Liposomal Delivery of Dexamethasone Attenuates Prostate Cancer Bone Metastatic Tumor Growth In Vivo

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Background. The inflammatory tumor microenvironment, and more specifically the tumor-associated macrophages, plays an essential role in the development and progression of prostate cancer towards metastatic bone disease. Tumors are often characterized by a leaky vasculature, which - combined with the prolonged circulation kinetics of liposomes - leads to efficient tumor localization of these drug carriers, via the so-called enhanced permeability and retention (EPR) -effect. In this study, we evaluated the utility of targeted, liposomal drug delivery of the glucocorticoid dexamethasone in a model of prostate cancer bone metastases.
Methods. Tumor-bearing Balb-c nu/nu mice were treated intravenously with 0.2–1.0–5.0 mg/kg/week free- and liposomal DEX for 3–4 weeks and tumor growth was monitored by bioluminescent imaging.
Results. Intravenously administered liposomes localize efficiently to bone metastases in vivo and treatment of established bone metastases with (liposomal) dexamethasone resulted in a significant inhibition of tumor growth up to 26 days after initiation of treatment. Furthermore, 1.0 mg/kg liposomal dexamethasone significantly outperformed 1.0 mg/kg free dexamethasone, and was found to be well-tolerated at clinically-relevant dosages that display potent anti-tumor efficacy.
Conclusions. Liposomal delivery of the glucocorticoid dexamethasone inhibits the growth of malignant bone lesions. We believe that liposomal encapsulation of dexamethasone offers a promising new treatment option for advanced, metastatic prostate cancer which supports further clinical evaluation. *Prostate 75:815–824, 2015.*
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KEY WORDS: bone metastasis; dexamethasone; drug delivery; liposomes; nanomedicine; prostate cancer

INTRODUCTION

Prostate cancer is the most common cancer type in males and the second leading cause of death from cancer [1]. If detected in early stage (i.e., exclusively localized in the prostate), prostatectomy and radiotherapy provide efficient treatment options. Bone metastases are prevalent in approximately 90% of patients with advanced prostate

cancer, and for this stage, no curative treatment options are currently available, stressing the need for novel treatment options.

In primary and metastatic cancers, tumor cells closely interact with different cell types and the extracellular matrix constituting the stromal compartment. It is increasingly recognized that tumor-associated inflammation plays a pivotal role in several stages of cancer carcinogenesis, dissemination, and metastasis [2–4], and multiple types of inflammatory cells have been described to contribute to prostate cancer tumorigenesis [5]. Neoplastic cells may activate various types of stromal cells and, conversely, activated stromal cells secrete additional growth factors, which further favor cancer cell proliferation and invasion. For instance, tumor-associated macrophages (TAM) have been shown to contribute to migration [6], angiogenesis [7], and chemotherapy-resistance [8]. Based on this tumorgrowth-stimulating nature of pro-inflammatory stromal cells, interference with tumor-associated inflammation provides a promising, yet underexplored, approach to combat cancer [9]. For this purpose, glucocorticoids (GC) [10], such as dexamethasone (DEX), are highly effective anti-inflammatory drugs that are also used as add-on in chemotherapy for palliative purposes in prostate cancer treatment. Strikingly, it has remained unclear whether GC indeed confer an additional therapeutic benefit by modulating tumor-associated inflammation. It has been speculated that high tumor concentrations of GC are needed to achieve such a specific anti-tumor effect [11]. Clearly, such tissue concentrations can only be achieved by high and frequent GC dosing, which inevitably entails the well-known range of detrimental GC-related side effects, providing a possible explanation for their limited use in cancer therapy [10].

Over the last few decades, tumor-targeted liposomal drug delivery has become an emerging therapeutic strategy. Many tumors are characterized by a leaky vasculature and poor lymphatic drainage. Specifically designed long-circulating liposomes have the ability to extravasate and slowly accumulate in tumor tissue after intravenous administration which is commonly referred to as the enhanced permeability and retention (EPR)-effect [12]. The abundance of TAM and their efficient phagocytizing capacity provide the rationale for the use of liposomes for the efficient delivery of anti-inflammatory drugs to the supportive tumor microenvironment. Liposomes typically reduce the exposure of healthy tissues to the encapsulated drug, which can significantly reduce the toxicity of the therapeutic. These characteristics therefore justify the use of long-circulating liposome drugs in the treatment of cancer, as exemplified by liposomal doxorubicin (i.e., Doxil/Caelyx) [13]. For prostate cancer, anti-tumor responses of liposomal cytotoxic drugs were observed in several relevant preclinical models in vivo [14] and in clinical studies [15] (reviewed in [16]).

