Application of vasoactive and matrix-modifying drugs can improve polyplex delivery to tumors upon intravenous administration

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Abstract

Low efficacy of cationic polymer-based formulations (polyplexes) for systemic gene delivery to tumors remains the crucial concern for their clinical translation. Here we show that modulating the physiological state of a tumor using clinically approved pharmaceuticals can improve delivery of intravenously injected polyplexes to murine melanoma tumors with different characteristics. Direct comparison of drugs with different mechanisms of action has shown that application of nitroglycerin or losartan improved extravasation and tumor uptake of polyplex nanoparticles, whereas angiotensin II had almost no effect on polyplex accumulation and microdistribution in the tumor tissue. Application of nitroglycerin and losartan caused from 2- to 6-fold enhanced efficacy of polyplex-mediated gene delivery depending on the tumor model. The results obtained on polyplex behavior in tumor tissues depending on physiological state of the tumor can be relevant to optimize delivery of polyplexes and other nanomedicines with similar physicochemical properties.

1. Introduction

Cationic polymers hold great promise for cancer gene therapy due to their simplicity of production and reduced immunogenicity in comparison with viral vectors. Some of these gene delivery systems are currently undergoing testing in clinical trials, although mainly for local application [1]. However, intravenous application of polyplexes in vivo is still limited mainly due to several transport obstacles including fast uptake by macrophages of the reticuloendothelial system, vascular,stromal, and intracellular barriers [2]. Additionally, at each step transferable nucleic acids are fraught with the risk of degradation by endogenous nucleases [3,4]. Being nanomedicines, polyplexes can passively accumulate in a tumor [5] due to the so-called enhanced permeability and retention (EPR) effect [6-10]. On the other hand, reduced blood flow in a tumor, as well as interstitial hypertension, vascular, and stromal barriers significantly impair tumor supply with intravenously administered nanomedicines, their transport across the vessel wall, and penetration [11]. As for polyplexes, very poor extravasation and short penetration distances of extravasated nanoparticles into tumor tissue were observed [5]. Despite the obviousness of this challenge, relatively little attention has been paid to it so far. We hypothesized that using safe and clinically approved drugs that modulate tumor blood flow as well as permeability of tumor vessels and extracellular matrix might improve EPR-mediated polyplex accumulation (Fig. 1) resulting in enhanced efficacy of gene delivery. The first approach is based on inducing mild hypertension by intravenous infusion of angiotensin-II (AT-II), which augments tumor blood flow [12] and convective influx of circulating nanoparticles to tumor tissue [13]. As a second approach, we used local application of nitroglycerin (NG) ointment onto the skin over a subcutaneous tumor. At low extracellular tumor pH, NG generates nitric oxide, which can cause an increase in tumor blood flow due to vasodilatation of adjacent normal vessels [14], and disruption of junctions between endotheliocytes [15] leading to enhanced tumor vasculature leakiness [16]. As the last strategy, we applied intraperitoneally losartan (LS), which inhibits angiotensin signaling in cancer-associated fibroblasts (CAFs), resulting in suppression of collagen I production and enhancement of extracellular matrix permeability [17]. Earlier, NG, LS, and AT-II have been successfully applied for facilitating delivery of some nanomedicines, although the question about the favorable drug for a particular nanof ormulation and a particular tumor remains open [18]. Here we directly compared the impact of these drugs on tumor-targeted delivery of one type of nanomedicine (polyplexes) with certain physicochemical characteristics.
upon intravenous administration using murine melanoma tumors with different properties, namely, Cloudman S91 and B16F1.

As a model gene delivery system, we used previously described polyplex nanoparticles [5,19,20] based on polyethylenimine-polyethylene glycol block-copolymer (PEI-PEG) and targeted with a ligand peptide MC1SP for melanocortin-1 receptors (MC1Rs) overexpressed in various melanoma cells and tumors [21–23]. Here we report the first success in use of drugs that alter tumor physiology for tumor-targeted gene transfer by an intravenous administration of nanoparticle gene delivery formulation. We also elucidated the effect of AT-II, NG, and LS on behavior of polyplex nanoparticles in tumor tissue. These data can be useful for the choice of the most suitable drug to improve delivery of nanomedicines with similar properties.

