Addition of low-dose tumor necrosis factor-α to systemic treatment with STEALTH liposomal doxorubicin (Doxil) improved anti-tumor activity in osteosarcoma-bearing rats

Saske Hoving[a], Ann L. B. Seynhaeve[a], Sandra T. van Tiel[a], Alexander M. M. Eggermont[a] and Timo L. M. ten Hagen[a]

Improved efficacy of Doxil (STEALTH liposomal doxorubicin) compared to free doxorubicin has been demonstrated in the treatment of several tumor types. We have shown that addition of low-dose tumor necrosis factor (TNF) to systemic Doxil administration dramatically improved tumor response in the highly vascularized rat soft tissue sarcoma BN175. Whether a similar enhanced efficacy can be achieved in less vascularized tumors is uncertain. We therefore examined the effect of systemic administration of Doxil in combination with low-dose TNF in intermediate vascularized osteosarcoma-bearing rats (ROS-1). Small fragments of the osteosarcoma were implanted s.c. in the lower limb. Treatment was started when the tumors reached an average diameter of 1 cm. Rats were treated with five i.v. injections at 4-day intervals with Doxil or doxorubicin and TNF. Systemic treatment with Doxil resulted in a better tumor growth delay than free doxorubicin, but with progressive diseases in all animals. The 3.5-fold augmented accumulation of Doxil compared to free doxorubicin presumably explains the enhanced tumor regression. Addition of low-dose TNF augmented the anti-tumor activity of Doxil, although no increased drug uptake was found compared to Doxil alone. *In vitro* studies showed that ROS-1 is sensitive to TNF, but systemic treatment with TNF alone did not result in a tumor growth delay. Furthermore, we demonstrated that treatment with Doxil alone or with TNF resulted in massive coagulative necrosis of tumor tissue. In conclusion, combination therapy of Doxil and low-dose TNF seems attractive for the treatment of highly vascularized tumors, but also of intermediate vascularized tumors like the osteosarcoma. *Anti-Cancer Drugs* 16:667–674 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:667–674

Keywords: Doxil, doxorubicin, osteosarcoma, systemic treatment, tumor necrosis factor-α, rat

[a]Department of Surgical Oncology, Erasmus MC Daniel den Hoed Cancer Center, Rotterdam, The Netherlands.

Sponsorship: The work was funded by grant DDHK 2000-2224 of the Dutch Cancer Society. Tumor necrosis factor was a generous gift from Dr G. Adolf (Bender Wien, Wien, Austria) and Doxil was kindly provided by Dr P. Working (ALZA, Mountain View, CA).

Correspondence to T. L. M. ten Hagen, Department of Surgical Oncology, Laboratory of Experimental Surgical Oncology, Erasmus MC, Room Ee 0102a, PO Box 1738, 3000 DR Rotterdam, The Netherlands.
Tel: +31 10 408 7682; fax: +31 10 408 9471;
e-mail: t.l.m.tenhagen@erasmusmc.nl

Received 30 November 2004 **Revised form accepted** 30 March 2005

Introduction

Encapsulation of anti-cancer agents in liposomes offers a potential means of manipulating drug distribution to improve anti-tumor efficacy and reduce toxicity. STEALTH liposomes are sterically stabilized liposomes that contain methoxy-polyethylene glycol (MPEG). Because of their small size, long circulation time and reduced interaction with formed elements of the blood, these liposomes tend to accumulate in tumors, presumably due to leakage through the often-compromised tumor vasculature [1–8]. Doxil (STEALTH liposomal doxorubicin) is effective in the treatment of several tumor types, including advanced or metastatic soft tissue sarcoma [9], AIDS-related Kaposi's sarcoma [10], metastatic breast cancer [11] and epithelial ovarian cancer [12].

Preclinical and clinical studies have shown impressive improvement of the anti-tumor activity of melphalan and doxorubicin in local treatment of different tumor types when tumor necrosis factor (TNF) was co-administered [13–18]. We demonstrated that the basis for the synergy is, on the one hand, a significant enhancement of tumor-selective melphalan and doxorubicin uptake, and, on the other hand, the subsequent destruction of tumor vasculature caused by TNF [14,19].

Successful application of TNF for systemic treatment of tumors, however, is seriously hampered by its severe toxicity and therefore only low dosages can be administered [20,21]. In previous studies we showed that systemic co-administration of Doxil and low-dose TNF resulted in a pronounced tumor response in both rat and mouse tumor models. In soft tissue sarcoma-bearing rats systemic treatment with Doxil in combination with low-dose TNF improved the anti-tumor activity dramatically, resulting in a tumor response (complete or partial regression) in most of the animals. Repeated injection

0959-4973

of Doxil combined with TNF resulted in augmented accumulation of the drug in tumor tissue which could explain the observed synergistic anti-tumor effect [22]. When B16BL6 melanoma-bearing mice were injected with Doxil combined with TNF, an increased drug accumulation was also found compared to liposomes alone [23]. TNF increases the leakiness of the vasculature by increasing the gaps between the endothelial lining in the tumor, possibly explaining the augmented accumulation after extravasation of liposomes [24,25].

In the experiments described here we evaluate whether the use of TNF in combination with Doxil not only results in a synergistic anti-tumor response in the highly vascularized soft tissue sarcoma, but also in the less-vascularized osteosarcoma.