Long-circulating liposomal delivery of GC is a therapeutic strategy that is under development for inflammatory diseases and has been demonstrated to exert beneficial effects in experimentally-induced arthritis [17] and preclinical models of cancer [11,18]. Surprisingly, the utility of liposomal GC in (pre) clinical prostate cancer studies has not been explored to date. Given the current use of GC in advanced prostate cancer as a chemotherapy add-on, the clinical translation of liposomal GC could be straightforward if proven efficacious. In this study, we evaluated the efficacy of liposomal versus free DEX in a preclinical model of metastatic bone disease in human prostate cancer. Furthermore, the pharmacokinetic profile and toxicology of liposomal and free DEX were established.

MATERIALS AND METHODS

Preparation of Liposomes

Liposomal DEX was prepared using the ethanol injection method [19], encapsulating dexamethasone disodium phosphate (Buma, Uitgeest, The Netherlands) with poly (ethylene glycol) 2000-distearoyl-phosphatidylethanolamine (DSPE-PEG-2000), dipalmitoylphosphatidylcholine (DPPC), (both Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (CHOL; Sigma, St Louis) in a 0.15:1.85:1.00 molar ratio. Multiple rounds of extrusion through polycarbonate membranes (final pore sizes of 100 nm/100 nm; Nuclepore, Pleasanton) were performed and unencapsulated DEX was removed with the tangential flow filtration unit (Pall minimate, Pall Millipore, Mijdrecht, the Netherlands). Mean particle size was determined using dynamic light scattering and the amount of encapsulated DEX was determined with high performance liquid chromatography as described previously [17].

Alexa-750-labeled liposomes were prepared by post-insertion of micelles. Briefly, micelles were prepared by mixing DSPE-NH_2-PEG-2000 and DSPE-PEG-2000 (both Avanti Polar Lipids, Birmingham) in a 1:1 molar ratio to enable covalent attachment of Alexa-750-succinimidyl to the NH_2-terminus of the PEG-conjugates. Subsequently, micelles were incubated with the liposomes at 60°C degrees to allow post-insertion of the PEG-conjugates.

Cell Lines and Culture Conditions

The human osteotropic prostate cancer cell line PC-3M-Pro4luc2, that stably express firefly luciferase-2, was cultured in Dulbecco's modified Eagle's medium (LifeTechnologies, Bleiswijk, the Netherlands) supplemented 10% FCII (ThermoScientific, Waltham) and 800 μg/ml G-418/neomycin (LifeTechnologies). Cell lines were maintained in a humidified incubator at 37°C and 5% CO_2.

Animal Studies

Seven-week old male athymic mice (Balb/c *nu/nu*, Charles River, L'Arbresle, France) were housed under sterile conditions. All animal experiments were approved by the local committee for animal health, ethics, and research of Leiden University (DEC12013).

Single cell suspensions of PC-3M-Pro4luc2 cells (50.000 cells/10μl PBS) were injected in both tibiae as described previously [20]. Tumor growth was monitored by bioluminescent imaging (BLI). Liposomal localization was examined by fluorescent imaging after intravenous injection of Alexa-750-labeled liposomes both using the Caliper Lumina imaging system (Perkin-Elmer, Groningen, the Netherlands). Tumors and other organs were measured ex vivo directly after sacrifice.

In the therapeutic studies, treatment with free- and liposomal DEX was initiated 15 days after tumor inoculation and administered by weekly intravenous injection (N = 5 mice per group with a tumor in both tibiae). Tumor growth was monitored for up to 21–26 days post -treatment. At the end of the experiment, mice were sacrificed and tibiae were collected for further histological analysis.

Histological Analysis

After fixation with 4% PFA for 24 hr, tibiae were decalcified in 10% EDTA for 10 days and tissues were paraffin-embedded. F4/80 and Goldner stainings were performed on 5 μM thick decalcified tissue sections [20]. For the F4/80 staining, sections were deparaffinated, rehydrated and treated with H2O2 before antigen retrieval using proteinase-K. After blocking, sections were incubated with rat-anti-mouse F4/80 primary antibody (Serotec, Oxford, UK) and secondary anti-rat immPRESS reagent (Vectorlabs, Burlingame). Goldner staining was performed as described previously [21].

