Journal of Bioenergetics and Biomembranes (2018) 50:53–58
https://doi.org/10.1007/s10863-017-9738-4

Study of the properties of doxorubicin-resistant cells affected by acute leucosis

Marina Yu. Skorkina[1] · Elena A. Shamray[1] · Victoria A. Salo[2] · Anatoly S. Buchelnikov[3] · Maxim P. Evstigneev[2]

Received: 26 April 2017 / Accepted: 28 November 2017 / Published online: 19 December 2017

Abstract
The stiffness of cell membrane was found to be one of the factors determining resistance of a cell in vitro to antibiotic doxorubicin action. Membranes of surviving cells are negatively charged (−35 – −30 mV) and have high values of stiffness (2.2–5.1 μPa) at the doxorubicin concentrations in the medium of 1–500 μg/ml. If the drug concentration and exposure time are being increased, only cells with 'soft' membrane (0.25–1 μPa) and positive surface potential (15–29 mV) survive. The data obtained have important prognostic value in studying drug resistance of tumour blood cells and can be used as objective markers of efficiency of the antitumor therapy.

Keywords Acute leucosis · Atomic force microscopy · Biomembrane · Doxorubicin · Membrane stiffness · Surface potential

Introduction

One of the challenges in modern oncohematology is the search of mechanisms responsible for resistance of malignant clones of hematopoietic system to a standard chemotherapy as well as development of strategies for its inhibition. According to statistical data ca. 10% of patients with acute myeloproliferative processes in blood system demonstrate poor results of initial standard chemotherapy and more than 60% of relapses are resistant to a treatment (Siegel et al. 2014).

The use of anthracycline drugs, such as doxorubicin, is widely spread in standard regimen of treatment of oncological blood diseases. Despite a number of studies learning intracellular molecular mechanisms of doxorubicin action (Bao et al. 2011; Denard et al. 2012; DiDonato et al. 2012; Du et al. 2012; Harati et al. 2012; Lerma-Díaz et al. 2006; Liu et al. 2008; Naci et al. 2012; Ortiz-Lazareno et al. 2014; Sun et al. 2011; Wang et al. 2012) so far there is no definite viewpoint on how multiple drug resistance of tumour cells is developed.

We assume transport properties of biomembranes playing crucial role in development of effective strategy to overcome drug resistance. Several studies demonstrate a surface tension of biomembrane being decreased under the influence of doxorubicin (Bell et al. 2013), which directly affects the intensity of endocytosis and drug transfer into cell (Rauch and Farge 2000). From the other hand, a vesicular transport of doxorubicin by endocytosis strongly depends on biochemical composition and fluidity of biomembranes. The increase of fluidity in chemoresistant tumour cells was experimentally proved by Wheeler et al. (1982). Thus, the mechanical properties of biological membrane significantly determine its transport function, in particular, the ability of drug to reach intracellular molecular targets. In other words, these properties affect drug resistivity of a cell.

Taking this as a starting point, we became interested to shed more light on how not only mechanical but also electrical properties of biomembranes influence drug resistivity of cell. The particular task was to investigate elastic and electrical properties of doxorubicin-resistant cell clones while lympho- and myeloproliferative processes are progressing in blood system.

Materials and methods

Cell cultivation Clones of tumour cells were obtained from whole blood of patients with acute lymphoblastic (15 persons)

✉ Marina Yu. Skorkina
skorkina@bsu.edu.ru

✉ Anatoly S. Buchelnikov
a.buchelnikov@spbu.ru

[1] Department of Biology, Belgorod State University, 85 Pobedy str, 308015 Belgorod, Russia

[2] Department of Physics, Sevastopol State University, 33 Universitetskaya str, 299053 Sevastopol, Russia

[3] Theodosius Dobzhansky Center for Genome Bioinformatics, Saint Petersburg State University, 41 Sredniy Avenue, 199034 Saint Petersburg, Russia

and myeloblastic leucosis (5 persons) to be further referred to as ALL and AML, respectively. The diagnosis for the patients was established for the first time and no treatment was done. The blood was venipunctured and was stored in vacuum vials (Vacuette K3E). Then we centrifuged it at 1500 rpm (152 *g*) for 5 min, washed off and re-suspended in RPMI-1640 medium (PanEco, Russia).

