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Intrinsically active nanobody-modified polymeric micelles for tumor-targeted combination therapy

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ABSTRACT

Various different passively and actively targeted nanomedicines have been designed and evaluated over the years, in particular for the treatment of cancer. Reasoning that the potential of ligand-modified nanomedicines can be substantially improved if intrinsically active targeting moieties are used, we have here set out to assess the in vivo efficacy of nanobody-modified core-crosslinked polymeric micelles containing covalently entrapped doxorubicin. Nanobody-modified polymeric micelles were found to inhibit tumor growth even in the absence of a drug, and nanobody-modified micelles containing doxorubicin were significantly more effective than nanobody-free micelles containing doxorubicin. Based on these findings, we propose that the combination of two therapeutic strategies within one nanomedicine formulation, i.e. the intrinsic pharmacological activity of ligand-modified carrier materials with the cytostatic activity of the incorporated chemotherapeutic agents, is a highly promising approach for improving the efficacy of tumor-targeted combination therapy.

1. Introduction

Chemotherapeutic drugs generally suffer from poor pharmacokinetics and from an inappropriate biodistribution. Because of their low molecular weight (M_w) for instance, intravenously (i.v.) administered anticancer agents tend to present with short circulation times and with low concentrations in tumors and metastases. To assist i.v. administered anticancer agents in achieving proper circulation times and tumor concentrations, and to at the same attenuate their accumulation in potentially endangered healthy organs and tissues, many different drug delivery systems have been designed and evaluated over the years [1–3]. Clinically relevant examples of such ~1–100 nm-sized carrier materials are liposomes, polymers and micelles. Various liposomal, polymeric and micellar nanomedicines have been approved for clinical use, and many others are in clinical trials or in preclinical development [4–6]. Virtually all of these formulations rely on the Enhanced Permeability and Retention (EPR) effect for improving the tumor localization of low M_w chemotherapeutic drugs, i.e. they exploit the physiological fact that solid tumors tend to present with leaky blood vessels and with defective lymphatics, thereby enabling them to efficiently accumulate in tumors over time [7–9].

Thus far however, the clinical performance of EPR-exploiting passively tumor-targeted nanomedicines has been relatively disappointing. They do generally substantially reduce the incidence and intensity of side effects, such as cardiotoxicity, bone marrow depression, alopecia and nausea, but to date, they have largely failed to really improve response rates and survival times [10,11]. To overcome this shortcoming, a number of efforts have been undertaken in recent years in which passively tumor-targeted nanomedicines are integrated in rationally designed combination regimens [12]. It has for instance been shown in this regard that polymeric nanomedicines interact synergistically with clinically relevant regimens of radiotherapy, and that they can be used to deliver multiple drugs to tumors simultaneously, leading not only to significant improvements in efficacy, but often also to substantial reductions in toxicity [13–15].

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An alternative strategy to improve the (pre-) clinical performance of nanomedicine formulations relies on the incorporation of targeting ligands, such as antibodies or peptides, which recognize receptor structures that are (over-) expressed at the target site [1–6]. Such actively targeted nanomedicines have long been thought to be able to enhance the overall tumor accumulation of drugs and drug delivery systems, but recent insights indicate that this is not the case: Kirpotin and colleagues, for instance, evaluated the (kinetics of) tumor accumulation of Her2-targeted immunoliposomes, and found no difference between actively and passively targeted liposomes [16]. Similarly, Choi and coworkers convincingly demonstrated that transferrin-targeted nanoparticles are equally (in-) efficient as transferrin-free nanoparticles in localizing to tumors [17]. Taking the basic principles of passive and active drug targeting into account [11], it indeed seems logical that active targeting cannot lead to substantially higher levels in tumors than passive targeting, since the initial accumulation of drug delivery systems in tumors exclusively relies on EPR, and not on binding to and/or uptake by tumor cells. Consequently, it can be reasoned that active targeting might only be useful for drugs and/or drug delivery systems which themselves are not being taken up by cancer cells, such as non-cationic DNA- and siRNA-containing nanomedicines, for which the incorporation of targeting ligands has indeed been shown to be indispensible for enabling efficient gene expression and/or gene silencing [18,19].