Toxicology and Pharmacokinetic Studies

Toxicity and pharmacokinetic studies were performed at Bracco Imaging SpA (Colleretto Giacosa, TO, Italy). Male, 5-week old, CD Sprague Dawley IGS BR rats were treated intravenously with vehicle, liposomal DEX (0.2–1.0–5.0 mg/kg/week) or free DEX (5 × 1.0 mg/kg/week) for a total of four weeks. Clinical signs, body weight, and clinical pathology were evaluated and blood was collected at several time points. Plasma concentrations of DEX were measured using a validated bioanalytical UPLC-MS assay. At the end of the study, animals were subjected to gross pathology examination and tissues were collected for histopathological analysis.

RESULTS

Liposomes Localize to Malignant Bone Lesions In Vivo

Liposomes have the propensity to localize to solid tumors due to the EPR-effect. In mice with established bone lesions (with presence of F4/80[+] TAM, Fig. 1a), we confirmed the EPR-effect in real-time by sensitive, non-invasive fluorescence imaging of intravenously administered Alexa-750-labeled liposomes (Fig. 1b). Besides metastatic lesions, also known sites for liposomal localization (i.e., liver and spleen) showed strong accumulation while other non-target organs were largely negative (Fig. 1c).

Liposomal Dex in an Intra-bone Tumor Model in Mice

Treatment of established malignant bone lesions in mice was performed by intravenous administration of vehicle, free- and liposomal DEX at day 15 after the intra-tibial inoculation of PC-3M-Pro4luc2 tumor cells (Fig. 2a). At all dose levels (i.e., 0.2, 1.0 and 5.0 mg/kg), both free- and liposomal DEX led to a significant inhibition of intra-bone tumor growth (Fig. 2b–d). For free DEX treatment, only the 5.0 mg/kg dosage seemed to stand out against the moderate effect seen at lower dosages, whereas liposomal DEX administration already demonstrated a peak response at 1.0 mg/kg. Strikingly, liposomal DEX outperformed free DEX administration at the 1.0 mg/kg dosage ($P < 0.01$ at d26, Fig. 2c, Fig. 3a), while similar (non-significant) effects were observed at the 0.2 and 5.0 mg/kg dosages (Fig. 2b and d). Histological evaluation confirmed the profound anti-tumor efficacy of (liposomal) DEX at the 1.0 mg/kg dosage, in which low tumor burden and intact trabeculae were observed in (liposomal) DEX-treated mice compared to vehicle-treated mice (Fig. 3b).

In parallel with the evaluation of the anti-tumor efficacy of free- and liposomal DEX in mice, changes in body weight were studied to assess the systemic
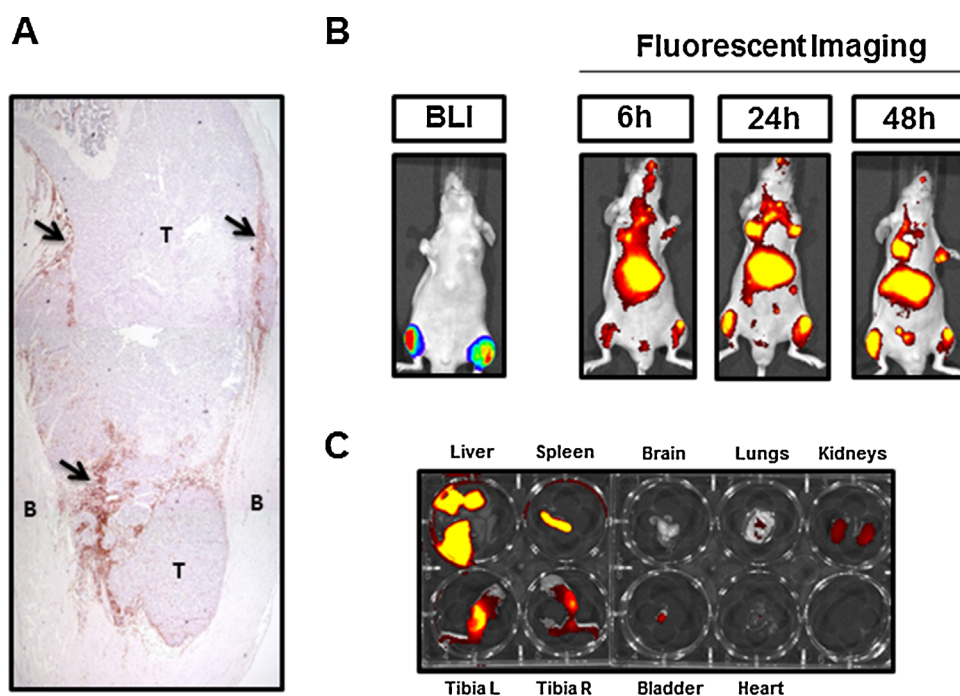
Fig. 1. The presence of tumor-associated macrophages (TAM) and localization of fluorescent liposomes in bone lesions of human prostate cancer. (A) Immunolocalization of F4/80$^+$ TAM in the tumor microenvironment of prostate cancer cells growing in bone (arrows). T = tumor, B = bone. (B) Alexa-750-labeled liposomes localize efficiently to malignant bone lesions (tibiae) at 6, 24, and 48 hr after intravenous injection. BLI = bioluminescent imaging. (C) Ex vivo fluorescence in organs of mice injected with Alexa-750-labeled liposomes show strong tumor, liver, and spleen localization.