2. Materials and methods

2.1. Polyplex nanoparticles

As a model gene delivery carrier, we used co-polymer synthesized on the basis of commercially available 25-kDa polyethylenimine (PEI) (Polysciences, Warrington, PA), 1.5-kDa heterobifunctional polyethylene glycol MAL-dPEG24-NHS ester (PEG) (Quanta BioDesign, Powell, OH), and MC1SP-oligopeptide CGYGPKKKRKVSIGSGSISISHFRWGKPV (Rusbiolink, Moscow, Russia) according to a previously described protocol [19]. The PEI/PEG and MC1SP/PEI molar ratios in the block-copolymer are 1.4 and 1.3, respectively. For polyplex preparation two plasmids, pEGFP-N3 (Clontech, Mountain View, CA) or pGL3-CMV (Promega, Madison, WI), encoding enhanced green fluorescence protein (EGFP) and luciferase, respectively, were used. For experiments with intravital microscopy of tumor-bearing mice, plasmid DNA (pEGFP-N3) for polyplex formation was additionally biotinylated and labeled with QD655 quantum dots (QDs) JTK Streptavidin Conjugate (Invitrogen/Molecular Probes, Eugene, OR) at DNA:quantum dot molar ratio 2:1 as described earlier [24]. Polyplexes were prepared by fast mixing of equal volumes of PEI-PEG-MC1SP solution with 10% d-glucose, 20 mM HEPES (pH 7.4), and DNA water solution, followed by 20 min incubation at room temperature prior to use. The final concentration of plasmid DNA in the polyplex solution was 20 μg ml⁻¹ for in vitro and 200 μg ml⁻¹ for in vivo experiments. The prepared polymeric nanoparticles were characterized in terms of their hydrodynamic diameters and ζ-potentials by dynamic light scattering methods using a ZetaPALS instrument (Brookhaven Instruments, Holtsville, NY).

2.2. Estimation of in vitro transfection efficacy and polyplex cytotoxicity

Briefly, 12,000 B16F1 or Cloudman S91 (clone M3) cells per well were seeded onto 48-well plates (Corning Costar Corp., Cambridge, MA) and cultivated in DMEM or DMEM/F12 medium, respectively, supplemented with 10% fetal bovine serum for 24 h. Then the growth medium was replaced, and polyplex solutions were added to the cells to final DNA concentration of 0.5 μg ml⁻¹. The percentage of EGFP-expressing cells was estimated by flow cytometry 48 h post-transfection. Propidium iodide staining was used to discriminate between vital and apoptotic cells. Per sample, 1 × 10⁶ gated events were collected. Cellular viability was evaluated using the MTT assay. All experiments were carried out in triplicate a minimum of three times.

2.3. Animal tumor models

Melanoma tumors were established in female C57BL/6 or DBA/2 mice (Stolbovaya, Moscow region, Russia) by subcutaneous injection of 1 × 10⁶ B16F1 or Cloudman S91 cells, respectively, into the flank region. During all experiments the animals were maintained under specific pathogen-free conditions at room temperature of 25 °C with free access to chow and water ad libitum. All experimental procedures were approved by the Institute Commission for Animals.

2.4. In vivo transfection

Gene delivery experiments were performed when the tumors reached a volume of 150–200 mm³. Mice treated with drugs (as described below) and non-treated control group (5–6 animals per group) were injected intravenously with polyplex solution containing 80 μg DNA encoding firefly luciferase and prepared at N/P ratio of 10 (the number of positive amino or imino groups in PEI per phosphate in plasmid DNA). After 24 h the mice were euthanized, and the tumors, livers, lungs, spleens, and hearts were excised, snap frozen in liquid nitrogen, and stored at −80 °C. Then the tissues were homogenized, and the tissue lysates were cleared by centrifugation. Luciferase activity of the supernatant was measured according to the protocol of Promega using Luciferase Assay System kits (Promega, Madison, WI). Protein concentration was measured using the Bradford assay.

