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Differences in doxorubicin release from polypeptide nanoparticles of various compositions during subcutaneous and intraperitoneal administration to rats

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Summary

High performance liquid chromatography (HPLC) was used to determine doxorubicin (DOX) contents in rat blood after subcutaneous or intraperitoneal administration of the drug loaded into the delivery systems (DS) based on nanoparticles of poly(amino acids) of various compositions. One variant of delivery system was prepared of self-assembled NPs based on the random copolymer of L-glutamic acid and D-phenylalanine (P[EF]). The second variant of DS was based on the poly-L-serine-*b*-poly(L-glutamic acid) block copolymer (P[SE]); this copolymer was used to introduce DOX into rat blood for the first time. Encapsulation of DOX in nano-sized DS provided the prolonged presence of DOX in rat blood (up to 2-3 weeks) regardless of the administration route.

Keywords

Doxorubicin, drug delivery system, poly(amino acid) nanoparticles, subcutaneous, intraperitoneal administration, HPLC.

Introduction

Doxorubicin is an efficient anticancer broad-spectrum antibiotic that, unfortunately, has such disadvantages as rapid clearance and cardiotoxicity [1]. Delivery systems for DOX that should help overcome these problems have been already developed for more than ten years. The efficiency of DOX loaded into a DS and its distribution in a body depend on many parameters. When the drug is introduced in equivalent amounts, these characteristics depend first of all on the DS structure and size [2, 3], method of administration [4-6] and, of course, on physiology of an organism.

In recent years, delivery systems based on nanostructures have attracted the most interest. Some of them increase stability and bioavailability of drugs, decrease toxicity of preparations. Unfortunately, a negative influence of nano-DS on cells of living organisms even at non-cytotoxic concentrations was reported [7]. In the opinion of some scientists [8], more detailed studies of nano drug DS, especially, on their toxicity, are necessary. The main requirement for new nanocarriers of antitumor drugs is a balance between efficiency and toxicity [9].

When using the DOX concentration in blood for comparison of efficiencies of different delivery systems, it is necessary to know the DOX concentration range in blood of the patients treated with this preparation. In the comprehensive survey involving women with breast cancer who received different variants of treatment with DOX, the drug concentrations in plasma were found to be within a range of 10-620 ng/mL [10].

The goal of this work was to compare the DOX contents in blood of healthy rats after subcutaneous (SC) and intraperitoneal (IP) administration of DOX delivery systems based on nanoparticles of poly(amino acids) (PANPs) at different structures.

Materials and methods

Reagents

Doxorubicin was purchased from Veropharm (Russia) as "Doxorubicin LENS" dosage form which contained 20% of doxorubicin hydrochloride (DOX) and 80% of mannitol. Before the experiment, dialysis of the preparation against water through a membrane with MWCO =1 kDa was performed for 2 days; then the substance was lyophilized. Poly(amino acids) were synthesized according to previously developed techniques based on commercial reagents (Sigma-Aldrich, Germany) [11, 12] with the use of solvents purchased from Vekton (Russia) and purified according to the standard protocols.

Determination of DOX amount

Spectrophotometric analysis

The absorbance values of DOX solutions at λ=485 nm were measured, and concentrations of solutions were calculated using the calibration curves. These graphs were plotted for various DOX-containing solutions.

HPLC analysis

Quantitative analysis of DOX in rat blood plasma was performed with the aid of a Prominence-I LC 2030C 3D Plus instrument (Shimadzu, Japan) equipped with an RF-20A fluorescence detector and a 5 μm Luna C18 column (Phenomenex, USA). The excitation wavelength was 475 nm, the emission wavelength was 555 nm. Analysis was performed in the gradient mode (with acetonitrile) in 0.01 N Na-formate buffer at pH = 3.68. The duration of the experiment was 20 min. The detection limit was 1 ng/mL. All measurements were performed three times.