Taking both of the above reasonings into account, i.e. that I) nanomedicine formulations are highly useful for combination therapies, and that II) more advanced strategies are needed to exploit the potential of actively targeted nanomedicines, we have here set out to provide proof-of-principle for an active targeting concept in which intrinsically active ligand-modified carrier materials are used to enhance antitumor efficacy. To this end, EGa1 nanobodies – which are currently under clinical evaluation for treating EGFR-overexpressing tumors [20] – were coupled to core-crosslinked polymeric micelles containing covalently entrapped doxorubicin [21], and the in vivo efficacy of nanobody-targeted polymeric micelles was evaluated in 14C tumor-bearing mice.

2. Materials and methods

2.1. Materials

6-Methacrylamidohexanohydrazide-DOX (DOX-MA) was synthesized as described in Ref. [22]. The EGa1 nanobody was produced and modified with *N*-succinimidyl S-acetylthioacetate (SATA) according to previously optimized procedures [23,24]. The macroinitiators with 4,4-azobis(4-cyanopentanoic acid) $mPEG_2ABCPA$ and $(PDP\text{-}PEG)_2ABCPA$ were synthesized as described in Ref. [24].

2.2. Synthesis and characterization of block copolymers

The block copolymers composed of pHPMAm-Lac_n (48% pHPMAm-Lac_1, 52% pHPMAm-Lac_2) and either $mPEG_{5000}$ (methoxy PEG) or $PDP\text{-}PEG_{5000}$ (pyridyldithio propionate PEG) were prepared by free radical polymerization, using $(mPEG_{5000})_2$-ABCPA or $(PDP\text{-}PEG_{5000})_2ABCPA$ as a macroinitiator, and subsequent methacrylation [24]. Polymer compositions and molecular weights were determined by ^1H NMR and GPC, and the critical micelle temperature (CMT) by means of light scattering [25].

2.3. Micelle preparation and characterization

Core-crosslinked micelles with covalently entrapped DOX were prepared as in Ref. [21]. All formulations contained 20 mg/mL of polymer and were concentrated if necessary. PDP-functionalized micelles for conjugation with EGa1 nanobody were prepared similarly, using a mixture of 20% (w/w) (PDP-PEG)-*b*-p(HPMAm-Lac_n) and 80% (w/w) mPEG-*b*-p(HPMAm-Lac_n). DOX and DOX-MA were determined by HPLC [21]. SATA-modified EGa1 nanobodies were coupled to the surface of empty or DOX loaded (PDP-PEG/mPEG)-*b*-p(HPMAm-Lac_n) micelles as described in Ref. [24]. Unreacted nanobody was removed by ultrafiltration. The final nanobody concentration was 0.8 mg/mL. The conjugation of the nanobody was confirmed using dot blot analysis, using a rabbit polyclonal anti-nanobody primary antibody, a peroxidase-conjugated secondary antibody, the SuperSignal West Femto Maximum Sensitivity Substrate (all from Thermo Fisher Scientific, The Netherlands), and ChemiDoc XRS chemiluminescence detection system (Bio-Rad Laboratories, Inc, USA).

2.4. Cell culture conditions and cytotoxicity experiments

Human UM-SCC-14C head and neck squamous cell carcinoma cells (abbreviated as 14C; developed by Dr. Carey, Ann Arbor, MI, USA [26]) were cultured in DMEM (Invitrogen, The Netherlands), supplemented with 2 mM L-glutamine, 7.5% v/v FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. For the cytotoxicity analyses, 5×10^3 cells/well were seeded into 96-well plates, allowed to adhere overnight, incubated for 4 h with increasing concentrations of the formulations (in 5 mM HEPES buffer; pH 7.4; in quadruplicates), washed, and cultured for another 68 h in fresh medium. Cell viability was determined using the WST-1 assay (Roche Diagnostics GmbH, Germany).