2.5. Immunofluorescent staining of tumor sections

Mice with 150–200 mm³ tumors were euthanized, and their tumors were excised, embedded into GSV 1 tissue-embedding medium (Slee...
Medical GmbH, Mainz, Germany), snap frozen in liquid nitrogen, and kept at −80 °C. Then the frozen tumor blocks were cut into 10-μm thick slices using a Leica CM1510 cryotome (Leica, Wetzlar, Germany), fixed in acetone–methanol (2:3) mixture for 15 min, and air-dried at room temperature. For vascularization studies, transverse tumor sections were immunostained with rat monoclonal anti-CD31 (PECAM-1, clone 390, dilution 1:200; BD Pharmingen, San Diego, CA) primary antibodies and FITC-labeled goat anti-rat IgG secondary antibodies (dilution 1:50; Abcam, Cambridge, UK). Collagen I was examined with rabbit polyclonal anti-type I collagen antibodies (dilution 1:50; Immtek, Moscow, Russia). Secondary antibodies were donkey anti-rabbit IgG labeled with Alexa Fluor 647 (dilution 1:200; Abcam, Cambridge, UK).

2.6. Tumor vascularization

For vascular density analysis, 10 fields (1300 μm × 1300 μm) from 6 tumors were captured using a Zeiss LSM 510 META NLO (Carl Zeiss, Oberkochen, Germany) confocal microscope equipped with a Plan-NEOFLAR ×10/0.30 lens. Using ImageJ software, we converted red–green–blue images to 8-bit gray scale images and subtracted autofluorescence level from areas without vessels (the background level was approximately the same). Then we defined rectangular selections of the same width from tumor edge towards center and plotted profiles, where the X-axis represents the horizontal distance through the selection and the Y-axis is the vertically averaged pixel intensity. In the next step, these profiles were divided into three parts corresponding to 0–300 μm, 300–600 μm, and 600–900 μm from the edge, followed by summing averaged pixel intensities within these parts. These values averaged between all rectangular selections were used for quantification of tumor vascularization.

2.7. Quantification of collagen I content

For vascular density analysis, we captured 12 random fields (220 μm × 220 μm) from 6 tumors using confocal microscopy. For autofluorescence subtraction, fluorescence from images of tissues stained only with secondary antibodies was considered as a background threshold. Using ImageJ, we calculated mean intensities in the collagen I channel and averaged the obtained values between all fields.

2.8. Scanning electron microscopy

Tumor-bearing mice were euthanized and prepared for tissue harvest by transcervical perfusion with 30 ml of phosphate-buffered saline (PBS) (pH 7.4) followed by 50 ml of 0.5% glutaraldehyde and 1% paraformaldehyde in 0.075 M sodium cacodylate buffer (pH 7.4). The tumors were excised and kept immersed in 2.5% glutaraldehyde in 0.075 M sodium cacodylate buffer for 2 h at room temperature. Serial 50-μm thick transverse sections were cut using a Leica VT1200 S vibratome (Leica, Wetzlar, Germany). The sections were rinsed with cacodylate buffer and immediately dehydrated with ethanol, in an acetone–methanol (2:3) mixture for 15 min, and air-dried at −80 °C. Then the frozen tumor blocks were cut into 10-μm sections. Tumor-bearing mice were euthanized and prepared for tissue harvest by transcervical perfusion with 30 ml of phosphate-buffered saline (PBS) (pH 7.4) followed by 50 ml of 0.5% glutaraldehyde and 1% paraformaldehyde in 0.075 M sodium cacodylate buffer (pH 7.4). The tumors were excised and kept immersed in 2.5% glutaraldehyde in cacodylate buffer for 2 h at room temperature. Serial 50-μm thick transverse sections were cut using a Leica VT1200 S vibratome (Leica, Wetzlar, Germany). The sections were rinsed with cacodylate buffer and immersed in aqueous 1% uranyl acetate for 1 h. In the next step, sections were dehydrated with ethanol, infiltrated with 100% t-butanol, and lyophilized. The lyophilized tissue sections were attached to the microscope holder by conductive double-sided adhesive tape and covered with ~10 nm Au-Pd layer using the Gatan precision etching coating system (Gatan, Pleasanton, CA). Scanning electron microscope images were acquired with a Zeiss Merlin microscope equipped with GEMINI II Electron Optics (Zeiss, Oberkochen, Germany) at acceleration voltage 1–5 kV and probe current 10–20 pA.