Materials and methods

Chemicals

Human recombinant TNF-α (specific activity 5×10^7 IU/mg) was kindly provided by Dr. G. Adolf (Bender, Wien, Austria) and stored at a concentration of 2 mg/ml at −80°C or under liquid nitrogen. Endotoxin levels (LAL) were below 0.624 EU/mg. Pegylated liposomal doxorubicin (Doxil) was kindly provided by Dr P. Working (ALZA, Mountain View, CA). Doxorubicin hydrochloride (adriblastina) was purchased from Pharmacia (Brussels, Belgium).

Animals and tumor model

Male inbred WAG/RIJ rats were used for the osteosarcoma model (ROS-1). This tumor originated spontaneously in the tibia of a rat. Rats were obtained from Harlan-CPB, (Austerlitz, The Netherlands) and weighed 250–300 g. Rats were fed a standard laboratory diet *ad libitum* (Hope Farms, Woerden, The Netherlands).

Small fragments (3 mm) of the syngeneic ROS-1 sarcoma or the soft-tissue sarcoma BN175 were implanted s.c. in the right hindleg as previously described [26]. Tumor growth was recorded by caliper measurement and tumor volume calculated using the formula $0.4(A^2 \times B)$ (where B represents the largest diameter and A the diameter perpendicular to B). Rats were sacrificed if tumor diameter exceeded 25 mm or at the end of the experiments. Animal studies were performed in accordance with protocols approved by the committee on Animal Research of the Erasmus MC.

Treatment protocol

Treatment was started when the ROS-1 tumors reached an average diameter of 9–11 mm. The rats were randomized into the following six groups: placebo liposomes (equivalent amounts of buffer or lipids), low-dose TNF, free doxorubicin, Doxil, doxorubicin plus TNF and Doxil plus TNF. Rats were injected 5 times i.v. with an interval of 4 days between the injections; first dose of 4.5 mg/kg Doxil or free doxorubicin and 1.0 mg/kg for consecutive doses. TNF was given at a concentration of 15 μg/kg for all five doses. When rats were treated with doxorubicin or Doxil combined with TNF, these agents were injected separately, shortly after each other.

The classification of tumor response was: progressive disease (PD) = increase of tumor volume (> 25%) over a 20 day period; no change (NC) = tumor volume equal to volume as start of treatment (−25 to +25%); partial remission (PR) = decrease of tumor volume (−25 to −90%); complete remission (CR) = tumor volume less than 10% of initial volume.

Measurement of doxorubicin accumulation in tumor tissue

The effect of TNF on Doxil accumulation in ROS-1 tumors was investigated. After three injections the tumor size of rats treated with Doxil in combination with TNF started to differ from the tumor size of rats in the other groups and for this reason we decided to compare doxorubicin tumor uptake not later than this time point. Rats received three injections of doxorubicin or Doxil with or without TNF with an interval of 4 days between the injections as described in the treatment protocol. Tumors were excised 24 h after the last injection, and tissues were analyzed for doxorubicin and its fluorescent metabolites as previously described [27]. Briefly, tumors were incubated in acidified isopropanol (0.075 N HCl in 90% isopropanol) for 24 h at 4°C and after that the tumors were homogenized (PRO200 homogenizer with 10-mm generator; Pro Scientific, Oxford, CT), centrifuged for 30 min at 2500 r.p.m. and supernatants were harvested. A Hitachi F4500 fluorescence spectrometer (excitation 472 nm and emission 590 nm) was used for measurement of the samples. A standard curve was prepared with known concentrations of doxorubicin diluted in acidified isopropanol. All measurements were repeated after addition of an internal doxorubicin standard.

Histology

Rats were treated with placebo liposomes, TNF, Doxil or Doxil + TNF. When the tumors reached an average diameter of 17 ± 1 mm, they were excised and fixed in 4% formaldehyde and embedded in paraffin. Tissue sections of 4 μm were cut and stained with hematoxylin & eosin, examined on a Leica DM-RXA and photographed using a Sony DXC950 camera. At least three different tumors in each experimental group were subjected to blind evaluation.

Immunohistochemistry

Untreated BN175 and ROS-1 tumors with a diameter of 9–11 mm were excised and immediately frozen in liquid nitrogen. Immunohistochemical studies were performed on acetone-fixed 7-μm cryostat sections. The tumor

sections were fixed for 30 min with 4% formaldehyde and, after rinsing with PBS, the endogenous peroxidase activity was blocked by incubation for 5 min in methanol/3% H_2O_2. The slides were incubated for 1 h with 1:50 mouse anti-rat-CD31 (Becton Dickinson, Alphen aan den Rijn, The Netherlands) diluted in 5% rat serum/PBS. Thereafter, sections were washed with PBS and incubated for 1 h with goat anti-mouse peroxidase-labeled antibody (Dako, ITK Diagnostics, Uithoorn, The Netherlands) diluted 1:100 in PBS with 5% rat serum. After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB solution (DAB kit; Dako) and counterstained lightly with hematoxylin (Sigma, St Louis, MO).

The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera. The number of vessels and the area of vessels per field of interest were measured in calibrated digital images (Research Assistant 3.0; RVC, Hilversum, the Netherlands). For quantification of the microvessel density (MVD) six representative fields of interest per slide (magnification × 16), three slides per tumor and four animals per group were examined.

