Bioencapsulated tumor spheroids as a 3D model to study cytotoxicity of novel antitumor drugs

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INTRODUCTION

Development of new effective antitumor drugs and delivery systems is one of the most important challenges for modern medicine. Various *in vitro* models based on cell cultures are becoming of particularly importance because of permanent expansion of a spectrum of compounds and tightening of requirements for models in experimental animals. As well known, classical *in vitro* models are suspension and monolayer cell cultures. However, it is obvious that cells under these conditions can not adequately mimic solid small size tumors *in vivo*. Therefore, it's necessary to develop more reliable methods for screening drug preparations. Instead of two-dimensional (2D) models various 3D systems, in particular multicellular tumor spheroids (MTS), have been proposed. MTS are considered as a 3D *in vitro* model of the small solid tumors *in vivo* (Sutherland 1988).Microencapsulation is rather new promising technique to generate MTS by cultivation of tumor cells in semi-permeable polymer microcapsules (Zaytseva-Zotova et al, 2011a). It allows to generate spheroids with a narrow size distribution, as well as spheroids on the basis of cell lines which are not capable to form aggregates in suspension culture, and therefore spheroids can not be prepared by classical techniques. Various biocompatible polymers, such as alginates, gums, carrageenans, pectin, gelatin, chitosan could be used for microencapsulation.

The aim of the study was to form MTS in alginate-chitosan microcapsules and to use them as a 3D test system for cytotoxicity study of new doxorubicin (DOX) derivatives.

MATERIALS AND METHODS

Reagents. Sodium alginate (3.5 cP at 25 °C, Sigma-Aldrich), DOX hydrochloride (JSC "ONOPB"), CaCl2 (Sigma, USA), EDTA, 3 - (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), PanEko, Russia, were used in this study. Oligochitosan (MM 3400 Da, DD 87%) was kindly provided by Prof. A. Bartkoviak, Poland. All solutions for cell immobilization were prepared in 0.9% NaCl solution.

Cell encapsulation and cultivation.
Human breast adenocarcinoma (MCF-7) cells were cultured in medium DMEM (PanEko, Russia) containing 10% of fetal calf serum (HyClone, USA) in a CO_2 incubator atmosphere containing 5% CO2 at 37°C.

Microencapsulation of cells was performed using an electrostatic bead generator, as described previously (Zaytseva-Zotova et al, 2011b). The cultivation of encapsulated cells was carried out in flasks of 25 or 75 cm^2 (Corning Inc.) in a CO_2 incubator atmosphere containing 5% CO2 at 37 ° C for 1-4 weeks to completely fill microcapsule internal volume.

Cytotoxicity study. Cell viability was assessed by MTT-test after 24, 48 and 72 hours. In the case of MTS, aliquots of the spheroids (12.5 µl of slurry) were incubated with 100 µl of drug solutions in the 96-well plates for 48 and 72 hours.

Synthesis of DOX conjugate with the bromide (4-4-carboxybutil) triphenylphosphonium (DOX-TPP)
EDAC (0.22 mmol) at 0°C was added to solutions of $(C_6H_5)_3P(Br)(CH_2)_4COOH$ and HONp in DMF (each substance to 0.22 mmol), mixed for 1 h and then added a solution of DOX * HCl (0.1 mmol) and 30 ml TEA (0.2 mmol). After 24 h stirring the mixture at room temperature and protecting from light, the solvent was evaporated and the product landed by adding MTBE. Further purification was performed on silica gel Chemapol 100/160 mm in A system. The yield was 38 mg (42%).

Synthesis of N-palmitoyl-doxorubicin (N-Palm-DOX). Palm-ONSu (66 mg, 0.187 mmol) and DIEA (65 ml, 0.374 mmol) were added to a solution of DOX * HCl in 2 mL of DMF (100 mg, 0.17 mmol) with stirring. After 18 h, protecting from light, the solvent was evaporated and the product was washed with water and planted by filtration. Purification was performed on silica gel (Sigma, 60A, 230-400 mesh) with chloroform: methanol, 10: 1. The fractions containing the final product were combined and evaporated. The yield was 56 mg (42%).

