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ORIGINAL CONTRIBUTIONS

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INFLUENCE OF DOXORUBICIN INCLUSION INTO PHOSPHOLIPID NANOFORMULATION ON ITS ANTITUMOR ACTIVITY IN MICE: INCREASED EFFICIENCY FOR RESISTANT TUMOR MODEL

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Aim: **The new formulation of doxorubicin on the base of phospholipid nanoparticles (particle size <30 nm) is elaborated in the Institute of Biomedical Chemistry (Russian Academy of Medical Sciences) on the base of plant phospholipids. The aim of study is to investigate an antitumor effect of this nanoformulation in mice with two cancer models with various sensitivity to chemotherapy — lymphoid malignancy P-388 and Lewis lung carcinoma (LLC).** ***Methods:*** **Nanophospholipid (NPh) formulation of doxorubicin was prepared by homogenization of soybean phosphatidylcholine and doxorubicin hydrochloride. The effect of this formulation was studied in experiments with single or threefold drug administration. Percents of tumor growth inhibition in mice under influence of free or NPh doxorubicin forms were compared.** ***Results:*** **Single administration of both free and NPh doxorubicin in mice with P-388 resulted in the same quick severe inhibition of tumor growth (60–90% depending from dose), with further gradual decrease of inhibition degree. However for more resistant tumor, LLC, the obvious advantage of NPh doxorubicin form was shown. The little effect of free doxorubicin began to reveal only after 11 days, but NPh formulation induced significant inhibition of tumor growth (40%) from the first experimental point (6 days after administration). The advantages of NPh doxorubicin was manifested particularly in low drug doses, 2 and 4 mg/kg. In other experiment design in mice with LLC, with threefold weekly drug administration, NPh doxorubicin appeared to be 2.5 times more active than free drug. The reason of the same actions of free and NPh doxorubicin form in P-388 is suggested the high drug sensitivity of this model, that gives quick high drug response for any doxorubicin form.** ***Conclusion*****: Doxorubicin in phospholipids nanoformulation revealed higher antitumor efficiency as compared with free doxorubicin in mice with LLC carcinoma. The mechanism of such changes is supposed to be caused by increase of doxorubicin availability for cancer cells.**

Key Words**: doxorubicin, phospholipids nanoparticles, P-388, LLC, antitumor action.**

Inclusion of drugs into different transport systems based on polymers or lipids, is the modern way to increase their bioavailability and efficiency [1, 2]. For oncology field such systems are considered to be particularly urgent, because they promote the overcoming of side effects that are relevant to antitumor drugs [3]. Many researches are devoted to developing of new forms of doxorubicin — an effective cytostatic, but with severe side effects, particularly cardiotoxicity [4, 5]. There are some liposomal forms of doxorubicin in pharmaceutical market, although they have also some disadvantages. So, the stabilized form of liposomal doxorubicin ("Doxil") may reveal some additional side effects as result of presence of polyethylene glycol [4], and other liposomal form, Myocet (TLC D-99), is removed rapidly from circulation by RES because of relatively large liposome sizes (150–180 nm) [5]. The new forms of doxorubicin in polymer-drug conjugates on base of the (hydroxypropyl)methacrylamide copolymers (PK1, PK2) are now in clinical trials and showed the decrease of side effects [6]. In the same time, the development of new technologies, that gave the new carrier type — phospholipid nanoparticles [7, 8], allows to return to phospholipids as to the most natural substances [9] with possibility of their usage as potential carriers for doxorubicin, but yet on new modern, nanomedicine, level. The use of phospholipid nanoparticles may give possibilities to utilize the advantages of liposomes as biocompatible carriers [9], but without their weaknesses [1, 3, 8].