2.5. In vivo therapeutic efficacy

Male athymic Balb/c nude mice (Charles River International Laboratories, Inc) were caged under standard conditions in different groups as depicted in Table 1. 14C cells were cultured as described above. 1×10^6 cells (dispersed in 100 μL medium) were inoculated subcutaneously into the right flank of each mouse. Tumors were measured every second day using a digital caliper. The tumor volume (in mm^3) was calculated using the formula $V = \pi/6 \times L \times S^2$, where L is the largest, and S is the smallest superficial diameter. When tumors reached a volume of ~100 mm^3, mice received i.v. (in the tail vein) injections of saline, free DOX, DOX-PM, EGa1-DOX-PM and drug-free EGa1-PM. DOX-PM and EGa1-DOX-PM were prepared as described above (in HEPES 180 mM pH 7.4 buffer) and concentrated to obtain the required dose (i.e. 3 and 9 mg DOX/kg). The EGa1 nanobody dose in all cases was 4 mg/kg. The formulations were injected every 3 days, for a total of 5 injections. In the high-dose groups, a total of 4 injections were given. The injection volume was 100 μL. When the tumors reached the humane end point (i.e. 1500 mm^3), the mice were sacrificed using cervical dislocation.

2.6. Statistical analysis

Results are presented as average ± standard deviation. Statistical significance was evaluated using the two-tailed student's *t*-test. $p < 0.05$ was considered to indicate significant differences.

3. Results and discussion

To provide initial experimental evidence for the use of intrinsically active ligand-modified nanomedicines, we used core-crosslinked polymeric micelles (PM) based on poly(ethylene glycol)-*b*-poly[*N*-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-pHPMAm-Lac_n; Fig. 1A). These carrier materials were selected because they circulate for prolonged periods of time [25], because they can be efficiently and stably loaded with doxorubicin via hydrazone-based and pH-responsive drug linkers (Fig. 1C), and because their functionalization with nanobody-based targeting ligands has already been established [24].

The biodegradable mPEG-*b*-p(HPMAmLac_1-*co*-HPMAmLac_2) and PDP-PEG-*b*-p(HPMAmLac_1-*co*-HPMAmLac_2) (Fig. 1A and B respectively) block copolymers used in this study were obtained upon radical polymerization using $(mPEG)_2ABCPA$ or $(PDP\text{-}PEG)_2ABCPA$

Table 1
Groups of Balb/c mice ($n = 6$) bearing s.c. 14C tumors used in the therapeutic efficacy study and the treatments they received. Administration started at the day when tumors reached a size of 100–200 mm^3 and was repeated every three days (EGa1 nanobody dose 4 mg/kg).

Group	Treatment	Dose
1	Free doxorubicin	5 × 3 mg DOX/kg
2	DOX micelles low dose	5 × 3 mg DOX/kg
3	DOX micelles high dose	4 × 9 mg DOX/kg
4	EGa1-DOX micelles low dose	5 × 3 mg DOX/kg
5	EGa1-DOX micelles high dose	4 × 9 mg DOX/kg
6	Empty EGa1 micelles	5 × 300 mg polymer/kg[a]
7	PBS	5 × 100 μL

[a] Polymer dose equal to the one of the groups treated with the high dose of DOX-MA micelles.

