced by IFN-γ (MIG)	44.7±19.0	29.0±11.6	ns

The anti-angiogenic factors are defined as proteins reported in literature to impede angiogenesis and tumor-associated inflammation The protein levels are compared to protein levels in control tumors (tumor from mice treated with PBS). The results were analyzed for statistically significant differences between the effects of LCL-BUP versus freeBUP at the level of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05); **, P<0.01; and ***, P<0.001. The results represent mean±SD of four independent measurements..*

***In vivo* production of COX-2**

The effect of LCL-GC on COX-2 production in tumors was determined by Western blot analysis. Tissue lysates of tumors from mice treated with the different LCL-GC showed different levels of COX-2 compared to controls (Figure 6). Among all LCL-GC tested, LCL-BUP had the strongest inhibitory effect on the production of COX-2 in tumors (by about 80% compared with control tumors). LCL-PLP also had a strong inhibitory effect (by about 60%), LCL-DXP a slight inhibitory effect and LCL-MPLP did not show any effect on COX-2 production (Figure 6).

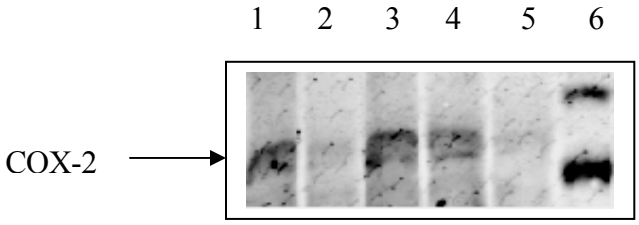

Figure 6. Effect of LCL-GC on the production of cyclooxygenase-2 (COX-2) in tumors analysed by Western blot. Lane 1= control tumors (tumors from mice treated with PBS); lane 2= treatment with LCL-PLP; lane 3= treatment with LCL-MPLP; lane 4= treatment with LCL-DXP; lane 5= treatment with LCL-BUP; lane 6= molecular weight markers: 90 kDa, and 55 kDa.

DISCUSSION

In the present study, we provide a follow-up of our earlier observation that LCL-PLP can inhibit solid tumor growth. To evaluate whether this finding can be generalized to other types of GC, we encapsulated GC other than PLP in LCL for targeting to tumor tissue. We selected three other types of synthetic GC (as disodium salts of the phosphate derivatives), i.e. budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), and methylprednisolone disodium phosphate (MPLP). The four GC differ in their potency to activate the human glucocorticoid receptor in the following order: budesonide > dexamethasone > methylprednisolone ~ prednisolone [19]. The antitumor effects of these LCL-GC were compared to those obtained with our earlier LCL formulation containing PLP. Our previous *in vitro* and *in vivo* studies indicate that the underlying mechanism of LCL-PLP responsible for inhibition of tumor growth is based on inhibition of angiogenesis, due to a strong reduction of intratumoral levels of *pro*-angiogenic factors with hardly any effect on *anti*-angiogenic factor levels [2].

All LCL-GC formulations inhibited tumor growth strongly, except for LCL-MPLP (Figure 4). The effects of LCL-GC are facilitated by the tumor-targeting property of the LCL formulation that increases the intratumoral drug concentration and enables the inhibitory effects of GC on tumor growth [2]. The most potent antitumor effect was achieved by LCL- BUP, reaching up to 85% inhibition of tumor growth compared to controls. This strong effect of LCL-BUP versus other three LCL-GC is likely related to the high potency of BUP. However, it is clear that potency is not the only factor that counts. LCL-MPLP performed worse than LCL-PLP whereas similar or better effects would have been expected based on the relative potency of both GC. The relative potency compared to cortisol, which is set at 1, is 5 for methylprednisolone and 4 for prednisolone [15-18]. Although the relative potency of dexamethasone is much higher than that of prednisolone (25 for dexamethasone, 4 for prednisolone) [16], their antitumor effects were similar (65%). The latter remarkable observation might be related to much stronger reduction of *anti*-angiogenic protein production by LCL-DXP when compared to LCL-PLP (Figure 4), and much higher degree of inhibition of COX-2 by LCL-PLP when compared to LCL-DXP (Figure 6).

When GC are administered in the free form, they are rapidly cleared from the circulation and therefore do not localize in the tumor to a substantial degree, with consequently lower antitumor activity as a result [1]. Only free BUP and free DXP induced a significant inhibition of tumor growth which again is likely a consequence of the high relative

potency of these GC [19]. Although free BUP and free DXP had similar effects on tumor growth, studies focused on the affinity of both GC for the human glucocorticoid receptor demonstrated a much higher receptor affinity for budesonide than for dexamethasone [19, 27, 28]. Nevertheless, both GC showed similar antitumor activity, as this potential therapeutic advantage of BUP over DXP might be lost due to the higher rate of clearance of budesonide from circulation as compared to dexamethasone [29, 30]. Tumor levels of budesonide are likely considerably lower than those of dexamethasone.

To gain more insight into the *in vivo* mechanisms of LCL-GC responsible for tumor growth inhibition, we studied the tumor levels of angiogenic proteins in the s.c. B16.F10 melanoma model using a protein array that detects both *pro*- and *anti*-angiogenic proteins. Previous results for LCL-PLP, obtained at a somewhat higher dose (20 mg/kg) and a higher dosing frequency than that employed in this study, pointed to a reduction of the production of *pro*-angiogenic/*pro*-inflammatory factors as the most likely mechanism of action [2]. This study confirms that LCL-PLP has a marked reducing effect on the level of most *pro*-angiogenic proteins, whereas the level of the majority of *anti*-angiogenic proteins was not affected. As a result, the balance between *pro*- and *anti*-angiogenic proteins is shifted in favor of inhibition of angiogenesis [2]. As compared to LCL-PLP, both LCL-BUP and LCL-DXP exerted a much stronger reducing effect on the production of the majority of *pro*-angiogenic proteins. In addition, administration of the two latter LCL-GC formulations decreased the production of *anti*-angiogenic proteins, as well.

The ranking order of the reducing effects of LCL-GC on the *pro*-angiogenic protein production was LCL-BUP > LCL-DXP > LCL-PLP > LCL-MPLP (Table 3).

Table 3. Ranking of LCL-GC in terms of antitumor actions in s.c. B16.F10 tumor model

LCL-GC	Inhibition of tumor growth	Reducing effect on *pro*-angiogenic factor levels	Reducing effect on *anti*-angiogenic factor levels	Inhibitory effects on COX-2 production
LCL-BUP	1	1	2	1
LCL-DXP	2	2	1	3
LCL-MPLP	3	4	3	4
LCL-PLP	2	3	4	2

The rank score is ranging from 1 to 4. Criteria for rank scores of different effects of LCL-GC: for ***inhibition of tumor growth*** *1,>80%; 2, 60-80%; 3, 40-60%; 4,< 40%; for* **reducing effects on *pro- and anti*-angiogenic factor levels,** *and* ***inhibitory effect on COX-2 production*** *1, > 60%; 2, 40-60%; 3, 20-40%; 4, <20%.*

At first sight, the activity of tested LCL-GC are in line with the potency of encapsulated GC type. However, although true for BUP and DXP versus both other GC types, it is clear that both GC types with similar low potency (MPLP and PLP) do differ in their effect on production of *pro*-angiogenic factors. LCL-PLP was clearly stronger than LCL-MPLP, with the latter hardly different from PBS treatment (Figure 5). Among all *pro*-angiogenic factors studied, bFGF and VEGF are key players in angiogenesis being involved in all tumor angiogenesis steps [31-33]. The tumor expression of bFGF was moderately to strongly reduced after treatment with all types of LCL-GC. Remarkably, production of VEGF was only strongly affected by treatment with the LCL-GC with the highest potency being LCL-BUP and LCL-DXP (Figure 5). Neither LCL-PLP nor LCL-MPLP had any effect on VEGF expression in tumors. Remarkably, also the level of two macrophage-derived cytokines, TNF α and IL-1α that stimulate VEGF overexpression in human melanoma cells [34], was strongly reduced only after treatment with LCL-BUP and LCL-DXP. This finding might explain the strong reducing effect of LCL-BUP and LCL-DXP on VEGF production, via their suppressive effect on the level of two *pro*-angiogenic factors that increase tumor cell-mediated production of VEGF.

Except for LCL-PLP, LCL-GC had a strong reducing effect on most of the *anti*-angiogenic protein levels in tumors, in the following ranking order: LCL-DXP > LCL-BUP > LCL- MPLP > LCL-PLP (Figure 5, Table 3). Interestingly, the production of two *anti*-angiogenic factors, IL-12p70 and PF4, was not affected after LCL-BUP treatment and LCL-PLP (Figure 5). In addition to anti-angiogenic effects, IL-12p70 is also known to induce cytotoxic effects on cancer cells [35-37]. The continuing presence of these *anti*-angiogenic factors likely strengthens the inhibitory effects resulting from the reduction of level of *pro*-angiogenic proteins induced by these two LCL-GC.

To link the anti-angiogenic effects of LCL-GC to inflammatory processes, we assessed their actions on cyclooxygenase (COX)-2 production in tumor tissue. It is known that COX-2 is the rate-limiting enzyme in the production of prostaglandin E_2 (PGE_2) a key mediator of inflammation [38-40]. Inhibition of the production of PGE_2 leads to a supplementary reduction of growth factors, cell adhesion molecules, and metalloproteinases involved in different steps of tumor angiogenesis [41, 42]. The ranking order of the inhibitory effects of LCL-GC on the COX-2 production was LCL-BUP > LCL-PLP > LCL-DXP > LCL-MPLP (Figure 6, Table 3). This outcome is in line with previous studies on A549 human adenocarcinoma cells which demonstrated the same order of inhibition of COX-2 expression induced by these GC [38].

The *in vitro* cytotoxicity studies on cell viability and proliferation support the high potency of LCL-BUP. LCL-BUP had a strong killing effect (up to 90%) on B16.F10 melanoma cells over 48h and also at substantially lower concentrations than the other GC tested (Figures 1 and 2). Therefore, a lethal effect on the cancer cells could contribute to the magnitude of the *in vivo* antitumor effects induced by LCL-BUP (Figure 4). This effect appears to be B16.F10 melanoma cell-specific, as C26 cells were less affected. In addition, all LCL-GC inhibited HUVEC proliferation, with again LCL-BUP being the most potent. This finding would suggest that direct inhibition of endothelial cell proliferation is also involved in inhibition of angiogenesis. It is remarkable that the liposomal drug appears to induce stronger effects than the free agent. These effects might be due to a higher intracellular drug concentration induced by liposomal encapsulation possibly as a result of endocytosis of the lipid particles by the endothelial cells.

In conclusion, all four selected GC encapsulated in LCL inhibit tumor growth, albeit to different degrees. The differences in antitumor activity correlate with their inhibitory activity towards the production of *pro*-angiogenic/*pro*-inflammatory factors involved in tumor angiogenesis and inflammation. Among the four LCL-GC types studied, LCL-BUP show the highest antitumor activity, which is likely related to the strong potency of this GC to reduce the production of *pro*-angiogenic and *pro*-inflammatory factors in tumors. In addition, the *in vitro* results presented herein suggest that LCL-BUP is strongly cytotoxic for B16.F10 melanoma cells and that anti-proliferative effects of all LCL-GC on angiogenic endothelial cells may play a role in their antitumor activity, as well. One of the future issues is to further identify the critical pathways involved in antitumor actions of different LCL-GC.

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Chapter 6

Antitumor activity and tumor localization of long-circulating liposomes containing glucocorticoids in B16 melanoma-bearing mice. Search for optimal glucocorticoid for encapsulation

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ABSTRACT

Prednisolone phosphate encapsulated in long-circulating liposomes (LCL-PLP) inhibited tumor growth by 80-90% after a single dose of 20 mg/kg, whereas PLP in the free form was completely ineffective at the same single dose. To generalize our findings with LCL-PLP, the antitumor activity and side effects of long-circulating liposomes (LCL) containing synthetic glucocorticoids (LCL-GC) other than PLP were investigated. In addition to PLP, budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), and methylprednisolone disodium phosphate (MPLP) were selected based on the difference in their potency to activate the human glucocorticoid receptor. The present study shows that the tumor localization of each GC is governed by the transport capacity of the LCL. The antitumor potency of the LCL-GC formulation strongly depends on the potency of the type of GC encapsulated. LCL-encapsulated BUP (LCL-BUP) had the highest antitumor activity which is likely due to the much higher potency of BUP encapsulated in LCL versus the other three GC types. The high potency of LCL-BUP confers the risk for occurrence of strong side effects. However, at a low dose of 3 mg/kg, LCL-BUP was highly efficacious without the occurrence of adverse effects. In line with their low potencies, all LCL-GC other than LCL-BUP showed hardly any differences in antitumor activity.

Keywords: long-circulating liposomes, glucocorticoids, antitumor activity, side effects, cancer

INTRODUCTION

Glucocorticoids (GCs) are potent and the most commonly used anti-inflammatory and immunosuppressive drugs. Reports in the last two decades demonstrate that GC can also inhibit solid tumor growth in experimental tumor models. These antitumor effects have been attributed to the inhibition of tumor angiogenesis, but can only be achieved by extremely high and frequent dosing of GC (cumulative weekly doses are in the range of 500-700 mg/kg). Antitumor effects of GC have been reported to be potentiated by additional administration of heparin [1-3]. However, GC cause severe side effects at the intensive dosage regimens required. The need for such intensive treatment schedules is explained by the pharmacokinetics of GC: they are rapidly cleared from the circulation, and accumulate at tumor sites only to a very limited extent. Therefore, the oncological application of GC could benefit greatly from the use of a tumor-targeted delivery system.

Indeed, our previous studies showed that liposomally-targeted PLP strongly inhibits tumor growth via inhibition of tumor angiogenesis [4, 5]. For efficient delivery of PLP into tumors by intravenous treatment, we used small-sized liposomes coated with poly(ethylene glycol) (PEG). PEG has been shown to be effective in reducing recognition and rapid removal of liposomes from the circulation by the mononuclear phagocyte system (MPS), enabling liposomes to stay in the circulation for a prolonged period of time. The long-circulation property provides the liposomes the opportunity to substantially extravasate and accumulate in tumors by virtue of the enhanced permeability of the tumor vasculature, as compared to healthy endothelium [4, 6, 7]. The antitumor effects of the PEG-liposomal PLP formulation are enabled by this tumor-targeting capability of the liposome particles, increasing intratumoral drug concentration and intensifying the inhibitory effects of PLP.

To generalize our findings with long-circulating liposomal PLP (LCL-PLP), we have investigated the antitumor activity and side effects of long-circulating liposomes (LCL) containing synthetic GC other than PLP. We have used their phosphate derivatives to ensure stable liposome encapsulation. In addition to PLP, the following GC were tested: budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), and methylprednisolone disodium phosphate (MPLP). They were selected due to their difference in the ranking order of their potency in terms of activation of the human glucocorticoid receptor: budesonide > dexamethasone > methylprednisolone ~ prednisolone [8-12]. These GC are on the market for the treatment of auto-immune and inflammatory disorders [13, 14] and they have not been explored as liposomal antitumor

agents. The choice for these GC was also inspired by a recent study on their *in vitro* antitumor effects towards A549 human adenocarcinoma cells showing differences in their genomic and non-genomic mechanisms of action [15].

The use of systemically administered GC confers the risk for occurrence of different side effects such as, amongst others, body weight loss, increase in blood glucose concentration, severe immune suppression, and alteration of prostaglandin metabolism [5, 16-18]. Encapsulation of GC in liposomes has been reported to reduce side effects [19].

The question addressed in this article is whether the liposomal GC tumor targeting concept can be exploited more efficiently by using other types of GC than PLP, with the ultimate aim to increase antitumor activity. To this end, it was investigated whether the potency of the LCL-encapsulated GC (LCL-GC) is an important determinant of the *in vivo* antitumor activity. In addition, we quantified the GC content in tumor tissue in an attempt to correlate antitumor activity with the amount delivered to the tumor.