In vitro cytotoxicity assay

The rat osteosarcoma ROS-1 cells were maintained in modified Eagle's medium supplemented with 10% fetal calf serum and 0.1% penicillin/streptomycin. Media and supplements were obtained from Life Technologies (Breda, The Netherlands).

ROS-1 cells were added in 100-µl aliquots to 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at a final concentration of 1×10^4 cells/well and allowed to grow as a monolayer. Cells were incubated at 37°C in 5% CO_2 for 72 h in the presence of various concentrations of TNF, doxorubicin and Doxil. The range of final drug concentrations was 0.1–10 µg/ml for TNF, 0.001–100 µg/ml for doxorubicin and 0.05–1000 µg/ml for Doxil. Growth of tumor cells was measured using the sulforhodamine B (SRB) assay according to the method of Skehan [28]. In short, cells were washed twice with phosphate-buffered saline, incubated with 10% trichloroacetic acid (1 h, 4°C) and washed again. Cells were stained with 0.4% SRB (15–30 min), washed with 1% acetic acid and allowed to dry. Protein-bound SRB was dissolved in Tris (10 mM, pH 9.4). The optical density was read at 540 nm. Tumor cell growth was calculated using the formula: cell growth = (test well/control) × 100%. The drug concentration reducing the cell growth to 50% of the control (IC_{50}) was determined from the growth curves. The experiments were repeated at least 5 times.

In vitro assessment of doxorubicin uptake in tumor cells

Intracellular doxorubicin levels in osteosarcoma ROS-1 cells were measured by flow cytometry to determine whether the observed *in vitro* toxicity correlated with cellular uptake of doxorubicin. ROS-1 cells were plated in 24-well plates at a final concentration of 5×10^4 cells/well and allowed to grow as a monolayer. Doxorubicin, Doxil and TNF, diluted in DMEM supplement with 10% fetal bovine serum, were added to the wells, after which cells were incubated for 10, 30, 60 and 120 min. The final drug concentration in the wells for all three drugs was 0, 0.1, 1.0 and 10 µg/ml. After incubation, cells were washed to discard non-incorporated drug and treated with trypsin/EDTA for 2 min. The cell suspensions were washed twice in DMEM supplemented with 10% FCS and resuspended in PBS. Cellular uptake was measured on a Becton Dickinson FACScan using CellQuest software on an Apple Macintosh computer. Excitation was set at 488 nm and emission at 530 nm. Fluorescence was corrected for cell size using the forward scatter (FSC) with the formula: corrected fluorescence (FLcor) = fluorescence at 530 nm (FL530)/FSC – $FL530_c/FSC_c$ ($FL530_c$ and FSC_c are fluorescence and forward scatter with no drug added to the cells).

Statistical analysis

Results were evaluated for statistical significance with the Mann–Whitney *U*-test. $p < 0.05$ was considered statistically significant. Calculations were performed on a personal computer using GraphPad Prism (version 3.0) and SPSS (version 10.0) for Windows 2000.

Results

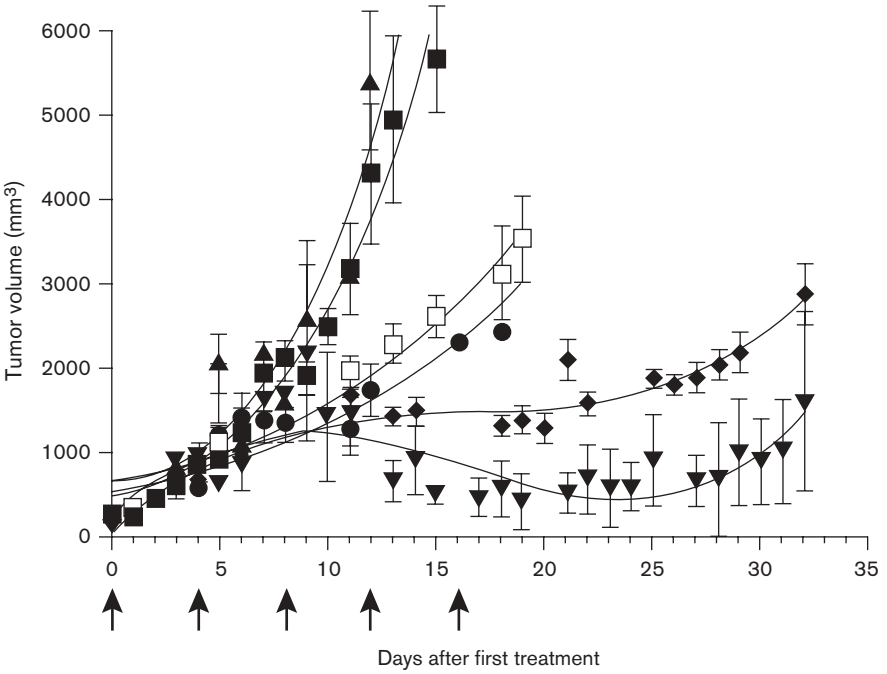
Effect of low-dose TNF on anti-tumor activity of Doxil

To evaluate the anti-tumor activity of Doxil in combination with low-dose TNF, osteosarcoma-bearing rats were treated with a total of five injections of doxorubicin or Doxil with or without TNF. Treatment with free doxorubicin resulted in a slight delay in tumor growth, with progressive diseases in all animals (Fig. 1). Progressive disease was also seen in all animals treated with Doxil, but with a better tumor growth delay than free doxorubicin. Addition of TNF to the Doxil treatment enhanced the anti-tumor response, resulting in a response rate of 50% (partial plus complete response). In contrast, addition of TNF to doxorubicin treatment did not improve the anti-tumor response. Treatment with TNF alone had no anti-tumor effect. No obvious systemic toxicity was observed in any of the treatments.

