



Method of hyperthermia and tumor size influence effectiveness of doxorubicin release from thermosensitive liposomes in experimental tumors

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ARTICLE INFO

Article history:

Received 26 October 2015

Received in revised form 30 November 2015

Accepted 1 December 2015

Available online 3 December 2015

Chemical compounds studied in this article:

doxorubicin hydrochloride (Pubmed CID 443939)

DPPC (Pubmed CID 452110)

DSPC (Pubmed CID 94190)

DPPG₂ (no Pubmed CID available)

CAS 495403-05-9)

gadodiamide (Pubmed CID 153921)

Keywords:

Thermosensitive liposomes

Phosphatidyloligoglycerol

Laser

Drug targeting

MRI

Mild hyperthermia

ABSTRACT

Systemic chemotherapy of solid tumors could be enhanced by local hyperthermia (HT) in combination with thermosensitive liposomes (TSL) as drug carriers. In such an approach, effective HT of the tumor is considered essential for successful triggering local drug release and targeting of the drug to the tumor. To investigate the effect of HT method on the effectiveness of drug delivery, a novel laser-based HT device designed for the use in magnetic resonance imaging (MRI) was compared systematically with the frequently used cold light lamp and water bath HT. Long circulating phosphatidylglycerol-based TSL (DPPG₂-TSL) with encapsulated doxorubicin (DOX) were used as drug carrier enabling intravascular drug release.

Experiments were performed in male Brown Norway rats with a syngeneic soft tissue sarcoma (BN 175) located on both hind legs. One tumor was heated while the second tumor remained unheated as a reference. Six animals were investigated per HT method. DPPG₂-TSL were injected i.v. at a stable tumor temperature above 40 °C. Thereafter, temperature was maintained for 60 min. Total DOX concentration in plasma, tumor tissue and muscle was determined post therapy by HPLC. Finally, the new laser-based device was tested in a MRI environment at 3 T using DPPG₂-TSL with encapsulated Gd-based contrast agent.

All methods showed effective DOX delivery by TSL with 4.5–23.1 ng/mg found in the heated tumors. In contrast, DOX concentration in the non-heated tumors was 0.5 ± 0.1 ng/mg. Independent of used HT methods, higher DOX levels were found in the smaller tumors. In comparison water bath induced lowest DOX delivery but still showing fourfold higher DOX concentrations compared to the non-heated tumors. With the laser-based applicator, a 13 fold higher DOX deposition was possible for large tumors and a 15 fold higher for the small tumors, respectively. Temperature gradients in the tumor tissue were higher with the laser and cold light lamp (−0.3 °C/mm to −0.5 °C/mm) compared to the water bath (−0.1 °C/mm and −0.2 °C/mm). Visualization of HT in the MRI demonstrated successful localized heating throughout the entire tumor volume by contrast agent release from DPPG₂-TSL.

In conclusion, HT triggered drug delivery by using DPPG₂-TSL is a promising tool in chemotherapy but effectiveness markedly depended on the method of heating and also on tumor size. Local HT using a cold light lamp or the new laser applicator allowed more efficient drug delivery than using a regional water bath heating. MR-compatibility of the new applicator gives the opportunity for future experiments investing drug delivery in more detail by MRI at low technical efforts.

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1. Introduction

Mild regional hyperthermia (HT) in combination with systemic chemotherapy [1,2] or radiation therapy [3–5] has been shown to enhance tumor therapy. A significant improvement in overall survival or in tumor control was obtained with a combined therapy compared to the standard therapy alone. Using thermosensitive liposomes (TSL) as drug carrier for further improvement of systemic

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chemothermotherapy seems feasible by increasing drug release in the tumor volume. This is achieved by externally controlled, temperature triggered release of chemotherapeutic drugs in the heated tumor tissue [6,7]. As a result of promising preclinical data, lysolipid-based TSL with encapsulated doxorubicin (DOX) have entered human clinical trials [8].

TSL are drug delivery carriers formed by a single bilayer membrane mainly composed of phospholipids surrounding an internal aqueous phase [9]. The membrane is characterized by its phase transition temperature (T_m) which is adjustable by the phospholipid composition [10,11]. Above this temperature, the phospholipids change their phase state from solid gel to liquid disordered and encapsulated drugs are released. In recent years, an improved TSL formulation for intravascular drug release based on the synthetic phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphodiglycerol (DPPG₂) was developed and showed a comparable fast drug release to lysolipid-based TSL combined with an improved vesicle stability [12–15]. Mechanism of action for TSL is their ultra-fast intravascular DOX release inside the heated tumor vasculature that significantly increases the concentration of bioavailable drug in the interstitial space when compared to conventional PEGylated liposomes or free drug [16,17]. TSL such as DPPG₂-TSL are very sensitive to temperature changes [21] and thus the achieved minimum temperature threshold is a significant factor for effective temperature triggered drug delivery. Alternative targeting concepts for TSL include a pre-HT administration to increase their intratumoral extravasation by the enhanced permeability and retention effect [18,19], combined with a second heat trigger to increase interstitial drug release [20].

