Journal of Controlled Release 151 (2011) 10–17

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Anti-tumor activity of liposomal glucocorticoids: The relevance of liposome-mediated drug delivery, intratumoral localization and systemic activity

Ewelina Kluza [a,*], Sin Yuin Yeo [a], Sophie Schmid [a], Daisy W.J. van der Schaft [a,c], Renate W. Boekhoven [a], Raymond M. Schiffelers [b], Gert Storm [b], Gustav J. Strijkers [a], Klaas Nicolay [a]

[a] Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands
[b] Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands
[c] Soft Tissue Biomechanics and Engineering, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

ARTICLE INFO

Article history:
Received 12 August 2010
Accepted 28 November 2010
Available online 3 December 2010

Keywords:
Anti-tumor therapy
Tumor-associated inflammation
Multifunctional liposomes
Magnetic resonance imaging
Monitoring of drug delivery

ABSTRACT

Tumor-associated inflammation has been recognized as an important tumor growth propagator and, therefore, represents an attractive target for anti-cancer therapy. In the current study, inspired by recent findings on the anti-tumor activity of liposomal glucocorticoids, we introduce paramagnetic and fluorescent liposomes, encapsulating prednisolone phosphate (PLP), to evaluate the local delivery of liposomal glucocorticoids to the tumor and its importance for the therapeutic response. The new multifunctional liposomes (Gd–PLP–L) (120 nm diameter, 5.8 mg PLP/60 μmol lipid, bioexponential blood-clearance kinetics ($T_{1/2\alpha} = 2.4 \pm 0.5$ h, $T_{1/2\beta} = 42.0 \pm 12.4$ h), drug leakage of 15%/72 h (*in vitro*)), containing 25 mol% Gd–DTPA–lipid and 0.1 mol% of rhodamine–lipid, were tested in B16F10 melanoma subcutaneously inoculated in C57BL/6 mice, and compared to the original PLP formulation (PLP–L). A single dose of Gd–PLP–L (20 mg PLP/kg/week, i.v.) was found to significantly inhibit tumor growth compared to non-treated mice ($P<0.05$), similarly to PLP–L. The accumulation efficacy of the liposomal agent in the tumor was assessed with MRI, using the increase in the longitudinal relaxation rate (ΔR_1) as a marker. Interestingly, large inter-tumor differences in ΔR_1 (0.009–0.063 s^{-1}, 24 h post-administration), corresponding to highly variable intratumoral Gd–PLP–L levels, did not correlate to the effectiveness of tumor growth inhibition. Uptake of liposomes by tumor-associated macrophages (TAM), determined by ex-vivo fluorescence microscopy, was limited to only 5% of the TAM population. Furthermore, the therapy did not lead to TAM depletion. Importantly, a 90% drop in white blood cell count both after Gd–PLP–L and PLP–L administration was observed. This depletion may reduce tumor infiltration of monocytes, which stimulate angiogenesis, and, thus, possibly co-contributes to the anti-tumor effects. In conclusion, MRI provides a powerful instrument to monitor the delivery of liposomal therapeutics to tumors and guided us to reveal that the activity of liposomal glucocorticoids is not limited to the tumor site only.

1. Introduction

The poor outcome of current anti-cancer therapies continuously stimulates the search for new treatment strategies [1,2]. One of the leading concepts aims at non-neoplastic constituents of the tumor microenvironment [3]. The idea underlying this therapeutic approach originates from the important role of the host response in tumor development. This includes the activation of the normal vasculature to form tumor blood vessels [4,5] and the involvement of immune response elements in tumor growth propagation [6,7]. The inhibition of these processes represents an attractive therapeutic strategy [8].

Following the example of angiogenesis inhibitors, anti-inflammatory drugs show the potential to become a new class of anti-cancer therapeutics. Among the investigated agents are the glucocorticoids (GC), a group of powerful anti-inflammatory drugs [9,10]. Early findings revealed that only very high daily doses of GC were capable of inducing the anti-tumor effects [11,12]. Such an intensive treatment regime led, however, to severe side effects, including morbidity and mortality, attributable to severe immunosuppression. Recently, the unfavorable therapeutic index was dramatically improved by using liposomes as GC delivery vehicles [9]. Long-circulating liposomes resulted in strong tumor growth inhibition, already after administration of a single weekly dose.