Histologic examination of anti-tumor activity of Doxil in combination with TNF

Histopathological examination was performed on tumors with the same size, shortly after regrowth occurred. Treatment of the rats with Doxil and Doxil in combination with TNF resulted in severe tumor necrosis and extensive cell death (Fig. 2). Massive coagulative necrosis of 68% of the tumor area was seen in Doxil-treated tumors and 40% in the Doxil + TNF-treated tumors. Less necrosis was seen in rats treated with placebo

Fig. 1



Growth curves of s.c. implanted ROS-1 osteosarcoma after systemic treatment with Doxil and low-dose TNF. Rats were injected 5 times i.v. with an interval of 4 days between the injections; first-dose 4.5 mg/kg Doxil or free doxorubicin and 1.0 mg/kg for consecutive doses and 15 μg/kg TNF for all five doses. Buffer alone (▲), TNF (■), doxorubicin (□), Doxil (♦), doxorubicin + TNF (●) and Doxil + TNF (▼). Mean tumor volumes are shown ± SEM.

liposomes or TNF (10 and 17%, respectively). In all four groups, infiltrated PMN were detected.

Doxil and doxorubicin accumulation in tumor tissue

To investigate whether the observed beneficial effect of TNF on the Doxil treatment was due to an increased liposome extravasation into tumor tissue, doxorubicin concentrations in tumors after three treatments were determined. Doxorubicin levels in tumors were 3.5-fold higher when Doxil was injected compared to free doxorubicin, although not significantly (Fig. 3). Addition of TNF did not induce a further accumulation of Doxil and TNF also had no effect on the tumor uptake of free doxorubicin.

MVD

Quantification of the MVD was performed by immunohistochemical staining of endothelial cells in frozen BN175 and ROS-1 tumor sections (Fig. 4A–D). The number of vessels as well as the total tumor vessel area was measured. The area per vessel is computed by dividing the total vessel area by the number of vessels. The number of vessels in the BN175 tumor was 2.9 times higher than in the ROS-1 tumor (Fig. 4A) ($p = 0.021$), although the vessels have the same size (Fig. 4B).

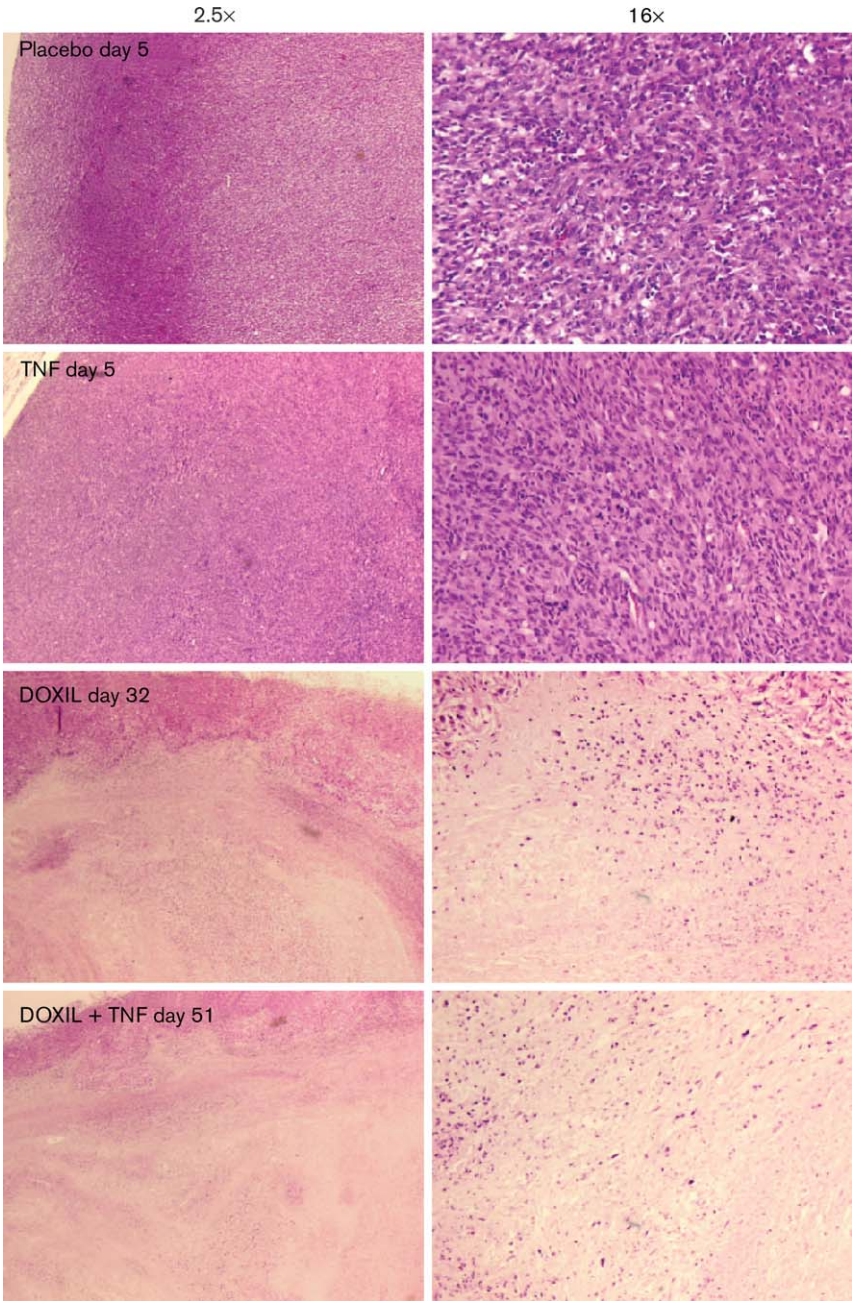
Direct effect of Doxil combined with TNF on ROS-1 cells *in vitro*