2.9. Treatment with vasoactive and matrix-modifying drugs

For AT-II treatment, tumor-bearing mice were anesthetized with 1.5% isoflurane by inhalation air at a heated stage at 37 °C. Immediately after polyplex injection, AT-II (Sigma, St. Louis, MO) solution was continuously infused into the tail vein at the rate of 2 μg kg⁻¹ min⁻¹ with a KD Scientific Model 200 infusion pump (KD Scientific Inc., Holliston, MA) for 25 min. For local treatment with NG, we used 0.2% NG ointment (Simplex, Moscow, Russia) application 20 min before polyplex injection to the skin overlying subcutaneous tumors at the dose of 0.04–0.10 mg per tumor. To reduce collagen I content in the extracellular tumor matrix, mice were treated with 30 mg kg⁻¹ LS (Sigma, Laramie, WY) by intraperitoneal injections of LS solution in Hanks’ balanced salt solution (HBSS) (pH 7.3) each day for 7 days. Polyplexes were administered on the day of the last treatment with LS.

2.10. Measurement of tumor blood microcirculation

Blood flow was measured in subcutaneous B16F1 and Cloudman S91 tumors when they reached a volume of 150–200 mm³. On the day before measurements, the hair on the skin in the area of the tumor was removed with tweezers under general anesthesia. Blood perfusion was measured with a LAKK-01 laser Doppler flowmeter (“LAZMA”, Moscow, Russia) with effective penetration depth of 1 mm. During measurements, the laser probe was fixed above the surface of the skin overlying the tumor. Mice were anesthetized with 1.5% isoflurane and kept for 10 min at rest before treatment with AT-II or NG ointment as described above.

2.11. Visualization of polyplex behavior in the tumor tissue

We observed polyplex microdistribution in drug-treated or control Cloudman S91 tumors with approximate volume of 10–20 mm³ implanted in the dorsal skinfold chamber of DBA/2 mice according to a previously described protocol [5]. Briefly, mice were anesthetized using 1.5% isoflurane, intravenously injected with 0.4 mg 500 kDa FITC-dextran (Sigma, St. Louis, MO) in PBS for visualization of tumor vessels, followed by administration of polyplexes with quantum dot-labeled plasmid DNA (80 μg DNA per mouse). Z-stack images were obtained using the Zeiss LSM 510 META NLO confocal laser scanning microscope 15, 30, 45, 60, 90, 120, 180, and 240 min after polyplex administration. Background images were captured before polyplex injection.

2.12. Analysis of polyplex microdistribution

We analyzed the obtained images using Image-Pro PLUS 5.0 software (Media Cybernetics Inc., Silver Spring, MD). Polyplex concentration in tumor tissue was calculated in relative units of “voxel volumes” (quantum dot-positive pixel areas summed over some superficial xy-plane images of one Z-stack) per mm³ of tissue. For analysis of polyplex penetration into the tumor tissue with time, binarized pixel areas of fluorescent objects in the quantum dot channel (after subtraction of background threshold) at the vessel wall surface, defined by FITC staining, and different distances from vessels were calculated for each time point and converted into voxel volumes per mm³ of tissue by multiplying by proportionality coefficient obtained from the previous correlation [5].

3. Results

3.1. Physicochemical and transfection properties of polyplex nanoparticles

In this study, we used model polyplex nanoparticles formed by mixing of PEI–PEG–MC1SP block-copolymers with plasmid DNA (Figs. 2a, S1a). Dynamic light-scattering measurements showed that polyplexes prepared at the various stoichiometric N/P ratios from 10 to 40 have slightly positive surface charge and hydrodynamic diameters around 40 nm (Figs. 2b,c, S1b). Furthermore, PEI–PEG–MC1SP-based polyplexes demonstrated good stability in the presence of serum for a minimum of 24 h [20].
Analysis of gene delivery properties of polyplex nanoparticles on B16F1 and Cloudman S91 (clone M3) melanoma cell lines possessing MC1Rs on their surfaces [23] demonstrated high transfection capacity and lack of apparent cytotoxicity at N/P ratios less than 40 (Fig. 2d,e). In general, transfection efficiency of Cloudman S91 cells with polyplexes was greater than in the case of B16F1 cells (Fig. 2d), which might be attributed to differences of intracellular behavior of polyplex nanoparticles, namely, efficiencies of internalization, endosomal escape, and nuclear translocation [24,25].

