Efficacy of doxorubicin thermosensitive liposomes (40˚C) and local hyperthermia on rat rhabdomyosarcoma

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Abstract. The efficacy of novel thermosensitive liposomes (40˚C) containing doxorubicin (Dox-Lip) together with local hyperthermia (HT) was studied on solid growing rat rhabdomyosarcomas. Tumor response and systemic toxicity were evaluated by comparing to free doxorubicin (Free Dox) with or without hyperthermia. Tumors were heated with infrared-A-radiation and drugs were infused intravenously after preheating the tumors followed by a further 60 min of heating at 42.5˚C. Recorded temperatures at various locations in the tumors indicated that all intratumoral temperatures, especially at the back rim, were definitely >40˚C. After single doses, tumor growth was further inhibited by Dox-Lip+HT compared to Free Dox+HT or Free Dox alone. Repeated treatments with Dox-Lip+HT (2x2.5 mg/kg+HT/2 weeks) resulted in a statistically significant tumor growth delay and was associated with a much lower systemic toxicity. Uptake studies of drugs in blood, tumor and normal tissues showed that Dox-liposomes (40˚C) are long circulating liposomes in the blood. However, the enhanced tumor response did not correlate with an increased uptake of Dox-Lip+HT in the tumor. The findings suggest that repeated applications of thermosensitive liposomal doxorubicin (40˚C) and local hyperthermia can control primary rat rhabdomyosarcomas while reducing the systemic toxicity of free doxorubicin.

Introduction

The disadvantage of chemotherapy is the lack of tumor-selective drug delivery after systemic application. Only a small fraction of the administered drug usually reaches the tumor. The main part of the drug is distributed throughout the body, producing dose-limiting systemic side effects. Significant barriers for the accumulation of drugs in the tumor are the chaotic tumor blood flow, the incomplete tumor vasculature and variable vessel permeability (1-4). To overcome this problem liposomes are promising carriers for chemotherapeutic agents in the treatment of tumors (5-9). Clinical studies, however, resulted in only a modest tumor response in a variety of cancers (10-15). In 1978, Yatvin *et al* designed liposomes that were stable at normal body temperature and permeable to encapsulated drugs when exposed to higher temperatures (16). Weinstein *et al* (17) were the first to show the additional effect of thermosensitive liposomes under hyperthermic conditions *in vivo* by an increase in intratumoral methotrexate delivery and a tumor growth delay compared to hyperthermia and the free drug (18) in an animal model. Since then, various types of thermosensitive liposomes differing in lipid chemistry, phase transition temperature (40-42˚C) and drug content have been intensively studied for a triggered drug release in tumors using local hyperthermia with temperatures of 40-45˚C (19-24). Studies on various tumor models pointed out an increased tumor-specific drug delivery and tumor growth delay when combining thermosensitive liposomes with local hyperthermia versus the free drug (24-34). Furthermore, local hyperthermia can trigger drug release from liposomes in the tumor vessel (35-39).

The release of drugs from thermosensitive liposomes in the heated tumor is closely related to temperature and temperature distribution in the tumor. Since a homogeneous heating of solid tumors is hard to achieve, thermosensitive liposomes with transition temperatures of 40-41˚C combined with mild hyperthermia should be of great advantage for tumor treatment. Therefore, we developed thermosensitive liposomes containing doxorubicin with a transition temperature of 40˚C (33). In an initial study the therapeutic efficacy of these liposomes on malignant brain tumors of rats in combination with interstitial RF-hyperthermia was investigated (33). It was found that doxorubicin concentrations in the middle hyperthermic areas of the tumor (>40˚C) were significantly enhanced following treatment with liposomal versus free doxorubicin. The aim of the present study was to investigate further the efficacy of the doxorubicin liposomes

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(40˚C) on solid growing rat rhabdomyosarcomas in combination with local hyperthermia, considering not only tumor response but also the systemic toxicity of the animals as compared to free doxorubicin. In particular, the efficacy after repeated administrations of doxorubicin liposomes (40˚C) and hyperthermia as well as the drug uptake in blood, tumor and normal tissues should be studied.