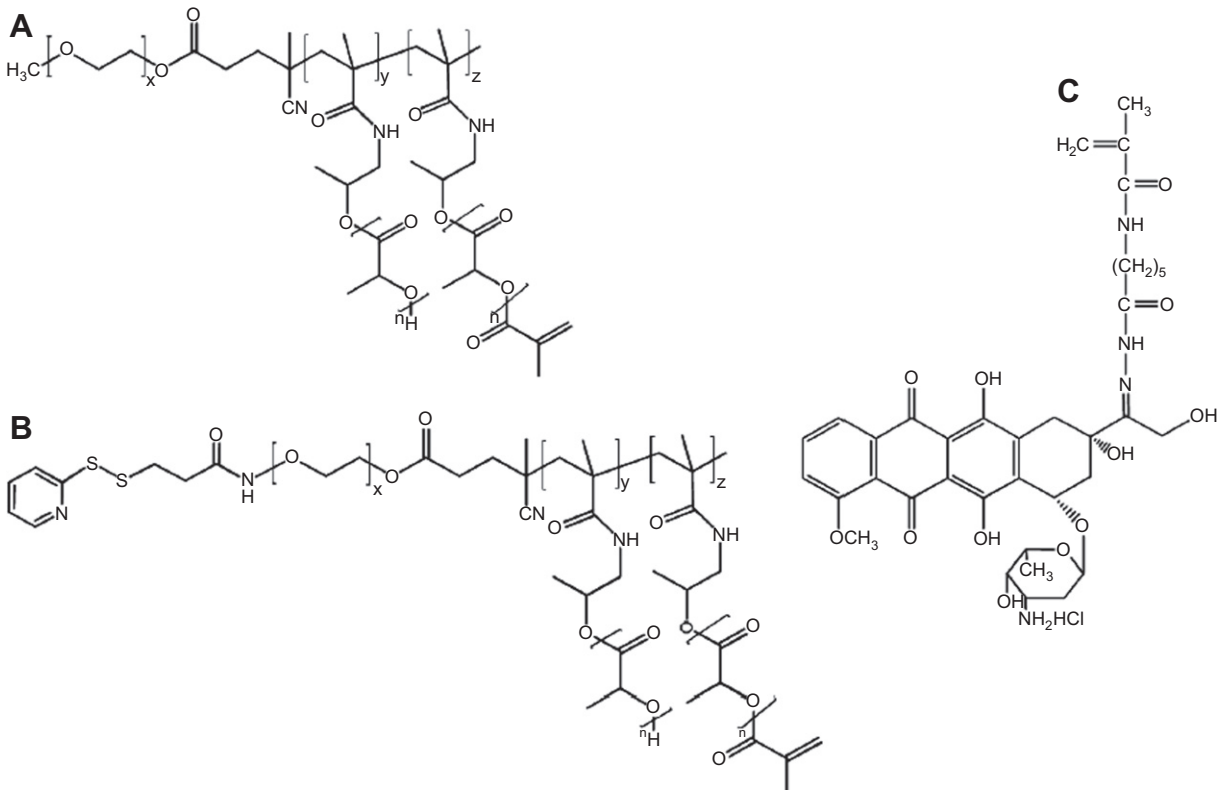

Fig. 1. Chemical structure of methacrylated mPEG-*b*-pHPMAmLac$_n$ (A), methacrylated PDP-PEG-*b*-pHPMAmLac$_n$ (B) and 6-methacrylamidohexanohydrazide-DOX (DOX-MA) (C).

macroinitiators, respectively. mPEG-*b*-p(HPMAmLac$_1$-*co*-HPMAmLac$_2$) polymers self-assembled into small monodisperse micelles with a mPEG corona and a pHPMAmLac$_n$ core when dissolved in aqueous solutions at concentrations above the critical micelle concentration (CMC) and at temperatures above the critical micelle temperature (CMT) [25,27] using the previously published rapid heating procedure [28], efficiently entrapping DOX-MA in their core (Figs. 1C and 2A and B). After this, they were core-crosslinked using KPS and TEMED, resulting in core-crosslinked micelles with covalently entrapped doxorubicin (Fig. 2C). The loading capacity of the obtained DOX-PM was 4% w/w (i.e. amount of covalently entrapped doxorubicin/amount of polymer), their size was 68 nm, and their PDI was 0.1.

In the case of nanobody-containing micelles, a mixture containing 80% mPEG-*b*-pHPMAmLac$_n$ and 20% PDP-PEG-*b*-pHPMAmLac$_n$ was used. EGa1 nanobody coupling to the surface of empty and DOX-MA-containing PDP-PEG/mPEG-pHPMAmLac$_n$ micelles was performed overnight using *N*-succinimidyl S-acetylthioacetate (SATA)-modified nanobodies in the presence of hydroxylamine hydrochloride (Fig. 2D). Nanobody-modified micelles were purified from free (unreacted) nanobodies via ultrafiltration. Nanobody coupling was confirmed using dot blot analyses: comparing the chemiluminescent signal intensity of nanobody-modified PM with that of free nanobody demonstrated close to quantitative conjugation (Fig. 3A–D). The size of drug-free EGa1-PM was 62 nm (PDI: 0.1) and that of EGa1-modified DOX-containing PM was 70 nm (PDI: 0.1). The loading capacity was 4% w/w. DOX-PM with or without EGa1 displayed similar release kinetics, showing that nanobody-functionalization does not negatively affect DOX release (Fig. 3E).

