Preparation and Investigation of Biological Activity of Thermosensitive Liposomes Loaded with Doxorubicin

E.V. Tazina, A.V. Polozkova, O.L. Orlova, E.V. Ignatieva, V.V. Mescherikova, A. Wainson, S.P. Yarmonenko, N.A. Oborotova

N.N. Blokhin Russian Cancer Research Center of RAMS, Moscow, Russia, el_tazina@yahoo.com

ABSTRACT

Purpose: Preparation of thermosensitive liposomes loaded with doxorubicin (Dox) and investigation of their effect on solid Ehrlich (line ELD) carcinoma and B-16 mouse melanoma in combination with hyperthermia. Results: Thermosensitive liposomes encapsulated 83-94 % of Dox. Mean diameter of thermoliposomes was 170 ± 10 nm. In experiments *in vitro* done with thermoliposomes loaded with Dox the cell death increased 5-fold when cell suspension was incubated at elevated temperature instead of 37 °C. The doubling time of tumors was 9 days after heating on a background of Dox administration at dose of 9 mg/kg, while heating of tumors after administration of thermoliposomes loaded with Dox at doses of 4.5 and 9 mg/kg increased tumor doubling time up to 12 and 16 days, respectively.

Keywords: doxorubicin, thermosensitive liposomes, hyperthermia, melanoma B-16, Ehrlich carcinoma

1 INTRODUCTION

Liposomes have been identified as promising carriers for therapeutic agents in the treatment of cancer. By encapsulating cancer chemotherapeutic agents within liposomes, preferential delivery to the tumor can be attained. The rationale behind using the combination of liposomes and local hyperthermia (42 ± 1) °C for drug delivery to tumors is several-fold. Hyperthermia has been shown to increase extravasation of liposomes out of tumor microvasculature. Thus, local elevation of temperature can increase preferential accumulation of liposomes to the heated tumor volume. In addition, elevated temperatures (~ 43 °C) have also been shown to trigger drug release from thermosensitive liposomes, making it possible to release liposome contents as desired. Using hyperthermia to target and trigger drug release may be advantageous because it is controlled by external means and, thus, more easily modified to the desired conditions. Lastly, besides targeting and triggering release in liposomes, hyperthermia itself has been shown to be directly cytotoxic. The therapeutic benefits from liposomes and hyperthermia individually, coupled with the potential advantages seen by their combination, make the use of the two modalities together an attractive method for drug delivery to tumors [1].

Doxorubicin, a potent antineoplastic agent against a wide range of human tumors, is used alone and in combination with other drugs for treatment of tumors including malignant lymphomas and leukemias. However, injection of doxorubicin hydrochloride (free drug) is associated with severe heart and gastrointestinal toxicity. Other side effects include hair loss, mouth inflammation, and so on. The encapsulation of doxorubicin into liposomes appears to reduce its cardiotoxicity [2].

The purpose of present work is preparation of lyophilized thermosensitive liposomes with encapsulated doxorubicin and investigation of their effect on cells *in vitro* and solid tumors *in vivo* in combination with hyperthermia.

2 MATERIALS AND METHODS

2.1 Preparation of Thermosensitive Liposomes Loaded with Doxorubicin

Thermosensitive liposomes were prepared by reverse evaporation method using dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC) (Lipoid), distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000 ammonium salt) and cholesterol (Avanti Polar Lipids) in molar ratio of 9:1:0.02:0.2. Tocopherol acetate (2 mol%) was added to protect the lipids against degradative processes. The extrusion was performed through 200 nm polycarbonate membranes (Whatman) at 50 °C using Avanti Mini-Extruder. Doxorubicin (Dox) was loaded into thermoliposomes by ammonium ion gradient for 1 hour at 50 °C. Drug-to-lipid ratio was 0.13:1 (w/w). The ammonium ion gradient was formed by dilution of the liposomal dispersion 20 times in 10 mM HEPES (pH 8.4). The thermosensitive liposomes with doxorubicin (Dox-TL) were lyophilized for better stabilization with 4 % sucrose added as a cryoprotectant. Vesicle size was measured by dynamic laser light-scattering measurements using Submicron Particle Sizer Nicomp-380. Thermoliposomes were separated from untrapped doxorubicin by gel filtration on column C 10/20 (Amersham Biosciences) with Sephadex G-50 sorbent and 0.15 M sodium chloride solution as an eluent. The concentration of Dox in thermoliposomes was determined spectrophotometrically at wavelength of 252 ± 2 nm.