Materials and methods

Tumor model. The studies were performed on isotransplanted rhabdomyosarcomas R1H growing subcutaneously in the right flank of male WAG/RijH albino rats [body weight 275±22 g (±SD)]. The rat rhabdomyosarcoma R1H tumor model was chosen because this type of soft tissue sarcoma also exists in humans and is treated among other modalities with doxorubicin (14,40). It is a well-established tumor model in our laboratory and has been widely used in various studies (41-44). The origin of the tumor and further details of the tumor/host system have previously been described (41). Animals were housed under conventional conditions: room temperature was at 21±1˚C (mean ± SD), relative humidity at 55±5% and a light-dark cycle for 12 h. Animals were fed a standard diet (Altromin™, Altromin, Germany) and acidified vitamin C-fortified water was given *ad libitum*. The transplantation of tumors was performed by implanting small samples of ~1 mm^3 subcutaneously in the right flank of anesthetised animals. The tumor size was determined *in situ* by measuring the three orthogonal diameters as a, b, and c using vernier calipers. Tumor volume was calculated using an ellipsoid approximation for the tumor $V_t=\pi/6(axbxc)$. Tumors with volumes of 1.1±0.1 cm^3 (V_0) and a diameter of ~1.2 cm at days 11-14 after transplantation (tumor doubling time 2.7±0.1 days) were selected for the studies. A standardization of the tumor volume was important since tumor vascularisation and hypoxia change with tumor size (45). In addition, the rhabdomyosarcoma R1H tumor is an isogeneic tumor on the WAG/RijH rat and shows no signs of specific immunogenicity. For treatment, animals were anesthetised by intramuscular (i.m.) injections of 50 mg/kg b.w. ketamine (Ketavet™, Parke-Davis, Germany) in combination with 6 mg/kg b.w. xylazine (Rompun™, Bayer, Germany). To prevent a decrease in body temperature during anesthesia animals were positioned prone on a temperature-controlled pad at 37˚C. At the end of the treatments, the rats were sacrificed by carbon dioxide asphyxiation when R1H tumors reached a volume of ~5 V_0.

The animal studies were approved by the Hamburg Ministry of Health Ethics Committee and were conducted according to the German Law for Animal Protection.

Preparation of doxorubicin thermosensitive liposomes. Thermosensitive liposomes were prepared by the reverse-phase evaporation method as was previously reported (33). Briefly, the lipid composition consists of phosphatidylcholine: dipalmitoyl phosphatidylcholine:dipalmitoyl phosphatidyl glycerol:cholesterol at a molar ratio of 10.5:4.5:15:20. It was dissolved in a chloroform:methanol:water mixture at a volume ratio of 65:25:4. The resulting lipid solution was evaporated to form a dry lipid film by a rotary evaporator between 45 and 50˚C for 30 min. The dry lipid film was dispersed with distilled water and evaporated again. The liposomal powder was mixed with a solution of doxorubicin (Adriablastin®, Pharmacia & Upjohn), dissolved in a sodium chloride solution (1 mg/ml) and homogenized with a sonicator (Branson B-12, model 250/450 Sonifier®, Braun Company, Germany) for 20 min. These thermosensitive liposomes containing doxorubicin (Dox-Lip) sharply release the cytotoxic drug doxorubicin at a transition temperature of 40˚C. The mean size of the Dox-liposomes was 319±106 nm (± SD) determined by a direct laser scattering device (Nicomp 380™, USA). For each treatment Dox-liposomes were newly prepared. For terminoIogy the drug doxorubicin alone is expressed as Free Dox in the study.

Administration of drugs. The drugs were administered to the animals via a catheter into a branch of the left femoral vein. The skin above the left iliac region was shaved and disinfected with Cutasept™ (Bode-Chemie, Germany). The femoral vein was prepared under an operation microscope (Zeiss Company, Germany). A microcatheter (0.6 mm in diameter, silicone tubing, Sedat™, France) filled with heparinized saline was inserted up to the main trunk of the femoral vein and fixed by suture. The open end of the catheter was connected with the syringe of the micropump (Harvard apparatus™, USA) containing the drugs for infusion. The drugs were always infused within 15 min after preheating the tumors for 15 min. At the end of treatment the catheter was removed and the vessel closed. A small amount of Terramycin™ (Pfizer, Germany) was given to the operation field. The skin was closed with three metal clamps (9 mm, Clay Adams, USA) and Nobecutan-Spray™ (Astra, Germany). Repeated administrations of the drugs were applied by re-opening the catheter which was previously closed with a metal pin after the first administration and which remained in a small pocket under the skin. No side effects, e.g. catheter occlusions, subcutaneous abscesses or loss of catheters were observed.

Hyperthermia treatment. Tumors were locally heated with water-filtered infrared-A-radiation (hydrosun 500, Hydrosun Medizintechnik, Germany) from a distance of 25 cm and a radiation field of 3 cm in diameter (46,47). The treatment temperature was at 42.5±0.2˚C over the 60-min heating period controlled by a master probe (Fig. 1). Recorded temperatures at various locations of the R1H tumor had shown that after a heating-up phase of 15 min all intratumoral temperatures, in particular, at the back rim of the tumor, were definitely above the level of 40˚C when the infusion of Dox-liposomes and free doxorubicin started. To prevent a decrease in body temperature during hyperthermia treatment, the anesthetised animal was positioned prone on a small perspex table heated by a waterbath at 37˚C. The relatively minor increase of the rectal temperature indicated that the tumor was only locally heated.