MATERIALS AND METHODS

Preparation of LCL-GC

LCL were prepared as described previously [5]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) 2000-distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol in a round-bottom flask. A lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone disodium phosphate (PLP), dexamethasone disodium phosphate (DXP) (both obtained from Bufa, Uitgeest, The Netherlands), budesonide disodium phosphate (BUP) or methylprednisolone disodium phosphate (MPLP) (synthesized by Syncom, Groningen, The Netherlands). Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of lipids with chloroform, according to Rouser [20]. Unencapsulated GC were removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. The aqueous phase after extraction was used for determining the glucocorticoid phosphate content by high performance liquid chromatography as described previously [21]. The type of column was RP18 (5 µm) (Merck) and the mobile phase consisted of acetonitril and water (1:3 v/v), pH 2. The eluent was monitored with an ultraviolet detector set at 254 nm. The detection limit for the high performance liquid chromatography setup was 20 ng/ml.

Cells

B16.F10 murine melanoma cells were cultured as monolayers at 37°C in a 5% CO_2-containing humidified atmosphere in DMEM medium (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).

Murine tumor model

Male C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (The Netherlands) and 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 the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the right flank of syngeneic C57Bl/6 mice. B16.F10 tumors became palpable at day 7 after tumor cell inoculation.

Antitumor effects of LCL-GC versus free GC

When the tumor became palpable (tumor volume ~20 mm^3) (at day 7) mice received two intravenous (i.v.) injections of LCL-GC and free GC at a dose of 10 mg/kg, at day 7, and 13 after tumor cell inoculation. Since day 7, tumor size was measured regularly and tumor volume was calculated according to the formula $V = 0.52 \times a^2 \times b$, in which *a* is the smallest and *b* the largest superficial diameter (in mm). Mice were sacrificed when the tumor volumes were larger than 2 cm^3.

Dose-response relationship

To compare the inhibitory effects of different doses of GC encapsulated in LCL on tumor growth, B16.F10 tumor-bearing mice received two i.v. injections of indicated formulations at days 7, and 13 after tumor cell inoculation. Tumor size was measured regularly, and tumor volume was calculated as described above.

Analysis of amount of GC in tumor tissue

At a tumor volume of approximately 1 cm^3, mice were injected i.v. with equivalent doses of LCL-GC that exerted the same inhibitory effect (by 50%) on tumor growth at day 15 after tumor cell inoculation. The same doses were used for free GC. Animals were killed and tumors were dissected 24h, 48h, 72h, and 96h after injection. The tissues were weighed and homogenized. 2 μg of each GC was added as an internal standard after which GC (phosphate) was extracted from the tissue with ethylacetate at pH 2, and evaporated until dryness under a nitrogen flow. Samples were reconstituted in methanol:water 1:1 (v/v) and analyzed by high performance liquid chromatography as described previously [22].

Adverse effects of liposomal and free GC

Adverse effects that are typical for systemic glucocorticoid treatment were investigated , such as body weight loss, weight loss of certain organs (spleen, liver), and weight gain of

the kidneys. Loss of body weight is due to the stimulatory effects of GC on protein degradation and lipolysis [23]. The effects of GC on liver and spleen are due to their suppressive effects on immune cells from these organs. Besides these actions, it is known that GC induced significant increase in kidney weight, possibly due to the alteration of prostaglandin metabolism [18]. Toxicity was evaluated by measurement of body weights every two days. Organs were weighed after mice sacrification.

Statistical Analysis

Data from different experiments were reported as mean ± SD. For statistical analysis, Student's t- test for independent means was used. A value of $P<0.05$ was considered significant. Statistical comparisons of the effects of different treatments on tumors and area under the tumor growth curves, were evaluated by one-way ANOVA with Dunnett's post- Test for multiple comparisons using GraphPad Prism version 4.02 for Windows software, GraphPad Software (San Diego, CA).

RESULTS AND DISCUSSION

Characterization of LCL-GC formulations

In this study, LCL were used which contained 5 mol% of PEG2000-conjugated distearoylphosphatidylethanolamine. PEG is a synthetic hydrophilic polymer that prolongs the circulation time of the liposomes when exposed on their surface [7]. The long-circulation property provides the liposomes with the opportunity to substantially extravasate and accumulate in tumors. The tumor targeting property of LCL is enabled by the enhanced permeability of tumor vasculature, as compared to healthy endothelium [4]. PEG-liposomes can extravasate through the "leaky" pathological vasculature and thereby accumulate into the malignant tissue (referred to as the "enhanced permeability and retention (EPR) effect") [19].

The following GC were encapsulated in LCL: budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), methylprednisolone disodium phosphate (MPLP), and prednisolone disodium phosphate (PLP). LCL-GC were characterized regarding particle size distribution, phospholipid concentration, and encapsulation efficiency. Mean particle size of the liposomes was found to be around 100 nm with a polydispersity value lower than 0.1. The low polydispersity values obtained indicate a narrow size distribution. The encapsulation efficiency was about 5% (7.5 μg GC/μmol phospholipid) in case of all four LCL-GC formulations, indicating that the degree of loading is independent from the type of GC.

Effect of encapsulated GC type on *in vivo* antitumor activity

To investigate whether the type of encapsulated GC is an important determinant of the *in vivo* antitumor activity, LCL-GC and free GC were injected i.v. at a dose of 10 mg/kg on days 7 and 13 after tumor cell inoculation. The antitumor activity induced by LCL-GC and free GC was analyzed by determining the area under the tumor growth curves (AUTC) until day 15 (the day when the first tumors from the control group reached the maximum size of 2 cm^3) (Figure 1, panels A and B) as well as inhibition of tumor growth compared to control tumors (tumors in mice treated with PBS) (Figure 1, panels C and D).

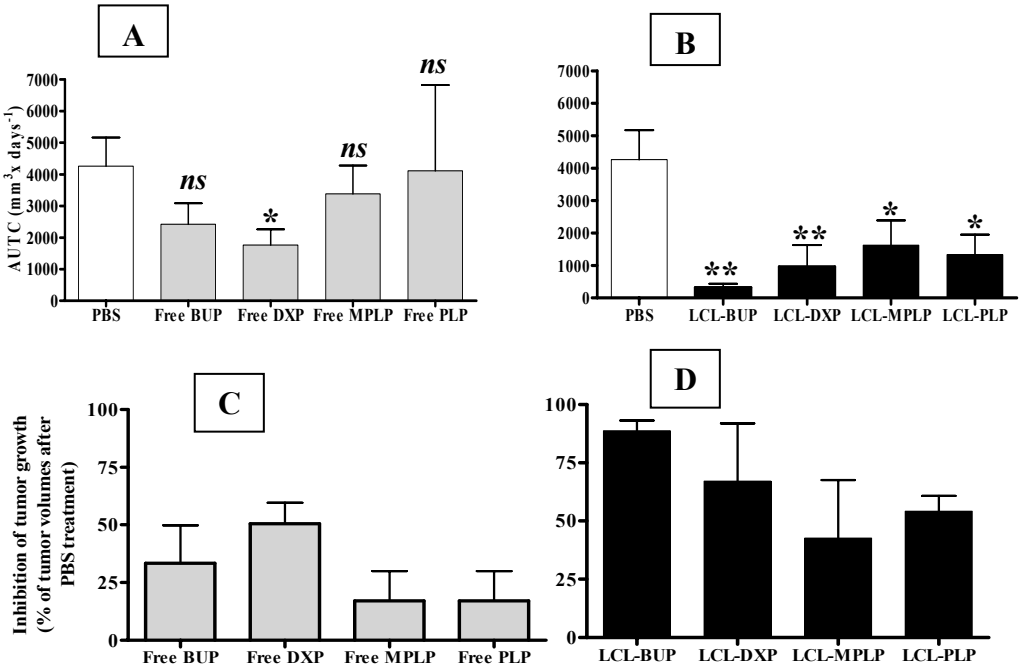

Figure 1. Antitumor activity of LCL-GC versus free GC until day 15 after tumor cell inoculation. The results are presented as area under the tumor growth curves (AUTC) until day 15 (the day when the first tumors from the control group reached the maximum volume of 2 cm^3) (panels A and B) and inhibition of tumor growth compared to control tumors (tumors in mice treated with PBS) at day 15 (panels C and D). Inhibition of tumor growth was obtained by comparing tumor volumes in mice treated with different GC formulations to tumor volumes in mice treated with PBS at day 15, and expressed as % of inhibition of control tumor growth (PBS – treated group).
The formulations were injected i.v. at a dose of 10 mg/kg on days 7, 13 in B16.F10 tumor-bearing mice. Mean±SD; n= 5 animals per experimental group; Free BUP= treatment with free BUP, Free DXP= treatment with free DXP, Free MPLP= treatment with free MPLP, Free PLP= treatment with free PLP, LCL-BUP= treatment with LCL-BUP, LCL-DXP= treatment with LCL-DXP, LCL-MPLP= treatment with LCL-MPLP, LCL-PLP= treatment with LCL-PLP. To compare the effects of different treatments on tumors *in vivo* with controls (tumors in mice treated with PBS), one-way ANOVA with Dunnett's Test for multiple comparisons was used and the *P* values are indicated as follows: ***ns,*** not significant ($P>0.05$); *, $P<0.05$; **, $P<0.01$.

Except for free DXP treatment, treatment with free GC did not result in significant inhibition of tumor growth compared to control tumors (Figure 1, panels A and C). When GC are administered in the free form, they are rapidly cleared from the circulation and therefore GC are not able to localize in the tumor sufficiently for achieving substantial antitumor activity [5]. Although only free DXP had an inhibitory effect on

tumor growth, studies focused on the affinity of human glucocorticoid receptor for both GC demonstrated a lower affinity of this receptor for dexamethasone than for budesonide [24]. However, free DXP showed antitumor activity, whereas free BUP lacks antitumor activity. This can be explained by higher rate of clearance of budesonide from the circulation as compared to dexamethasone (in rats: clearance values are 1.5 $L \cdot h^{-1} kg^{-1}$ for budesonide [25] and 0.2 $L \cdot h^{-1} kg^{-1}$ for dexamethasone [26]). Consequently, tumor levels of budesonide are likely lower than those of dexamethasone.

Importantly, all LCL-GC significantly inhibited tumor growth (Figure 1, panel B) with LCL-BUP being the most active. In view of the lack of or low antitumor activity of free GC, it is clear that antitumor effects of LCL-GC are enabled by the tumor-targeting property of the liposome formulation. The strong inhibition of tumor growth induced by LCL-BUP (90%) as compared to LCL-DXP (70%), LCL-PLP (55%) and LCL-MPLP (40%) (Figure 1, panel D) seems related to the strong potency of BUP versus the other GC types.

Antitumor activity of LCL-GC: dose -response relationship

To assess dose–response relationships, B16.F10 tumor-bearing mice received two i.v. injections of different doses of the four different LCL-GC formulations, at days 7 and 13 after tumor cell inoculation. For all LCL-GC, tumor volumes were smaller with increasing doses of liposomal GC (Figure 2) and a significant correlation between dose and response was noted (Spearman correlation coefficient >0.9). In case of LCL-BUP and LCL-DXP, maximal effects were already achieved at a dose of 10 mg/kg (inhibition of tumor growth 90% for LCL- BUP and 70% for LCL-DXP, respectively). At the highest dose tested (30 mg/kg), inhibition of tumor growth was 70% for LCL-MPLP and 85% for LCL-PLP. Again LCL-BUP was the most potent formulation in line with the much higher potency of budesonide when compared to the other GC types tested. LCL-BUP administered at a dose of 3 mg/kg exerted the same inhibitory effect on tumor growth (about 75%) as LCL- PLP at a dose of 20 mg/kg.

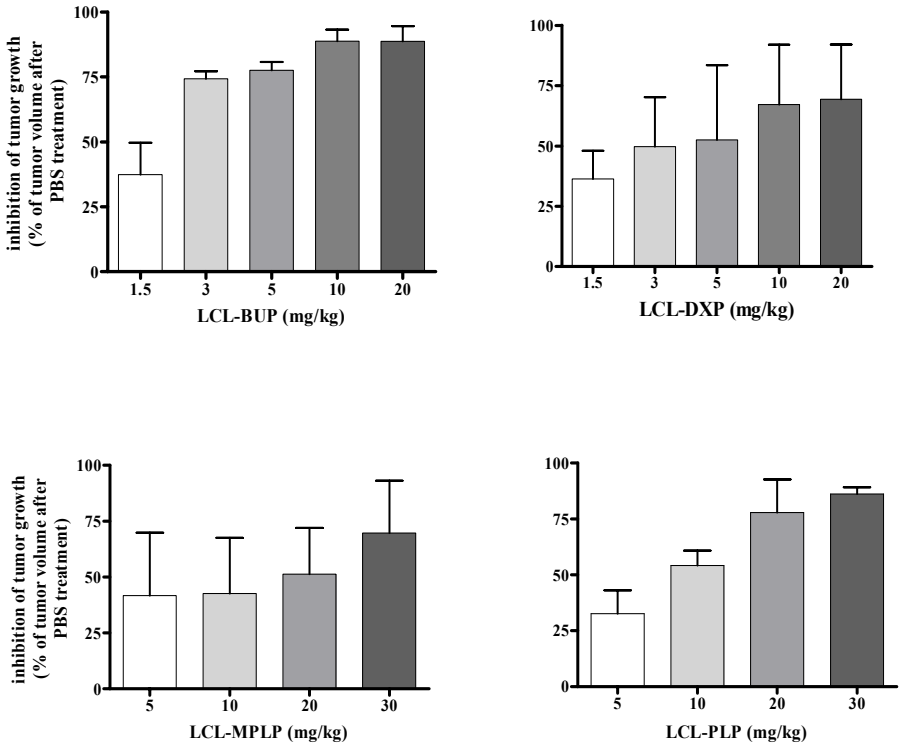

Figure 2. Antitumor activity of different LCL-GC: dose–response relationship. The results are presented as inhibition of tumor growth compared to control tumors (tumors of mice treated with PBS). Inhibition of tumor growth induced by LCL-GC was obtained by comparing tumor volumes in mice treated with LCL-GC formulations to tumor volumes in mice treated with PBS at day 15, and expressed as % of inhibition compared to tumor volumes after PBS treatment. Tumor volume was reported at day 15 (the day when the first tumors from the control group reached the maximum volume of 2 cm^3) after tumor cell inoculation. Mice received two injections of either formulation at days 7, and 13 after tumor cell inoculation. Mean ± S.D, n=5 animals/ experimental group, LCL-BUP= treatment with LCL-BUP, LCL-DXP= treatment with LCL-DXP, LCL-MPLP= treatment with LCL-MPLP, LCL-PLP= treatment with LCL-PLP.

Adverse effects

Except for LCL-MPLP, all LCL-GC induced a strong loss of spleen weight. Spleen weight loss appeared to be reversible, except for LCL-BUP at a dose of 20 mg/kg and LCL-PLP at a dose of 30 mg/kg. In the latter cases, spleen weight did not recover at the day of sacrifice (day 21 after tumor cell inoculation). In case of LCL-BUP, strong body weight loss was only observed at the highest dose (20 mg/kg) tested.

Additionally, mice treated with LCL-BUP at doses ranging from 5 to 20 mg/kg showed side effects like kidney stones, abdominal alopecia, and loss of equilibrium.
Apparently, LCL-BUP being the most potent LCL-GC formulation in terms of antitumor activity, also carries the highest risk for occurrence of side effects. Nevertheless, at the low dose of 3 mg/kg, LCL-BUP was highly efficacious without the occurrence of adverse effects.

Quantification of GC in tumor tissue

The LCL-GC formulations were injected at the doses at which tumor growth was inhibited by 50%, compared to the growth of control tumors at day 15 after tumor cell inoculation. In ascending order these doses were about 2.5 mg/kg for LCL-BUP, 5 mg/kg for LCL-DXP, 10 mg/kg for LCL-PLP, and 20 mg/kg for LCL-MPLP. The tumor-associated GC (phosphate) levels were determined at 24, 48, 72 and 96 hours after intravenous injection by high performance liquid chromatography as described previously [22]. Free GC were injected at the same doses, with GC levels being determined at 24h after intravenous injection. At this time point, total GC (phosphate) (P) levels in the tumor tissue were detectable only after injection of free MPLP and free PLP at levels 100 times lower than those after administration of LCL-MPLP and LCL-PLP, respectively (data not shown). At 24h, total GC (P) concentrations in B16.F10 tumor tissues were 1± 0.3 μg/g for LCL-BUP, 3± 1 μg/g for LCL-DXP, 9 ± 3 μg/g for LCL-PLP, and 15 ± 4 μg/g for LCL- MPLP (n = 3, mean ± SD), levels which all correspond to approximately 3.5 ± 1% of the injected dose (%ID) (Figure 3). These results suggest a dose-independent tumor localization for each of the LCL-GC formulations. The data are in accordance with our previous results for LCL-PLP with 2-5% ID of prednisolone in tumor tissue recovered [5]. At 48h, approximately 50% of the GC level in the tumor at 24h was cleared (Figure 3). In case of all the LCL-GC formulations the GC levels were approximately 1.6 ± 0.5% ID.