In vitro experiments were performed to define whether direct cytotoxicity contributed to the improved tumor response of Doxil in combination with TNF. Figure 5 shows that exposure of ROS-1 tumor cells to doxorubicin resulted in a dose-dependent cell growth inhibition, with an IC_{50} of 3.8 μg/ml. Doxil appeared to be less cytotoxic to osteosarcoma cells with an IC_{50} of 25.7 μg/ml. ROS-1 cells were moderately sensitive to TNF with a maximum growth inhibition of 32% at 10 μg/ml TNF. Addition of TNF to doxorubicin or Doxil had no effect on the cytotoxicity of the anti-tumor agents; comparable IC_{50} values were found.

In vitro uptake of Doxil or doxorubicin in tumor cells *in vitro*

We examined if TNF augmented the intracellular accumulation of doxorubicin or Doxil, which could explain the improved tumor response. Increased intracellular concentrations of doxorubicin or Doxil were observed when incubated with increasing concentrations of doxorubicin or Doxil (ranging from 0.1 to 10 μg/ml) and increasing time (ranging from 10 to 120 min). Addition of TNF up to 10 μg/ml did not result in an increased uptake of doxorubicin or Doxil (data not shown).

Fig. 2



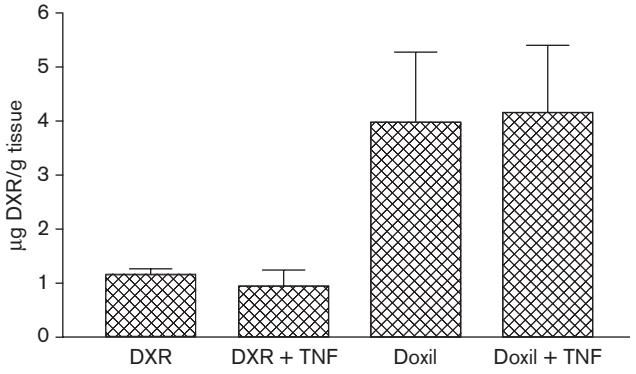
Paraffin sections of the rat osteosarcoma after systemic treatment, hematoxylin & eosin stained. Rats were treated with placebo liposomes, TNF (15 μg/kg), Doxil (4.5 mg/kg first dose and 1 mg/kg doses 2–5) or TNF combined with Doxil. Representative pictures are shown. Original magnification × 2.5 and × 16.

Discussion

In the present study we demonstrated that STEALTH liposomal doxorubicin (Doxil) resulted in a better tumor growth delay than free doxorubicin in osteosarcoma-bearing rats. However, tumor control was not achieved as progressive disease was observed in all animals. Addition of low-dose TNF augmented the anti-tumor activity of Doxil resulting in a response rate of 50%. These experiments are in agreement with earlier studies where we demonstrated that TNF improved the anti-tumor activity of Doxil in a rat soft tissue sarcoma and a mouse melanoma model [22,23].

Transport of small drugs across the blood vessel wall involves diffusion, whereas transport of macromolecules involves convection. Diffusion is the random motion of small molecules. Convection is mediated by the movement of fluid [29–32]. In highly vascularized tumors more tumor cells will be reached by the chemotherapeutics. In general, tumor vessels are more permeable than normal vessels and the maximum size of particles that can cross the tumor vessel wall is called the pore cutoff size. There is a large variance in cutoff sizes in different tumor types. Vessels of some, but not all, primary brain tumors are nearly impermeable, while in other tumors cutoff pore sizes between 100 nm and 1.2 μm are found. Vascular permeability may depend on tumor type and microenvironment, and increases with tumor size. It is believed that it is not the MVD that is the limiting factor of macromolecule drug delivery to solid tumors, but the permeability of the tumor vessels [33,34].