We have previously shown the increase of antitumor activity of doxorubicin by means of its inclusion into 50–60 nm phospholipid nanoparticles, stabilized by glycyrrhizic acid (the drug Phosphogliv) [10]. Then, the inclusion of doxorubicin into other nanophospholipid (NPh) transport system, without other additive components and with particle size 20–30 nm, was described [11]. The embedding of a number of drugs into such nanoparticles resulted in improvement of their pharmacokinetics or/and changes of interaction with blood components [8]. For doxorubicin such inclusion decreased drug association with erythrocytes in incubation *in vitro* with blood, that increased the part of available drug in plasma [12]. Drug redistribution from plasma proteins (albumin) fraction to high density lipoproteins (HDL) was also shown [12]. It may be suggested to stimulate doxorubicin delivery to cancer cells — through interaction with receptors — SRB1 or, after drug redistribution, B,E-receptors, that is known to be highly expressed in a number of tumors [13, 14]

The aim of this study was to investigate the antitumor activity of doxorubicin embedded in 20–30 nm phospholipid nanoparticles on tumor models. In order to evaluate possible influence of tumor resistance variability on its response to NPh doxorubicin formulation, two tumor mice models with known

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Abbreviations used: LLC – Lewis lung carcinoma; NPh formulation (or NPh doxorubicin) – nanophospholipid formulation of doxorubicin.

different response to chemotherapy were used — Lewis lung carcinoma (LLC) and lymphoid malignancy P-388. The later is known as one of the most sensitive tumors — fewer than 2% of all agents active against P388 showed significant effects in other more resistant models [15, 16]. LLC revealed higher resistance for a number of drugs [17, 18]. Different responses of these two models for the same drugs suggested the necessity to study their responses for new doxorubicin nanoformulation — for the more distinct elucidation of the field of its efficiency.

MATERIALS AND METHODS

NPh formulation of doxorubicin was prepared according to the original technology with homogenization, as detailed in the invention [11]. Soybean phosphatidylcholine Lipoid S100 (Lipoid GmbH, Germany) and doxorubicin hydrochloride (Dian Jiang, Chong Qing, China) were used. The particle size was not more than 30 nm. The degree of binding of doxorubicin with phospholipid particles was 98% [11]. The medicinal preparation Doxorubicin-LANS ("LANS-Pharm", Russia) was used as free drug.

Antitumor effects of NPh doxorubicin were studied on Balb/c mice weigting 20–25 g inoculated with two tumor strains — LLC and lymphoid leukemia P-388 (solid form), obtained from N.N. Blokhin Russian Oncology Center and maintained in the same animals in P.A. Gertsen Moscow Scientific Oncology Institute. Tumors were transplanted to mice subcutaneously: for P-388 — 106 tumor cells in 0.1 ml of 0.9% NaCl, for LLC — 25 mg of tumor tissue in 0.3 ml of 0.9% NaCl.

Doxorubicin preparations in NPh or free forms were administered by single intravenous injections in the doses of 2; 4; 8 or 10 mg/kg to mice with P-388 in 24 h after tumor cell inoculation and to mice with LLC — in 48 h after tumor transplantation. Antitumor efficacy was monitored by inhibition of tumor growth (% from control) within 14–18 days. Experiments with long-term treatment regimen were also carried out in mice with LLC. Both doxorubicin preparations (free or NPh) in dose 5 mg/kg were administered intraperitoneally weekly — three times, starting 1 week after tumor transplantation. Tumor dimensions were measured on 22nd day after transplantation. The percent of inhibition of tumor growth in comparison with control (untreated) animal group was calculated. Each group of animals included 10–13 animals.

The statistical analysis of the obtained data has been carried out using standard Student's t-criterion. All experiments were carried out in accordance with demands of Russia National Bioethic Committee, on the base of "Ethical and law problems of clinical trials and scientific experiments with human and animals" (M., 1994), that corresponds to international standards of animal welfare.

RESULTS AND DISCUSSION

The effect of doxorubicin treatment appeared to be substantially different for two tumor models from the very beginning of experiment (Fig. 1, *a*, *b*).