adverse effect of GC. At clinically relevant, lower doses (i.e., 0.2 and 1.0 mg/kg), no significant differences between treatment groups were observed in mice (Fig. 4a and b), with the exception of 1.0 mg/kg liposomal DEX at day 9 of treatment. This, however, recovered at later time points (Fig. 4b). At the highest 5.0 mg/kg dose level, significant weight loss was observed in mice treated with liposomal DEX, but not free DEX ($P < 0.001$ at day 19, Fig. 4c). This enhanced weight loss of liposomal DEX at high dosages compared to free DEX may be explained by the delayed clearance of liposomal DEX, as opposed to the rapid clearance of free DEX (Table I).

Pharmacokinetics and Toxicity Assessment of Liposomal Dex in Rats

In order to characterize the pharmacokinetics and the toxicology of liposomal DEX, we subsequently performed a multiple-dose study in healthy rats comparing weekly administration of liposomal DEX (0.2, 1.0, and 5.0 mg/kg) with daily administration of 1.0 mg/kg free DEX (cumulative dose 5.0 mg/kg weekly; equal to highest dose level of liposomal DEX) (Suppl. Table 1). When comparing the pharmacokinetics of 1.0 mg/kg liposomal DEX with a single dose of 1.0 mg/kg free DEX, a strong increase in initial plasma concentration (C_{max}, 3-fold increase), circulation half-life ($t_{1/2}$) and the area under the curve (AUC, 100 times larger) was observed (Table I).