Next, the in vitro uptake and cytotoxicity of the formulations was evaluated. This was done using the EGFR-expressing squamous cell carcinoma cell line UM-SCC 14C. As shown in Fig. 3F–G, in line with the in vitro principles of active targeting and with previous observations, Ega1-modified PM were much more efficiently taken up upon 4 h of incubation at 37 °C than were untargeted micelles [11,24,29,30]. It is important to take into account in this regard that both nanobody-modified and nanobody-free PM possessed a neutral surface charge (i.e. a zeta potential of ~0 mV), thereby excluding charge-related differences in cellular uptake, and exemplifying that the enhanced uptake resulted from EGFR-mediated uptake mechanisms. Subsequently, the in vitro efficacy of nanobody-targeted and nanobody-free PM was investigated, and compared to that of free doxorubicin. To this end, 14C cells were exposed to the formulations for 4 h, after which the cells were washed, and allowed to grow for another 68 h in the absence of drug. As shown in Fig. 3H, nanobody-modified DOX-PM were found to be significantly more effective in killing cancer cells than were untargeted DOX-PM ($p < 0.01$), which was due to their more efficient cellular uptake (as shown in Fig. 3F–G), leading to higher accumulation in endo- and lysosomes, and to pH-responsive DOX release. As expected, free DOX was found to be the most effective formulation in vitro (Fig. 3H).

In vivo, this situation was reversed. When administered at its maximum tolerated dose (MTD; 5 × 3 mg/kg [31]), free DOX did induce tumor growth inhibition in mice bearing 14C xenografts, but both untargeted and nanobody-modified DOX-PM were significantly more effective (Fig. 4A; $p < 0.001$ for both comparisons). This can be explained by the prolonged circulation times of core-crosslinked PM [25], which most probably led to significantly higher levels of DOX accumulating in tumors via the EPR effect. Strikingly, also drug-free nanobody-modified PM significantly inhibited tumor growth ($p < 0.001$ vs. control group) and they did so to an extent similar to that observed for free DOX administered at its MTD ($p > 0.05$; Fig. 4A). In line with the concept put forward in the Introduction, in which it is suggested that targeting ligands