2.2 Effect of Dox-TL on B-16 Melanoma Cells *in Vitro*

Dox-TL and free Dox *in vitro* were administered to B-16 melanoma cell suspension at concentrations corresponding to 1-15 μg of Dox per ml. Cell suspension with drugs was incubated during 30-40 min at 37 °C or during 60 min at 42.5 °C. The suppression of cell growth and cell survival (clonogenicity) were used as the end points.

2.3 Effect of Dox-TL on Transplantable Tumors under Hyperthermia

Dox-TL or free Dox were administered to C57BL/6j mice with B-16 melanoma and (CBA x C57BL) mice with Ehrlich (line ELD) carcinoma on the 8-th day after transplantation. Tumors were transplanted in the shank muscle of mice. Dox-TL or free Dox were injected in retroorbital sinus of animals at doses of 9 and 4.5 mg Dox per kg body weight. Hyperthermic (HT) treatment of animals bearing transplanted tumors was performed at 43°C for 30 min using water bath. Each treated group included 6 animals. There were slight differences in the arrangement of B-16 melanoma and Ehrlich carcinoma experiments.

The animals bearing B-16 melanoma were divided into 8 groups:

- Control untreated group;
- Animals administered 4.5 mg/kg of Dox-TL;
- Animals administered 9 mg/kg of Dox-TL;
- Animals administered 9 mg/kg of Dox;
- Animals treated with HT;
- Animals administered 4.5 mg/kg of Dox-TL + HT;
- Animals administered 9 mg/kg of Dox-TL + HT;
- Animals administered 9 mg/kg of Dox + HT.

The animals with Ehrlich carcinoma received the following treatment:

- Control untreated group;
- Animals administered 9 mg/kg of Dox-TL;
- Animals treated with HT;
- Animals administered 9 mg/kg of Dox-TL followed by HT in 10-12 min;
- Animals administered 9 mg/kg of Dox-TL followed by HT in 15-20 min;
- Animals administered 9 mg/kg of Dox-TL followed by HT in 35-40 min;
- Animals administered 9 mg/kg of Dox followed by HT in 15-20 min.

The tumors were measured three times a week and mean tumor volume in the group was calculated. Dynamics of tumor regression and regrowth was assayed as ratio (Vt/Vo) of the mean tumor volume at the day of measurement (Vt) to the mean tumor volume at the day of experiment (Vo). Animals were followed till their death.

3 RESULTS

The method of Dox-TL preparation is developed. Thermosensitive liposomes encapsulated 83-94 % of Dox. The efficacy of Dox encapsulation after the process of lyophilization was 73-80 %. Mean diameter of lyophilized thermoliposomes was 170 ± 10 nm. According to this technology the appropriate amount of drug "Lyophilized thermosensitive liposomal Doxorubicin for injection 0.7 mg" is formed and handed for pharmaceutical and pre-clinical studying.

In experiments *in vitro* done with Dox-TL the cell death increased 5-fold when cell suspension was incubated at elevated temperature instead of 37 °C (Fig. 1).