Treatment and evaluation. Animals with tumors of 1.1±0.1 cm^3 were randomly divided into four groups according to the treatment protocols: controls (no treatment), Free Dox, Free Dox+HT and Dox-Lip+HT. The number of animals per

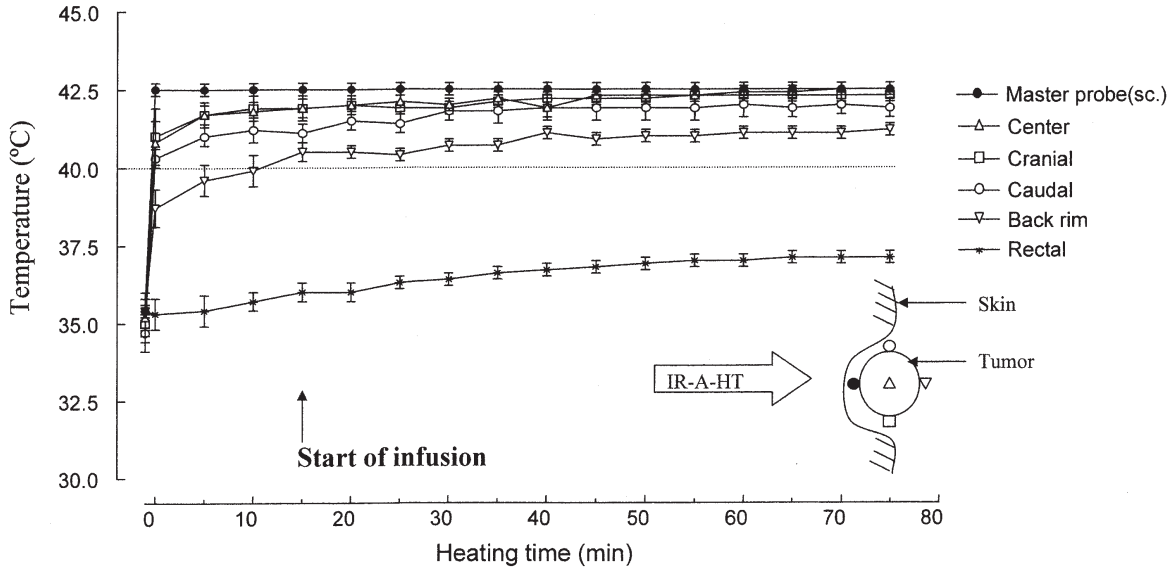

Figure 1. Recordings of temperatures at various locations in the R1H tumor during a heating period for 75 min controlled by the master probe at 42.5˚C. After preheating for 15 min, the tumor temperatures at the indicated locations were >40˚C when the drug infusion started. The slow increase of rectal temperature up to 37.1±0.3˚C represents a very local heating of the tumor by the infrared-A-radiation source. (n=10, ± SEM).

Table I. Tumor growth delay of rat rhabdomyosarcomas.[c]

	Tumor growth delay[a] (days)		
Treatment[c]	2.5 mg/kg	3.5 mg/kg	5.0 mg/kg
Free Dox	2.2±0.4	5.5±1.2	23.8±2.1
(No. of tumors)	(10)	(10)	(10)
Free Dox+HT	6.7±1.0	11.1±1.9	32.7±2.1
(No. of tumors)	(10)	(10)	(10)
Dox-Lip+HT	7.9±1.5	9.1±0.8	33.7±2.6
(No. of tumors)	(10)	(10)	(10)

[a]Determined at 2-fold of the treatment volume (V_0 = 1.1 cm^3). [b]Mean ± SEM. [c]R1H after treatment with Free Dox, Free Dox+HT and Dox-Lip+HT and doses of 2.5, 3.5 and 5.0 mg/kg body weight. Heating of tumors (HT) was performed at 42.5˚C for 60 min. The number of tumors/animals is indicated by No.

group was 10. Doxorubicin was applied to the animals in doses of 2.5, 3.5 and 5.0 mg/kg with or without hyperthermia as described above. Tumor volumes were measured 3 times per week and animals were weighed once a week. The tumor response was evaluated by analysing individual tumor volume curves and determing tumor growth delay with respect to untreated controls at the level of 2 V_0, that is 2-fold the initial treatment volume. The systemic toxicity was evaluated by changes in body weight and survival of the animals.

Distribution of drugs in the tumor and body. The uptake of drugs in tumor, blood, liver, spleen, kidney, heart and lung of rats has been studied on small tissue samples using high performance liquid chromatography (HPLC). Blood and tissue samples were taken at the end of single and repeated treatments with 2.5 mg/kg Free Dox, Free Dox+HT and Dox-Lip+HT, and were immediately frozen in liquid nitrogen. Samples were stored at -70˚C until the HPLC measurements were performed. Preparation of the samples and details of the HPLC system have previously been described (48).

Statistical analysis. All measurements are expressed as mean values ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Fisher's PLSD test (protected least significant difference) for multiple comparison. The level of significance was set at $p<0.05$.