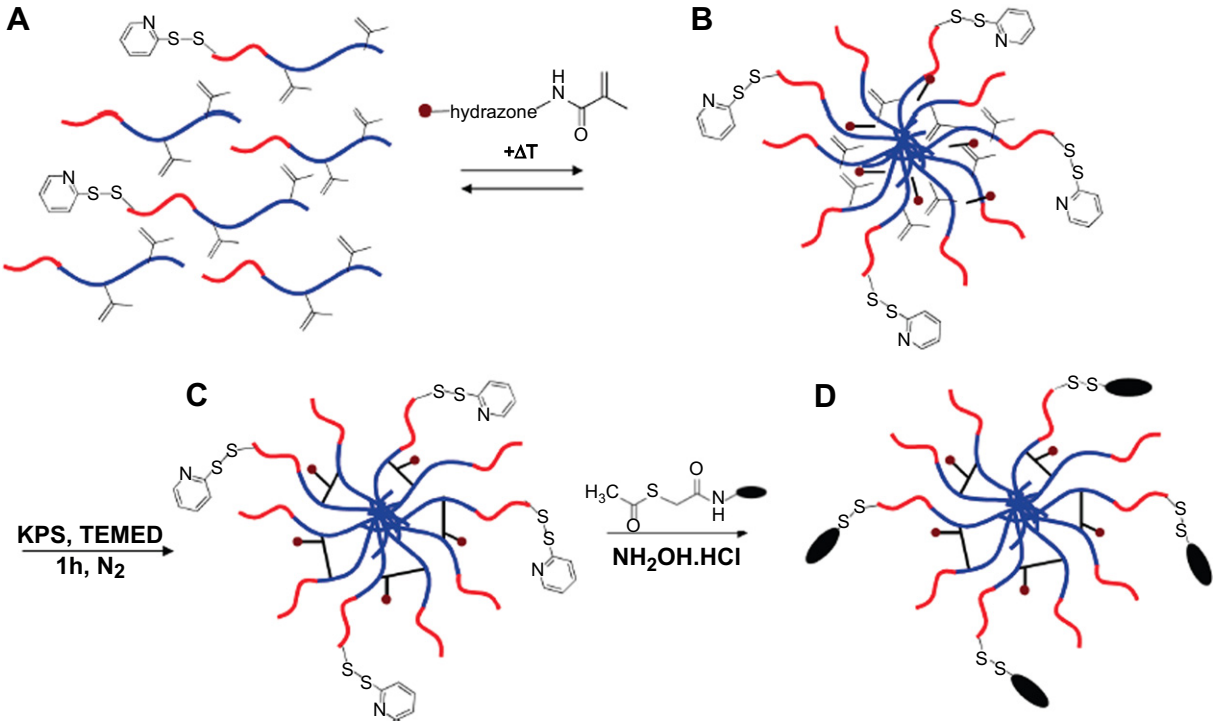

Fig. 2. Synthetic scheme for the preparation of nanobody-modified PM. Initially, mPEG-*b*-pHPMAmLac$_n$ and PDP-PEG-*b*-pHPMAmLac$_n$ copolymers (A) were mixed with DOX-MA, and upon employing the rapid heating procedure, they formed DOX-MA loaded micellar self-assemblies (B) with a size of ~70 nm and a low PDI. These self-assemblies were then core-crosslinked (C), assuring not only longevity in blood upon i.v. administration but also stable drug encapsulation (via co-crosslinking of DOX-MA in the micellar core). Finally, Ega1 nanobodies were conjugated to the surface of the core-crosslinked micelles, to enhance cellular uptake and confer intrinsic antitumor activity (D).

can be used to confer intrinsic antitumor activity and thereby facilitate tumor-targeted combination therapy, these findings demonstrate that nanobody-modified carrier materials are able to inhibit tumor growth even in the absence of a drug.

The above insights were confirmed and extended by comparing the therapeutic activity of untargeted DOX-PM with that of Ega1-modified DOX-PM, showing that the latter were significantly more effective, not only in inhibiting tumor growth