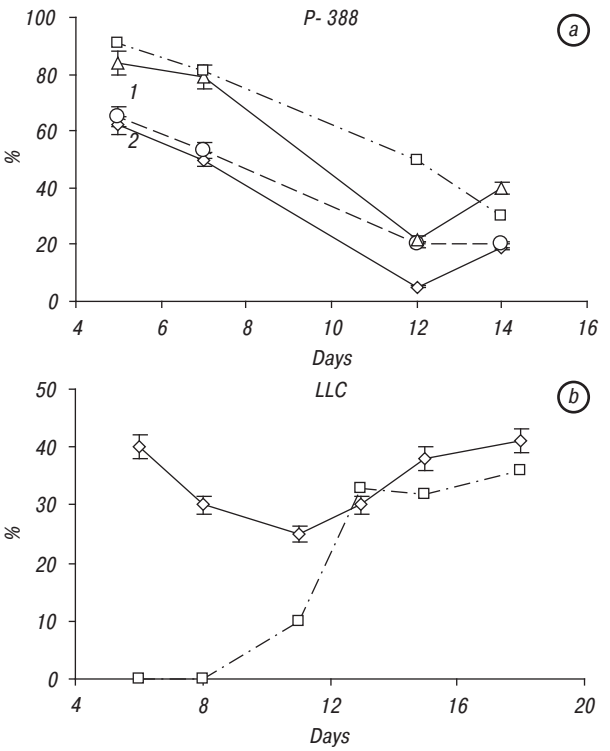

Fig. 1. Inhibition of tumor growth in mice as compared with control animals after single administration of free or NPh doxorubicin forms: *a*) lymphoid malignancy P-388 (1 — 10 mg/kg, 2 — 2 mg/kg), *b*) LLC (4 mg/kg). Solid curves — NPh doxorubicin, dashed curves — free doxorubicin

For P-388 (Fig. 1, *a*) both drug forms induced significant inhibition of tumor growth in the first experimental point (5 days after administration) — 62–65% at dose 2 mg/kg, and 88–90% at 10 mg/kg. The effects of intermediate doses, 4 and 8 mg/kg, were similar (data not shown). The use of NPh form did not increase doxorubicin efficiency in this tumor model. It may be caused possibly by the fact that the limit of maximal doxorubicin effect for this sensitive tumor has been already reached quickly in the case of free drug. This assumption is confirmed by the decrease of inhibition percent in the next days — till 10–20% to 12th days (Fig. 1, *a*), as result of possibility of quick maximal response of this tumor model to administered drug [15, 16].

In contrary, for mice with LLC (Fig. 1, *b*) substantial differences in efficiency of two doxorubicin forms were observed, and effect of NPh doxorubicin appeared to be significantly higher. Free doxorubicin (4 mg/kg) had any effect within 8–9 days of experiment, and it was only from 11th day, when a little inhibition of tumor growth began (11%). But NPh doxorubicin induced significant inhibition of tumor growth (40%) from the first experimental time point (6 days after administration). Unlike to P-388 tumor (Fig. 1, *a*), LLC growth inhibition (Fig. 1, *b*) in next experimental days did not decrease, but oppositely — gradually elevated. It testifies that the beginning of doxorubicin effect in LLC reflects gradual drug accumulation in tumor tissue, and NPh drug form accelerates this process.

One of the mechanisms of increase of antitumor activity of NPh doxorubicin as compared with free

drug is suggested the ability to modulate doxorubicin delivery in tumor. The quantity of drug in cancer cells, that is necessary for beginning of its action, is reached earlier and at lower doses for doxorubicin in phospholipid nanoparticles, than for free drug. But for the sensitive tumor P-388 even little drug quantity is sufficient for the great growth inhibition. It is not excluded, that possible differences in response for NPh doxorubicin could be also revealed for this tumor, but at lower drug doses. The mechanism of different sensitivity of tumor models are suggested to be associated with some morphological and/or biochemical characteristics [19–21]. For example, there are data about possible association of tumor sensitivity with such factors as activity of cathepsins B, L and D, causing degradation of cellular matrix [19], or vascular endothelial growth factor [20], or P-glycoprotein [21], or enzyme dihydrodiol dehydrogenase (DDH) [18].

For treatment of LLC the advantage of NPh doxorubicin form as compared with free doxorubicin has been revealed particularly at low drug doses (2 and 4 mg/kg), when the action of free doxorubicin didn't yet manifest itself (Fig. 2). It should be noted, that such doses are more close to those used in the clinic (40–60 mg/m^2 every 3 weeks, i. e., 1.4–1.6 mg/kg [22, 23]). Fig. 2 demonstrates also, that the same extent of tumor growth inhibition (~30–50% after 8 days) is achieved for NPh doxorubicin at 1.5–2 times lower doses (4–6 mg/kg), than for free drug (6–8 mg/kg). It is important because of known doxorubicin side effects [4, 5].