Results

Tumor response and effects on body weight. Treatment of the R1H tumors with single doses of 2.5, 3.5 and 5.0 mg/kg Free Dox, Free Dox+HT and Dox-Lip+HT showed a dose-dependent inhibition of tumor growth which was greatest after treatment with Dox-Lip+HT (Fig. 2, top panel). Whereas Free Dox has only a minor effect on the tumor, an additionally hyperthermic treatment considerably increased the efficacy of Free Dox+HT on the tumor (Fig. 2B). A dose of 5.0 mg/kg showed partial tumor remission. Treatment with 5.0 mg/kg Dox-Lip+HT (Fig. 2C) also resulted in partial tumor remission and in a further increase in tumor growth delay. Compared to Free Dox the difference in tumor growth delay was statistically significant ($p<0.05$). Table I summarizes the tumor growth delays obtained after the various treatments. In addition, a single heat treatment at 42.5˚C for 60 min had no effect on tumor growth (control: 2.7±0.1 and HT: 3.0±0.4 days). The administration of Dox-Lip (40˚C) alone neither affected R1H tumor growth nor changed the animal body weight compared to the untreated tumors.

The efficacy of the drugs applied led to systemic toxicity resulting in a decrease in animal body weight (Fig. 2, bottom

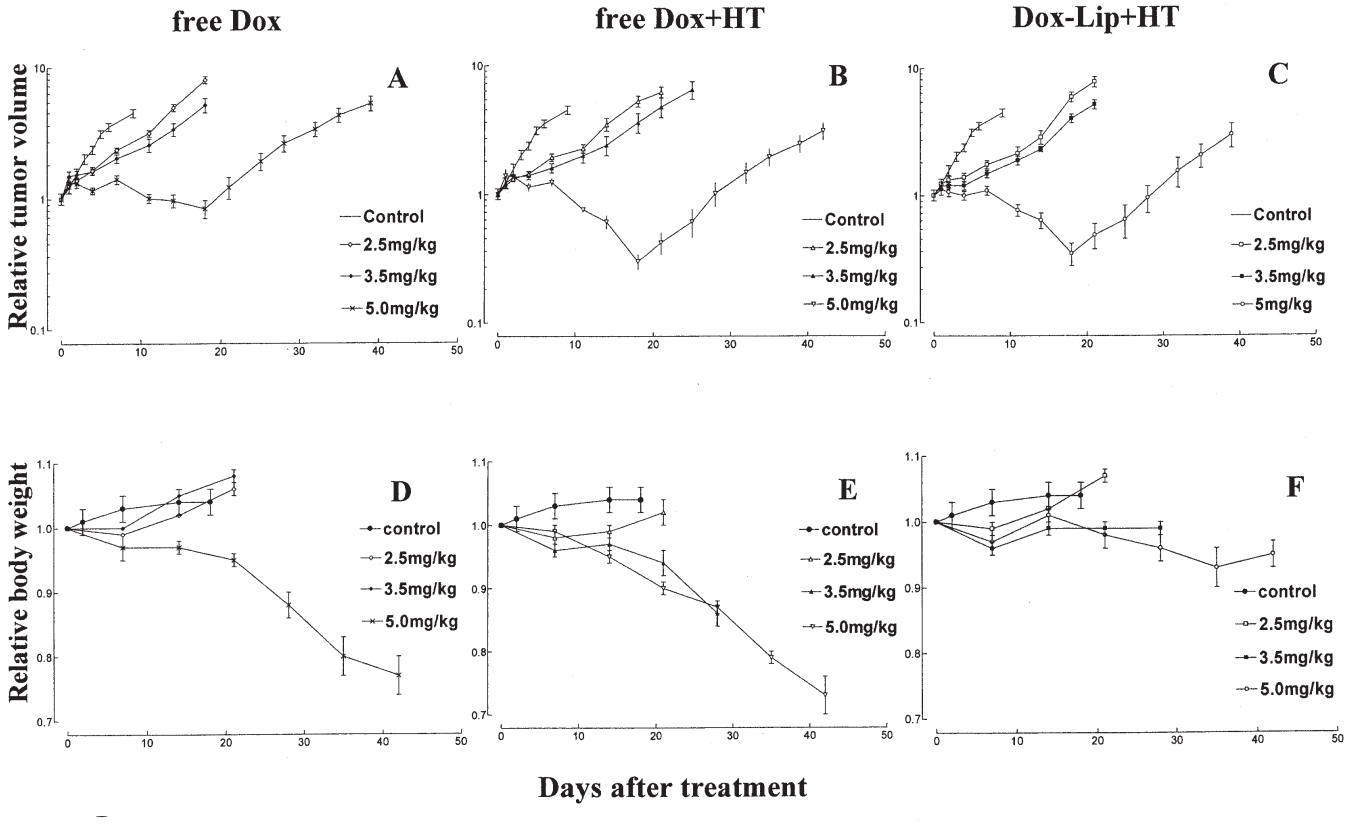

Figure 2. Relative changes of the R1H tumor volume after treatment with 2.5, 3.5 and 5.0 mg/kg b.w. of Free Dox, Free Dox+HT and Dox-Lip+HT (HT = 42.5˚C, 60 min) versus the untreated controls (top panels) and the corresponding relative changes in animal body weight (bottom panels). The number of tumors/animals studied per group was 10.