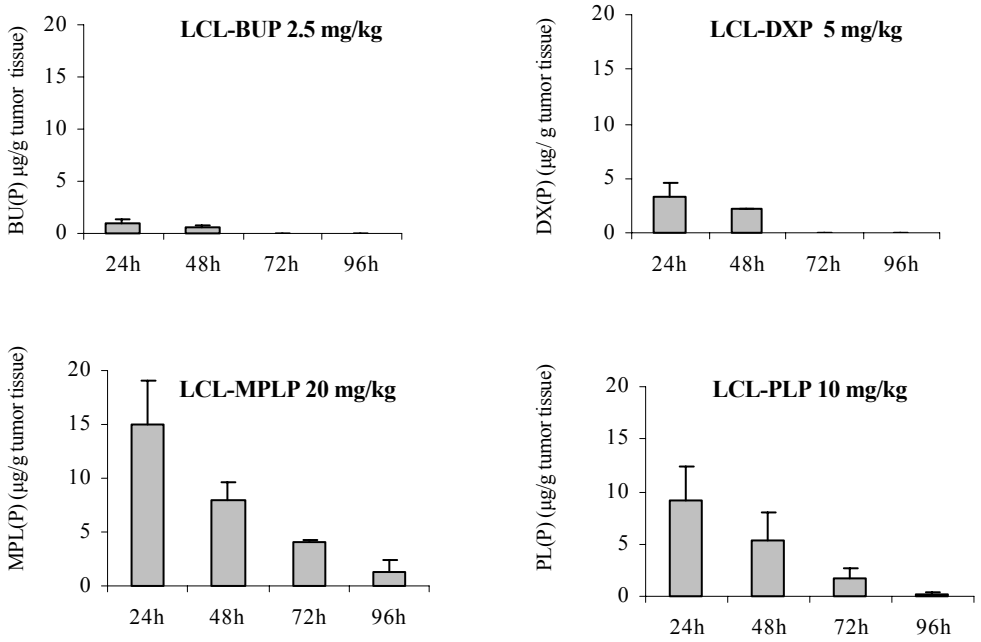

Figure 3. Quantification of GC (phosphate) in tumor tissue after LCL-GC treatment. LCL-GC were administered i.v. at the dose that results in 50% inhibition of tumor growth compared to the growth of control tumors (20 mg/kg for LCL-MPLP, 10 mg/kg for LCL-PLP, 5 mg/kg for LCL-DXP, and 2.5 mg/kg for LCL-BUP). Mean ± S.D, n=3 animals/experimental group, LCL-BUP= treatment with LCL-BUP, LCL-DXP= treatment with LCL-DXP, LCL-MPLP= treatment with LCL-MPLP, LCL-PLP= treatment with LCL-PLP.

The accumulation of GC in tumor tissue is enabled by the use of LCL as well as the phosphate derivative of GC. In line with the similar degree of tumor accumulation of the various LCL-GC formulations, LCL have been shown not to leak encapsulated water-soluble drug in the circulation [21]. The water-soluble phosphate derivative of GC used in our experiments ensures stable encapsulation within LCL. Previous experiments showed that unmodified prednisolone, although showing high initial encapsulation efficiency, was rapidly released from the liposomes upon intravenous injection [5].

In conclusion, encapsulation of GC in LCL offers an increased antitumor activity irrespective of the type of GC. The increased intratumoral drug levels are governed by the transport capacity of the liposomes, making the antitumor efficacy only dependent on the differences in GC potency. Indeed, the data show that the antitumor potency of the LCL-GC formulation can strongly depend on the potency of the type of GC encapsulated, as the antitumor activity induced by LCL-BUP is much higher when compared to that of the other LCL-GC formulations (budesonide has 250-fold higher potency compared to cortisol which is set at 1) [12]. LCL-GC other than LCL-BUP show hardly any differences in their antitumor activity which is in line with their low potencies (25 for dexamethasone, 5 for methylprednisolone, and 4 for prednisolone compared to cortisol which is set at 1) [8-11]. The relatively high potency of BUP within LCL-BUP confers the risk for occurrence of strong side effects. However, at the dose of 3 mg/kg, LCL-BUP was highly efficacious without the occurrence of adverse effects.

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Chapter 7

Antitumor activity of pravastatin after tumor targeted delivery

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Submitted for publication

ABSTRACT

Statins possess biological activities that extend beyond their cholesterol-lowering capability. These so-called pleiotropic effects result from the altered post-translational modification of GTP-binding proteins, which regulate many intracellular pathways. Pre-clinical studies suggest that this could make statins effective anticancer agents. However, required doses are generally higher than those needed to lower cholesterol levels. Therefore, targeted delivery is attractive to improve activity. Furthermore, as GTP-binding proteins affect many pathways, target site-specific delivery can minimize side effects. In this study, we investigated tumor-specific delivery of pravastatin (PRV) using long-circulating liposomes (LCL). A single i.v. dose of 5 mg/kg liposome-encapsulated PRV (LCL-PRV) inhibited murine B16.F10 melanoma growth over 70% as compared to free PRV, which was ineffective. Neither treatment affected serum cholesterol levels. *In vitro* studies on the effects of LCL-PRV on viability and proliferation of tumor cells, endothelial cells, and macrophages revealed that macrophages were the most sensitive cell type towards LCL-PRV treatment. Measurement of intratumoral levels of 24 proteins involved in inflammation and angiogenesis showed that LCL-PRV treatment effectively inhibited production of *pro*-inflammatory/*pro*-angiogenic mediators as compared to free drug. Taken together, targeted delivery of statins can improve their antitumor activity. This activity is primarily resulting from local inhibition of tumor inflammation and angiogenesis.

Keywords: statins, liposomes, inflammation, angiogenesis

INTRODUCTION

Statins are amongst the most widely prescribed drugs. Statins have originally been designed as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway leading to de novo cholesterol synthesis [1]. They form the basis for treatment of patients with, or at risk for, cardiovascular diseases, with the objective of reducing plasma cholesterol levels. Apart from their action on cholesterol synthesis, it is known that statins exhibit actions other than cholesterol reduction. These, so called pleiotropic, effects of statins include improvement of endothelial function, enhancement of antioxidant activity, and inhibition of inflammation [2, 3]. The pleiotropic effects of statins are related to the reduced formation of isoprenoids, which in turn are responsible for post-translational modification of proteins. Especially the modification of small GTP-binding proteins like Rho, Rac, and Ras seem important as they affect a multitude of intracellular pathways [4-6].

The observation that statins have pleiotropic activities has stimulated extensive research on the possible beneficial and harmful effects of these drugs, especially with regard to cancer. Newman and Hulley reported that statins were carcinogenic in rodents at levels close to those prescribed in humans [7]. Epidemiological studies have shown a protective, neutral, as well as inducing effect of statin use on the development of various cancers [8, 9]. With regard to treatment of tumors, evidence of in vitro and preclinical studies generally seem to indicate beneficial effects, albeit that they are obtained at relatively high doses that go beyond the doses needed for inhibition of cholesterol synthesis [3, 10, 11].

To exploit the pleiotropic effects of statins in cancer treatment, improving the pharmacokinetics and tissue distribution of statins seems important to promote the desired activity and limit adverse effects. Also, as the inhibitory effects of statins on tumor growth are only obtained at high doses, site-specific delivery may intensify antitumor effects, while reducing the doses that are required. In this study, we used long-circulating liposomes to target pravastatin (PRV) to tumor tissue after intravenous administration. The effects on tumor growth in a murine B16.F10 melanoma model are investigated.

MATERIALS AND METHODS

Preparation of LCL-PRV

LCL were prepared as described previously [12]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly (ethylene glycol) (PEG)2000-distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol in a round-bottom flask. After lipid film formation, by evaporation of the organic solvent, the film was hydrated with a solution of 5 mg/ml PRV sodium, (Sigma, St. Louis, USA). PRV is one of the few hydrophilic statins, and therefore it can be stably encapsulated in the liposome's aqueous interior. Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering and found to be 100 nm with a polydispersity value below 0.1. The polydispersity value indicates limited variation in particle size. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of liposomal preparations with chloroform, according to Rouser [13]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. The aqueous phase after chloroform extraction was used for determining the PRV content by high performance liquid chromatography as described previously for glucocorticoids [14]. The type of column used was an RP18 (5 μm) (Merck, Darmstadt, Germany) and the mobile phase consisted of acetonitril and water (35:65 (v/v)), brought to pH 2 with trifluoric acid. The eluent was monitored with an ultraviolet detector set at a wavelength of 239 nm. The detection limit for the high performance liquid chromatography setup was 20 ng/ml. The liposomal preparation contained approximately 500 μg PRV/ml, and 60 μmol lipid/ml. The liposome suspension was stored at 4°C. The liposomes displayed limited leakage of contents, less than 10% of the encapsulated drug was lost over a 3 week storage period. Liposomes were used within one week of preparation.

Cells

Cells were cultured as monolayers at 37°C in a 5% CO_2-containing humidified atmosphere. B16.F10 murine melanoma cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin,

100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, and 4 mM L-glutamine (all Gibco, Breda, The Netherlands). Human umbilical vein endothelial cells (HUVEC) (Cambrex, East Rutherford, NJ) were cultured in complete EGM endothelial cell growth medium (Cambrex). J774A.1 cells are a reticulum sarcoma cell line, which displays a number of characteristics of primary macrophages [15]. J774A.1 cells were cultured in DMEM supplemented with 10% heat-inactivated horse serum, 100 IU penicillin/ml, 100 µg streptomycin/ml, 0.25 µg amphotericin B/ml, 2 mM L-glutamine (all Gibco), and10 mM Hepes (Acros).

Murine tumor model

Male C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (The Netherlands) and 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 the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the flank of syngeneic C57Bl/6 mice. B16.F10 tumors had a diameter of approx. 4 mm at day 8 after tumor cell inoculation. Treatment was started at this time-point. Animals received 5 m/kg PRV intravenously (i.v.) via the tail vein either in free or liposome-encapsulated form at day 8 and 11.

Cell viability *in vitro*

To determine whether LCL-PRV and free PRV had a direct cytotoxic effect on cells, 5 x 10^3 B16.F10, HUVEC, J774 cells/well were plated in a 96-well plate for 24h. Then, empty liposomes or PRV formulations were added in PBS and incubated for 24h, 48h, or 72h. After exposure time, cell viability was determined by XTT-assay (Sigma, St. Louis, USA) according to the manufacturer's instructions. Cells were incubated with tetrazolium salt XTT and electron-coupling reagent (N-methyl dibenzopyrazine methylsulfate) for 1 h at 37 °C in the CO_2-incubator. Absorbance was measured at 490 nm with a reference wavelength of 655 nm.

Cell proliferation *in vitro*

To determine the effect of LCL-PRV and free PRV on cell proliferation, 10^3 B16.F10, HUVEC, or J774 cells/well were plated in a 96-well plate for 24h. After that, empty liposomes or PRV formulations were added in PBS. The anti-proliferative effect of each

treatment was determined after 24h, 48h, and 72h of incubation by ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The technique is based on the incorporation of the pyridine analogue bromodeoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. Cells were incubated with BrdU solution for 24h. Then medium was completely removed from the wells, and cells were fixed and DNA was denatured. To detect BrdU incorporated in synthesized cellular DNA, a monoclonal antibody conjugated with peroxidase was added. After 90 minutes of incubation, unbound antibody was removed and cell lysates were washed three times with PBS. The immune complexes were detected by adding a substrate of peroxidase (tetramethyl-benzidine). The reaction product was quantified by measuring the absorbance at 450 nm with a reference wavelength of 655 nm.

Serum cholesterol levels

Serum cholesterol levels were measured spectrophotometrically using the cholesterol/cholesteryl ester quantification kit (BioVision, Mountain View, CA) according to manufacturer's instructions. Animals were treated with 5 mg/kg PRV either in free or LCL form at day 8 and 11 after tumor cell inoculation. Blood was obtained at day 12 from the tail vein. Individual samples were measured in duplicate.

Production of angiogenic factors *in vivo*

To compare the effects on angiogenic factors, LCL-PRV and free PRV was administered i.v. at a dose of 5 mg/kg at day 8 and 11 after tumor cell inoculation. 4 animals were used per experimental group. As controls, tumors from mice treated with PBS or empty liposomes were used. On day 12, mice were sacrificed and tumors were isolated. A screening of angiogenic/inflammatory proteins present in tumor tissues was performed using a RayBio® Mouse Angiogenic protein Antibody Array membrane 1.1 (RayBiotech Inc. Norcross, GA) as described previously [14]. In brief, each membrane contains 24 types of primary antibodies against specific angiogenic proteins. To detect the levels of angiogenic factors, the tumor tissue for each group was lysed. Protease Inhibitor Cocktail (Sigma) was added to the lysis buffer. After obtaining the pooled tumor tissue lysates for each group, the protein content of the lysates was determined according to Peterson [16]. One array membrane was used per pooled tumor tissue lysate. The array membranes were incubated with 250 µg of protein from tissue lysate for 2h, at room temperature.

Membranes were subsequently incubated with a mixture of secondary biotin-conjugated antibodies against the same angiogenic factors as those bound onto the membranes. Then, membranes were incubated with HRP-conjugated streptavidin for 2h, followed by two detection buffers, for 1 minute. Each step of membrane incubation was followed by five washing steps. The membranes were exposed to x-ray film for 40 seconds and signal detected using film developer. Using GelPro Analyzer software, version 3.1, the color intensity was determined for each spot in comparison to positive control spots already bound to the membrane. Angiogenic protein levels in tumors treated with free PRV, and LCL-PRV was expressed as percentage of protein levels in vehicle treated tumors. The final results represent mean values ± standard deviation of two measurements.

Statistical Analysis

For statistical analysis, Student's t- test for independent means was used. A value of $P<0.05$ was considered significant. The differences between the overall effects of different treatments on tumor growth and serum cholesterol levels were analyzed by one-way ANOVA. The differences between the effects of different treatments on angiogenic factor production were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Effect on tumor growth

To compare the effects of LCL-PRV and free PRV on growth of subcutaneous (s.c.) B16.F10 melanoma, mice received a single i.v. administration of 5 mg PRV per kg bodyweight of either formulation at the moment that the tumor had a diameter of approximately 4 mm (day 7). No differences were observed between the growth of the tumors in mice treated with empty liposomes and PBS (data not shown). Tumor volumes were smaller for LCL-PRV as compared to PBS-treated tumors ($P<0.01$) (Figure 1). The free drug had no effect indicating that targeting is essential to achieve therapeutic effects at this dose level.

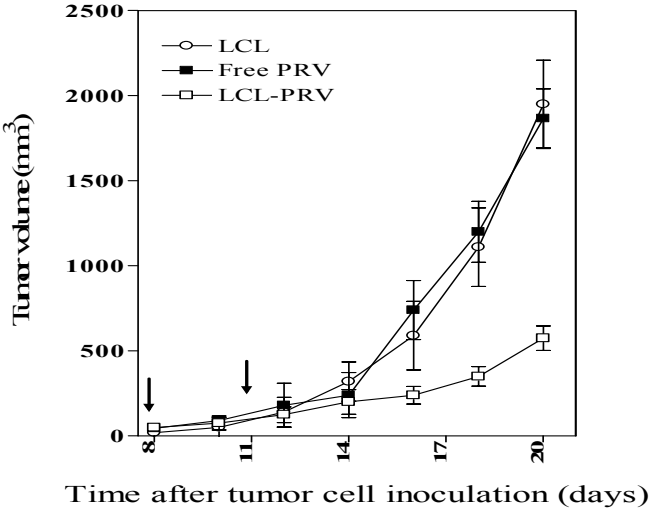

Figure 1. Antitumor effect of LCL-PRV. Subcutaneous B16.F10 melanoma-bearing mice received a single injection of 5 mg/kg PRV as free drug or encapsulated in LCL, or an equivalent dose of empty liposomes at the moment that the tumor diameter measured 4 mm (day 7). Comparison of area-under-the-tumor-growth-curves showed that tumor growth was decelerated after LCL-PRV treatment ($p<0.01$, one way ANOVA).