Despite the increase in total exposure to DEX, our study did not reveal increased toxicity attributable to the liposomal DEX formulation: both 5.0 mg/kg liposomal DEX and 5×1.0 mg/kg free DEX showed comparable GC-related toxicity in terms of reduced weight gain (Suppl. Fig. 1), creatinine levels (Suppl. Table 2), white blood cell count (WBC) (Suppl. Table 3), and organ weight (Suppl. Table 4), which were all anticipated at these dose levels. Compared to equal doses of free DEX, high dose (5.0 mg/kg) liposomal DEX increased serum levels of liver enzymes (Glutamic-pyruvic transaminase [GPT] and glutamic-oxaloacetic transaminase [GOT]) (Suppl. Table 2) and showed slightly higher liver toxicity in histopathologic evaluation ('increased mild infarct and necrosis') (Suppl. Table 5), which likely is a consequence of the well-known redistribution and preferred localization of intravenously administered liposomes to the liver. Taken together, liposomal DEX induces a similar toxicity profile compared to free DEX while enhancing the anti-tumor efficacy in experimentally-induced bone metastases, thus increasing the therapeutic index.
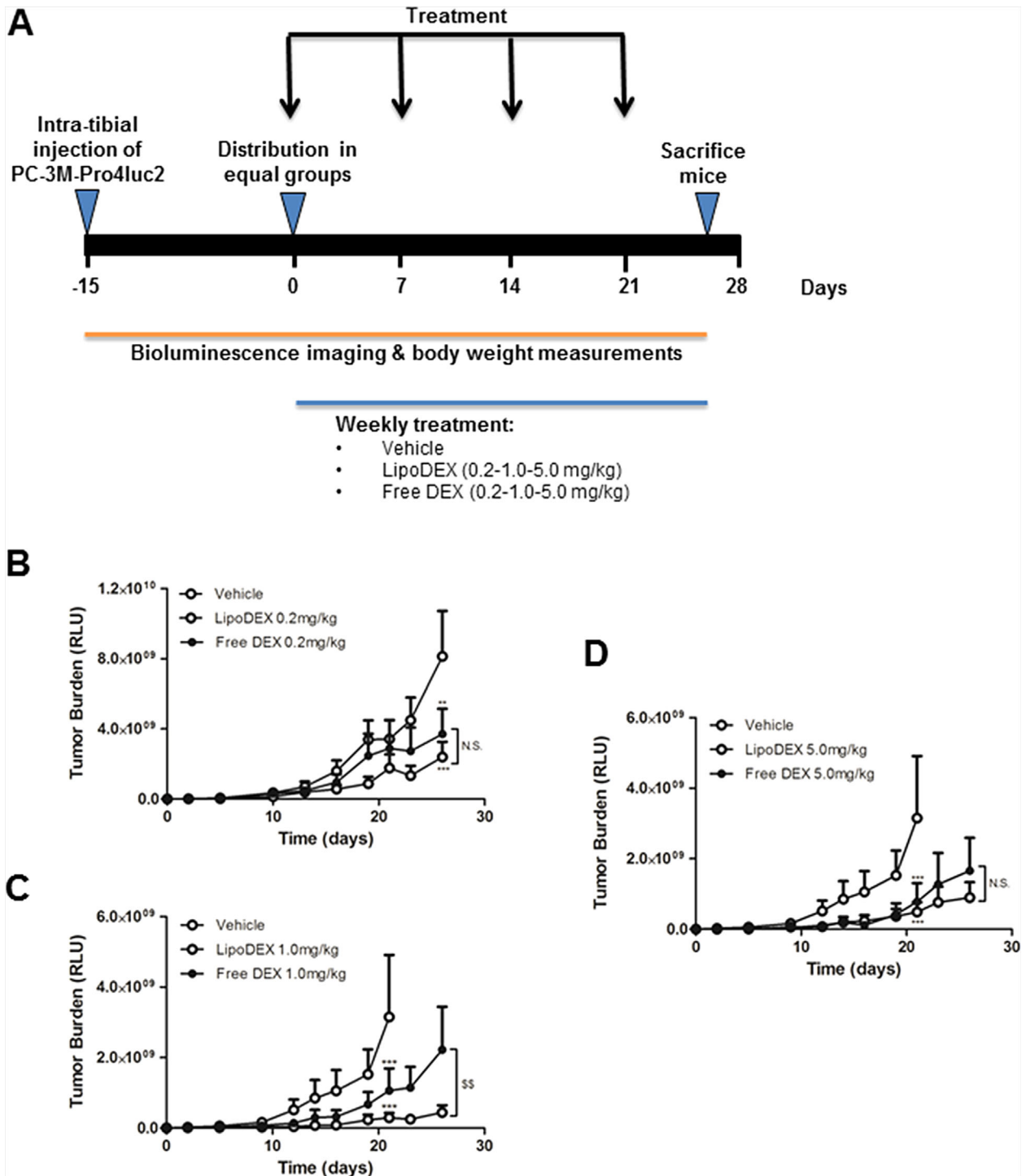
Fig. 2. Treatment with (liposomal) DEX attenuates the growth of experimentally-induced bone metastases. (A) Experimental schedule; arrows indicate time of treatment. The anti-tumor effect of liposomal- and free dexamethasone was compared for the dosages (B) 0.2 mg/kg, (C) 1.0 mg/kg, and (D) 5.0 mg/kg. **$P < 0.01$ versus vehicle; *** $P < 0.001$ versus vehicle; $\$\$$ $P < 0.01$; N.S. = not significant.