Table II. Tumor growth delay of rat rhabdomyosarcomas.

	Tumor growth delay[a] (days)	
Treatment[d]	lx2.5 mg/kg	2x2.5 mg/kg
Free Dox	2.2±0.4[b]	10.4±3.3[b]
(No. of tumors)	(10)	(10)
Free Dox+HT	6.7±1.0	15.5±4.0
(No. of tumors)	(10)	(9)
Dox-Lip+HT	7.9±1.5	32.8±3.0[c]
(No. of tumors)	(10)	(9)

[a]Determined at 2-fold of the treatment volume (V_0 = 1.1 cm^3). [b]Mean ± SEM. [c]Significant p-value ($p<0.05$) to others (Fisher's PLSD). [d]R1H after treatment with one fraction of Free Dox, Free Dox+HT and Dox-Lip+HT (1x2.5 mg/kg) or two fractions for 2 weeks (2x2.5 mg/kg). Tumor heating (HT) was at 42.5˚C for 60 min. The number of tumors/animals is indicated by No.

panels). Whereas for 5.0 mg/kg Free Dox and Free Dox+HT (Fig. 2D and E) a continuous decline in body weight to 28% was observed the treatment with Dox-Lip+HT showed only a transient reduction of body weight (8%) followed by recovery (Fig. 2F). No death of animals was observed during the observation time. The results of the single treatments suggest that a treatment of rat rhabdomyosarcomas R1H with thermosensitive liposomal doxorubicin (40˚C) and local IR-A-hyperthermia can significantly inhibit tumor growth and reduce the systemic toxicity of the animals versus free doxorubicin with or without hyperthermia.

The efficacy of a repeated application of Dox-liposomes (40˚C) and hyperthermia was investigated by treating the tumors with 2 fractions of 2.5 mg/kg compared to Free Dox+HT or Free Dox alone. Drugs were applied once weekly for two weeks (Fig. 3, top panel). The treatment of tumors with Dox-Lip+HT resulted in a significant tumor growth delay (32.8±3.0 days, $p<0.05$) as compared to Free Dox+HT (15.5±4.0 days) and Free Dox alone (10.4±3.0 days). However, after repeated treatment partial tumor remission versus a single treatment with 5.0 mg/kg Dox-Lip+HT, was not achieved (Fig. 2). Tumor growth delays obtained after single and repeated treatments are summarized in Table II.

With regard to systemic toxicity (Fig. 3, bottom panel) the application of 2 fractions of Dox-Lip+HT reduces animal body weight only transiently (5%), followed by recovery. This is in contrast to the toxicity of 2 fractions of Free Dox+HT or Free Dox alone where body weight declined continuously without causing the death of animals during the observation time. The results after repeated application of Dox-Lip+HT support the findings after a single treatment.