Effect on lipid metabolism

To investigate whether the antitumor effects correlated with the primary mechanism of action of PRV, serum cholesterol levels were measured before treatment and one week after treatment with 5 mg/kg LCL-PRV, free PRV, or an equivalent dose of empty liposomes. Cholesterol levels were not significantly different before and after treatment or between treatments (Figure 2).

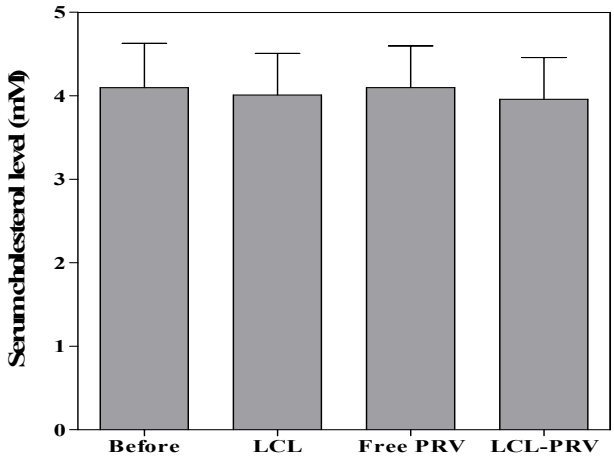

Figure 2. The effect of LCL-PRV and free PRV on serum cholesterol level. Before= before treatment, LCL=treatment with empty LCL, Free PRV=treatment with free PRV, LCL-PRV=treatment with LCL-PRV. No changes in serum cholesterol levels after different treatments compared to the cholesterol levels before treatments were noted (P =0.98, one way ANOVA).

Effects on cell viability and proliferation *in vitro*

Subsequently, direct cytotoxic effects of PRV formulations were evaluated. To evaluate the cytotoxic effects of LCL-PRV versus free PRV, tumor cells (B16.F10), proliferating primary endothelial cells (HUVEC), and macrophages (J774), were incubated *in vitro* for 24, 48, and 72 hours with increasing PRV concentrations ranging from 5 to 200 µg/ml. PBS-treated cells were used as control. There were no changes in viability of cells incubated with PBS, empty liposomes, or cell culture medium. Viability of cells was marginally affected during the first 48h of incubation with any treatment. Differences were more pronounced at the 72h time-point. The 72h data are shown in Figure 3.

Viability of B16.F10 cells was in general unaffected (<15%) by LCL-PRV or free PRV treatment for all concentrations. For HUVEC, viability of cells was slightly affected at the highest concentrations for both liposomal and free drug. For J774 cells, LCL-PRV resulted in a moderate reduction in viability, by approximately 40% at the highest concentrations tested (100 μg/ml and 200 μg/ml), while free PRV induced a 20% inhibition.

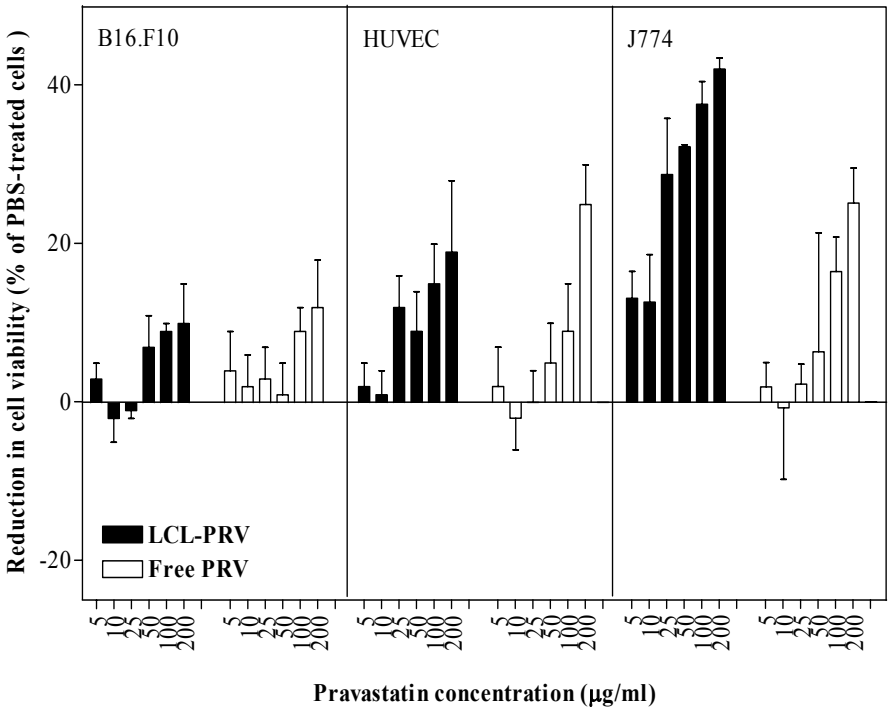

Figure 3. Effect of LCL-PRV and free PRV on cell viability. Only data obtained at the latest time point (72h) are shown. Mean±SD; n= 3 measurements; free PRV= treatment with free PRV, LCL-PRV=treatment with LCL-PRV

Apart from direct cytotoxic effects, PRV could also affect proliferation of these cell types in the tumor. To study the anti-proliferative effects of PRV formulations, B16.F10, HUVEC, and J774 cells were incubated up to 72 hours with increasing concentrations of PRV ranging from 5 to 200 μg/ml. Similarly as for the cytoxicity data, proliferation was hardly affected during the first 48 h (data not shown).

At 72h, the effects were more pronounced, the strongest inhibition of cell proliferation (by 50-60%) was found after LCL-PRV treatment of J774 cells at concentrations of 100-200 µg/ml (Figure 4). HUVEC showed again a less significant inhibition of cell proliferation, while B16.F10 cells were hardly affected by PRV formulations.

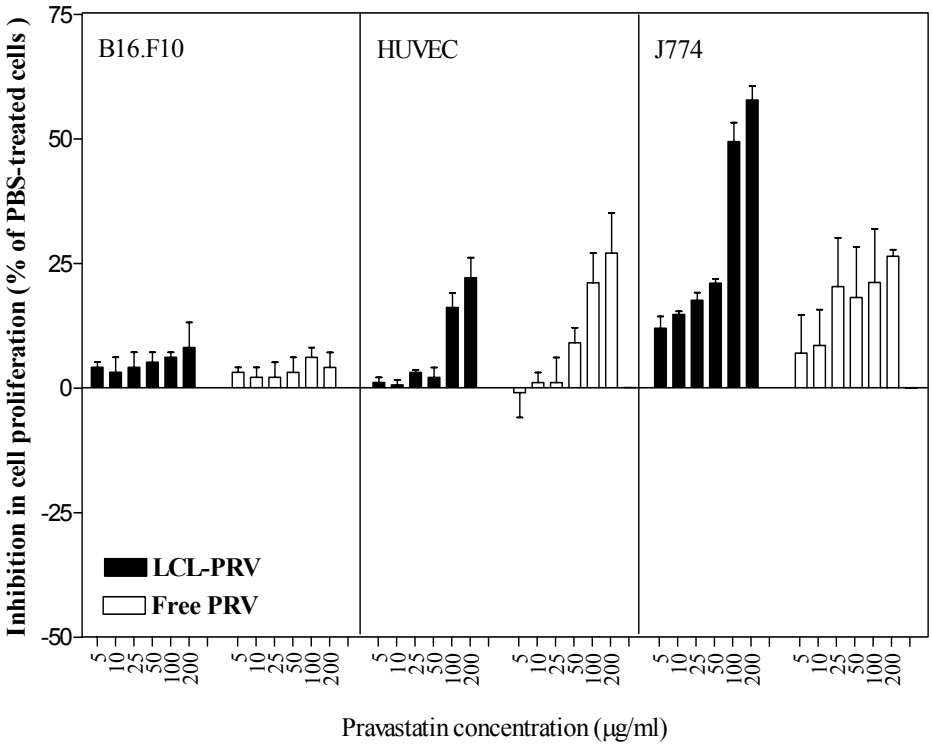

Figure 4. **Effect of LCL-PRV and free PRV on cell proliferation.** Only data obtained at the latest time point (72h) are shown. Mean±SD; n= 3 measurements; free PRV= treatment with free PRV, LCL-PRV=treatment with LCL-PRV

Effects on production of angiogenic factors *in vivo*

The *in vitro* studies suggest that the macrophage is the most sensitive cell type towards PRV treatment. Inhibition of macrophages could inhibit tumor growth by inhibiting the inflammatory response. Macrophages secrete many *pro*-inflammatory/*pro*-angiogenic factors that can stimulate tumor expansion. Therefore, we investigated the effects of LCL-PRV and free PRV treatments on *pro*-inflammatory/*pro*-angiogenic protein levels in tumor tissue. A screening of 24 angiogenic/inflammatory proteins present in tumor tissue was performed using an angiogenic protein array. Tumor-bearing mice were treated with free PRV, LCL-PRV or empty liposomes. With this array, tumor levels of factors involved in angiogenesis, inflammation and apoptosis can be determined. There were no significant differences in protein levels in tumors treated with PBS and those in tumors treated with empty liposomes (P=0.76). PRV formulations decreased the production of factors involved in angiogenesis/inflammation. The effect of LCL-PRV on *pro*-angiogenic protein levels in tumors was much stronger (P= 0.01) than the effect of free PRV. The effects of liposomal and free PRV on the intratumoral production of *pro*-angiogenic proteins and *anti*-angiogenic proteins are shown in more detail in Tables 1 and 2. For 7 out of 17 *pro*-angiogenic proteins studied, reduction was stronger after treatment with LCL-PRV than with free PRV. More specifically, LCL-PRV treatment reduced expression of the *pro*-angiogenic factors M-CSF, IGF-II, IL-1α, IL-1β, leptin (by 30-60%) and IL-6, TNFα, eotaxin, (by 60-90%). Tumor levels of eotaxin were strongly reduced after both treatments (Table 1). The level of the majority of *anti*-angiogenic proteins was only slightly suppressed after liposomal and free PRV treatment (Table 2).

Table 1. Effects of i.v. administered LCL-PRV and free PRV on *pro*-angiogenic protein levels in subcutaneous B16.F10 melanoma in mice

***Pro*-angiogenic factors**	Reduction induced by LCL-PRV (% of reduction as mean±SD)	Reduction induced by free PRV (% of reduction as mean±SD)	Statistical differences
Granulocyte- colony stimulating factor (G-CSF)	23.5 ± 0.1	13.7 ± 2.0	ns
Granulocyte- macrophage-colony stimulating factor (GM-CSF)	17.8 ± 0.8	-12.7 ± 1.5	***
Monocyte-colony stimulating factor (M-CSF)	37.2 ± 4.0	26.6 ± 0.6	ns
Insulin growth factor II (IGF-II)	31.7 ± 0.1	7.4 ± 0.1	***
Interleukin 1α (IL-1α)	32.5 ± 2.0	-0.2 ± 0.5	***
Interleukin 1β (IL-1β)	42.0 ± 0.4	-14.1 ± 3.6	***
Interleukin 6 (IL-6)	60.4 ± 3.9	34.8 ± 4.4	***
Interleukin 9 (IL-9)	18.4 ± 3.3	12.5 ± 1.8	ns
Interleukin 12p40 (IL-12 p40)	1.4 ± 5.7	-5.1 ± 2.0	ns
Tumor necrosis factor α (TNF α)	73.7 ± 11.4	29.6 ± 19.0	***
Monocyte chemoattractant protein-1 (MCP1)	26.0 ± 0.3	17.2 ± 1.0	ns
Eotaxin	82.5 ± 1.6	71.6 ± 1.5	ns
Fas ligand (FasL)	7.8 ± 1.0	2.2 ± 0.3	ns
Basic fibroblast growth factor (bFGF)	15.7 ± 0.2	2.2 ± 0.3	ns
Vascular endothelial growth factor (VEGF)	4.9 ± 3.1	4.1 ± 0.9	ns
Leptin	47.4 ± 8.1	5.2 ± 0.1	***
Thrombopoietin (TPO)	13.5 ± 0.5	8.5 ± 0.0	ns

Pro-angiogenic factors are defined as proteins that are reported in literature to favor angiogenesis and tumor-associated inflammation. The protein levels are compared to control protein levels in tumors from mice treated with PBS. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P-values are indicated as follows: ***ns,*** *not significant (P>0.05), and* *******, P<0.001. The results represent mean±SD of two measurements.*

Table 2. Effects of i.v. administered LCL-PRV and free PRV on *anti*-angiogenic protein levels in subcutaneous B16.F10 melanoma in mice

***Anti*-angiogenic factors**	Reduction induced by LCL-PRV (% of reduction as mean±SD)	Reduction induced by free PRV (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	17.4 ± 10.5	13.0 ± 3.9	ns
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	7.9 ± 4.6	0.4 ± 4.9	ns
Platelet factor 4 (PF4)	17.6 ± 4.0	9.7 ± 1.0	ns
Interleukin 12 p70 (IL-12 p70)	-5.0 ± 0.0	-4.6 ± 0.9	ns
Interleukin 13 (IL-13)	25.3 ± 9.4	9.0 ± 2.9	*
Interferon γ (IFN-γ)	30.8 ± 2.5	6.8 ± 0.2	***
Monokine induced by IFN-γ (MIG)	12.5 ± 10.4	-1.04 ± 6.1	ns

The anti-angiogenic factors are defined as proteins that are reported in literature to impede angiogenesis and tumor-associated inflammation. The protein levels are compared to control protein levels in tumors from mice treated with PBS. The results were analyzed for statistically significant differences between the effects of different treatments on the levels of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P-values are indicated as follows: ***ns,*** *not significant (P>0.05);* *******, P<0.05; and* *********, P<0.001. The results represent mean±SD of two measurements.*

DISCUSSION

LCL-PRV can inhibit growth of s.c. B16.F10 melanoma in mice after a single i.v. administration of 5 mg/kg. At this dose, free PRV failed to inhibit tumor growth. As serum cholesterol levels were unaffected by this dosing schedule, the pleiotropic effects of statins seem responsible for the antitumor activity.

In our *in vitro* cytotoxicity studies, we observed slight inhibitory effects on tumor cells and endothelial cells and stronger inhibitory effects on macrophages in case of (liposomal) PRV. Statins have been shown to induce apoptosis in B16 melanoma cells, albeit that the strongest activity was noted for lovastatin and, similarly as in our studies, PRV failed to affect melanoma cells [17]. The hydrophilic nature of PRV could prevent efficient tumor cell membrane passage. For its effects on lipid metabolism, PRV relies on the hepatic organic anion transporter polypeptide 1B1 to arrive intracellularly in the hepatocytes [18]. The absence of this transporter on melanoma cells may account for the differences in activity. Alternatively, a lower potency of PRV compared to lovastatin could be responsible for the difference. Statins have been reported to have biphasic effects on endothelial cells [19, 20]. They appear to stimulate endothelial cell division, reduce apoptosis, and promote capillary tube formation at low concentrations, but at higher concentrations they inhibit the same processes. At the high concentrations used in this study, we also observed small negative effects of PRV on endothelial cell viability and proliferation, which could contribute to antitumor effects by inhibiting angiogenesis. The macrophage cell line used in this study appeared most sensitive to treatment with LCL-PRV. Previously, a direct inhibitory effect of cerivastatin was demonstrated on macrophage proliferation [21, 22], which appears to be similar to the effects obtained here for PRV.

The observed sensitivity of macrophages *in vitro*, to particularly LCL-PRV, could translate in reduced tumor angiogenesis and inflammation *in vivo*. Macrophages are important for tumor progression as they stimulate (lymph)angiogenesis and metastasis, remodeling of the extracellular matrix, and suppress functions of the adaptive immune system [23, 24]. B16.F10 melanoma tissue isolated from mice contained many *pro*-angiogenic/*pro*-inflammatory cytokines. In line with the lack of antitumor activity, free PRV generally failed to reduce levels of these molecules, but LCL-PRV selectively inhibited certain *pro*-inflammatory cytokines to a much higher degree than the free drug, most notably for M-CSF, IGF-II, IL-1α, IL-1β, leptin, IL-6, and TNF α.

These results support an anti-inflammatory/anti-angiogenic mechanism of antitumor action for LCL-PRV.

Taken together, LCL-PRV locally inhibits expression of tumor–associated *pro*-inflammatory cytokines. This is likely the result of the encapsulation into the LCL. By virtue of the liposome-encapsulation, target tissue accumulation is increased and intracellular uptake by inflammatory cells in the tumor is promoted [12], thereby markedly changing the biological activity of the agent.