Preparation of the drug delivery systems

Two variants of nanoparticles prepared of amphiphilic copolymers of amino acids with different structures were used for DOX encapsulation. P[EF] are self-assembled nanoparticles based on the random copolymer of L-glutamic acid and D-phenylalanine with molar ratio of monomer units Glu/Phe = 2.8. Synthesis of this copolymer, preparation of nanoparticles and their properties are described elsewhere [10,13]. Nanoparticles of different structure (P[SE]) with molar ratio between monomer units Glu/Ser = 1.5 were formed by self-assembly according to the technique used for the P[EF] particles.

DOX loading into the delivery systems

DOX was loaded into nanoparticles by the diffusion technique. A certain amount of DOX was added into the suspension containing nanoparticles; the suspension was stirred for 30 min on a shaker at room temperature, then left to stand overnight at 4°C. After encapsulation, free DOX was separated from nanoparticles by centrifugation at 12000 rpm for 10 min. Nanoparticles were washed with water three times, then all portions of the supernatant were combined, lyophilized, dissolved in 1.5 mL of buffer solution and analyzed spectrophotometrically at 480 nm. The drug load (L) was determined as a difference between the initial DOX contents in the suspension and the amount of DOX not included into nanoparticles over the mass of nanoparticles in the suspension.

Cytotoxicity of the delivery systems

Cytotoxicity in cell samples was studied with the use of a cell analyzer RTCA iCELLIgence System (ACEA Biosciences, USA), which allows registering the state of cell cultures over time (without additional staining of cells) by measurement of electrical impedance of microsensors in wells.

Dynamics of MCF7 cell proliferation during conditioning with PANPs was assessed under culture conditions. The cells were cultivated in the complete DMEM nutrient medium (Paneco, Russia) supplied with 1% of L-glutamine 200 mM, 10% of fetal bovine serum and 1% of antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin), 1% of antifungal agent (amphotericin B 250 μg/mL). Cell cultures were carried out in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37°C, 5% CO_2 and 100% humidity. The aliquots of 35 000 MCF7 cells were placed in each well with the nutrient medium (well volume: 0.2 mL). At 24 hr, empty PANPs (SE and EF) were added to wells. Cell index was measured by the cell analyzer continuously. The data obtained from three wells were averaged.

In vivo experiments with rats

Healthy female outbred rats with body mass of 256 to 300 g obtained from "Rappolovo" farm for laboratory animals were admitted to the experiments. The animals were caged (2-5 individuals in a cage), had free access to water and food (all-in-one pelleted formula for prolonged keeping of rodents 4R F18 from Mucedonia, Italy). All procedures involving animals were in compliance with Ethical Standards approved by the Russian State Standard (#33216-2014) and the principles of the Basel Declaration. All manipulations with animals were performed using anesthesia: Sol. Zoletil 50 (0.05 mL *per* 0.1 kg of body mass) and Sol. Rometarum 20 mg/mL introduced intramuscularly (0.0125 mL *per* 0.1 kg of body mass).

The drug (DOX in both PANPs, 4 mg of DOX *per* 1 rat) was injected intraperitoneally (IP) or subcutaneously (SC) in 1.0 mL of 5% glucose solution using 21-gauge needles. 3 to 5 animals were used in each experiment.

To determine DOX concentration in rat blood after administration of the drug by two routes for various delivery systems, the blood samples (1.0 mL) were taken from the rat tail vein at different intervals after injection. Before drawing a blood sample, an animal was anesthetized and fixed in a holder for rodents. Blood plasma was separated in the following way: 10 min after blood sampling, blood was centrifuged for 15 min at 1500 rpm. The supernatant was frozen and kept in a covered vessel at – 40°C for subsequent HPLC analysis.