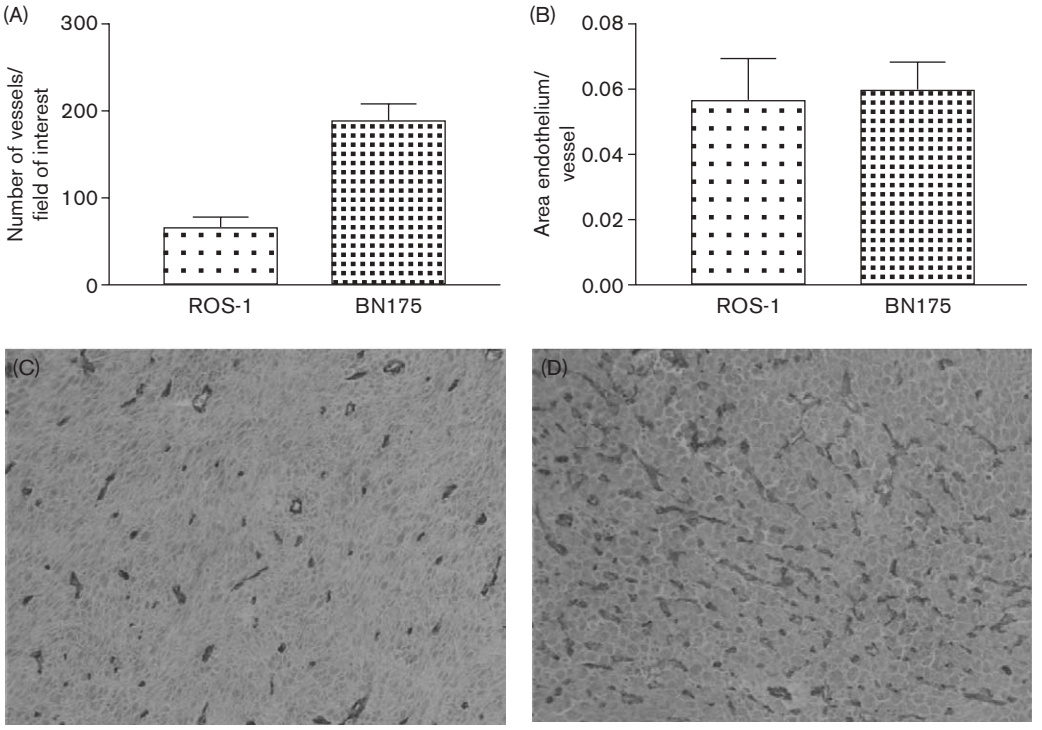
Fig. 3



Concentrations of doxorubicin in ROS-1 tumors after systemic administration of three injections of doxorubicin (DXR) or Doxil (4.5 mg/kg first dose and 1 mg/kg doses 2 and 3) with or without TNF. Tumors were excised 24 h after the last injection. The mean of three to six rats is shown ± SEM.

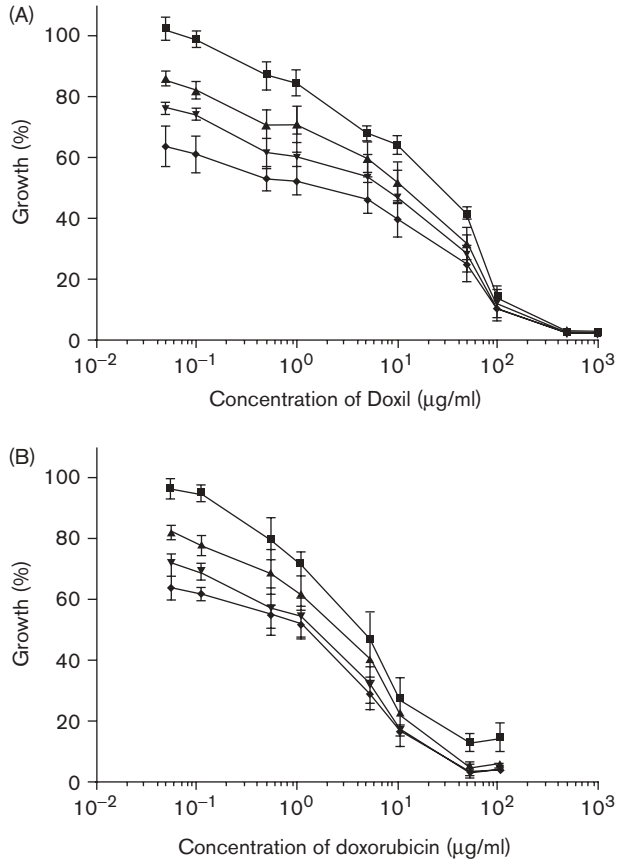
Several studies have shown that encapsulation of anticancer agents in liposomes can reduce systemic toxicity while retaining or even improving *in vivo* efficacy [1,4,7,35]. Doxil is effective in the treatment of several tumor types, including advanced or metastatic soft tissue sarcoma [9], AIDS-related Kaposi's sarcoma [10], metastatic breast cancer [11] and epithelial ovarian cancer [12]. STEALTH liposomes have a long circulation time

Fig. 4



MVD of untreated ROS-1 and BN175 tumors 9–11 mm in diameter was assessed by immunohistochemical staining for CD31. (A) Number of vessels per field of interest. The BN175 tumor has 2.9 times more vessels than the ROS-1 tumor ($p = 0.021$). (B) area endothelium per vessel. The vessels of the BN175 and ROS-1 tumors are of the same size. Six representative fields of interest per slide (magnification × 16), three slides per tumor and four animals per group were examined. The mean ± SEM is shown. Photographs of cryostat sections of untreated tumors 8–10 mm in diameter stained for CD31. (C) ROS-1, (D) BN175. Representative images are shown. Original magnification × 16.