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Chapter 8

Investigation into the role of tumor-associated macrophages in the antitumor activity of Doxil

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ABSTRACT

Tumor-associated macrophages (TAM) are most important in driving tumor angiogenesis and inflammation. Our recent studies show specific localization of long-circulating liposomes (LCL) within the endosomal/lysosomal compartment of TAM. Based on this finding, this study aims to investigate whether clinically applied LCL formulations such as Doxil (poly(ethylene glycol) (PEG)-liposomes with encapsulated doxorubicin), may have alternative mechanisms of action additionally to their cytotoxicity towards tumor cells. Our results suggest that the antitumor activity of Doxil does not depend on the presence of functional TAM in tumor tissue, although a mild degree of suppression of TAM-mediated production of angiogenic factors was induced by Doxil.

Keywords: Doxil, tumor-associated macrophages, angiogenic proteins, tumor cells

INTRODUCTION

Poly(ethylene glycol) (PEG)-coated liposomes possess a passive tumor-targeting property [1, 2]. By virtue of the enhanced permeability of the tumor microcirculation, intravenously (i.v.) administered PEG-liposomes can extravasate into tumor tissue. The long-circulating character of PEG-liposomes enables them to exploit this enhanced permeability more efficiently. Once extravasated into the tumor, long-circulating liposomes (LCL) appear to accumulate in the interstitium area surrounding capillaries and tend to be taken up by tumor-associated macrophages (TAM) [3]. TAM are the cell type that drive tumor angiogenesis and inflammation [4-6]. As a result of this natural tropism of LCL for TAM, angiogenic and tumor-associated inflammatory processes are possibly efficiently affected by properly designed LCL-encapsulated drugs. Our previous studies showed that prednisolone phosphate (PLP) encapsulated in LCL (LCL-PLP) exerts strong inhibitory effects on tumor growth via inhibition of tumor angiogenesis in subcutaneous (s.c.) B16.F10 melanoma and C26 colon carcinoma murine tumor models [3, 7]. Recent results strongly suggest that LCL-PLP act via their uptake by TAM leading to suppression of TAM-mediated production of *pro*-angiogenic factors (see Chapter 4). Indeed specific localization of LCL within the endosomal/lysosomal compartment of TAM has been observed [3]. This also raises the question whether clinically applied LCL formulations, such as Doxil™ (Caelyx™ in Europe) (PEG-liposomes with encapsulated doxorubicin), may have alternative mechanisms of action additionally to their cytotoxicity towards tumor cells [8]. Therefore, this study aims to investigate whether the mechanism of antitumor activity of Doxil involves an anti-angiogenic/anti-inflammatory action via potential suppressive effects on functions of TAM in B16.F10 melanoma-bearing mice. It is even not excluded that intracellularly accumulating Doxil particles kill TAM, as it has been reported that doxorubicin-containing liposomes could efficiently deplete part of the liver macrophage population after i.v. administration to rats [9, 10]. To evaluate whether TAM play an important role in the antitumor action of Doxil, clodronate-containing liposomes were used as a tool to deplete macrophages [11, 12]. Clodronate-containing liposomes as macrophage-suppressive agents have already been used in inflammatory and auto-immune diseases, where macrophages have been suggested to be involved in pathological processes [13]. To study the antitumor activity of Doxil against tumors with suppressed TAM functions, tumor-bearing animals were pretreated with clodronate-liposomes before the actual treatment with Doxil.

The effect of Doxil treatment on the levels of *pro*-angiogenic and *anti*-angiogenic factors was determined in B16.F10 melanoma-bearing mice with and without pretreatment with liposomal clodronate (Lip-CLOD). To suppress TAM functions in tumors, clodronate was encapsulated in LCL (mean size around 100 nm) [3, 12]. In addition, to reduce chemoattraction of new monocytes in tumors, clodronate-containing large negatively charged liposomes (mean size around 1 μm) were co-injected [14]. As positive control, the same experiments were conducted with LCL-PLP, a tumor-targeted formulation with known strong anti-angiogenic/anti-inflammatory activity in tumors [7, 15].

MATERIALS AND METHODS

Preparation of LCL-PLP

LCL were prepared as described previously [3]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma, St. Louis, USA), and poly(ethylene glycol) 2000 - distearoylphosphatidylethanolamine (Lipoid GmbH) in a molar ratio of 1.85:1.0:0.15, respectively, were dissolved in ethanol in a round-bottom flask. After lipid film formation, the film was hydrated with a solution of 100 mg/ml prednisolone disodium phosphate (PLP), (obtained from Bufa, Uitgeest, The Netherlands). Liposome size was reduced by multiple extrusion steps through polycarbonate membranes (Nuclepore, Pleasanton, USA) with a final pore size of 50 nm. Mean particle size of the LCL was determined by dynamic light scattering and found to be 0.1 μm with a polydispersity value lower than 0.1. The polydispersity values obtained indicate limited variation in particle size. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of liposomal preparations with chloroform, according to Rouser [16]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. After extraction, the aqueous phase was used for determining the glucocorticoid phosphate content by high performance liquid chromatography as described previously [17]. The type of column was RP18 (5 μm) (Merck) and the mobile phase consisted of acetonitril and water (1:3 v/v), pH 2. The eluent was monitored with an ultraviolet detector set at 254 nm. The detection limit for the high performance liquid chromatography setup was 20 ng/ml. The liposomal preparation contained about 5 mg PLP /ml and ~60 μmol phospholipid/ml.

Preparation of clodronate-containing liposomes

To deplete TAM, clodronate-containing LCL [3, 12] (mean size about 100 nm) were prepared as described previously for LCL-PLP. After lipid film formation, the film was hydrated with a solution of dichloromethylene bisphosphonate, disodium clodronate (Bonefos™ infusion (conc. 60 mg/ml)), (obtained from Schering, Weesp, The Netherlands). To reduce recruitment of new monocytes in tumors, large negatively charged liposomes (mean size about 1 μm) were used [14]. Appropriate amounts of egg phosphatidylcholine and egg phosphatidylglycerol (both obtained from Lipoid GmbH, Ludwigshafen, Germany) cholesterol (Sigma, St. Louis, USA) in a molar ratio of

1.85:0.3:1 were dissolved in ethanol. The hydration of lipid film was performed with 10 ml of clodronate or Bonefos infusion (60 mg/ml). Liposomes were extruded twice through a filter with a pore size of 8 μm. Phospholipid content was determined with a phosphate assay, performed on the organic phase after extraction of liposomal preparations with chloroform, according to Rouser [16]. Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette with a molecular weight cut-off of 10 kDa at 4°C with repeated changes of buffer. After extraction, the aqueous phase was used for determining the clodronate content by ultraviolet spectrophotometry at 238 nm after formation of clodronate complex with $CuSO_4$ solution [18]. Both types of liposomes contained about 5 mg clodronate/ml and ~70 μmol phospholipid/ml.

Cells

B16.F10 murine melanoma cells were cultured as monolayers at 37°C in a 5% CO_2-containing humidified atmosphere in DMEM medium (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).

Murine tumor model

Male C57Bl/6 mice (6 – 8 weeks of age) were obtained from Charles River (The Netherlands) and 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 the national regulations and were approved by the local animal experiments ethical committee. For tumor induction, 1 x 10^6 B16.F10 melanoma cells were inoculated subcutaneously (s.c.) in the right flank of syngeneic C57Bl/6 mice. B16.F10 tumors became palpable at day 7 after tumor cell inoculation.

Effect of liposomal clodronate pretreatment on the antitumor activity of Doxil

At 7 days after tumor cell inoculation, tumor size was measured and tumor volume calculated according to the formula $V = 0.52 \times a^2 \times b$, in which a is the smallest and b, the largest superficial diameter (in mm).

The effect of Doxil and free doxorubicin in presence or absence of liposomal clodronate pretreatment on the growth of B16.F10 melanoma in mice were studied.

As positive control, the same experiments were conducted with LCL-PLP, a tumor-targeted formulation with known strong anti-angiogenic/anti-inflammatory activity in tumors [7, 15].

To suppress macrophages a mixture of both clodronate liposomes (ratio 1:1 (w/w)) (Lip-CLOD) at a dose of 25 mg/kg [19] was injected i.v. at day 7 (when tumors became palpable). Doxil and free doxorubicin were administered i.v. at a dose of 2 mg/kg at days 8 and 11 after tumor cell inoculation. LCL-PLP and free PLP at a dose of 20 mg/kg were injected i.v. using the same dosing schedule as for Doxil. As controls, tumor-bearing mice treated with PBS which did not receive Lip-CLOD treatment were used. 4-5 animals were used per experimental group. On day 12, mice were sacrificed and tumor volumes were measured.

Effect of liposomal clodronate pretreatment on the anti-angiogenic actions of Doxil

To assess the effect of Doxil on TAM-mediated production of angiogenic factors, the same experimental setup as described above for testing antitumor activity of Doxil was used. At day 12 after tumor cell inoculation, mice were sacrificed and tumors were isolated. A screening of angiogenic proteins present in tumor tissues was performed using an angiogenic protein array (RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1 (RayBiotech Inc. Norcross, GA)), according to manufacturers instructions [11]. Each membrane contains 24 types of primary antibodies against certain angiogenic proteins. The tumor tissues for each group were lysed in 30 min with cell lysis buffer (RayBiotech), containing protease inhibitor cocktail (Sigma). After obtaining the pooled tumor tissue lysates, the protein content of the lysates was determined according to Peterson [12]. Subsequently, the array membrane was subjected to different incubation steps, each for 2h at room temperature followed by five washing-steps. First, the array membrane was incubated with 250 µg of protein from tissue lysates. Each membrane was incubated with a mixture of secondary biotin-conjugated antibodies, after which membranes were incubated with HRP-conjugated streptavidin. Thereafter, membranes were incubated with a mixture of two detection buffers (RayBiotech) for 1 minute. X-ray film was exposed to the membranes for 4 minutes and then the film was developed. The experiment was perfomed in duplicate. Protein levels were quantified measuring the color intensity of each spot using GelPro Analyzer software, version 3.1, in comparison to positive

control spots already bound to the membrane. Angiogenic protein levels in tumors were expressed as percentage of the levels of the same proteins in tumors from mice treated with PBS. The final results represent mean±SD of two measurements.

Statistical Analysis

Data from different experiments were reported as mean±SD. For statistical analysis, Student's *t*- test for independent means was used. A value of $P<0.05$ was considered significant. The differences between the overall effects of different treatments on tumor growth were analyzed by one-way ANOVA with Dunnett's Multiple Comparison Test. The differences between the effects of different treatments on angiogenic factor levels were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Effect of Lip-CLOD pretreatment on antitumor activity of Doxil

To investigate whether the antitumor activity of Doxil on B16.F10 melanoma model is dependent on the presence of TAM in tumor tissue, B16.F10 melanoma-bearing mice were pretreated with Lip-CLOD before i.v. administration of Doxil. The purpose of Lip-CLOD pretreatment is to create suppressed TAM functioning in the tumor before treatment with Doxil. To this end, we prepared a mixture of two types of clodronate liposomes (Lip-CLOD) in a ratio 1:1 (w/w). To deplete TAM, clodronate was encapsulated in LCL (mean size about 100 nm) [3, 12]. In addition, to reduce chemoattraction of new monocytes in tumors, clodronate-containing large negatively charged liposomes (mean size about 1 μm) were co-injected [14]. The capability of Lip-CLOD to suppress TAM activity after i.v. administration of 25 mg/kg at day 7 after B16.F10 melanoma cell inoculation has been shown by us earlier (see Chapter 4). Doxil treatment started 24h after Lip-CLOD pretreatment and involved an i.v. dose of 2 mg/kg at days 8 and 11 after tumor cell inoculation. In addition, a separate animal group was treated with LCL-PLP used as positive control, as it has been shown recently that the antitumor activity of LCL-PLP is largely based on inhibition of tumor angiogenesis and inflammation [7]. LCL-PLP treatment (i.v. dose of 20 mg/kg at days 8 and 11 after tumor cell inoculation) started also 24h after Lip-CLOD pretreatment. The antitumor activity of Doxil was compared to that of LCL-PLP, in the presence and in the absence of Lip-CLOD pretreatment (Figures1 and 2).

When Lip-CLOD pretreatment was not given, Doxil treatment alone inhibited tumor growth by 80% ($P<0.01$) compared to the growth of control tumors (tumors in mice receiving only PBS). Similar inhibition of tumor growth (by 70%, $P<0.01$) was induced by LCL-PLP treatment (Figure 1).

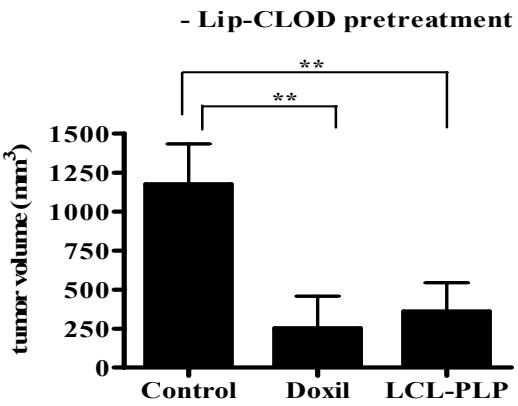

Figure 1. **Antitumor activity of Doxil and LCL-PLP in B16.F10 murine melanoma model when animals were not pretreated with Lip-CLOD.** Tumor volumes at day 12 (day of sacrification) were compared to volumes of tumors from mice treated only with PBS. One-way ANOVA with Dunnett's Multiple Comparison Test was used; **, P<0.01. The results represent mean±SD of 4-5 mice. -Lip-CLOD = no pretreatment with Lip-CLOD, Control= treatment with PBS, Doxil= treament with Doxil, LCL-PLP= treatment with LCL-PLP

Lip-CLOD alone (i.e. not followed by Doxil or LCL-PLP treatment) inhibited tumor growth by 65% (P<0.05) compared to control tumors. When Lip-CLOD administration is followed by LCL-PLP treatment, no additional growth inhibitory effect was seen (Figure 2). When Lip-CLOD pretreatment is followed by Doxil treatment, clearly Doxil had a strong additional antitumor effect (by 73% *P*=0.0436) when compared to that induced by Lip-CLOD-treated animals (Figure 2).

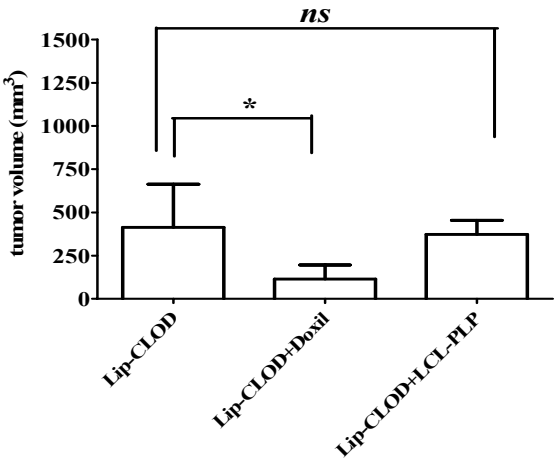

Figure 2. Effect of Lip-CLOD pretreatment on antitumor activity of Doxil and LCL-PLP in murine B16.F10 melanoma model. All groups were pretreated with Lip-CLOD 24h before the actual treatment. Tumor volumes at day 12 (day of sacrification) were compared to tumor volumes in mice treated with PBS. One-way ANOVA with Dunnett's Multiple Comparison Test was used; ***ns*,** not significant ($P>0.05$); *****, $P<0.05$. The results represent mean±SD of 4-5 mice. Lip-CLOD= treatment only with Lip-CLOD, Lip-CLOD+Doxil= pretreament with Lip-CLOD followed by Doxil treatment, Lip-CLOD+LCL-PLP= pretreament with Lip-CLOD followed by LCL-PLP treatment

Effect of Doxil on angiogenic protein production; influence of Lip-CLOD pretreatment

To assess the effect of Doxil on angiogenic protein production in the B16.F10 melanoma model, with and without Lip-CLOD pretreatment, angiogenic protein levels in tumor tissue were studied. A screening of 24 angiogenic proteins involved in angiogenesis, inflammation and apoptosis present in tumor tissue was performed using an angiogenic protein array of RayBio® Mouse Angiogenic protein Antibody Array membranes 1.1 (RayBiotech Inc.Norcross, GA) [20]. The effect of TAM suppression on the production of angiogenic factors was verified in tumors from mice which were treated with Lip-CLOD alone (i.e. not followed by Doxil or LCL-PLP administration). Again LCL-PLP was used as positive control, as this tumor-targeted formulation has strong reducing effects on production of *pro*-angiogenic/*pro*-inflammatory factors in tumors [7].