DISCUSSION

In this study we show, for the first time, a pronounced and sustained anti-tumor efficacy of liposome-encapsulated DEX in a preclinical model of metastatic prostate cancer. We demonstrate a considerable EPR-effect in experimentally-induced bone metastases as liposomes preferentially and efficiently localize at the site of malignant bone lesions. Furthermore, liposomal encapsulation of
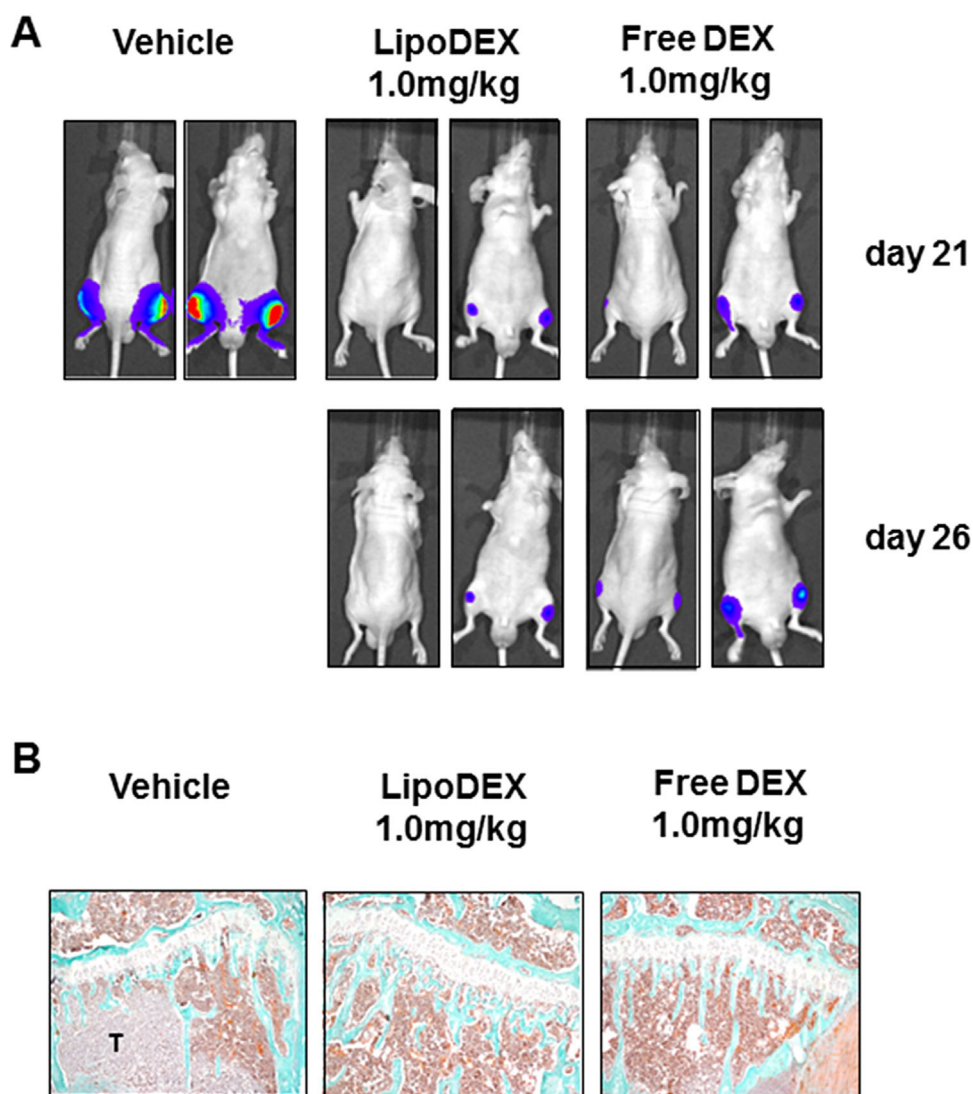
Fig. 3. Representative whole-body bioluminescent images and Goldner staining of tumor-bearing tibiae treated with 1.0 mg/kg (liposomal) DEX. (A) Bioluminescent images and (B) Goldner-staining of tibiae upon treatment with (liposomal) DEX reveal decreased intra-bone tumor burden. T = tumor

DEX significantly enhanced its anti-tumor efficacy at dose level 1.0 mg/kg/week (compared to non-encapsulated DEX), whereas similar trends were observed for other dosages. Our data in metastatic disease are in line with the enhanced anti-tumor efficacy of liposomal GC reported in other unrelated tumor models [11,22]. In those studies, liposomes predominantly localize to TAM, indicating a key role of these cells in mediating the anti-tumor activity of liposomal GC [11,23]. The glucocorticoid receptor (GR), the receptor for dexamethasone, was highly expressed in 50% of prostate cancer specimens [24], but is predominantly associated with cancer-associated stromal cells, while the actual prostate cancer cells only expressed low levels of GR [25]. This supports the notion that mainly stromal cells, including TAM, are targeted with liposomal drug delivery.