3.2. Polyplex-mediated transfection and characterization of B16F1 and Cloudman S91 melanoma tumors

To examine efficiency of gene transfer upon intravenous administration, mice with B16F1 and Cloudman S91 tumors were injected into the tail vein with polyplexes containing pGL3 plasmid DNA (80 μg). According to our recent data [5], the maximum of reporter gene expression in the tumor was observed 24 h after polyplex injection. Therefore, we carried out luciferase assay after the same time interval post-injection.

It has been shown that polyplexes cause comparable luciferase expression in B16F1 and Cloudman S91 tumors (Fig. 3a), although cultured Cloudman S91 cells are more transfectable according to the above-mentioned data (Fig. 2d). This fact can have various possible explanations. For example, it might be assumed that in tumor polyplexes are mainly internalized by non-cancer cells (endotheliocytes, fibroblasts, immune cells) resulting in MC1R-independent transfection. However, the presence of a ligand moiety associated with polyplexes is essential for systemic gene transfer to at least Cloudman S91 tumors, because in that case MC1SP-containing polyplexes caused 2-fold increase in reporter gene expression as compared with non-targeted nanoparticles (tumor uptake was the same for both types of polyplexes) [5]. Another explanation of the observed discrepancy is possible differences in the architecture of B16F1 and Cloudman S91 experimental tumors. To clarify this issue, we compared their morphological characteristics. First, we determined the extent of tumor vascularization using immunofluorescent staining of tumor sections with anti-CD31 antibodies and confocal microscopy. We found a gradual decrease in blood vessel network density towards the center of the tumor in both cases (Fig. 3b,c). These observations agree with previous data on other tumor models and human tumors [26–28]. However, B16F1 tumors were found to be 2–3-fold more vascularized than Cloudman S91 tumors, especially in the peripheral regions (Fig. 3c). Most probably, this fact reveals better supply of B16F1 tumors with polyplex nanoparticles. The second morphological characteristic we examined is endothelium ultrastructure in B16F1 and Cloudman S91 tumor vessels. As far as the process of angiogenesis during tumor progression is disturbed [29], tumor endothelium has multiple defects including transcellular fenestrae and intercellular openings [30]. It has been shown using scanning electron microscopy that tumor endothelium contains numerous fenestrae with diameters up to 800 nm as compared with normal vessels (Fig. 3d). The size frequency distribution of fenestrae in tumor vessels demonstrated that the number of large fenestrae (>300 nm) in the B16F1 endothelium is much higher than in the case of Cloudman S91 tumors (Fig. 3e). We also found a few intercellular openings between endotheliocytes in both tumor models (Fig. S2). Hence, endothelium of B16F1 tumors appears to be more permeable for nanoparticles than the Cloudman S91 vessels owing to increased number of large transvascular holes in the endothelium. Finally, we compared collagen I content in tumor tissues of both models using immunofluorescent staining of tumor sections with anti-collagen I antibodies. Collagen I is the key component of tumor interstitium, being responsible for elevated stromal viscosity and a steric barrier functioning for nanomedicines [31]. The role of collagen as a steric barrier has been demonstrated for diffusion of viral nanoparticles for gene delivery [32], whose artificial analogs (polyplexes) were used in this study. Moreover, collagen I impairs hydraulic conductivity of tumor interstitium and contributes to solid stress, which additionally limits interstitial transport of nanoparticles [33]. We found higher collagen I content in Cloudman S91 tumors in comparison with B16F1 (5351 ± 302 a.u. versus 4535 ± 230 a.u., respectively; means ± S.E.M., N = 12), although the difference is statistically insignificant (p = 0.06, Mann-Whitney U test). Considering this fact together with higher density of the vessel network, tumor cells in B16F1 tumors seems more exposed to nanomedicines. This may explain comparable to Cloudman S91 tumors reporter gene...
expression despite the difference in transfection efficiency of the cultured cells.