Morphological studies

Morphological experiments were carried out according to the standard technique described earlier [4]. The materials for histological studies were fixed in 10% neutral formalin in phosphate buffer (pH = 7.4) for, at least, 24 hrs, dehydrated in a series of ethanol solutions at increasing concentrations, and embedded into paraffin blocks according to the standard histological technique. To obtain comparable results, the samples were treated simultaneously under similar conditions. The paraffin sections (5 μm thick) were prepared by means of an Accu-Cut SRT 200 microtome (Sakura, Japan) and stained with Mayer hematoxylin and Eosin (BioVitrum, Russia). Microscopic analysis was performed using a Nikon Eclipse E200 light microscope (Nikon, Japan) with a 10× ocular and 4, 10, 20, and 40× objectives.

Statistical evaluation

The standard errors in determining the average DOX contents in blood plasma at distinct time points were in the range of 10-23%.

Results and discussion

The poly(amino acid) nanoparticles used as delivery systems for DOX have various structures and compositions, namely: P[EF] is a random copolymer of L-glutamic acid and D-phenylalanine with molar ratio between monomer units Glu/Phe = 2.8, and P[SE] is a block copolymer of L-glutamic acid and L-serine with molar ratio between monomer units Glu/Ser = 1.5. Molecular masses of the copolymers and hydrodynamic diameters of nanoparticles prepared of them are 6500 Da, 148 nm for P[EF], and 7750 Da, 295 nm for P[SE], respectively. The general characteristics of the used nanoparticles have been determined earlier [12, 14].

Usage of PANPs as delivery systems for DOX suggests their distribution not only in the tumor area, but throughout the entire body. The absence of toxicity of the P[EF] nanoparticles towards healthy cells of various organisms (human embryonal cells (HEK-293), mouse fibroblasts (NIH-3T3) and human lung epithelial cells (BEAS-2B)) at concentrations up to 1.0 mg/L has been demonstrated earlier [15].

In the present work, cytotoxicity of nano-sized PANPs towards the MCF7 line cells was determined by the iCELLIgence technique that allows real-time monitoring of cell proliferation or death. Cell index was determined by the analyzer continuously.

One may see from the profile of cell index growth over time (curve 3) that the number of cells *per* well decreases after 100 hr of culture. This is explained by high initial amount of cells in a well. By 100 hrs, there are too many cells *per* a single well; they do not have enough nutrients and start to die. Higher cell indices obtained with poly(amino acid) nanoparticles may be explained by their composition, since they contain amino acids released in culture medium.

The release of DOX into rat blood was studied after administration of PANPs of both structures containing 4 mg DOX. The maximum tolerated dose (MTD) of DOX for rats is 5 mg *per* 1 kg of animal body mass [16]. The average mass of the

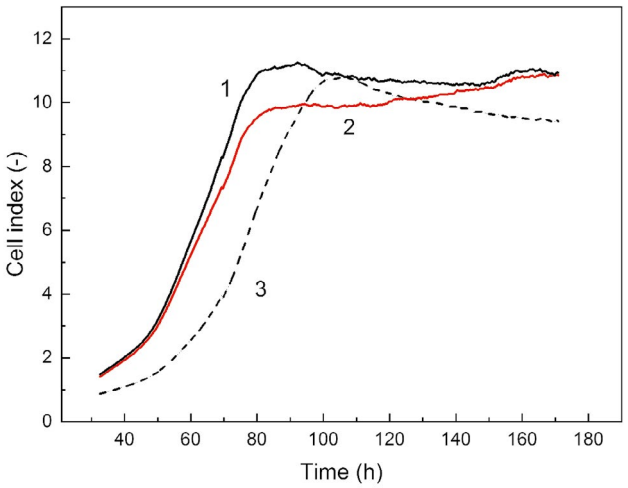

Figure 1. Growth rates of MCF7 cells in the presence of the P[SE] (1) and P[EF] (2) nanoparticles and in the free state (3); the initial number of cells: 35 000/sample. Concentration of nanoparticles was 5 mg/mL