Fig. 5



In vitro growth of the ROS-1 tumor cells as a function of the added amount of Doxil (A) or doxorubicin (B) in combination with 0 (■), 0.1 (▲), 1 (▼) and 10 (♦) μg TNF/ml. The means of at least three individual experiments performed in duplicate are shown ± SEM.

and tend to accumulate in tumors, presumably due to leakage through the tumor vasculature [1,3,4,6–8,36]. In the present study we showed that systemic treatment with Doxil resulted in a better tumor growth delay than free doxorubicin and that the 3.5-fold increased drug accumulation could explain the observed enhanced anti-tumor effect. The anti-tumor activity of Doxil in the osteosarcoma model is comparable to the anti-tumor activity in the soft-tissue sarcoma BN175, although *in vitro* studies showed a clear difference. BN175 cells are 10 times more sensitive to Doxil than ROS-1 cells [22]. Four times more Doxil is found in ROS-1 tumor tissue than in the BN175 tumor after three i.v. injections of Doxil alone. Most likely this indicates that the tumor vessels of the ROS-1 tumor are more permeable than the vessels of the BN175 tumor.

Previously we reported on the improved anti-tumor activity of systemically injected Doxil when combined with low-dose TNF in soft tissue sarcoma-bearing rats. Repeated injections of Doxil + TNF resulted in augmented tumor uptake of doxorubicin which could explain the observed enhanced anti-tumor effect [22]. Also in the B16 melanoma-bearing mice an augmented accumulation of Doxil is seen when combined with TNF. TNF likely increases the leakiness of the vasculature by increasing the gaps between the endothelial lining in the tumor, possibly explaining the augmented accumulation after extravasation of liposomes [24,25].

In the present study we did not observe an increased drug accumulation when Doxil was combined with TNF in the osteosarcoma ROS-1 model. TNF has an effect on tumor endothelial cells and the ROS-1 tumor is less vascularized than the BN175 tumor. Although in TNF-based isolated hepatic perfusions a highly vascularized tumor results in a good tumor response and *vice versa*, no such effect was found in the TNF-based isolated limb perfusion. Both the BN175 and the ROS-1 tumor showed a synergistic anti-tumor response when TNF is added to the ILP [18,37].

In vitro studies demonstrated a higher sensitivity of the osteosarcoma cells for TNF as compared to the BN175 cells. The dose of TNF used in the systemic treatment is probably too low to have an effect on its own, but may switch the balance to a better response when combined with Doxil. Also, administration of TNF may trigger local production of TNF and other cytokines improving tumor response when combined with Doxil.

We found less coagulative necrosis in tumor tissue after treatment with Doxil and TNF than with Doxil alone. We think this is due to the fact that the tumors were excised at the same size. As the tumors from Doxil + TNF-treated rats regressed more, they also regrew more to gain the same size as the Doxil alone-treated tumors.

We demonstrated for the first time that TNF augments the anti-tumor activity of Doxil not only in highly vascularized tumors like the soft-tissue sarcoma BN175, but also in the intermediate vascularized ROS-1. These findings confirm the promising role for TNF as an enhancer of systemic therapy of solid tumors with STEALTH liposomes.

References

1 Allen TM, Newman MS, Woodle MC, Mayhew E, Uster PS. Pharmacokinetics and anti-tumor activity of vincristine encapsulated in sterically stabilized liposomes. *Int J Cancer* 1995; **62**:199–204.
2 Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA* 1988; **85**:6949–6953.
3 Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumors. *Drugs* 1997; **54**(**suppl 4**): 15–21.
4 Lasic DD. Doxorubicin in sterically stabilized liposomes. *Nature* 1996; **380**:561–562.

5 Maeda H, Fang J, Inutsuka T, Kitamoto Y. Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications. *Int Immunopharmacol* 2003; **3**:319–328.

6 Mayer LD, Cullis PR, Bally MB. Designing therapeutically optimized liposomal anticancer delivery systems: lessons from conventional liposomes. In: Lasic DD, Papahadjopoulos D (editors): *Medical Applications of Liposomes*. Amsterdam: Elsevier; 1998, pp. 231–257.

7 Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, *et al.* Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA* 1991; **88**:11460–11464.

8 Wu NZ, Da D, Rudoll TL, Needham D, Whorton AR, Dewhirst MW. Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumor tissue. *Cancer Res* 1993; **53**:3765–3770.

9 Judson I, Radford JA, Harris M, Blay JY, van Hoesel Q, Le Cesne A, *et al.* Randomised phase II trial of pegylated liposomal doxorubicin (DOXIL/CAELYX) versus doxorubicin in the treatment of advanced or metastatic soft tissue sarcoma: a study by the EORTC Soft Tissue and Bone Sarcoma Group. *Eur J Cancer* 2001; **37**:870–877.

10 Northfelt DW, Dezube BJ, Thommes JA, Miller BJ, Fischl MA, Friedman-Kien A, *et al.* Pegylated-liposomal doxorubicin versus doxorubicin, bleomycin, and vincristine in the treatment of AIDS-related Kaposi's sarcoma: results of a randomized phase III clinical trial. *J Clin Oncol* 1998; **16**:2445–2451.

11 O'Brien ME, Wigler N, Inbar M, Rosso R, Grischke E, Santoro A, *et al.* Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX/Doxil) versus conventional doxorubicin for first-line treatment of metastatic breast cancer. *Ann Oncol* 2004; **15**:440–449.