Approximately 1 million of cells were seeded in 6-well plates in RPMI-1640 medium in 1:1 cells-to-medium volume ratio. Five wells were filled with doxorubicin solution (Ebewe, Austria) in different concentrations (1, 10, 100, 500 and 1000 μg/ml) in a ratio of 1 μl of doxorubicin solution per 10 μl of cell suspension in RPMI-1640 medium. The sixth well plate served as a control without addition of doxorubicin solution. The cells were incubated for 1 h and 24 h at 37 °C in a CO_2 incubator.

The purpose of revealing the effect of doxorubicin on biophysical properties of biomembranes determines the choice of drug concentration. The range of concentrations is therefore quite wide, exceeding the physiological doses.

Assessment of viability was accomplished by coloration of 1 μl of cell suspension by 0.4% trypan blue solution in phosphate buffer (pH 7.2–7.3) and further calculation of dead cells by means of light microscope (Olympus, USA). The percentage of surviving cells is given as:

$$N = \left(1 - \frac{\text{number of stained cells}}{\text{total number of cells}}\right) \cdot 100\%$$

Doxorubicin-resistant cells were washed off thrice using Hanks medium (pH 7.4) in order to remove the incubation solution. Then we became able to study elastic and electric properties of cell surface by means of various scanning modes of atomic force microscope.

Atomic force microscopy Following to Skorkina et al. (2015) we investigated elastic properties of blastic blood forms using Integra-Vita instrument (configuration based on inversed optical microscope Olympus IX-71 (NT-MDT, Russia)) when applied a load of 25 local cell surface regions (Fig. 1). 20 cells from each plate well were scanned in semi-contact mode using NSG03 cantilevers (Nanoworld, USA). Local Young's modulus was calculated based on Sneddon's modification of Hertzian contact problem. Within the framework of this model, a cell is assumed an elastic isotropic medium with a Poisson coefficient $\nu = 0.5$, and the cantilever needle is modeled by a solid cone. The experimental curves were processed using Ef3 software (NT-MDT, Russia).

Electrical properties of blasts were studied by measuring of surface potential. Cell suspension was prepared according to a protocol published by Sladkova and Skorkina (2014). Again, 20 cells from each plate well were scanned in Kelvin probe mode using NSG03/TiN cantilevers (Nanoworld, USA) with conductive titanium coating. The obtained scans were further processed in Nova software (NT-MDT, Russia).

Statistical data treatment Experimental results were treated using variational statistics methods. All the data are given as mean values along with their average standard errors. Statistical analysis was accomplished with an aid of Student's *t*-criterion at $p < 0.05$.

Results

The results of incubation are shown in Figs. 2 and 3. The fraction of viable blood cells for both types of acute leucosis demonstrates monotonic decrease with increasing concentration of doxorubicin (Figs. 2 and 3).

After 1-h incubation of plate with ALL blood, the fractions of viable cells have decreased almost two and four times in comparison with control (doxorubicin concentrations were 500 and 1000 μg/ml, respectively). Also 97% and 24% of tumour lymphoblasts survived at the drug concentrations of 1 μg/ml and 1000 μg/ml, respectively. After 24-h incubation of ALL blood, the fraction of doxorubicin-resistant cells has decreased in similar way: 92% and 10% of tumour cell population survived at the concentrations of doxorubicin of 1 μg/ml and 1000 μg/ml, respectively (Fig. 2).

Blood cells from AML patients were found to be more resistant to doxorubicin, particularly, to its high doses. After 1-h and 24-h incubation, at least 55% and 40% of tumour myeloblasts, respectively, maintained their viability despite even the highest concentration of doxorubicin (Fig. 3).