The effectiveness of the liposomal formulation was attributed to enhanced GC accumulation in the tumor, caused by the long blood circulation of liposomes and the so-called enhanced permeability and retention effect [13]. In the tumor microenvironment, the inhibition of macrophage activity by GC has been proposed as a key mechanism of the anti-tumor activity, leading to down-regulation of pro-angiogenic factors, which are produced by functional tumor-associated macrophages [9,14]. Nevertheless, the significance of liposome-mediated drug accumulation in the tumor for the therapeutic efficacy was not fully understood.

* Corresponding author. b2.03, N-laag, Den Dolech 2, 5612 AZ Eindhoven, The Netherlands. Tel.: +31 40 247 5836; fax: +31 40 243 2598.
E-mail address: E.Kluza@tue.nl (E. Kluza).

0168-3659/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2010.11.031

Moreover, *in vitro* data suggested a direct cytotoxic and anti-proliferative activity to both endothelial and tumor cells [10,15]. Importantly, there was little evidence that the activity of liposome-encapsulated GC is restricted to the tumor microenvironment. Considering these uncertainties, the exact mechanism of the anti-tumor effects of liposomal GC remained unclear.

In the current study, we therefore determined the efficacy of local delivery of liposomal glucocorticoids to the tumor and its importance for the tumor growth inhibition. For this purpose, inspired by the original formulation of liposomal prednisolone phosphate (PLP–L) [16], we designed MRI- and fluorescence-detectable liposomes, which encapsulate the aforementioned GC. A unique multiparametric approach, which included *in vivo* magnetic resonance imaging (MRI), fluorescence microscopy as well as immunological analyses, allowed us to extensively evaluate the treatment.

First, we characterized the physicochemical properties and the therapeutic activity of the new multifunctional liposomes. Subsequently, by using *in vivo* MRI, we were able to assess the delivery efficacy of the liposomal drug to the tumor. The non-invasive character of MR measurements enabled us to reveal the relation between the intratumoral liposome uptake and the therapeutic outcome. Moreover, MRI was applied to accurately measure tumor volumes over time.

Next, we performed an extensive *ex vivo* analysis in order to determine the precise localization of drug-carrying liposomes within the tumor microenvironment. Particularly, we examined the liposome uptake by tumor-associated macrophages (TAM), as the proposed main cellular target of PLP–L in the tumor. In addition, we investigated the effect of the treatment on the size of TAM population.

Finally, we studied the systemic activity of liposomal GC. Considering the nature of the applied therapy, immunosuppressive effects were expected [17] that could affect tumor growth via angiogenesis inhibition, and therefore, be involved in the anti-tumor activity [6,7]. As an indicator of systemic effects, the levels of circulating white blood cells were monitored after liposomal PLP administration.

2. Materials and methods

Materials, preparation of PLP–L and Gd–PLP–L and characterization of liposome formulations are described in the Supplementary material.

2.1. Murine tumor model

B6F10 murine melanoma cells were cultured as a monolayer at 37 °C and 5% CO_2, in DMEM medium (1 g glucose/L) (Invitrogen, Breda, The Netherlands), supplemented with 10% fetal bovine serum (FBS) (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), 2 mmol/L L-glutamine (Lonza Bioscience, Visp, Switzerland), 50 U/mL penicillin/streptomycin (Lonza). B16F10 murine melanoma cell line originates from American type Culture Collection (ATCC, CRL-6475). Passages between 9 and 15 of the original ATCC batch were used for inoculation. 6–8 weeks-old C57BL/6 mice (Charles River, The Netherlands) were inoculated subcutaneously with 1×10^6 B16F10 cells in the right flank. Between day 7 and 9, the tumors became visible in all mice. The animal experiments were approved by the Institutional Ethical Review Committee for animal experiments of Maastricht University.