When Lip-CLOD pretreatment was not administered, Doxil reduced the level of the majority of intratumoral *pro*-angiogenic factors only slightly (Table 1 and Figure 3). The strongest reduction after Doxil treatment was noted for eotaxin, FasL, and VEGF (by 50-70%).

Table 1. Effects of i.v. administered Doxil and LCL-PLP on *pro*-angiogenic protein levels in s.c. B16.F10 melanoma when Lip-CLOD pretreatment was not given

***Pro*-angiogenic factors**	Reduction induced by Doxil (% of reduction as mean±SD)	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Statistical differences
Granulocyte-colony stimulating factor (G-CSF)	42.6 ± 4.0	85.2 ± 1.1	***
Granulocyte-macrophage-colony stimulating factor (GM-CSF)	7.3 ± 2.0	73.4 ± 3.0	***
Monocyte-colony stimulating factor (M-CSF)	8.0 ± 1.0	50.0 ± 0.1	***
Insulin growth factor II (IGF-II)	24.5 ± 5.3	70.5 ± 0.7	***
Interleukin 1α (IL-1α)	34.3 ± 0.0	69.1 ± 0.6	***
Interleukin 1β (IL-1β)	17.2 ± 6.4	76.2 ± 5.2	***
Interleukin 6 (IL-6)	21.5 ± 9.2	69.9± 1.2	***
Interleukin 9 (IL-9)	15.5 ± 2.4	47.4 ± 9.2	***
Interleukin 12p40 (IL-12 p40)	8.9 ± 1.6	63.0± 1.3	***
Tumor necrosis factor α (TNF α)	37.3 ± 2.3	22.0 ± 9.8	**
Monocyte chemoattractant protein-1 (MCP1)	6.3 ± 1.2	47.6± 0.2	***
Eotaxin	55.5 ± 2.6	96.4 ± 5.1	***
Fas ligand (FasL)	66.1 ± 8.3	86.0 ± 8.9	***
Basic fibroblast growth factor (bFGF)	39.7 ± 3.5	52.6 ± 1.8	*
Vascular endothelial growth factor (VEGF)	62.5 ± 0.5	7.0 ± 4.3	***
Leptin	24.7 ± 0.7	14.9 ± 0.4	ns
Thrombopoietin (TPO)	38.0 ± 0.9	3.3 ± 2.0	***

Pro-angiogenic factors are defined as proteins reported in literature to favor angiogenesis and tumor-associated inflammation. The protein levels are compared to protein levels in control tumors (tumors from mice treated with PBS when the Lip-CLOD pretreatment was not given). The results were analyzed for statistically significant differences between the effects of Doxil and LCL-PLP on the levels of pro-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05) *, P<0,05, **,P<0.01, and ***, P<0.001. The results represent mean±SD of two measurements.*

The production of most of the *anti*-angiogenic factors in tumors was also only slightly affected by the Doxil treatment (Table 2 and Figure 3).

Table 2. Effects of i.v. administered administered Doxil and LCL-PLP on *anti*-angiogenic protein levels in s.c. B16.F10 melanoma when Lip-CLOD pretreatment was not given

***Anti*-angiogenic factors**	Reduction induced by Doxil (% of reduction as mean±SD)	Reduction induced by LCL-PLP (% of reduction as mean±SD)	Statistical differences
Tissue inhibitor of metalloproteinase 1 (TIMP-1)	38.1 ± 7.4	8.4 ± 1.5	***
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	40.5 ± 2.5	17.6 ± 1.2	***
Platelet factor 4 (PF4)	19.4 ± 0.8	6.7 ± 2.5	ns
Interleukin 12 p70 (IL-12 p70)	14.2 ± 1.0	4.3 ± 0.1	ns
Interleukin 13 (IL-13)	40.5 ± 2.5	27.0 ± 4.3	*
Interferon γ (IFN-γ)	36.0 ± 1.0	60.2 ± 3.6	***
Monokine induced by IFN-γ (MIG)	38.7 ± 0.6	25.6 ± 4.4	*

The anti-angiogenic factors are defined as proteins reported in literature to impede angiogenesis and tumor-associated inflammation. The protein levels are compared to protein levels in control tumors (tumors from mice treated with PBS when the Lip-CLOD pretreatment was not given). The results were analyzed for statistically significant differences between the effects of Doxil and LCL-PLP on the levels of anti-angiogenic factors. A two-way ANOVA with Bonferroni correction for multiple comparisons was used and the P values are indicated as follows: ***ns,*** *not significant (P>0.05); *, P<0.05 and ***, P<0.001. The results represent mean±SD of two measurements.*

The LCL-PLP formulation, however, exerted strong reducing effects on the intratumoral *pro*-angiogenic protein production, in the absence of Lip-CLOD pretreatment (Figure 3). More specifically, LCL-PLP treatment reduced expression of the *pro*-angiogenic factors GM-CSF, M-CSF, IGF-II, IL-1α, IL-6, IL-12p40, bFGF (by 50-75%), and G-CSF, IL-1β, eotaxin, FasL (by 75-100%) (Table 1). The reduction exerted by LCL-PLP on most of the *pro*-angiogenic proteins was much stronger (P=0.0086) when compared to the result obtained with Doxil (Figure 3). The level of the majority of *anti*-angiogenic proteins was slightly suppressed after LCL-PLP (Table 2 and Figure 3). Only the intratumoral production of IFNγ was strongly reduced (by 60%) by LCL-PLP treatment.

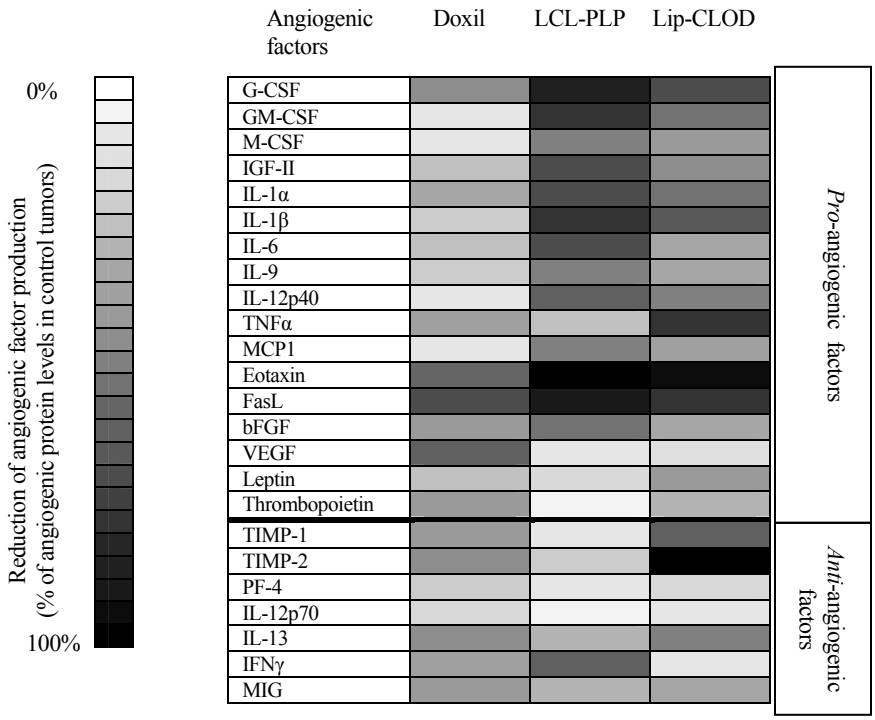

Figure 3. Comparison of the anti-angiogenic actions of Doxil, LCL-PLP, and Lip-CLOD in murine B16.F10 melanoma model. Results are presented as % reduction of the levels of tumor angiogenic factors ranging from 0% (white) to 100% (black) compared to the level of angiogenic factors in control tumors. Control tumors are defined as tumors from mice treated only with PBS. Doxil= treatment only with Doxil; LCL-PLP= treatment only with LCL-PLP; Lip-CLOD= treatment only with Lip-CLOD

Lip-CLOD treatment alone strongly reduced the level of most of the *pro*-angiogenic tumor proteins (on average by 50%, $P<0.0001$ compared to control tumors) (Figure 3 and 4). Interestingly, the level of two *anti*-angiogenic factors (TIMP-1 and TIMP-2) was also strongly reduced (about 60-100%, $P<0.0001$) (Figure 3). When Lip-CLOD pretreatment was given, Doxil treatment had no additional reducing effects on *pro*-angiogenic and *anti*-angiogenic factor levels (Figures 4 and 5). However, when Lip-CLOD pretreatment was followed by treatment with the positive control, LCL-PLP formulation, the reduction of *pro*-angiogenic protein production was somewhat enhanced (by 16%, $P= 0.0001$) (Figure 4). No additional effect of LCL-PLP on the level of *anti*-angiogenic tumor proteins was seen (Figure 5).

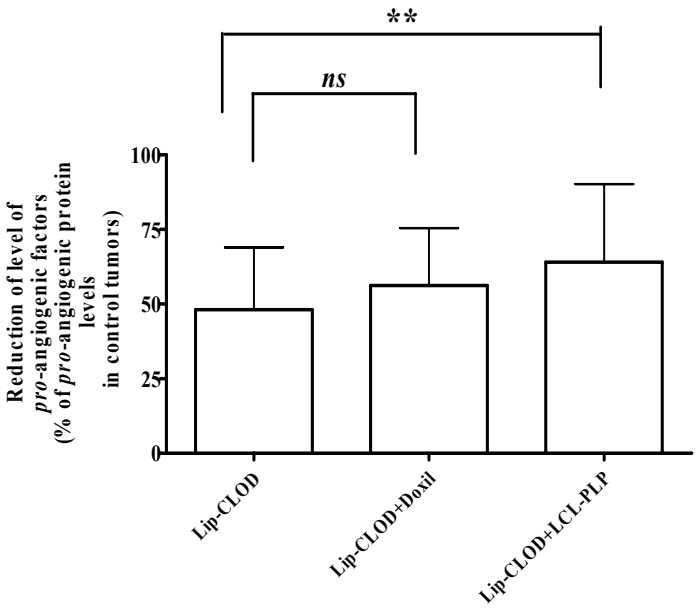

Figure 4. The influence of Lip-CLOD pretreatment on the effect of Doxil and LCL-PLP on production of *pro*-angiogenic factors in tumors. Results are presented as % average reduction of the level of tumor *pro*-angiogenic factors compared to the level of *pro*-angiogenic factors in control tumors. Control tumors are defined as tumors from mice not treated with Lip-CLOD but treated with PBS. Mean±SD; n= 17 *pro*-angiogenic factor levels determined in duplicate per experimental group, Lip-CLOD= treatment only with Lip-CLOD, Lip-CLOD+Doxil= pretreament with Lip-CLOD followed by Doxil treatment, Lip-CLOD+LCL-PLP= pretreament with Lip-CLOD followed by LCL-PLP treatment. One-way ANOVA with Dunnett's Multiple Comparison Test was used; ***ns*,** not significant ($P>0.05$), and**, $P<0.01$.

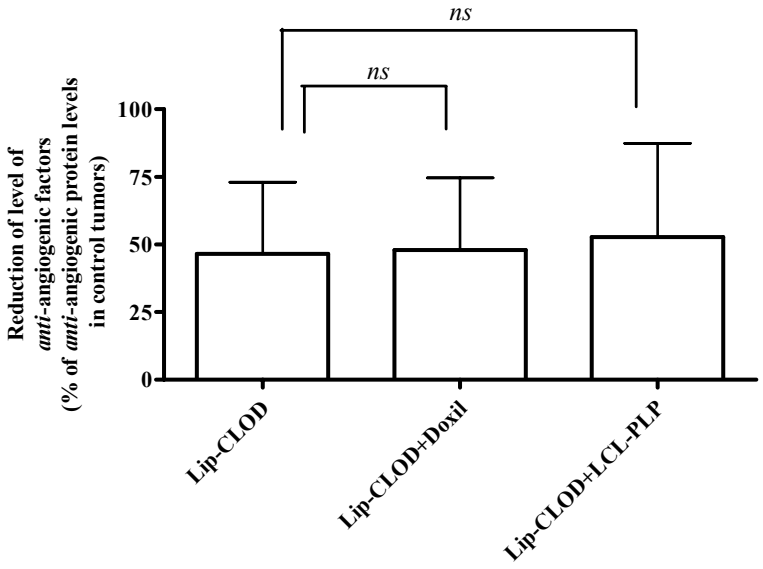

Figure 5. The influence of Lip-CLOD pretreatment on the effect of Doxil and LCL-PLP on production of *anti*-angiogenic factors in tumors. Results are presented as % average reduction of the level of tumor *anti*-angiogenic factors compared to the level of *anti*-angiogenic factors in control tumors. Control tumors are defined as tumors from mice not treated with Lip-CLOD but treated with PBS. Mean±SD; n= 7 *anti*-angiogenic factor levels determined in duplicate per experimental group, Lip-CLOD= treatment only with Lip-CLOD, Lip-CLOD+Doxil= pretreament with Lip-CLOD followed by Doxil treatment, Lip-CLOD+LCL-PLP= pretreament with Lip-CLOD followed by LCL-PLP treatment. One-way ANOVA was used; ***ns*,** not significant (P>0.05).

DISCUSSION

Doxil is a commercially available LCL formulation containing the well-known antitumor agent, doxorubicin. It has been shown to enhance significantly the doxorubicin levels in tumor and thereby antitumor activity, in various mouse and human xenograft tumor models [10, 21-24]. This study aimed to investigate whether the mechanism of the antitumor activity of Doxil involves inhibition of tumor angiogenesis through suppressive and possibly even lethal effects on TAM, as i.v. administered LCL extravasating in solid tumors have been shown to substantially localize in TAM [3]. The anti-angiogenic actions of Doxil were compared with those induced by LCL-PLP, a tumor-targeted formulation with known strong anti-angiogenic/anti-inflammatory activity in tumors [7, 15]. Recent studies reported that i.v. administration of LCL-PLP results in strong inhibition of tumor growth [3]. The mechanism of antitumor action of LCL-PLP appeared to be primarily based on a reduction of intratumoral level of *pro*-angiogenic factors [7]. The anti-angiogenic effects exerted by LCL-PLP are enabled by the tumor-targeting capability of LCL, which is a combined result of the long circulation time of the liposomal formulation and the enhanced permeability of tumor vasculature as compared to healthy endothelium [7, 25]. LCL can extravasate through the permeable pathological vasculature and thereby accumulate into the malignant tissue (referred to as the "enhanced permeability and retention (EPR) effect") [15]. Once extravasated into the tumor, LCL were observed to localize in the immediate vicinity of tumor blood vessels and in the endosomal/lysosomal compartment of TAM [3]. It is known that TAM have a main role in tumor growth progression being an important source of inflammatory and angiogenic factors involved in all steps of tumor angiogenesis [11, 25-28].

To investigate whether Doxil in addition to direct cytotoxic effects on tumor cells, also exerts antitumor activity via suppression of TAM, we investigated the effects of pretreatment with Lip-CLOD [12, 29, 30] on the antitumor activity of Doxil. Previous studies already showed the feasibility of clodronate encapsulated in liposomes for suppression of TAM activity from s.c. tumor tissue [11]. As already shown in Chapter 4, Lip-CLOD alone inhibited strongly tumor growth. Furthermore, Lip-CLOD induced strong reduction of the production of most of the *pro*-angiogenic factors as well as of certain *anti*-angiogenic factors (Figure 3). These results clearly suggest that TAM play a vital role in tumor growth by producing angiogenic factors critical for tumor growth progression.

In the absence of Lip-CLOD pretreatment both Doxil and LCL-PLP had strong tumor growth inhibitory effect. Tumor growth was inhibited by 70-80% compared to the growth of control tumors (Figure 1).

In the presence of Lip-CLOD pretreatment, Doxil had a strong additional antitumor effect. However, an additional antitumor effect was not observed in case of LCL-PLP treatment (Figure 2).

With Lip-CLOD already establishing antitumor activity via TAM suppression, the lack of any additional effect induced by the subsequent LCL-PLP treatment suggests that the LCL-PLP localizing in the tumor area is not able to further downregulate the functioning of the already suppressed TAM, illustrating the effectiveness of the Lip-CLOD treatment. The observation that Doxil though is able to induce additional tumor growth inhibition, would indicate that the antitumor activity does not depend on the presence of functional TAM in tumor tissue, and that Doxil is killing tumor cells via other mechanisms, such as direct cytotoxic effects of doxorubicin on the tumor cells [31-33].