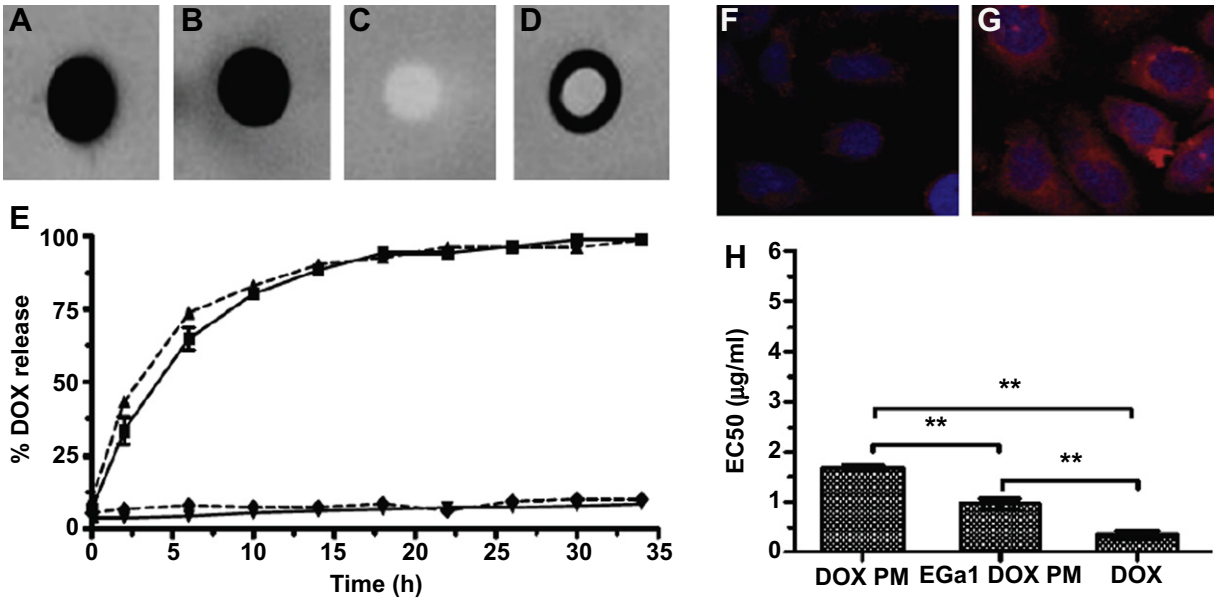

Fig. 3. Characterization of nanobody-modified PM. A–D: Dot blot of free EGa1 nanobody (A), drug-free EGa1-modified (PDP-PEG/mPEG)-*b*-pHPMAmLac$_n$ polymeric micelles (B), DOX-containing mPEG-*b*-pHPMAmLac$_n$ polymeric micelles (C) and EGa1-modified DOX-containing (PDPPEG/mPEG)-*b*-pHPMAmLac$_n$ polymeric micelles (D). The white core in panels C and D is caused by interference of doxorubicin fluorescence with the chemiluminescent detection method employed. E: Release of DOX from untargeted PM at pH 5 (continuous line, ■) and pH 7.4 (continuous line, ▼), and from nanobody-modified PM at pH 5 (discontinuous line, ▲) and pH 7.4 (discontinuous line, ◆). F–G: Uptake of untargeted (F) and nanobody-modified (G) PM containing rhodamine (as a model drug; in red) by 14C cells upon 4 h of incubation at 37 °C. H: In vitro cytotoxicity, evaluated using the WST-1 assay. Cells were pulse-incubated with the indicated formulations for 4 h, followed by medium replacement and allowed to grow for another 68 h. Results are expressed as the DOX-equivalent concentration required for inhibiting the viability of 14C cells by 50%. ** indicates $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