The viability of tumour cells is closely related to their functional activity; therefore, we measured surface potential of doxorubicin-resistant cell membranes. No reliable changes of biomembrane charge was detected at the doxorubicin concentration of 1 μg/ml (Table 1). After 1-h incubation of ALL blood, 8%, 38% and 91% ($p < 0.05$) decrease of surface potential in resistant populations of tumour cells were observed at the drug concentrations of 10, 100 and 500 μg/ml, respectively. At the highest drug concentration of 1000 μg/ml doxorubicin-resistant cells have lost negative charge and their surface potential have been essentially increased. After 24-h incubation, similar phenomena were observed. However, at the drug concentration of 500 μg/ml it was detected 40% ($p < 0.05$) increase of surface potential of resistant cells. At the concentration of 1000 μg/ml the charge of surviving cells increased by 85% ($p < 0.05$) as compared with the charge of cells after 1-h incubation.

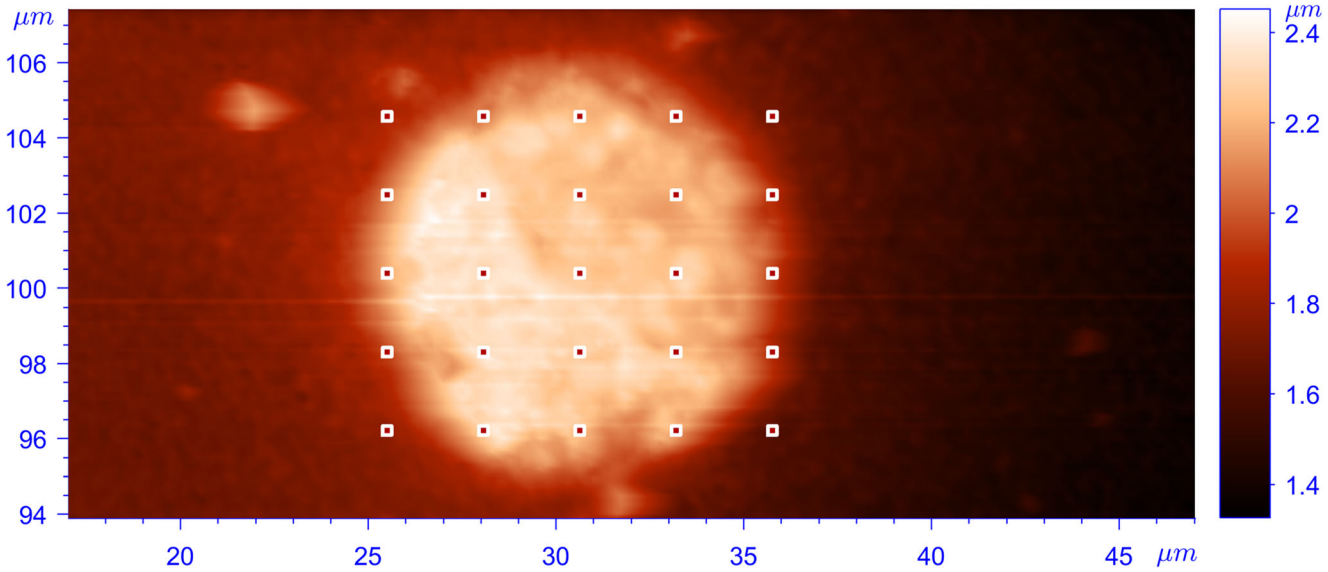

Fig. 1 Local points of force apposition on cell surface

The properties of surviving AML blood cells under the influence of doxorubicin were found to be similar to those of ALL blood cells. Surface potentials of cell membranes have decreased after 1-h incubation in the concentration range of 100–500 μg/ml. The most significant decrease by 97% ($p < 0.05$) has been detected at the drug concentration of 500 μg/ml. In the samples with 1000 μg /ml of doxorubicin cell membrane was positively charged (see Table 1). After 24-h incubation, similar phenomena was observed: positive charge of membranes of doxorubicin-resistant cells at 1000 μg/ml drug concentration had increased by 68% ($p < 0.05$) as compared with charge of cells after 1-h incubation.