3.3. Influence of vasoactive and matrix-modifying drugs on tumor physiology, polyplex behavior in tumor tissue, and gene delivery

To improve both the EPR effect and efficiency of polyplex-mediated systemic gene delivery to tumor tissue, we applied three drugs, namely, AT-II, NG, and LS, which affect tumor physiology in terms of blood flow and vascular and stromal permeability.

3.3.1. Angiotensin-II

The rationale of AT-II use is that mild hypertension selectively increases blood flow and the convective component of transport across the vessel wall in tumor, which would improve uptake and distribution of nanomedicines. It is worth noting that in human tumors augmentation of tumor blood flow does not always occur [34]. In our case, we detected relative 20–30% increase in tumor blood flow according to laser Doppler flowmetry measurements (Fig. S3a).

To assess the impact of AT-II on polyplex transvascular transport in tumor tissue, we used real-time intravital microscopy. For this purpose, we implanted Cloudman S91 cells in dorsal skinfold window chambers and observed generated tumors for polyplex uptake with a laser scanning confocal microscope. To visualize nanoparticle behavior, polyplex DNA was labeled with quantum dots, whereas vessels were stained by intravenous injection of FITC-dextran (Fig. 4a). Earlier, we showed using the same technique the a significant contribution of the EPR effect to polyplex accumulation in Cloudman S91 tumors [5]. We observed very fast accumulation of polyplexes in the tumor followed by subsequent 75% decrease in concentration from 15 min up to 4 h after intravenous administration. We found that a major part of the polyplexes initially accumulated in the tumor were retained on the vessel wall surface and subsequently were washed out back to the blood circulation. Only a minor fraction of the polyplexes primarily bound to the vessel endothelium extravasated and penetrated into the tumor tissue (Fig. 4b; Fig. S4). Therefore, what we mean by the term “tumor uptake” is concentration of extravasated polyplexes.

Unexpectedly, AT-II infusion just after polyplex injection did not enhance interstitial polyplex uptake (Fig. 4c). As compared with control (non-treated mice after polyplex injection), we observed only insignificant acceleration of polyplex influx to tumor tissue from blood vessels (Figs. 4c; 5). Perhaps the magnitude of blood flow increase was insufficient for considerably higher polyplex deposition. In this connection, for better results infusion of AT-II via the tumor-feeding artery might be
applied [35–37], although the use of this technique can be restricted due to its invasiveness. Another possible explanation is relatively fast elimination of polyplexes from the bloodstream (elimination half-life is around 18 min) [5], whereas earlier application of this approach succeeded in increased tumor uptake only in the case of macromolecules and nanoparticles with prolonged circulation [13,38,39]. Thus, interstitial accumulation would be magnified if polyplexes had prolonged circulation time.

As a result, treatment of mice with AT-II did not affect the level of polyplex-mediated reporter gene expression in tumors as compared with control tumors (Fig. 6a,b). Moreover, AT-II increased off-target reporter gene expression in liver and spleen (Figs. 6c; S5). It is important to emphasize that the barrier function of splenic and hepatic vasculature is decreased because vessels lack continuous basement membrane and have openings and/or transcellular fenestrae in their endothelium [40]. Perhaps AT-II-induced hypertension leads to enhanced accumulation of polyplexes in these organs and increased transfection, whereas in tumor high interstitial fluid pressure along with dense extracellular matrix impair hypertension-driven convective influx of polyplexes, resulting in no change in reporter gene expression in comparison with tumors of mice not treated with AT-II.

3.3.2. Nitroglycerin

Physiological effects of NG are explained by its ability to generate nitric oxide. Although nitric oxide is released in many types of tumors and induce tumor progression, at high local doses nitric oxide can promote cancer cell death and tumor regression [41,42]. Here we assessed the impact of local NG application on tumor physiology only after short-term exposure in the context of EPR effect magnification. NG has a strong vasodilation effect. As far as tumor vasculature is almost incapable of autoregulation due to the absence of smooth-muscle lining cells, alterations in tumor blood flow can vary between tumor models depending on organization of the NO-responsive vessels into normal tissues adjacent to the tumor [14]. In the case of Cloudman S91 and B16F1 tumors, topical application of NG ointment caused 50% augmentation in relative tumor blood flow (Fig. S3). Besides affecting tumor blood flow, NG can diminish the barrier function of tumor vessels [15], which was confirmed by intravital microscopy. In NG-treated tumors, polyplexes demonstrated enhanced extravasation into tumor tissue in comparison with control tumors, resulting in 1.5-fold higher total uptake (Fig. 3c).