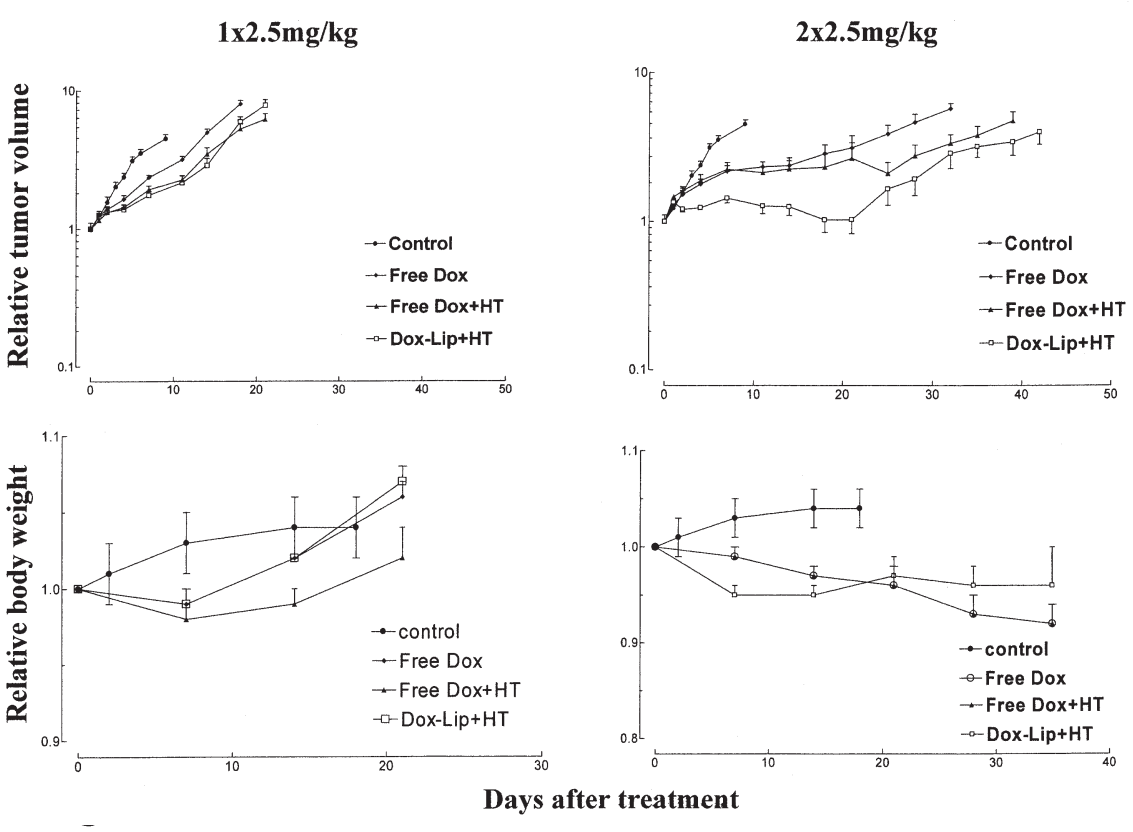

Figure 3. Relative changes of R1H tumor volume, after treatment with 2.5 mg/kg b.w. of Free Dox, Free Dox+HT and Dox-Lip+HT, given in one fraction (1x2.5 mg/kg) or two fractions (2x2.5 mg/kg) once a week for 2 weeks (top panels) and the relative changes in animal body weight (bottom panels). The number of tumors/animals studied was 10.

Thus, it can be stated that repeated applications of doxorubicin thermosensitive liposomes (40˚C) in combination with local hyperthermia led to a significant higher efficacy on the R1H tumor and was much better tolerated by the animals as compared to free doxorubicin with or without local hyperthermia.

Distribution of drugs. The concentration of doxorubicin after the intravenous administration of Free Dox and Dox-Lip under local hyperthermia or of Free Dox is presented in Table III for tumor, blood and normal tissues after single and repeated treatments with 2.5 mg/kg of doxorubicin. After a single treatment with Dox-Lip+HT the concentration of doxorubicin in the tumor was significantly higher than in Free Dox (3.7±1.5 versus 1.8±0.3 μg/g, $p<0.05$) and as compared to Free Dox+HT, only a minor enhancement was noted (3.0±0.4 μg/g). The concentration of Dox-Lip+HT in blood was substantially higher (12.2±5.0 μg/g) with respect to the tumor whereas the concentrations of Free Dox and Free Dox+HT in the blood were at the level of detection. This indicates a slower clearance of Dox-liposomes (40˚C) from the blood and a longer lifetime in the blood circulation. The concentration of Dox-Lip+HT in liver, lung and especially in the spleen was considerably enhanced with a minor reduction in kidney, but no change in the heart. The lower doxorubicin concentration in the heart indicates that Dox-liposomes (40˚C) reduce cardiotoxicity as compared to Free Dox. The concentrations of Dox-liposomes (40˚C) in normal tissues were consistently higher compared to the tumor which is due to structural and functional abnormalities in the tumor microcirculation (4).

After repeated treatment the concentration of Dox-Lip+HT in the tumor was surprisingly lower as compared to Free Dox+HT (2.2±0.3 versus 2.8±0.5 μg/g) but still enhanced in relation to Free Dox (1.6±0.4 μg/g) (Table III). With regard to a single treatment the uptake after repeated treatment is reduced to ~40%. In blood, the concentration of Dox-Lip+HT had more than doubled as compared to the single treatment. Again, this is an indication of the long lifetime of the Dox-liposomes (40˚C) in blood circulation. The repeated treatment with Dox-Lip+HT did not generally change the uptake in normal tissues versus the single treatment with the exception of the liver. The special distribution of Dox-liposomes (40˚C) in the body may be responsible for the lower systemic toxicity of the animals after repeated treatment.