TAM are known to secrete a wide range of cytokines that support tumor growth, and a notable example is interleukin-6 (IL-6) [4]. In addition to production of IL-6 by cancer cells [26], malignant cells can also induce IL-6 secretion by reactive stromal cells in the bone microenvironment, which in turn facilitates metastatic outgrowth [27]. Generally, IL-6 is involved in proliferation, apoptosis, migration, invasion, angiogenesis, and the maintenance of prostate cancer stem cells [28,29], thereby strongly contributing to prostate carcinogenesis. It has been documented that DEX [30] and liposomal DEX [31,32] inhibit expression and secretion of IL-6 in macrophages and monocyte-like cells. Decreased IL-6 secretion by stromal could contribute to the antitumor
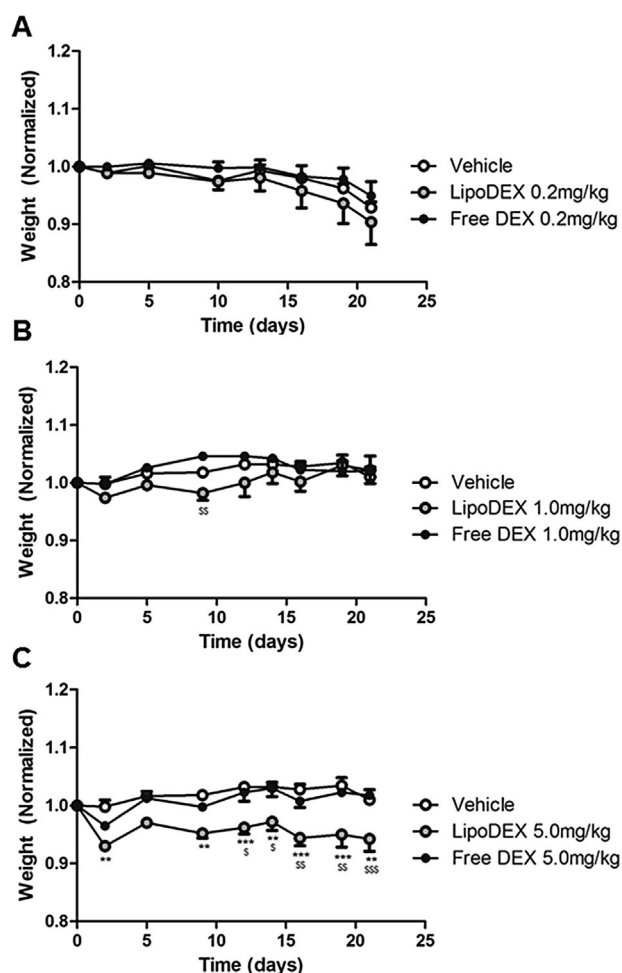
Fig. 4. The effect of (liposomal) DEX on body weight of nude mice. The effect of (A) 0.2 mg/kg, (B) 1.0 mg/kg, and (C) 5.0 mg/kg (liposomal) DEX on the body weight of nude mice. Treatment at day 0, 7, and 14. **$P < 0.01$ versus vehicle; ***$P < 0.001$ versus vehicle; $ $P < 0.05$ versus free DEX; $$ $P < 0.01$ versus free DEX; $$$ $P < 0.001$ versus free DEX.

effect on malignant bone lesions we observe with liposomal DEX.

In the context of bone metastases, the effects of DEX on cells of the bone microenvironment are of special interest. GC have been described to reduce osteoblast function, induce osteocyte apoptosis, and enhance osteoclast differentiation [33]. In contrast to the anti-tumor effect observed in our studies, DEX may thus be potentially stimulatory for tumor growth by enhancing osteoclastic bone resorption, leading to the release of bone-matrix bound growth factors beneficial for bone metastasis growth [34]. Interestingly, liposomal encapsulation of GC was previously found to block osteoclast differentiation and to reduce osteoclastic bone resorption [35], which may also contribute to the superior anti-tumor effects of our liposomal formulation of DEX versus free DEX administration.

Although the apparent uptake of liposomal DEX in the bone lesions seems to be mediated by TAM, direct effects of (liposomal) DEX on tumor cells cannot be fully excluded as PC-3M-Pro4luc2 cells express the glucocorticoid receptor (GR). At present, the use of free GC in the treatment of prostate cancer is controversial and subject of a vivid clinical debate. On the one hand, direct, growth-inhibitory effects of GR-agonists (including DEX) have been found in GR-positive prostate tumors [36] and clinical studies demonstrated anti-tumor efficacy of DEX treatment in advanced prostate cancer patients [37]. Recent contrasting evidence indicates that resistance to anti-androgens may be mediated by GR-overexpression [38] and that (non-liposomal) GC can induce resistance to chemotherapy [39]. Liposomal encapsulation may, however, prevent this as TAM are the main target cells. Despite potential involvement in therapy resistance, GC are still the standard-of-care in advanced prostate cancer in combination with chemotherapy.