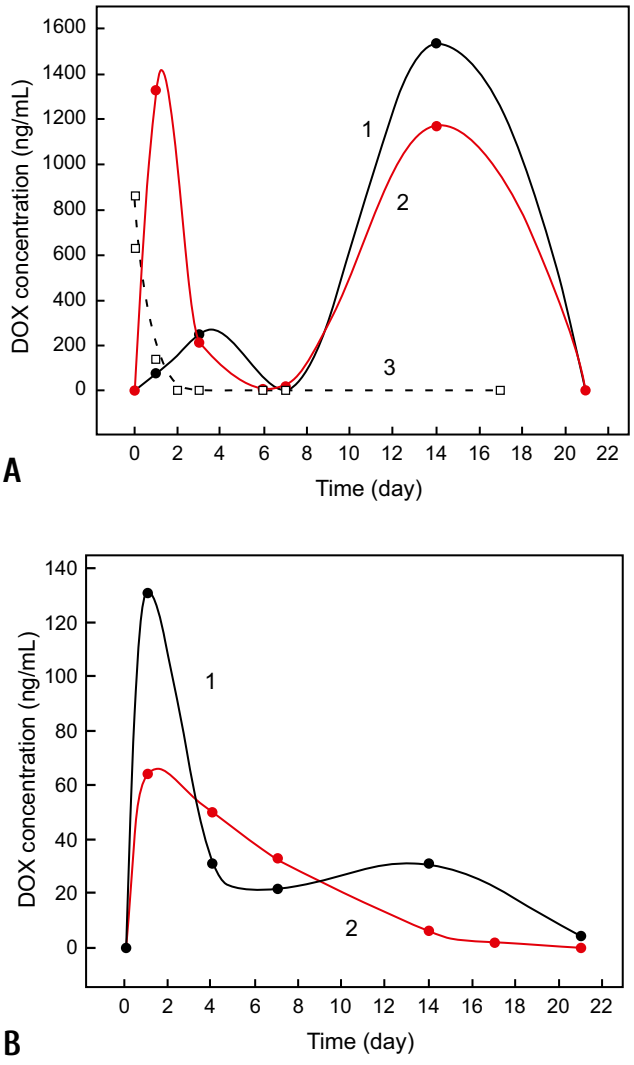

Figure 2. DOX concentration in rat plasma after intraperitoneal (A) and subcutaneous (B) injections. Delivery systems: P[SE] (1), P[EF] (2); carrier-free DOX (3)

rats used in the experiments was 278±22 g. Therefore, each animal received the dose that was approximately 3 times higher than MTD. However, the animals were active; no changes in their behavior and dietary pattern were observed.

Pharmacokinetic profiles of DOX concentration in rat blood after intraperitoneal and subcutaneous administration of both types of DS are presented in Fig. 2. In all cases, the prolonged presence of DOX in rat blood (up to 2-3 weeks) is observed. Curve 3 in Fig. 2A illustrates the DOX concentration in blood after IP administration of the carrier-free drug. DOX is removed from blood as soon as 2 days after administration. The efficiency of the used delivery systems based on polypeptide nanoparticles may be compared with that of nanostructured lipid carriers. E.g., Ansar et al. [17] compared the contents of antioxidant substance Thymoquinone after intravenous and oral administration of a nanostructured lipid carrier. In both cases, the preparation was present in blood for not more than 8 hrs, the contents of Thymoquinone in blood being considerably lower upon oral administration.

It is not possible to obtain the release profile for free DOX following SC injection since local inflammation and tissue necrosis are observed after SC injection of carrier-free DOX [18].

In the experimental studies involving animals, IP administration is considered as equivalent to intravenous injection [19]. SC injection shows some different features as compared to intravenous or intraperitoneal routes. After subcutaneous administration, the drug absorption proceeds rather slowly, due to lower blood diffusion rates.