The observed enhancement of tumor accumulation is probably a result of increase in both tumor blood flow and vascular permeability. As for interstitial transport of polyplexes, increase in their concentration occurs only in close proximity to the tumor vessels (Figs. 5; S4b).
We found that LS application lead to 30% and 20% decrease in collagen I content in Cloudman S91 and B16F1 extracellular tumor matrix, respectively (Figs. 7; S6). Indeed, treatment with LS significantly improved permeability of tumor interstitium for polyplexes (Figs. 5; S4b), which led to a 2-fold increase in tumor uptake (Fig. 4c) and 3-fold enhancement of reporter gene expression in the tumor (Fig. 6a). At the same time, there is a discrepancy between tumor models in the magnitude of increase in luciferase expression (Fig. 6a,b). In spite of greater drop in collagen I content in Cloudman S91 tumor interstitium, we unexpectedly detected higher relative enhancement in transcription of B16F1 tumors. Perhaps this fact could be explained by the extent of LS-mediated alleviation of growth-induced solid stress in Cloudman S91 and B16F1 tumors. It is important to emphasize that extracellular matrix components, including not only collagen I but also glycosaminoglycans (such as hyaluronan), also contribute to solid stress and compression of tumor vessels [11]. Because both collagen I and hyaluronan levels may vary in different tumors, LS is able to promote decompression and reperfusion of vessels differently in different tumor models [17]. As a result, the extent of change in nanomedicine supply and uptake after LS application will vary between tumor models depending on both collagen I and hyaluronan content decrease.

4. Discussion

In recent decades the main efforts to improve polyplex-mediated gene delivery have been addressed to development of polymer chemistry aiming at more effective internalization by target cancer cells, overcoming intracellular barriers resulting in high transfection, and decrease in cytotoxicity [45]. Furthermore, special attention has been focused on biodegradability and biocompatibility of polyplex nanoparticles designed for in vivo systemic applications [45], while the problems of poor polyplex extravasation and limited penetration into tumor tissue were almost set aside. Only a few strategies have been proposed to improve transvascular influx and uniform distribution of polyplexes [46–48]. We tested here a new approach for optimization of systemic polyplex-mediated gene delivery to tumors based on modulating their physiological state with low-cost and safe small-molecule drugs.

These drugs have been applied earlier for optimization of nanomedicine delivery. AT-II has been successfully applied for tumor-targeted delivery of antibodies [13,38] and liposomal doxorubicin [39]. Moreover, AT-II was clinically tested in humans for delivery of the polymeric drug SMANCS, which resulted in enhanced tumor accumulation and improved therapeutic response [37]. Application of NG to enhance delivery and therapeutic efficiency of intravenously injected polymeric drugs has also been reported recently [49]. As for LS, it has been successfully used for potentiation of anti-cancer efficiency of nanotherapeutics, including intratumorally injected oncolytic herpes simplex virus and PEGylated liposomal doxorubicin upon intravenous administration [50]. Additionally, LS-mediated inhibition of angiotensin receptor-1 signaling can attenuate growth and progression of different types of tumors per se, including melanomas [51–53].