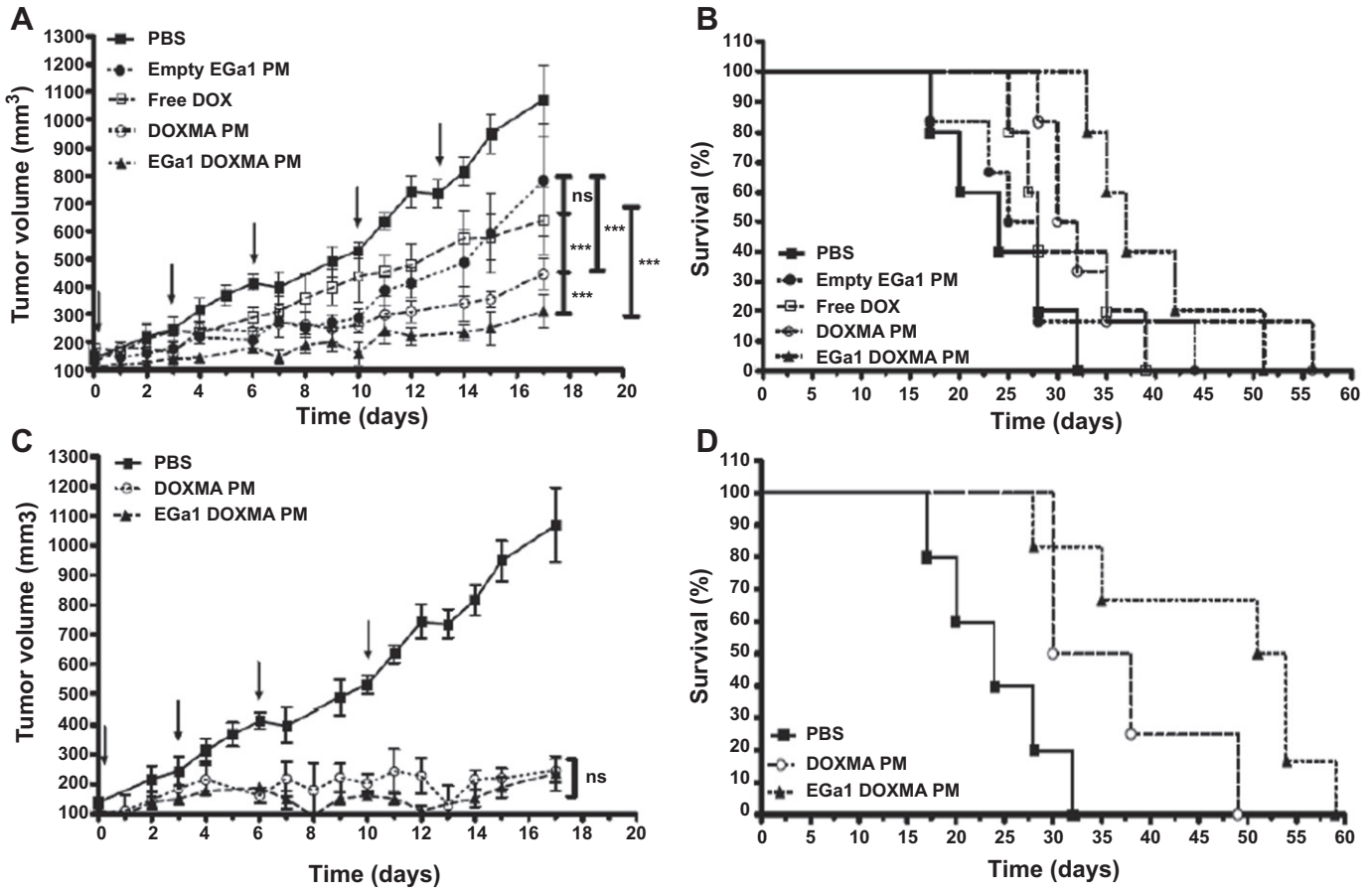

Fig. 4. In vivo efficacy of nanobody-modified PM. A–B: Mean tumor volumes (A) and survival times (B) of mice bearing subcutaneous 14C xenografts after the i.v. administration of PBS, drug-free EGa1-modified PM, free doxorubicin (3 mg/kg), DOX-containing PM (3 mg DOX/kg) and EGa1-modified DOX-containing PM (3 mg DOX/kg). Mice were treated every 3 days for a total of 5 injections (as indicated by the arrows). C–D: Mean tumor volumes (C) and survival times (D) of 14C tumor-bearing mice upon treatment with high-dose DOX-containing PM (9 mg DOX/kg) and EGa1-modified DOX-containing PM (9 mg DOX/kg). Mice were treated every 3 days for a total of 4 injections (as indicated by the arrows). *** indicates $p < 0.001$.

($p < 0.001$; Fig. 4A), but also in prolonging the survival of the animals (median survival time: 31 vs. 37 days; Fig. 4B). Moreover, since in a previous study in mice bearing B16F10 melanomas, no signs of toxicity were observed upon treatment with untargeted DOX-containing PM in this dose range [21], we also assessed the efficacy of both micellar nanomedicines at a higher dose (i.e. 4 × 9 mg/kg). In this case, as exemplified in Fig. 4C–D, both untargeted and targeted DOX-PM almost completely suppressed tumor growth. At several time points during initial follow-up (i.e. up to day 17) however, the average tumor volume in animals treated with nanobody-modified DOX-PM tended to be lower than that in animals treated with standard DOX-PM (Fig. 4C). In addition, also during long-term follow-up (i.e. up until day 60), animals treated with Ega1-modified DOX-PM survived longer than did animals treated with standard DOX-PM (median survival time: 53 vs. 32 days; Fig. 4D). This enhanced efficacy is expected to be due to the enhanced uptake of nanobody-modified PM as compared to untargeted PM, to improved cancer cell specificity of nanobody-modified PM upon accumulating in tumors via EPR (vs. accumulation primarily in macrophages; see Refs. [16,17]), and to an additive or synergistic interaction of DOX, which is efficiently delivered to tumors using PM, with the intrinsic pharmacological activity of the nanobodies on the surface of the PM, thereby enabling a rational and a highly promising form of tumor-targeted combination therapy.

4. Conclusion

We here present therapeutic results obtained with nanobody-targeted core-crosslinked polymeric micelles with covalently entrapped doxorubicin. In vitro, nanobody-modified DOX-PM were significantly more effective in killing cancer cells than was untargeted DOX-PM. In vivo, nanobody-modified PM inhibited tumor growth, even in the absence of a drug. As a result of this intrinsic anticancer activity, nanobody-modified DOX-PM were found to be more effective than were untargeted DOX-PM, not only in inhibiting tumor growth, but also in prolonging animal survival. Therefore, it can be concluded that the combination of two therapeutic strategies in one nanocarrier formulation – i.e. the intrinsic pharmacological activity of nanobody-targeted PM with the cytostatic activity of micellar doxorubicin – is a highly promising strategy for improving the treatment of advanced solid malignancies.

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