In general, liposomal delivery can reduce toxicity of the encapsulated drug. However, one needs to be aware of the manifestation of potential new toxicities due to the altered drug distribution as a result of liposomal encapsulation. An illustration of this is the use of PEGylated liposomal doxorubicin (i.e., Doxil/Caelyx) in clinical prostate cancer. Typically, increased skin- and mucosal toxicities are observed upon liposomal encapsulation of doxorubicin, whereas a decrease of cardiotoxicity was found, which is the major problem with systemic free doxorubicin treatment [40]. Based on this, it is imperative to monitor new toxicities related to liposomal GC. So far, our results show that liposomal encapsulation of DEX does not yield unexpected safety issues beyond the well-known toxicity of exogenous GC exposure. Lower dosages (0.2–1.0 mg/kg) of liposomal DEX were generally well tolerated in both mice and rats. Indeed, toxicities with high dosages of liposomal GC were reported (i.e., body weight loss, decreased number of WBC) [22,23], but these can mostly be attributed to systemic GC exposure in general. Our toxicity studies in rats showed comparable toxicity (changes in several serum parameters and organ sizes) with equal cumulative dosages of free and liposomal DEX (5.0 mg/kg).

Only for the liver, an organ with substantial liposome uptake via Kupffer cells, increased toxicity was observed. Despite the dramatically increased AUCs of liposomal DEX, there was no significant increase of overall toxicity of liposomal DEX versus an equal cumulative dose of free DEX. These observations support the notion that the majority of the liposomal DEX measured in plasma indeed remains entrapped in inactive form within the liposomes thus avoiding

TABLE I. Pharmacokinetics of (liposomal) Dex in a Multiple Dose Study in CD Sprague Dawley Rats

	LipoDEX 1.0 mg/kg		Free DEX 1.0 mg/kg	
	Male	Female	Male	Female
Day 1 (first treatment)				
Tmax (hours)	0.083	0.083	0.083	0.083
Cmax (μg/ml)	17.5	21.0	6.48	5.91
AUC0-t (μg min/ml)	10581	8071	103.8	89.6
Terminal half-life (T$_{1/2}$) (mins)	439	283	0.083	0.42
Clearance (ml min^{-1} kg^{-1})	439	0.124	8543	3968
Volume of distribution Vz (ml/kg)	62.0	50.2	688	2161
Day 22/28 (last treatment)				
Tmax (hours)	0.083	0.083	0.083	0.083
Cmax (μg/ml)	23.0	20.9	7.94	5.92
AUC0-t (μg min/ml)	15089	9890	129.0	86.6
AUC0-t/dose (μg h/ml) (mg/kg)	914	700	0.143	0.207
Clearance (ml min^{-1} kg^{-1})	0.0663	0.1011	6885	4104
Volume of distribution Vz (ml/kg)	48.2	47.6	27.6	26.2

potential systemic adverse effects. Anticipating that the tumor tissue is directly accessible from the blood compartment (a consequence of enhanced local vascular permeability) we postulate that the elevated plasma levels of liposomal DEX also lead to increased DEX concentrations in the tumor microenvironment (i.e., TAM), thus explaining the augmented anti-tumor response in our study. Locally present enzymes may be responsible for release and conversion of the inactive liposomal drug into high tissue levels of active DEX. These high levels of DEX in the tumor microenvironment may subsequently dampen tumor-associated inflammation and thereby inhibit the growth of bone metastatic disease.

CONCLUSION

Our preclinical study shows more potent, and sustained, anti-tumor effects of liposomal DEX versus free DEX in metastatic bone disease from human prostate cancer. The beneficial effects of DEX in a liposomal formulation are presumably mediated via the supportive tumor microenvironment, in particular TAM, rather than via a direct effect on the metastatic tumor cells. Moreover, liposomal DEX delivery is well-tolerated at dose levels that display strong anti-tumor efficacy. We believe these experimental data warrant the clinical evaluation of liposomal DEX in metastatic prostate cancer.

ACKNOWLEDGMENTS

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