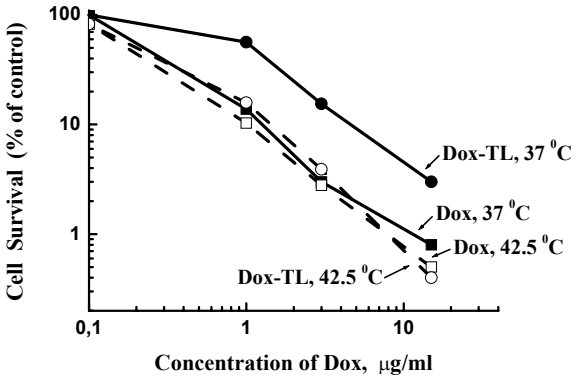

Figure 1: Survival of B-16 melanoma cells after their incubation with free Dox or Dox-TL at 37 °C and 42.5 °C.

The doubling time of tumors treated 7-9 days after inoculation was 9 days after heating on a background of Dox administration at dose of 9 mg/kg, while heating of tumors after administration of Dox-TL at doses of 4.5 and 9 mg/kg increased tumor doubling time up to 12 and 16 days, respectively (Fig. 2).

On the 34-th day after treatment 3 mice survived in the group administered 9 mg/kg of Dox-TL and treated by HT. In the group administered 4.5 mg/kg of Dox-TL + HT only one mouse survived. The 50 % survival was 34 and 29 days for these groups, respectively (Fig. 3). For group administered 9 mg/kg of free Dox + HT the 50 % survival was 21 days.

The slowest growth rate of Ehrlich carcinoma was observed in group administered 9 mg/kg of Dox-TL followed by HT in 15-20 min. The doubling time of tumors increased from 3 days in control group up to 14 days for this group (Fig. 4). Tumor growth inhibition after Dox-TL injection followed by HT in 10-12 min and 35-40 min was less significant.

On the 22nd day after treatment animal survival was 30% in group administered 9 mg/kg of free Dox followed by HT in 15-20 min. The highest survival was noted in the group of animals administered 9 mg/kg of Dox-TL

followed by HT in 15-20 min. 100 % survival for this group was observed during 38 days. On the 50-th day survival was 40 % while all the animals in control group have died by this day (Fig. 5).

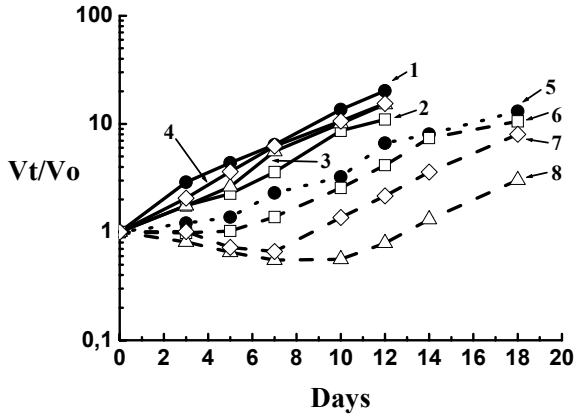

Figure 2: Dynamics of B-16 melanoma growth after HT treatment on a background of Dox and Dox-TL administration.

1 – Control; 2 – Dox (9 mg/kg); 3 – Dox-TL (9 mg/kg);
4 – Dox-TL (4.5 mg/kg); 5 – HT (43 °C, 30 min);
6 – Dox (9 mg/kg) + HT; 7 – Dox-TL (4.5 mg/kg) + HT;
8 – Dox-TL (9 mg/kg) + HT.

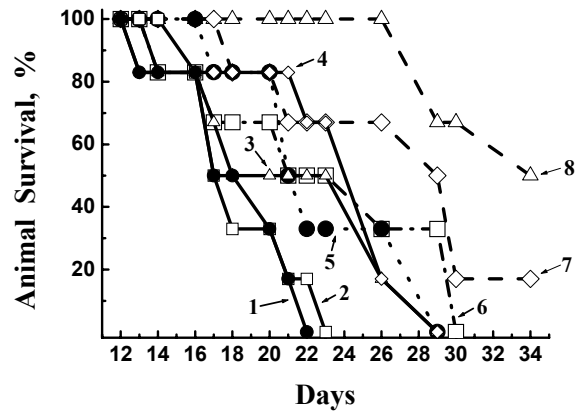

Figure 3: Survival of animals with B-16 melanoma after HT treatment on a background of Dox and Dox-TL administration.