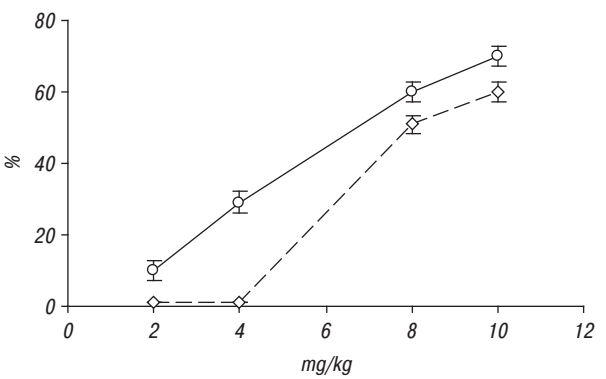

Fig. 2. Dependence of tumor growth inhibition in mice with LLC tumor from doxorubicin doses in free and in NPh forms (8 days after single administration). Solid curves — NPh doxorubicin, dashed curves — free doxorubicin

The results for LLC model demonstrated also the gradual smoothing of distinctions between free and NPh drug forms with increase either of doses (Fig. 2) or of experiment duration (Fig. 1, *b*). It confirms the assumption, that the reason of stimulating influence of phospholipid nanoparticles is their ability to modulate doxorubicin accumulation in tumor by increase of drug bioavailability [1, 3, 12]. The quantity of drug in cancer cells, that is necessary for beginning of its action is reached earlier and at lower doses for doxorubicin in phospholipid nanoparticles, than for free drug.

Pronounced advantage of NPh form of doxorubicin was observed more notably in other experimental setting — after three times weekly administration of drugs in the dose of 5 mg/kg to mice with LLC (Table 1). After treatment with free doxorubicin the average tumor volume was 5390 mm^3 (compared to 6713 mm^3 in the control group), i. e. growth inhibition was only 20%. But administration of NPh doxorubicin resulted in more than 2-fold greater effect, causing growth inhibition by 56% (tumor volume 2934 mm^3).

There are several possible mechanisms promoting doxorubicin delivery to cancer cells after inclusion into phospholipids nanoparticles. It may be related to its decreased binding with blood cells and proteins and correspondent redistribution to lipoproteins, that we have shown earlier for this doxorubicin form [12]. As others possible contributing factors one can assume the penetration of phospholipid nanoparticles through defects of tumor vessels (EPR effect) [24], or the overcoming of multidrug resistance (MDR) barrier, as it was shown recently for phospholipid-coated solid lipid nanoparticles (SLN) [25]. The possibility of endocytosis of nanoparticles because of their nanosize [26] may also be supposed. The positive influence of phospholipid particles nanosize is confirmed by more pronounced effect of NPh doxorubicin in this study (2-fold, Table 1) as compared with our previous results for doxorubicin in other phospholipid nanoparticles, with the size of 50–60 nm, in the same experimental conditions (tumor model, dose and treatment scheme) — only 30% [10].

Table. Inhibition of tumor growth in mice with LLC tumor after 3-times weekly administration of free doxorubicin or its NPh form

Animals groups	Tumor volume, mm^3	Tumor growth inhibition percent* (%)
1. Control (n = 10)	6713 ± 453	–
2. Administration of free doxorubicin (n = 11)	5390 ± 389	20.0 ± 5.6
3. Administration of NPh doxorubicin (n= 13)	2934 ± 182	56.1 ± 3.8
	P_{3-2} <0.001	P_{3-2} <0.001

Notes: Doxorubicin preparations were administered intraperitoneally in the dose of 5 mg/kg weekly – three times, starting 7 days after tumor transplantation. Tumor dimensions were measured on 22nd day after transplantation. * differences are significant as compared with control group values

Thus, treatment with doxorubicin in nanoformulation of natural phospholipids, obtained as the ultra-fine emulsion with a particle size less than 30 nm [11], has demonstrated in mice with LLC substantially higher antitumor efficiency as compared with free doxorubicin, despite of absence of such effect in more sensitive tumor model, lymphoid malignancy P-388. Preferential effect of NPh doxorubicin as compared with free drug in LLC tumor was especially notable at low drug doses and particularly at the regimen of repeated weekly administrations. The results confirm the necessity to take into consideration the sensitivity or resistance of various used tumor models in the elaboration of new forms of drug delivery [15], and also testify to possible potential prospectivity of NPh form of doxorubicin for tumors with poor response to therapy. The treatment of such tumors may require high doses of cytostatics, which may be often impossible because of their severe side effects, and in this case replacement of free doxorubicin for its NPh form could be advisable.

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