12 Stebbing J, Gaya A. Pegylated liposomal doxorubicin (Caelyx) in recurrent ovarian cancer. *Cancer Treat Rev* 2002; **28**:121–125.

13 Bickels J, Manusama ER, Gutman M, Eggermont AM, Kollender Y, Abu-Abid S, *et al.* Isolated limb perfusion with tumour necrosis factor-alpha and melphalan for unresectable bone sarcomas of the lower extremity. *Eur J Surg Oncol* 1999; **25**:509–514.

14 Eggermont AM, Schraffordt KH, Lienard D, Kroon BB, van Geel AN, Hoekstra HJ, *et al.* Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. *J Clin Oncol* 1996; **14**:2653–2665.

15 Eggermont AM, Schraffordt KH, Klausner JM, Kroon BB, Schlag PM, Lienard D, *et al.* Isolated limb perfusion with tumor necrosis factor and melphalan for limb salvage in 186 patients with locally advanced soft tissue extremity sarcomas. The cumulative multicenter European experience. *Ann Surg* 1996; **224**:756–764.

16 Lienard D, Lejeune FJ, Ewalenko P. In transit metastases of malignant melanoma treated by high dose rTNF alpha in combination with interferon-gamma and melphalan in isolation perfusion. *World J Surg* 1992; **16**:234–140.

17 Olieman AF, Lienard D, Eggermont AM, Kroon BB, Lejeune FJ, Hoekstra HJ, *et al.* Hyperthermic isolated limb perfusion with tumor necrosis factor alpha, interferon gamma, and melphalan for locally advanced nonmelanoma skin tumors of the extremities: a multicenter study. *Arch Surg* 1999; **134**:303–407.

18 van Der Veen AH, de Wilt JH, Eggermont AM, van Tiel ST, Seynhaeve AL, ten Hagen TL. TNF-alpha augments intratumoural concentrations of doxorubicin in TNF-alpha-based isolated limb perfusion in rat sarcoma models and enhances anti-tumour effects. *Br J Cancer* 2000; **82**:973–980.

19 de Wilt JH, ten Hagen TL, de Boeck G, van Tiel ST, de Bruijn EA, Eggermont AM. Tumour necrosis factor alpha increases melphalan concentration in tumour tissue after isolated limb perfusion. *Br J Cancer* 2000; **82**:1000–1003.

20 Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, *et al.* Shock and tissue injury induced by recombinant human cachectin. *Science* 1986; **234**:470–474.

21 Spriggs DR, Sherman ML, Michie H, Arthur KA, Imamura K, Wilmore D, *et al.* Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study. *J Natl Cancer Inst* 1988; **80**:1039–1044.

22 ten Hagen TL, van Der Veen AH, Nooijen PT, van Tiel ST, Seynhaeve AL, Eggermont AM. Low-dose tumor necrosis factor-alpha augments antitumor activity of stealth liposomal doxorubicin (DOXIL) in soft tissue sarcoma-bearing rats. *Int J Cancer* 2000; **87**:829–837.

23 Brouckaert P, Takahashi N, van Tiel ST, Hostens J, Eggermont AM, Seynhaeve AL, *et al.* Tumor necrosis factor-alpha augmented tumor response in B16BL6 melanoma-bearing mice treated with stealth liposomal doxorubicin (Doxil) correlates with altered Doxil pharmacokinetics. *Int J Cancer* 2004; **109**:442–448.

24 Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J Exp Med* 1989; **169**:1977–1991.

25 Partridge CA, Horvath CJ, Del Vecchio PJ, Phillips PG, Malik AB. Influence of extracellular matrix in tumor necrosis factor-induced increase in endothelial permeability. *Am J Physiol* 1992; **263**:L627–L633.

26 Manusama ER, Nooijen PT, Stavast J, Durante NM, Marquet RL, Eggermont AM. Synergistic antitumour effect of recombinant human tumour necrosis factor alpha with melphalan in isolated limb perfusion in the rat. *Br J Surg* 1996; **83**:551–555.

27 Mayer LD, Tai LC, Ko DS, Masin D, Ginsberg RS, Cullis PR, *et al.* Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. *Cancer Res* 1989; **49**:5922–5930.

28 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**:1107–1112.

29 Boucher Y, Baxter LT, Jain RK. Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: implications for therapy. *Cancer Res* 1990; **50**:4478–4484.

30 Yuan F. Transvascular drug delivery in solid tumors. *Semin Radiat Oncol* 1998; **8**:164–175.

31 Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol* 1988; **133**:95–109.

32 Jain RK. Transport of molecules, particles, and cells in solid tumors. *Annu Rev Biomed Eng* 1999; **1**:241–263.

33 Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, *et al.* Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc Natl Acad Sci USA* 1998; **95**:4607–4612.

34 Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, *et al.* Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res* 1995; **55**:3752–3756.

35 Sparano JA, Winer EP. Liposomal anthracyclines for breast cancer. *Semin Oncol* 2001; **28**:32–40.

36 Newman MS, Colbern GT, Working PK, Engbers C, Amantea MA. Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. *Cancer Chemother Pharmacol* 1999; **43**:1–7.

37 Manusama ER, Stavast J, Durante NM, Marquet RL, Eggermont AM. Isolated limb perfusion with TNF alpha and melphalan in a rat osteosarcoma model: a new anti-tumour approach. *Eur J Surg Oncol* 1996; **22**:152–157.