Discussion

The treatments of R1H rat rhabdomyosarcomas with doxorubicin thermosensitive liposomes (40˚C) and hyperthermia have clearly shown that this drug (Dox-Lip+HT) is effective on the R1H tumor and that this treatment modality has some advantages compared to doxorubicin alone (Free Dox) or doxorubicin and hyperthermia (Free Dox+HT). Tumor growth was significantly inhibited after a single treatment compared to Free Dox and approximately equal to that of Free Dox+HT. However, the systemic toxicity of the animals was considerably reduced (Fig. 2). Repeated

Table III. Concentration of doxorubicin (Dox) in R1H tumor, blood and in normal tissues after treatment with one fraction of Free Dox, Free Dox+HT and Dox-Lip+HT (1x2.5 mg/kg) or two fractions (2x2.5 mg/kg) for 2 weeks.[a]

	Concentration (μg/g) of lx2.5 mg/kg[a]			Concentration (μg/g) of 2x2.5mg/kg. 2 weeks[a]		
Tissue	Free Dox	Free Dox+HT	Dox-Lip+HT	Free Dox	Free Dox+HT	Dox-Lip+HT
Tumor	1.8±0.3	3.0±0.4	3.7±1.5	1.6±0.4	2.8±0.5	2.2±0.3
Blood	0.1±0.0	0.1±0.0	12.2±5.0	0.1±0.0	0.1±0.0	26.4±4.0
Liver	7.1±0.3	8.0±0.7	16.4±2.6	4.3±1.1	4.7±0.0	9.6±0.5
Spleen	5.3±0.8	9.1±2.5	55.1±9.4	3.8±0.6	4.6±0.3	56.2±2.4
Kidney	12.6±1.1	14.6±1.4	10.3±2.1	7.5±1.3	13.6±1.7	12.8±1.2
Heart	6.7±0.6	4.7±0.5	5.4±1.1	3.4±0.8	5.8±0.1	6.3±1.0
Lung	6.7±0.7	9.1±0.3	13.7±3.9	5.0±1.3	7.4±0.3	15.3±1.2

[a]Tumor heating (HT) was at 42.5˚C for 60 min. Concentrations of doxorubicin (μg/g wet tissue) were determined in 3-5 samples of each tumor/animal using HPLC. Mean values ± SEM, n= 6.

treatments of Dox-Lip+HT suppressed the tumor growth significantly in contrast to the other two strategies with Free Dox and Free Dox+HT (Fig. 3). Again, the higher efficacy on the R1H tumor was associated with a lower systemic toxicity.

Since tumor response and side effects are closely related to the uptake of drugs the higher R1H tumor response of Dox-Lip+HT after a single treatment can be attributed to a higher uptake of Dox-liposomes (40˚C) during hyperthermia in the tumor (Table III). This is in agreement with other studies where an increased uptake of drugs containing thermosensitive liposomes in various tumor models was found (24-29,33,35,36). After repeated application, however, the uptake of Dox-Lip+HT in the tumor was lower than Free Dox+HT (2.2±0.3 versus 2.8±0.5 μg/g), but still higher than Free Dox (1.6±0.4 μg/g). With regard to the single treatment a decrease in uptake of ~40% was obtained. To the best of our knowledge, it is the first time that repeated applications of Dox-liposomes+HT and local hyperthermia leading to a lower drug uptake in the tumor have been noted. This is an indication of a reduced blood flow in the tumor and a damage of tumor microvasculature. Chen *et al* (49) have shown that a rapid release of doxorubicin during hyperthermia (42˚C) considerably reduced RBC velocity in tumors as well as microvascular density which had a statistically significant decrease 24 h after treatment. The studies were performed on a human squamous carcinoma xenograft (FaDu) implanted in dorsal skinfold chambers in nude mice. Under the assumption that this is also true for the microvasculature of R1H tumors the first treatment of Dox-Lip+HT already damaged the R1H tumor blood vessels. The consequence is a reduced tumor blood flow and a lower uptake of Dox-liposomes (40˚C) in the R1H tumor one week later (Table III). In addition, the first treatment also changes the tumor micromilieu (e.g. pH, oxygenation, nutrient supply and cell loss), thereby modifying the release and extravasation of Dox-Lip (40˚C) in the tumor during the second heat treatment. Since direct hyperthermic effects on tumor blood vessels in a temperature range of 40-42˚C can almost be excluded (50,51), the higher tumor response after repeated treatments with Dox-liposomes (40˚C) and hyperthermia may be due to cytotoxic effects on the tumor and to a damaged tumor microvasculature.

The efficacy of drugs containing thermosensitive liposomes in combination with hyperthermia depends not only on the lipid composition of the liposomes but also on the size of the nanoparticles (38,39,52). It was found that the magnitude of hyperthermia-induced extravasation from tumor vasculature was inversely proportional to particle size (38). Since we used Dox-liposomes (40˚C) of a large size (319±106 nm), it must be assumed that the extravasation of Dox-liposomes (40˚C) considerably differed from that of small PEG-liposomes (90 nm) (25).