This suggestion derived from the tumor growth inhibition results is confirmed by the results obtained at the level of the intratumoral production of angiogenic proteins. Lip-CLOD treatment alone appeared to strongly reduce the production of particularly the *pro*-angiogenic factors, which is in good agreement with its potent antitumor activity (Figure 3). LCL-PLP treatment alone induces a similar strong degree of suppression, albeit the intensity of the suppressive effect of both formulations varies with the type of angiogenic factor. If the LCL-PLP is administered after the anti-angiogenic Lip-CLOD treatment, only a slight additive effect was seen, which is in line with the similar anti-angiogenic mode of action of LCL-PLP via suppressive effects on TAM (Figure 4). Doxil, however, was much less effective in reducing the angiogenic protein levels as compared to LCL-PLP and Lip-CLOD, suggesting that the strong antitumor activity of Doxil is not caused by TAM-related effects, although the mild degree of suppression of angiogenic factor production observed might have been caused by Doxil particles localizing in TAM and inhibiting their function. That Doxil mainly acts via direct cytotoxic effects on tumor cells, is indicated by the observation that only Doxil treatment was able to strongly reduce the intratumoral level of VEGF, a key angiogenic protein produced in high amounts by melanoma cells [34]. Both the anti-angiogenic LCL-PLP and Lip-CLOD formulations did not show this reducing effect on VEGF, supporting that both formulations lack direct cytotoxic effects on melanoma cells.

In conclusion, the present data suggest that the antitumor activity of Doxil in the B16.F10 melanoma tumor model is not dependent on the presence of functional TAM in tumor tissue. Although localization of extravasated Doxil particles in TAM is a realistic possibility, the antitumor activity is likely for a large part based on other mechanisms. Doxorubicin may be released from extracellularly localized Doxil particles and subsequently entering tumor cells [35, 36]. In addition, Doxil particles are likely being taken up by TAM [3]. Intracellular processing of Doxil particles within TAM involves degradation of the LCL bilayers within the endosomal/lysosomal compartment. This degradation process likely leads to liberation of doxorubicin molecules within TAM [31]. As they have been reported to be chemically stable in the harsh environment encountered, they may pass cellular membranes, and act intracellularly by inhibiting the functionality and even viability of TAM explaining the observed mild suppressive effects on the production of angiogenic proteins. Alternatively, the liberated doxorubicin molecules may be released in the extracellular tumor interstitium, followed by passive diffusion into tumor cells, and in this way contributing to the cytotoxicity of Doxil towards tumor cells [31-33].

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Chapter 9

Summarizing discussion

INTRODUCTION

Glucocorticoids (GC) are a class of steroid hormones that bind the glucocorticoid receptor. In vertebrates, glucocorticoid receptors are present in nearly all cells and these receptors regulate a variety of physiological processes. As pharmacological agents, GC are best known for their anti-inflammatory and immunosuppressive actions. In the 1980's, studies were published that showed that GC could also inhibit solid tumor growth in experimental tumor models [1]. When evaluating the preclinical antitumor studies with GC, it appears that antitumor effects are achieved at substantially higher doses than the doses needed to achieve inhibition of an extra-tumoral inflammatory response. More specifically, to obtain antitumor effects cumulative weekly doses are in the range of 500-700 mg/kg [1-3]. In contrast, their effects on inflammatory disorders like rheumatoid arthritis are achieved at doses approx. 100-1000-fold lower (Figure 1).

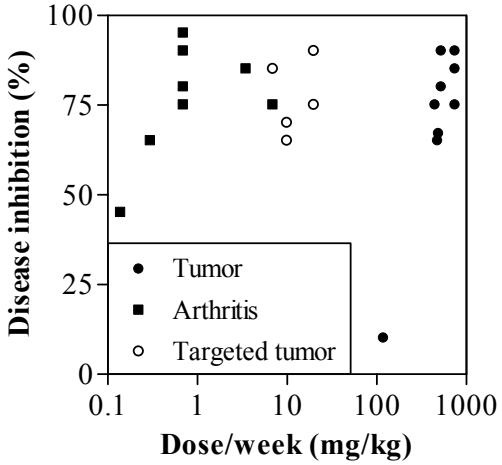

Figure 1. Inhibition of tumor growth or arthritis in rodent disease models by corticosteroids. Cumulative weekly doses of glucocorticoids with corresponding effects on disease are shown. Closed symbols represent untargeted drugs, the open symbols show the enhancement of anti-tumor efficacy achieved by selective delivery of the glucocorticoids to the tumor. (Results are adapted from the following references: [2-16])

The high doses of free GC needed to achieve therapeutic effects in the antitumor studies even resulted in death of some of the animals due to opportunistic infections, indicating that severe systemic side effects occur at these dose levels [3]. As GC are rapidly cleared from the circulation and localize poorly at tumor sites, targeted delivery of GC to tumor tissue is an attractive strategy to increase intratumoral drug concentrations to intensify the antitumor effects [17]. In this manner, it may be feasible to achieve antitumor effects at clinically relevant doses.

Long-circulating liposomes (LCL) are currently the only targeting system being explored for this purpose as is discussed in **Chapter 2**. For efficient delivery of GC into tumors after intravenous treatment, 100 nm-sized liposomes coated with poly(ethylene glycol) (PEG) were used. The long-circulation property and small size allow them to exploit the enhanced permeability and retention-effect. This effect, which is based on the hyperpermeability of tumor vasculature compared to healthy endothelium is driving preferential tumor localization of small sized long-circulating colloidal drug carriers [18-21]. Using LCL delivery, antitumor activity of GC was already obtained at doses that were approximately 25 to 50-fold lower than for the free GC (Figure 1). The primary aim of this thesis was to investigate the mechanisms of tumor growth inhibition by GC encapsulated in LCL (LCL-GC). An overview of the possible mechanisms of glucocorticoid action that could be involved in tumor growth inhibition is also provided in **Chapter 2**. In addition to the postulated direct effects on tumor angiogenesis, the beneficial effects of inhibiting several pro-inflammatory pathways were discussed.

ANTITUMOR ACTIVITY OF LONG-CIRCULATING LIPOSOME-ENCAPSULATED GLUCOCORTICOIDS

Angiogenic factors

Chapter 3 focused on the effects of long-circulating liposome-encapsulated prednisolone phosphate (LCL-PLP) and free PLP on angiogenic protein production in vivo in tumor tissue and on viability and proliferation of tumor cells and endothelial cells in vitro. The most important effect of LCL-PLP appeared to be a strong reduction of most pro-angiogenic protein levels, whereas the levels of the majority of anti-angiogenic proteins were not affected.

These beneficial effects were specific for the liposome formulation as the free drug affected the pro-angiogenic protein levels to a much lesser extent. This is likely the result of the enhanced delivery of PLP to the tumor tissue increasing tumor drug levels several orders of magnitude [8]. In addition, the *in vitro* studies pointed to a strong anti-proliferative effect of LCL-PLP on endothelial cells. Again, free PLP showed limited effects. It may be regarded as counterintuitive that the free drug incubated with cells shows minor effects as compared to the PEGylated liposome-encapsulated form that lacks a signal for cellular uptake. We have shown that the level of phosphatases in the incubation medium is sufficient to activate the free PLP pro-drug (unpublished observations). This excludes lack of cleavage of the phosphate group as a possible explanation. Alternative explanations might be circumvention of inactivating interactions of PL(P) in the incubation medium as a result of liposome encapsulation or a differential cellular processing of liposomal and free drug resulting in stronger effects for PLP delivered by the liposomal route. As the effects are clearest at later time points (72h), loss of the PEG-lipid facilitating cellular uptake of liposomes may contribute to enhanced intracellular drug levels [22]. Taken together, these results pointed to a strong inhibitory effect of LCL-PLP on tumor angiogenesis by reduction of the intratumoral production of several important *pro*-angiogenic factors and a direct inhibition of endothelial cell proliferation.

Tumor-associated macrophages

The effects on *pro*-angiogenic factors, together with our observation that LCL localize in the immediate vicinity of tumor blood vessels and are taken up by macrophages via the endosomal/lysosomal route [5], stimulated focused investigations on the role of tumor–associated macrophages (TAM) in the therapeutic activity of LCL-GC in **Chapter 4**. Among the immune cell populations present in tumor tissue, TAM are most important in promoting and coordinating tumor growth by driving tumor angiogenesis and inflammation [23-26] and they are an important source of the *pro*-inflammatory and *pro*-angiogenic factors that were shown to be inhibited by LCL-PLP [19, 23-25, 27, 28].

To evaluate whether TAM play a crucial role in B16.F10 melanoma growth, mice were treated with liposomal clodronate (Lip-CLOD). Liposomally delivered clodronate has an ability to deplete macrophages [29].

Previous studies already showed the feasibility of clodronate encapsulated in liposomes for elimination of TAM from s.c. tumor tissue [30]. In our study, a mixture of two types of clodronate liposomes (Lip-CLOD) was used: LCL-encapsulated clodronate [5, 29] to deplete TAM and large negatively charged liposomes to prevent chemoattraction of blood monocytes into tumor tissue [31]. Our results show strong antitumor effects induced by Lip-CLOD treatment, which indeed supports a net promoting role of TAM in the growth of B16.F10 melanoma. Likely, TAM stimulate B16.F10 melanoma growth via the production of *pro*-angiogenic factors as these factors were suppressed after Lip-CLOD treatment. A particular finding was that Lip-CLOD treatment also strongly reduced the tumor levels of two *anti*-angiogenic factors TIMP-1 and TIMP-2, which are also produced by TAM. This observation supports the dual role of TAM in tumor development where they fulfill both tumor promoting and tumor inhibiting roles.

To evaluate the involvement of TAM in the antitumor action of LCL-PLP, animals were pretreated with Lip-CLOD and subsequently received LCL-PLP treatment. In the presence of TAM, LCL-PLP strongly inhibited tumor growth whereas depletion of TAM abolished this inhibitory effect. These results indicate that the antitumor effects of LCL-PLP are enabled by their inhibitory effects on TAM-mediated production of *pro*-angiogenic factors in tumors. These suppressive effects are supported by immunohistochemical observations. The microscopic images of macrophage antigen F4/80-stained tumor sections show that in tumors from mice treated with LCL-PLP, macrophages are clustered in small spots, which could be the result of limited chemo-attraction and infiltration by suppression of chemoattractant production in tumors [27, 32, 33]. Interestingly, in the presence of TAM, LCL-PLP treatment did not affect levels of TIMP-1 and TIMP-2 whereas the production of these two *anti*-angiogenic factors was drastically reduced after TAM depletion. This result indicates that LCL-PLP preferentially affects pro-inflammatory/pro-angiogenic pathways in TAM as compared to Lip-CLOD treatment. In conclusion, LCL-PLP had a strong inhibitory effect on the level of *pro*-angiogenic factors produced mainly by TAM whereas the *anti*-angiogenic function of these cells was less affected.

Antitumor effects of different LCL-GC

To evaluate whether the antitumor activity of LCL-PLP can be generalized to other types of GC, we investigated the effects of other GC encapsulated in LCL in **Chapters 5** and **6**. We selected three synthetic GC (as disodium salts of the phosphate derivatives to ensure stable encapsulation), i.e. budesonide disodium phosphate (BUP), dexamethasone disodium phosphate (DXP), and methylprednisolone disodium phosphate (MPLP). Together with PLP, these GC differ in their potency to activate the human glucocorticoid receptor in the following order: budesonide > dexamethasone > methylprednisolone ~ prednisolone [34].

To gain more insight into the mechanism of activity of the different LCL-GC formulations, **Chapter 5** investigated the production of *pro*- and *anti*-angiogenic /inflammatory factors in tumor tissue as well as the viability and proliferation of tumor cells and endothelial cells *in vitro*. Except for LCL-MPLP, all LCL-GC formulations strongly inhibited tumor growth. Among the four LCL-GC types studied, LCL-BUP show the highest antitumor activity, which seems related to the strong potency of this GC to reduce the production of *pro*-angiogenic/*pro*-inflammatory factors in tumors. In addition, the *in vitro* results suggest that LCL-BUP has a strong cytotoxic effect on B16.F10 melanoma cells, which may contribute to the overall antitumor effect. Apart from GC-potency, other factors appear to play a role. LCL-MPLP performed worse than LCL-PLP whereas similar or better effects would have been expected based on the relative potency of both [35-38]. Furthermore, antitumor activity of LCL-DXP was equivalent to that of LCL-PLP, although the relative potency of dexamethasone is much higher than that of prednisolone [36]. These observations could be related to a stronger reduction of *anti*-angiogenic protein production by LCL-DXP when compared to LCL-PLP, and a much higher degree of inhibition of COX-2 by LCL-PLP when compared to LCL-DXP or LCL-MPLP.

In **Chapter 6** the antitumor activity of these four LCL-GC is investigated and related to the potency of each GC. In addition, we quantified the GC content in tumor tissue to correlate antitumor activity with the amount of GC delivered to the tumor. Adverse effects that are typical for systemic GC treatment were also assessed. It appeared that the tumor levels of each GC is governed by the transport capacity of the liposomes and are not affected by the clearance profile of the individual drug. The differences in the antitumor efficacy of liposomal GC were strongly correlated to GC potency: BUP

coupled highest potency to highest antitumor activity, but also demonstrated the highest risk for occurrence of side effects. At 3 mg/kg LCL-BUP was highly efficacious without the occurrence of adverse effects.

ALTERNATIVE ANTI-INFLAMMATORY AGENTS ENCAPSULATED IN LCL

The strong antitumor activity of LCL-GC based on angiogenesis/inflammation inhibition offers promise to create novel tumor-targeted therapeutics, which inhibit other pathways that drive tumor angiogenesis and inflammation [39, 40] (Table 1).

Table 1 shows that a wide variety of drug classes have been investigated for antitumor effects based on an anti-inflammatory mechanism of action. It also shows that inflammation can be inhibited along many different pathways [41].

Table 1. Drug classes with anti-inflammatory properties investigated for antitumor effects. *Different classes of clinically applied drugs with different anti-inflammatory actions that have been investigated for cancer treatment are shown. This list is not complete, many more exist. Interestingly, many drugs with a primary activity not related to inflammation (e.g. cholesterol lowering in cardiovascular diseases for statins) also possess some anti-inflammatory activity.*

Class *(example)*	**Mechanism of action** *(reference)*
NSAIDs *Aspirin, Ibuprofen*	Inhibition of cyclooxygenase (COX)-1/-2/-3/Inhibition of 5-lipoxygenase/Newly identified mechanisms like neutralization of radicals, generation of resolvins and protectins [42]
COX-2-inhibitors *Celecoxib*	Inhibition of COX-2/Newly identified mechanisms like inhibition of Ca^{2+} ATPase, protein-dependent kinase 1, cyclin-dependent kinases [43]
Statins *Pravastatin, Atorvastatin*	Reduction of cholesterol levels in circulation (probably not involved in anti-inflammatory action)/Influence on geranylgeranylation of Rho-proteins/Direct effects on immune cell function [44]
Biologicals *Etanercept, Infliximab*	Inhibition of receptor-interaction of pro-inflammatory cytokines (like TNF-alpha and IL-1) as soluble receptors or antibodies [45]
Corticosteroids *Prednisolone*	Interaction of glucocorticoids to their cytosolic receptor complex reduces inflammatory gene expression and causes rapid intracellular signaling through other components of the complex (e.g. Src). /Nonspecific interaction with cellular membranes through intercalation altering cation transport via plasma membrane and increasing proton leak from mitochondria/Interaction with membrane bound receptors [46]
ACE-inhibitors *Captopril*	Inhibition of angiotensin-converting enzyme suppresses angiotensin II reducing pro-inflammatory cytokines and adhesion molecules [47]
Tetracyclins *Doxycyclin, Minocyclin*	Inhibition of bacterial protein synthesis (probably not related to inflammation inhibition)/Inhibition T-cell activation/ Inhibition phospholipase-A2/ Inhibition matrix metalloproteinases (MMP)/ Inhibition nitric oxide synthase (NOS) [48]
PPAR-γ-agonists *Thiazolidinedione*	Regulation of lipid metabolism in adipocytes and insulin sensitivity (probably not related to inflammation inhibition)/ Inhibition of NOS, TNF-α and MMP9/ Inhibition of NF-κB, AP1 and STAT transcription factors [49]

The relevance of each of these pathways for inhibition of tumor growth is unclear. In addition, most drug classes affect multiple pathways, but the relative inhibition of each pathway is different for each drug molecule within that class. Rational selection of the most appropriate drug is impossible without information on the importance of specific pathways in tumor progression. This is an important area of future research.