Here we showed that application of vasoactive and matrix-modifying drugs alters tumor physiology and differently affects polyplex uptake and microdistribution in the same tumor model. For example, AT-II has almost no effect on polyplex delivery, whereas treatment with LS resulted in 2-fold increase in polyplex accumulation and improvement of polyplex distribution in tumor tissue (Figs. 4c; 5). At the same time, other parameters of polyplex behavior in the tumor such as kinetics of polyplex binding to the vessel wall and further washout remain almost unaffected by different drugs (Fig. S4a). It should be noted that intravital microscopy reflects the real polyplex distribution only in small tumors, or in the peripheral vasculature of large tumors with similarly high vessel density. However, the results obtained using this technique together with relatively high fraction of peripheral vessels in the considered tumor models clearly show the link between polyplex uptake in tumor and improved polyplex-mediated transfection delivery under the
influence of different physiologically active drugs. For example, AT-II has no impact on tumor uptake and gene delivery, whereas NG and LS improved both of them (Figs. 4c; 6). Moreover, NG and LS increased polyplex-mediated gene expression only in tumor but not in non-target organs (Fig. 6c). We found that LS displays the maximal impact on efficiency of gene delivery, resulting in 3- and 6-fold enhancement of reporter gene expression in Cloudman S91 and B16F1 melanoma tumors over control (polyplexes only) (Fig. 6a,b).

Most likely, modulating tumor physiology with the mentioned and other physiologically active drugs could be applied to any nanomedicines independently of the type of nanocarrier, because pathophysiological barriers in tumor tissue are relevant one way or another for all intravenously injected nanotherapeutics. Furthermore, it seems reasonable to test in future an approach based on simultaneous application of some drugs with different mechanisms of action, for example, LS together with NG, in an attempt to augment nanomedicine uptake in an additive manner. Another aspect, which should also be noted, is the development of reliable criteria for the use of these drugs in certain clinical cases.

5. Conclusion

In this study, we compared the influence of different vasoactive and matrix-modifying drugs on delivery of model polyplex nanoparticles with certain physicochemical properties to melanoma tumors. We found that LS is a more promising agent leading to 2-fold augmentation of tumor uptake and improved microdistribution of polyplexes in tumor tissue, resulting in up to 6-fold higher reporter gene expression. We also assume that variation of tunable features of polyplexes could alter the outcome of the proposed approach. In this connection, our data might be relevant only for nanoparticles with similar physicochemical properties and pharmacokinetics.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary Figures

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.04.011.

References


Fig. S1. Physicochemical properties of polyplexes. (a) Chemical structure of PEI-PEG-MC1SP block-copolymer. (b) Normalized size distribution of polyplexes prepared at different N/P ratios and measured by dynamic light scattering methods. Measurements were performed in 40 µl quartz cuvettes at 25 °C with count rates between 10 and 40 kCps in ten or more runs of 240-s duration each and analyzed by multimodal size distribution analysis.
**Fig. S2.** SEM images of vessels in B16F1 and Cloudman S91 melanoma tumors. Transcellular pores in tumor endothelium (black arrowheads) and openings between endotheliocytes (white arrowheads) are present in both tumor models. Scale bar, 2 µm.
Fig. S3. Tumor blood flow modulation with physiologically active drugs. Influence of (a) AT-II and (b) NG treatment on tumor blood microcirculation measured by laser Doppler flowmetry. Time point “0 min” corresponds to the onset of treatment with drugs. All values are expressed as mean ± S.E.M. Animal number per group N = 4.
Fig. S4. Influence of physiologically active drugs on polyplex pharmacokinetics in Cloudman S91 tumor tissue. (a) Microdistribution of polyplexes in non-treated (black) and treated with AT-II (red), NG (green), and LS (blue) tumors after i.v. injection. Curves with circle symbols represent kinetics of bound with the vessel wall surface polyplexes, whereas curves with square symbols show kinetics of polyplex uptake. Values are expressed as mean ± S.E.M. (b) Final distribution of extravasated polyplexes in Cloudman S91 tumor tissue 240 min post-injection. Animal number per group N = 3–6.
Fig. S5. Influence of physiologically active drugs on off-target luciferase gene delivery in tumor-bearing DBA/2 mice. In contrast to AT-II, NG and LS do not affect off-target reporter gene expression. Results are expressed as mean ± S.E.M. (*P < 0.05, **P < 0.0001, one-way ANOVA followed by a post hoc Dunnett’s t-test).
Fig. S6. Influence of treatment with LS on collagen I production in B16F1 tumors. B16F1 tumors after treatment with LS in comparison with control tumors of mice treated with HBSS. Histology images showing the effect of LS on tumor collagen I expression. Collagen I was defined with anti-collagen I antibodies (green), whereas cell nuclei were stained with DAPI (blue). Scale bar, 20 µm.