So far, in pre-clinical small animal studies markedly different localized heating methods have been applied making a quantitative comparison of results challenging. The influence of the heating method on efficacy of drug delivery remains unclear. Frequently used experimental HT methods include non-invasive methods like immersion of the tumor in a temperature controlled water bath [18,22–26] or using external resistive heating coils [17,19], different heating lamps [15,27–29], ultrasound devices [30–32], laser [33], or radiofrequency (RF) antennas [35]. Invasive methods such as a catheter flushed with heated water through the tumor also have been applied [34].

Besides using TSL for targeting, a second approach to improve the clinically used combination of the systemic chemotherapy with technically challenging HT is to develop control and steering of HT by using MRI for visualization and thermometry. To explore such an approach in future studies a new MRI-compatible laser light applicator was developed for tumor selective non-invasive HT.

The aim of the present study was to investigate effectiveness of mild HT induced drug release from TSL in small animals with subcutaneous implanted syngeneic tumors using different preclinical HT methods. For this purpose widely applied and simple to use HT methods such as water bath and cold light lamp were characterized and compared to HT induced by a newly developed MR-compatible laser-based device.

2. Material and methods

2.1. Chemicals

The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were obtained from Corden Pharma Switzerland LLC (Liestal, Switzerland). DPPG₂ was synthesized as described before [36]. DOX was purchased from Sigma Aldrich GmbH (Munich, Germany) and Gd-DTPA-BMA (Omniscan™) was from GE Healthcare Buchler GmbH & Co. KG (Braunschweig, Germany). All other chemicals were either from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich GmbH (Munich, Germany). Purified, deionized water generated with the Milli-Q Advantage A10 system from Merck Millipore (Billerica, MA, USA).

2.2. Preparation of TSL

Lipid composition of all TSL formulations used in the present study was DPPC/DSPC/DPPG₂ 50:20:30 (mol/mol) (DPPG₂-TSL). TSL encapsulating either 300 mM citrate pH 4.0 or 250 mM Gd-DTPA-BMA, pH 7.4 were prepared by the lipid film hydration and extrusion method as described in detail elsewhere [13,37]. DOX was actively loaded to the citrate containing liposomes [13].

All preparations have been characterized as previously described [10,13,15,37]. In brief, the hydrodynamic diameter (z average), size intensity distribution plot and zeta potential were measured by DLS (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, United Kingdom). The instrument was calibrated with a size standard (Nanosphere™, 125 nm, Thermo Fisher Scientific, Waltham, MA, USA). Phospholipid composition was measured with thin layer chromatography (TLC). TLC plates were developed with chloroform/methanol/acetic acid (97.5%)/water 100:60:10:5 (vol/vol). A lipid standard containing DPPC, DPPG₂ and lyso-phosphatidylcholine was applied in every TLC run to check the separation quality. Phospholipid concentration was quantified by a phosphate assay using a 1 g/l phosphate solution (Merck KGaA, Darmstadt, Germany) as reference standard. DOX concentration was measured by fluorescence spectroscopy (Cary Eclipse, Varian Inc., Palo Alto, CA, USA) after disruption of the liposomes with Triton X-100. Commercially available non-thermosensitive PEGylated liposomal DOX (Caelyx®, Janssen-Cilag GmbH, Neuss, Germany) was used as reference standard. The concentration of gadodiamide was quantified by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a gadolinium ICP standard solution (Merck KGaA, Darmstadt, Germany) as reference standard. The thermosensitivity of each preparation was verified in vitro by measuring the temperature dependent release profile of DOX and gadodiamide with fluorescence spectroscopy (Cary Eclipse, Varian Inc., Palo Alto, CA, USA) and a 0.47 T NMR-analyzer (Minispec NMS120, Bruker BioSpin GmbH, Rheinstetten, Germany), respectively. The characterization of the liposomes is reported in detail in Supplementary Tables 1–3.

2.3. Animals

All experiments have been performed in accordance to approved protocols (Regierung von Oberbayern, Az. 55.2.1.54–2532.3-21-11, Germany). Experiments were performed on rats weighing 200–300 g (Charles River GmbH, Sulzfeld, Germany) under anesthesia (isoflurane 2–5% and oxygen 1.5 l/min). The animals were housed in cages in groups of four, in a temperature-controlled environment with a 12-h light/dark cycle and with free access to standard chow (ssniff Spezialdiäten GmbH, Soest, Germany) and water ad libitum.

2.4. Tumor model

The soft tissue sarcoma cell line BN175, growing syngeneic in male Brown Norway rats was used [15]. Cells were grown in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany) (vol/vol), 100 U/ml penicillin and 100 µg/ml streptomycin, respectively. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Tumors were generated as previously published [15]. In brief, $1.5 \times 10^6/50 \mu\text{l}$ medium BN175 cells were injected subcutaneously in both hind legs. Tumor growth was recorded by caliper measurements every second day and the volume was calculated using an ellipsoid approximation $V = \pi/6(a * b * c)$. Tumors were allowed to grow to either 0.7 to 1 cm³ or >2 cm³, respectively. Depending on the tumor volume, the rat was then either assigned to the so-called small or large tumor treatment group, respectively.

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