Chapter 7 describes the antitumor effects of LCL containing pravastatin. Statins are used in cardiovascular disease and were originally designed as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway leading to *de novo* cholesterol synthesis [50]. Apart from their action on cholesterol synthesis, it is known that statins exhibit anti-inflammatory actions [51-53]. Newman and Hulley reported that statins were carcinogenic in rodents [54]. These actions of statins appear to be the result of the reduced formation of isoprenoids, which in turn are responsible for post-translational modification of small GTP-binding proteins (Rho, Rac, and Ras). These proteins are crucial for a multitude of intracellular signaling pathways [55, 56] involved in angiogenic and inflammatory processes in tumors. With regard to statin antitumor activity, evidence of *in vitro* and preclinical studies generally seem to indicate beneficial effects of statins, albeit that they are obtained at higher doses than those needed for inhibition of cholesterol synthesis [44, 52, 57]. Using a similar rationale as for GC, LCL encapsulation may intensify antitumor effects while reducing the doses that are required.

Liposomal pravastatin inhibited B16.F10 tumor growth by 70% after a single dose of 5 mg/kg, which (corresponding to LCL-GC observations) occurs likely via reduction of expression of tumor–associated *pro*-angiogenic/*pro*-inflammatory factors. This appears to be the result of the encapsulation into LCL, as free drug was ineffective.

COMPARISON BETWEEN DOXIL AND LCL-PLP

The capability of LCL to deliver cytostatic drugs to sites of malignancy is already successfully applied in the clinic. An example is Doxil™ (PEG-liposomes with encapsulated doxorubicin) [58]. Based on the suppressive effects of LCL-PLP and LCL-encapsulated clodronate on TAM, we hypothesized that Doxil could also act in a similar way on these cells. Doxil is already known to cause toxicity and depletion of resident macrophages in liver [59, 60].

Chapter 8 focuses on this alternative mechanism of action of Doxil to their cytotoxicity towards tumor cells. To evaluate whether TAM play an important role in the mode of action of Doxil, Lip-CLOD pretreatment was again used as a tool [29, 30]. As positive control, the same experiments were conducted with LCL-PLP, a tumor-targeted formulation with known strong anti-angiogenic/anti-inflammatory activity in tumors as reported in **Chapters 3** and **4**.

In the absence of TAM resulting from Lip-CLOD pretreatment, Doxil had a strong additional antitumor effect. However, an additional antitumor effect was not observed in case of LCL-PLP treatment. These results suggest that Doxil does not act primarly through TAM. In line with these observations, TAM-mediated production of *pro*-angiogenic factors was only slightly reduced after Doxil treatment. Therefore, antitumor activity of Doxil seems to be primarily the result of cytotoxic effects on tumor cells.

CONCLUSION AND PERSPECTIVES

It is clear from this thesis that LCL-GC offer promise as novel antitumor agents.

For obvious reasons cancer therapy has always focused on the primary tumor(s) and its metastases. The three conventional ways to fight the primary and secondary tumors are 1) surgery, 2) radiation therapy, and 3) chemotherapy. Instead of focusing on the tumors themselves, the novel treatment concept studied in this thesis is directed towards inflammatory cells in and around the tumor(s) aiming at locally silencing tumor-induced angiogenesis/inflammation. Besides being a valuable new treatment in itself, it could also show added value complementary to the three conventional anti-tumor treatment strategies. Until now, this promising concept of silencing tumor-associated angiogenesis/inflammation has not yet been translated into new anticancer therapeutics. However, enough preclinical evidence is now available to warrant the start of clinical investigation of liposomal glucocorticoids in cancer therapy.

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Appendices

SAMENVATTING VAN HET PROEFSCHRIFT

Verrassend genoeg kunnen glucocorticoïden (GC) de groei van solide tumoren remmen [1]. Helaas zijn de benodigde doses voor deze antitumoreffecten vele malen hoger dan de doses die voldoende zijn voor de bekende immuunsuppressieve effecten. De doses die vereist zijn, zijn zelfs dermate hoog dat in preklinische studies ernstige bijwerkingen optraden en dieren zelfs overleden als gevolg van het optreden van opportunistische infecties [2]. Omdat GC bovendien snel uit de bloedbaan geklaard worden en een groot verdelingsvolume hebben, lokaliseren ze maar in beperkte mate in het tumorweefsel. Gerichte aflevering van GC aan tumorweefsel('targeting') lijkt daarom een aantrekkelijke strategie om intratumorale farmaconconcentraties te verhogen en bijwerkingen te verminderen. Eén mogelijke aanpak om deze gerichte aflevering te bewerkstelligen is het verpakken van GC in lang-circulerende liposomen. We hebben aangetoond dat intraveneuze toediening van liposomaal ingekapseld prednisolon fosfaat inderdaad tumorgroei kan remmen [3].

Het mechanisme bleef echter grotendeels onbekend. Het voornaamste doel van dit promotie-onderzoek was daarom om meer inzicht krijgen in het werkingsmechanisme van liposomale GC bij tumoren, om zo de formulering verder te kunnen verbeteren.

In onze studies vonden we een krachtige remming van de productie van pro-angiogene/pro-inflammatoire eiwitten door liposomaal prednisolon fosfaat in vergelijking met het vrije geneesmiddel [4]. De pro-angiogene eiwitten bevorderen de aanmaak van nieuwe bloedvaten en voorzien daarmee de tumor van meer voedingsstoffen en zuurstof. Daarnaast stimuleert de ontstekingsrespons overleving en metastasering van tumorcellen dankzij secretie van groeifactoren en bindweefselafbraak. Met name de macrofagen die in de tumor aanwezig zijn werden sterk geremd in hun productie van deze eiwitten. Vervolgens werd gekeken of, behalve prednisolon fosfaat, ook andere GC profiteerden van inkapseling in liposomen. Alle door ons geteste GC, te weten budesonide, dexamethason en methylprednisolon lieten een verhoogde antitumor activiteit zien. De kracht van tumorremming correleerde met de mate van inhibitie van angiogene/inflammatoire eiwitproductie. Omdat met name de onstekings- en angiogenese remmende effecten van GC verantwoordelijk lijken voor de tumorinhbitie zijn ook andere geneesmiddelen met een mogelijk anti-inflammatoire werking getest zoals statines.

Ook statines remmen tumorgroei als ze worden ingekapseld in liposomen en deze activiteit correleert weer met remming van de productie van anti-angiogene eiwitten. Omdat de effecten gemedieerd lijken te worden door tumor-geassocieerde macrofagen is getest of een cytotoxische verbinding (doxorubicine) ingekapseld in liposomen (in de handel bekend onder de naam Doxil^{TM}/Caelyx^{TM}) ook ontstekingsprocessen in de tumor zou kunnen remmen door een negatief effect op de functionaliteit van macrofagen. Het bleek dat de sterke tumorremming door deze formulering niet gerelateerd was aan de reming van pro-angiogene/pro-inflammatoire eiwitten. Daardoor is juist de combinatie van een cytotoxische en anti-inflammatoire strategie zeer interessant dankzij de verschillende werkingsmechanismen.

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REZUMAT IN LIMBA ROMANA

Glucocorticoizii (GC) au capacitatea de a inhiba cresterea tumorilor solide datorita actiunii lor inhibitoare asupra celor doua procese vitale pentru dezvoltarea tumorilor: angiogeneza si inflamatia [1, 2]. Studiile preclinice au demonstrat ca efectele antitumorale ale GC sunt exercitate la doze mult mai mari decat cele eficiente in terapia bolilor antiinflamatorii. Aceste doze insa sunt acompaniate de efecte secundare severe, conducand la decesul animalelor de experienta, datorita fenomenului de imunosupresie [3]. Pe de alta parte, dozele terapeutice obisnuite nu sunt corespunzatoare, deoarece GC sunt eliminati rapid din circulatie si se localizeaza in cantitati ineficiente la nivelul tesuturilor tumorale. De aceea, administrarea GC sub forma unui sistem de transport la tinta ar putea fi o strategie promitatoare pentru a asigura concentratiile de GC eficiente pentru inhibarea cresterii tumorale. Una dintre terapiile la tinta investigate este reprezentata de utilizarea glucocorticoizilor incorporati in lipozomi cu timp de circulatie lung (din engleza: "long-circulating liposomes" (LCL)). Studiile anterioare au demonstrat ca fosfatul sodic de prednisolon (FP) incorporat in LCL (LCL-FP) inhiba drastic cresterea tumorala in doua modele tumorale la soarece, melanomul B16.F10 si adenocarcinomul de colon C26, dupa administrarea intravenoasa [4]. Acest efect antitumoral este facilitat de sistemul de transport lipozomal, care are capacitatea de tintire pasiva a tumorilor. Mecanismul prin care LCL-FP inhiba dezvoltarea tumorilor nu este elucidat. De aceea, principalul scop al acestei cercetari a fost clarificarea mecanismului actiunii antitumorale a GC incorporati in lipozomi cu timp de circulatie prelungit. Efectele FP incapsulat in lipozomi au fost comparate cu efectele administrarii FP in forma libera (neincorporat in lipozomi). Au fost studiate actiunile celor doua formulari asupra producerii intratumorale de proteine implicate in angiogeneza *in vivo,* precum si asupra viabilitatii si proliferarii celulelor tumorale si endoteliale ale vaselor sanguine *in vitro*. Rezultatele obtinute indica un efect antiangiogenic puternic al LCL-FP prin inhibarea producerii de proteine proangiogenice de catre macrofagele prezente in tesutul tumoral.

Pentru a generaliza rezultatele obtinute cu prednisolon in tratamentul cancerului, trei alti GC diferiti au fost testati dupa incorporarea lor in LCL: fosfatul disodic de budesonida, fosfatul disodic de dexametazona si fosfatul sodic de metilprednisolon. Acesti GC au fost selectati datorita diferentelor in afinitate pentru receptorul uman de GC. In legatura cu acesti GC incorporati in LCL au fost investigate: eficacitatea antitumorala, mecanismele de actiune si efectele secundare.

Toti GC incorporati in lipozomi au inhibat semnificativ cresterea tumorala, comparativ cu formele lor libere. Surprinzator, administrarea GC in lipozomi evidentiaza diferente in ceea ce priveste eficacitatea lor asupra inflamatiei si angiogenezei asociate tumorilor.

Tintirea pasiva cu LCL a fost evaluata si in cazul altor clase de compusi activi, ca de exemplu statinele. Statinele, cu o actiune farmacologica principala care nu este asociata cu angiogeneza tumorala (efect hipocolesterolemiant in afectiunile cardiovasculare) prezinta activitate antiangiogenica asupra tumorilor dupa administrarea sub forma lipozomala.

Ultimul capitol experimental al tezei prezinta o comparatie intre efectele antitumorale ale produsului comercial Doxil^{TM} sau CaelyxTM (in Europa) (doxorubicina incapsulata in LCL) si LCL-FP. Rezultatele noastre sugereaza ca Doxil inhiba progresia cresterii tumorii datorita unui efect direct asupra celulelor tumorale si numai partial datorita efectului antiangiogenic, asupra macrofagelor intratumorale. In cazul LCL-FP, principalul mecanism implicat in efectul antitumoral este un mecanism antiangiogenic.

In concluzie, cercetarea prezentata in aceasta teza sugereaza noi strategii in terapia cancerului prin utilizarea unor noi formulari cu efect antiangiogenic/ antiinflamator care pot completa tratamentele clasice antitumorale: tratamentul chirurgical, radioterapia si chimioterapia. Trebuie subliniat ca, pana in prezent conceptul de inhibare a angiogenezei si inflamatiei asociate tumorii, nu a fost exploatat ca si tratament anticanceros. Studiile preclinice prezentate ofera date suficiente pentru o viitoare cercetare clinica asupra potentialului anticanceros al glucocorticoizilor incapsulati in LCL.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ANOVA	Analysis of variance
AP-1	Activator protein 1
AREs	Adenylate/ uridylate-rich elements
AUC	Area under the concentration-time curve
AUTC	Area under the tumor growth curve
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BUP	Budesonide disodium phosphate
CBP	cAMP response element binding protein-binding protein
cGCR	Cytosolic glucocorticoid receptor
Ch	Chaperones
coCh	Co-chaperones
COX-2	Cyclooxygenase 2
$cPLA_2$	Cytosolic phospholipase A_2
CREB	cAMP response element binding protein
DMEM	Dulbecco's Modified Eagle's medium
DXP	Dexamethasone disodium phosphate
EGM	Endothelial cell growth medium
EPR	Enhanced permeability and retention
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
GC	Glucocorticoids
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GRE	Glucocorticoid response elements
HAH	Heparin adipic hydrazide
hsp	Heat shock proteins
HUVEC	Human umbilical vein endothelial cells
ID	Injected dose
IFN-γ	Interferon γ
IGF-II	Insulin growth factor II
IKK	Kinase of NF-κB inhibitor
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-9	Interleukin 9
IL-12 p40	Interleukin 12 p40
IL-12 p70	Interleukin 12 p70
IL-13	Interleukin 13
IP3	Inositol triphosphate

i.v.	Intravenous administration
LCL	Long-circulating liposomes
LCL-BUP	Long-circulating liposome-encapsulated BUP
LCL-DXP	Long-circulating liposome-encapsulated DXP
LCL-GC	Long-circulating liposome-encapsulated GC
LCL-MPLP	Long-circulating liposome-encapsulated MPLP
LCL-PLP	Long-circulating liposome-encapsulated PLP
LCL-PRV	LCL-encapsulated PRV
Lip-CLOD	A mixture of two types of clodronate liposomes: clodronate-containing LCL and clodronate-containing large negatively charged liposomes (ratio 1:1 (w/w))
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein-1
M-CSF	Monocyte-colony stimulating factor
mGCR	Membrane glucocorticoid receptor
MIG	Monokine induced by IFN-γ
MMP	Matrix metalloproteinases
MPCE	Murine pulmonary capillary endothelial cells
MPLP	Methylprednisolone disodium phosphate
MPS	Mononuclear phagocyte system
NF-AT	Nuclear Factor of activated T cells
NF-κB	Nuclear factor κB
nGRE	Negative glucocorticoid response elements
NK cells	Natural killer cells
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PEG	Poly(ethylene glycol)
PF4	Platelet factor 4
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PLP	Prednisolone disodium phosphate
PRV	Pravastatin
RA	Rheumatoid arthritis
SAPK/JNK	Stress-activated protein kinase/c-Jun N-terminal kinase
s.c.	Subcutaneous administration
SD	Standard deviation
Src-1	Steroid receptor co-activator 1
STAT	Signal Transduction and Activator of Transcription family transcription factors
TAM	Tumor-associated macrophages
TIMP-1	Tissue inhibitor of metalloproteinase 1
TIMP-2	Tissue inhibitor of metalloproteinase 2
TNF α	Tumor necrosis factor α
TPO	Thrombopoietin
VEGF	Vascular endothelial growth factor

CURRICULUM VITAE

Manuela Banciu was born on 26th May 1974, in Sighişoara, Romania. From 1989 to 1992, she attended the secondary school at "Gheorghe Marinescu" College in Târgu Mureş, Romania. In 1997 she graduated Biology at the Faculty of Biology and Geology, "Babes-Bolyai" University of Cluj-Napoca, Romania. One year later, she obtained a Master Degree in Cell and Molecular Biology at the same University. During 5 years (1997 – 2002) she was employed as microbiologist, at "S.C. Terapia S.A.", one of the largest pharmaceutical companies in Romania. Since 2002 she has been appointed as teaching assistant in Biochemistry and Cytology at the Department of Experimental Biology, Faculty of Biology and Geology, Cluj-Napoca, Romania. In December 2004 she was awarded with a Traveling Grant (Company of Biologists, UK) at the Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands. In January 2005 she was granted with a 1-year Galenos Marie Curie Fellowship at the same University. In June 2006 she has started her PhD project on antitumor effects of glucocorticoids encapsulated in long-circulating liposomes under the supervision of Professor Dr Gert Storm and Dr Raymond Schiffelers, at the same Department. The results of her PhD research are described in this thesis.

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