2.2. Study design

The pre-treatment MRI examination was performed on the day when tumors became palpable (Day 0). One group of animals (n = 10) received an intravenous injection of Gd–PLP–L (20 mg PLP/kg) during scanning to enable longitudinal MRI monitoring of liposome accumulation in the tumor. The other animals received either PLP–L (20 mg PLP/kg) (n = 10) or saline (n = 8) just after the pre-treatment MRI scans were accomplished. 24 h post-administration mice treated with Gd–PLP–L were scanned again to assess the intratumoral levels of Gd–PLP–L (n = 5). The following MRI examination, on Day 7, was performed for all treatment groups to assess post-treatment tumor volumes. After these measurements, the mice were sacrificed and dissected tumors were immunohistochemically analyzed. Besides the above-described protocol, two groups of mice (n = 6/group) were sacrificed either 2 h or 24 h after administration of Gd–PLP–L, immediately after MRI examination, and tumor tissues were analyzed with fluorescence microscopy.

2.3. MRI

MRI measurements were performed with a 6.3 T scanner (Bruker Biospin, Ettlingen, Germany), using a 3 cm-diameter birdcage coil (Rapid Biomedical, Rimpar, Germany). The mice were anesthetized with isoflurane and placed in a cradle, equipped with a mask for anesthetic gas and a warm water pad. For the animals treated with Gd–PLP–L, an infusion line with Gd–PLP–L was placed in the tail vein for injection during MRI experiment. During measurements, respiration was monitored with a balloon sensor connected to an ECG/respiratory unit (Rapid Biomedical). The imaging protocol included a fat-suppressed multislice T_2-weighted spin-echo sequence (TE/TR = 35/4200 ms, number of averages (NA) = 4), a diffusion-weighted spin-echo sequence (TE/TR = 35/2000 ms, b-value = 0 or 400 s/mm^2, NA = 2). The intratumoral accumulation of Gd–PLP–L was monitored using T_1-weighted imaging and T_1 mapping. T_1-weighted images were acquired using a fat-suppressed multislice spin-echo sequence (TE/TR = 8.8/800 ms, NA = 6), pre- and post-contrast (up to 1.5 h). All pre-contrast parameters and slice positions were cloned for post-contrast imaging. T_1 maps were calculated from a series of T_1-weighted spin-echo images with different TR (TR = 400, 600, 800, 1000, 1500, 2500, and 3500 ms, TE = 8.8 ms, NA = 1). The latter measurements were performed pre-, 2 h post- and 24 h post-contrast. For all scans, matrix = 128 × 128, FOV = 3 × 3 cm^2, and slice thickness = 1 mm.

2.4. MRI data analysis

MR images were analyzed in Mathematica 6.0 (Wolfram Research Inc., Champaign, IL). Regions of interest (ROI) were manually defined by drawing contours around the tumor in all image slices of the diffusion-weighted acquisition. T_2-weighted images served as an additional reference. Tumor volumes were calculated by multiplying the number of pixels in the tumor ROIs with the pixel volume. Tumor contrast enhancement in T_1-weighted images was analyzed on a pixel-by-pixel basis. The enhancement was considered significant when the signal intensity was increased by at least 5 times the SD of the noise. T_1 maps were generated in each slice from the images acquired as a function of TR. T_1 was calculated in each pixel from the best fit of the signal intensity versus repetition time TR, according to $S \sim 1 - \exp(-TR/T_1)$.

2.5. Ex vivo localization of Gd–PLP–L in tumor tissue

In order to assess the precise localization of the rhodamine-labeled Gd–PLP–L in the tumor tissue, immunohistochemical analysis was performed in tumors dissected 2 h and 24 h after Gd–PLP–L administration. First, 5 μm-thick sections of frozen tumors were cut and fixed with ice-cold acetone. Tumor-associated macrophages (TAM) were stained with FITC-conjugated rat anti-mouse CD68 antibody (Serotec). For staining of tumor cells, rat anti-mouse CD44 antibody (BD Biosciences) and FITC-conjugated goat anti-rat secondary antibody (Invitrogen) were used. Endothelial cells of tumor blood vessels were stained with rat anti-mouse CD31 antibody (Dako) and