Synthesis of N-palmitoyl-hydrazone of doxorubicin (Palm-N_2H-DOX). Solutions of Palm-N_2H_3 (77 mg, 0.2 mmol) and 30 ml (0.4 mmol) TFA in 5 mL of methanol were added to solutions of DOX * HCl (12 mg, 0.02 mmol) and 10 ml TFA (0.13 mmol) in 10 mL of methanol with stirring. After 8 h, protecting from light, the mixture was evaporated and the product was precipitated by adding acetonitrile and washed on the filter MTBE. The yield was 15 mg (94%).

RESULTS AND DISCUSSION

In order to form tumor spheroids, the cells were entrapped in semi-permeable microcapsules and cultured for 2-4 weeks. One of the main advantages of this approach is the possibility to generate spheroids in microcapsules with a narrow size distribution. To form the microcapsule membranes oppositely charged polyelectrolytes, namely alginate (polyanion) and chitosan (polycation) were selected. Alginate-chitosan microcapsules were stable in the medium at least for 1 month. The cells grew and proliferated, completely filling the microcapsule volume within 2-4 weeks. The generated spheroids were used to study cytotoxicity of new derivatives. In this research, we used three DOX preparations modified either by palmitic acid (N-Palm-DOX, and Palm-N_2H-DOX), or by triphenylphosphine (DOX-TPP).

DOX residue attachment to palmitic acid BAD! allows to get complexes with albumin circulating in the blood which can accumulate in the tumor tissue due to EPR (enhanced permeability and retention). In addition, it was found that palmitic acid is able to induce apoptosis in tumor cells, so it may contribute to the drug citotoxicity effect. As well known, modification of the DOX amino group changes its intracellular localization. Such derivatives could accumulate predominantly in the mitochondria. As a result it could allow to overcome cell resistance towards drugs. Using lipophilic cations, such as triphenylphosphonium, for this purpose could result in better drug penetration through the cell membrane and subsequent accumulation in mitochondria. More over, a number of mitochondria in tumor cells is higher then in normal ones, and therefore their negative charge is also higher.

Study of DOX derivatives on the MTS-based model showed that in the case of MTS higher doses are needed to achieve the similar effect like in the 2D model (Table 1).

Table 1 : The IC50 values for new derivatives of doxorubicin.

Drug	IC50, uM		
	24 h	48 h	72 h
	2D		
DOX	0,8	0,5	0,4
Palm-N_2H-DOX	23	5	4
N-Palm-DOX	30	9	4
DOX-TPP	70	35	20
	3D		
DOX	n/a	28	7
Palm-N_2H-DOX	n/a	48	28
DOX-TPP	n/a	100	35

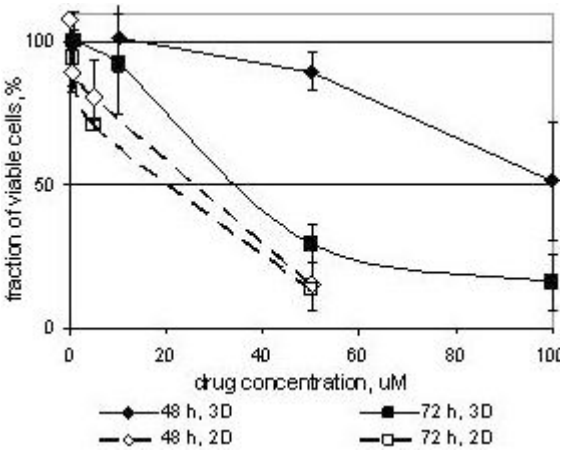

Fig. 1 : Cytotoxic effect of DOX-TPP on the MCF-7 cells in the MTS (3D) and in the monolayer culture (2D).

A typical dependence of the cell survival after treating with DOX-TPP is shown in Figure 1. As can bee seen, cells in MTS are more resistant towards the drug than in monolayer. Thus, after 48 h IC50 for 2D model was 26 uM whil for MTS it was much higher (100 uM). However after 72 h IC50 was already 20 uM and 35 uM respectively.It is obvious that the MTS due to 3D structure represent a more appropriate *in vitro* model, than the 2D model based on monolayer cultures.

CONCLUSIONS

New DOX derivatives were synthesized. MTS were obtained in polyelectrolyte alginate-chitosan microcapsules. Cytotoxicity of novel DOX derivatives was evaluated using 3D model based on microencapsulated MTS compared to 2D model (monolayer culture). It was demonstrated that in the case of MTS the higher doses of all DOX derivatives were needed to achieve the effect similar to this in the monolayer culture. Microencapsulated spheroids could be considered as new promising 3D in vitro model for novel drugs screening.

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