Comparison of Fig. 2A and 2B indicates higher absorption of DOX after IP administration. Pharmacokinetic profiles of DOX release into blood upon SC administration are almost similar for both delivery systems.

Naturally, one should bear in mind that humans and rats have different physiology. However, one should note that the DOX concentrations in rat blood in all our experiments, i.e., DOX loaded in PANPs administered in two ways, are within a range close to the concentrations found in the blood of women with breast cancer receiving this preparation [10].

The fate of DOX following its intravenous administration into mice is described by Zhang et al. [20]. The authors compared time profiles of DOX concentration in blood plasma of HeLa-bearing mice after intravenous administration of carrier-free DOX and DOX incorporated into nanoparticles of different composition (mean particle size of 200 nm). The amounts of DOX in different organs were determined by HPLC. 48 hrs later, the DOX concentrations in plasma were close to zero for all variants of administration. The information about amounts of DOX found in the studied organs and tissues allowed the authors to estimate the proportion of DOX present in blood plasma for 24 hrs. This value was approximately 5% of the DOX amount introduced into animal organism.

Pharmacokinetic profiles of DOX obtained in our work were used to estimate the fraction of drug released into blood upon its IP administration for the both delivery systems. These values (percentages of the DOX mass injected into rat organism, 4 mg *per* animal) were equal to 0.93% for P[SE] and 1.35% for P[EF]. Even lower values were obtained in the case of SC administration of DOX (tenths of a percent). When comparing these results, it is necessary to take into account the DS structure, differences in rat and mouse organisms. Moreover, one should consider different administration routes of DOX carriers (direct injection into bloodstream, or its introduction *via* hypodermis during SC administration).

Qualitative information about the presence of P[EF]+DOX in connective tissue at the site of subcutaneous administration may be obtained by morphological studies. In the absence of pathologically changed epidermis, numerous macrophages were observed in dermis and hypodermis layers at 23-48 days after injection of P[EF]+DOX. Their cytoplasm was filled with DOX granules. Local aseptic inflammation is the normal consequence of the drug injection, with absence of detectable necrosis. Morphological study of rat liver and lungs after administration of DOX in EF carrier still revealed toxic manifestations typical of DOX, which, however, were reversible.

Conclusions

Nanoparticles based on poly(amino acids) of different compositions were used as delivery systems for introducing DOX into rat organism. Intraperitoneal and subcutaneous administrations facilitated the sustained DOX release into blood over a period of 2-3 weeks in therapeutic amounts. It was shown that subcutaneous administration of DOX in the amount 3 times exceeding its maximal tolerated dose (MTD, loaded into the developed DS) caused neither irritation nor necrosis in the injection area. Further studies will be aimed at determination of DOX distribution in organs and tissues following its introduction into organism by means of the developed PANPs. Subcutaneous administration of DOX delivery systems appears to be an interesting approach, in order to deposit a toxic drug in the body.

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Conflict of interest

The authors declare no conflict of interest.

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Различия в высвобождении доксорубицина из полипептидных наночастиц различного состава при подкожном и внутрибрюшинном введении крысам

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Резюме

При помощи метода высокоэффективной жидкостной хроматографии определено содержания ДОХ в крови крыс после его введения подкожно или внутрибрюшинно в системах доставки (СД), представляющих собой наночастицы на основе полиаминокислот разного состава. Один вариант структуры СД – самоорганизованные наночастицы на основе статистического сополимера L-глутаминовой кислоты и D-фенилаланина (P[EF]). Второй вариант – блок-сополимер L-серина и L-глутаминовой кислоты (P[SE]), впервые используемый в качестве систем доставки DOX. Введение ДОХ с помощью наноразмерных DS обеспечивает пролонгирование пребывания ДОХ в крови крыс независимо от способа введения до 2-3х недель.

Ключевые слова

Доксорубицин, полиаминокислотные наночастицы, системы доставки, внутрибрюшинное введение, подкожное введение, высокоэффективная жидкостная хроматография.