1 – Control; 2 – Dox (9 mg/kg); 3 – Dox-TL (9 mg/kg);
4 – Dox-TL (4.5 mg/kg); 5 – HT (43 °C, 30 min);
6 – Dox (9 mg/kg) + HT; 7 – Dox-TL (4.5 mg/kg) + HT;
8 – Dox-TL (9 mg/kg) + HT.

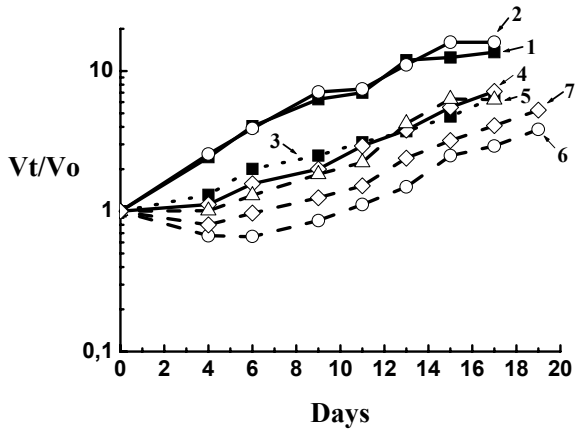

Figure 4: Dynamics of Ehrlich carcinoma growth after HT (43 °C, 30 min) treatment on a background of Dox and Dox-TL administration at dose of 9 mg/kg.

1 – Control; 2 – Dox-TL; 3 – HT;
4 – Dox + HT in 15-20 min;
5 – Dox-TL + HT in 10-12 min;
6 – Dox-TL + HT in 15-20 min;
7 – Dox-TL + HT in 35-40 min.

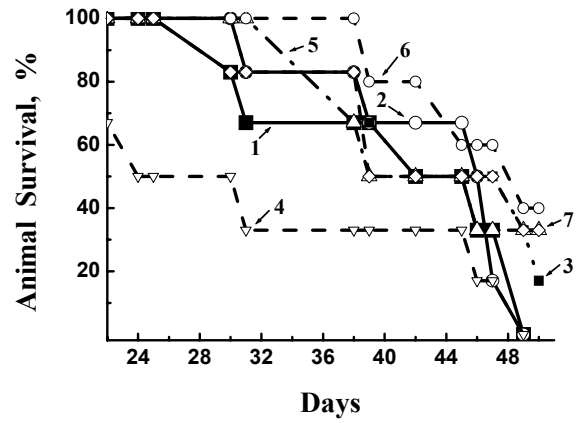

Figure 5: Survival of animals with Ehrlich carcinoma after HT (43 °C, 30 min) treatment on a background of Dox and Dox-TL administration at dose of 9 mg/kg.

1 – Control; 2 – Dox-TL; 3 – HT;
4 – Dox + HT in 15-20 min;
5 – Dox-TL + HT in 10-12 min;
6 – Dox-TL + HT in 15-20 min;
7 – Dox-TL + HT in 35-40 min.

4 CONCLUSION

- The method of thermoliposomes preparation with Dox encapsulation by ammonium ion gradient allowing produce the drug form with high trapping efficacy of Dox (83-94 %) is developed.
- Sucrose is chosen as the appropriate cryoprotectant for lyophilization of Dox-TL.
- The technique of quantitative determination of Dox concentration in thermoliposomes and Dox encapsulation efficacy in thermoliposomes is developed.
- The appropriate amount of drug for biological and pharmaceutical studying has been prepared. Some series of drug are placed on storage at −18 °C to define an expiration date.
- It is shown that Dox-TL in combination with HT is more effective than free Dox in suppression of growth both *in vitro* and *in vivo*.

ACKNOWLEDGEMENT

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