A possible mechanism of liposomal drug delivery to tumors in combination with local hyperthermia is that liposomes extravasate from pores in the tumor vessel walls. Hyperthermia increases pore size and thus increases tumor liposome extravasation (29). Our electron microscopic studies on R1H tumor blood vessels show that tumor blood vessels consisted of a continuous lining of flattened endothelial cells devoid of fenestrate with broad overlapping cell contacts and tight junctions overlying a delicate continuous basal lamina (53). The angioarchitecture of primary R1H tumors showed extremely great variations between blood vessels and vascular density in the tumor periphery and centre (44,54). Therefore, it raises the question of whether the proposed mechanisms based on findings of a human squamous cell carcinoma xenograft (FaDu) can also be transferred to tumor blood vessels of the R1H tumor which is an isogeneic tumor model.

For the application of liposomes in tumor therapy it is important to know whether the liposomes will be trapped in the liver and spleen and in the reticuloendothelial system (RES). For that reason methods of coating the liposomal surface were developed using various substances such as polyethyleneglycol (PEG) (22,55,56). Doxorubicin containing PEG-liposomes [e.g. doxil(r), caelix(r) and myocet(r)] are commercially available and are applied for the chemotherapy of cancers (12-15). These liposomes can escape from the RES and thus, have a longer circulation in the blood. Laverman *et al* (57,58) reported that long-circulating (PEG)-liposomes were cleared rapidly from the

blood circulation when injected repeatedly in the animal (rat). They pointed out that this should be a general phenomenon for liposomes. It was considered that hepatosplenic macrophages play an essential role in this process. Our studies on the repeated application of Dox-liposomes (40˚C) and hyperthermia at a one-week interval did not confirm an enhanced clearing of liposomes from blood (Table III). In contrast, we found an increase of Dox-Lip+HT concentration in the blood compared to the first application. Drug accumulation in the liver was also lower with respect to the first application, but accumulation in the spleen still remained high. Laverman *et al* (57,58) supposed further that the enhanced clearance of liposomes was based on a gradual decrease within one generation of macrophages such as Kupffer cells in the liver. Since one generation of macrophages exists for ~7 days and our second treatment with Dox-Lip+HT was given one week later, the RES may not have sufficiently recovered to produce a new generation of macrophages. This means, that the Dox-liposomes (40˚C) will not be trapped in the RES after two applications (Table III). However, the pharmacokinetics of Dox-liposomes (40˚C) containing doxorubicin are still complex in R1H tumor, especially with the interaction of hyperthermia, and needs to be studied further.

Moreover, the delivery and extravasation of Dox-liposomes (40˚C) to the R1H tumor during local IR-A-hyperthermia is also determined by the intratumoral temperature distribution during heating time at 42.5˚C (Fig. 1). In the relatively small R1H tumors (1.1 cm^3) differences in temperature existed between the tumor surface and back rim of 1.5˚C and between the cranial and caudal locations of 0.4˚C at the end of heating. Since homogeneous heating was not achieved the thermally induced extravasation of Dox-liposomes (40˚C) and Free Dox in the tumor will be non-uniform, and is associated with the heterogeneous tumor blood flow and the incomplete and chaotic tumor microvasculature. Therefore, homogeneous heating of solid tumors is a prerequisite for a high drug delivery of thermosensitive liposomes to the tumor.

The efficacy of the thermosensitive liposomes (40˚C) was not only studied on tumor response but also with regard to systemic toxicity of the animals using loss of body weight as an endpoint (Figs. 2 and 3, bottom panels). For a single treatment, a maximum dose of 5 mg/kg doxorubicin was very well tolerated by the rats when given as Free Dox or Dox-liposomes (40˚C) combined with hyperthermia (Fig. 2). No severe symptoms of sickness were observed. That was the reason for splitting the dose of 5 mg/kg into 2 fractions of 2.5 mg/kg for the repeated treatments. It is well known that high doses of doxorubicin lead to cardiotoxicity and nephrosis. The accumulation in the heart of Free Dox, Free Dox+HT and Dox-Lip+HT after single and repeated treatments was almost at the same level for all drugs (Table III) and did not indicate a higher stress to the heart by Dox-Lip+HT as compared to Free Dox. In kidney a relatively high uptake of the three drugs was found which contributed to systemic toxicity by urinary dysfunction of the animal. In the lung and especially spleen, a high uptake of Dox-Lip+HT was obtained but it appears that the high accumulation of the drug in the two organs did not lead to severe systemic toxicity of the animal. However, it is noteworthy that the time of 6 weeks after treatment was certainly not long enough to observe side effects of the drugs, especially of Dox-Lip+HT, through changes in the behaviour of the animal. Even under loss of body weight the mobility of the rats was not modified.

In conclusion, doxorubicin liposomes (40˚C) in combination with local hyperthermia were effective in the treatment of rhabdomyosarcomas. Repeated treatments resulted in a higher tumor response and were associated with a lower systemic toxicity compared to free doxorubicin with or without hyperthermia. The higher efficacy and great tolerance of doxorubicin thermosensitive liposomes (40˚C) together with hyperthermia is a promising treatment modality for soft-tissue sarcomas.

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