Not only electrical, but also elastic properties of cell surface were studied for doxorubicin-resistant cells. After 1-h incubation, in the samples from ALL patients the average stiffness of cell surface has increased by 26%, 95% and 189% ($p < 0.05$) at the drug concentrations of 10, 100 and 500 μg/ml, respectively. However, at the highest concentration of 1000 μg/ml in the rest population of doxorubicin-resistant cells the stiffness of cell surface has decreased by 67% ($p < 0.05$) (see Table 2). After 24-h incubation the doxorubicin-resistant cells were found having stiffness significantly lower than that of the control values (see Table 2).

For AML blood we observed the similar picture as for ALL blood after 1-h incubation. The stiffness of cell surface has increased by 42%, 74% and 94% ($p < 0.05$) in the drug concentration range of 10, 100 and 500 μg/ml, respectively. However, at the concentration of 1000 μg/ml the cell stiffnesses have decreased by 45% and 86% ($p < 0.05$) after 1-h and 24-h incubation, respectively (see Table 2).

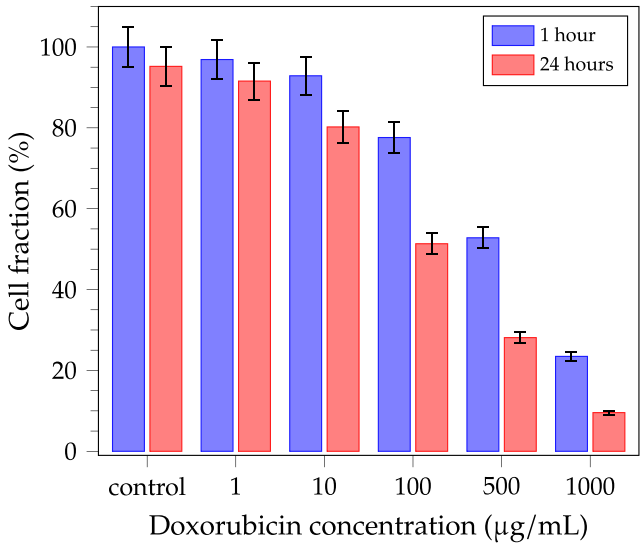

Fig. 2 The fraction of doxorubicin-resistant cells from patients with acute lymphoblastic leucosis

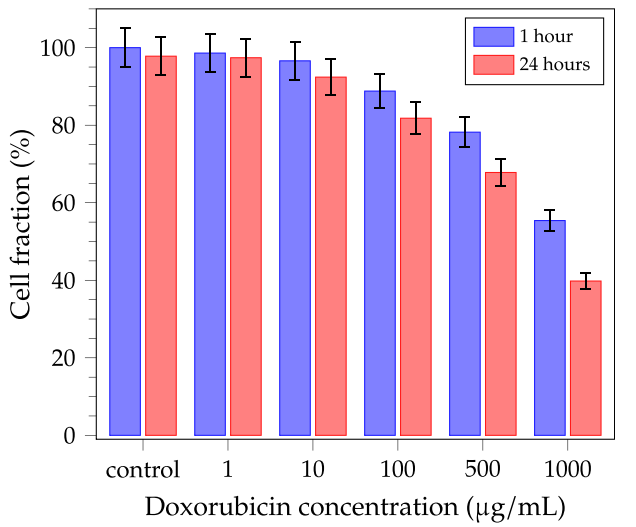

Fig. 3 The fraction of doxorubicin-resistant cells from patients with acute myeloblastic leucosis

Table 1 The value of the surface potential (mV) of the doxorubicin-resistance cells

Samples	Acute lymphoblastic leucosis		Acute myeloblastic leucosis	
	1 h	24 h	1 h	24 h
Control	−28.36 ± 0.12		−24.18 ± 0.10	
1 μg/ml	−26.71 ± 0.34	−29.36 ± 0.38	−24.65 ± 0.19	−27.53 ± 0.26
10 μg/ml	−30.71 ± 0.25*	−35.82 ± 0.17*	−29.33 ± 0.24*	−33.12 ± 0.08*
100 μg/ml	−39.18 ± 0.71*	−51.27 ± 0.24*	−35.21 ± 0.04*	−40.18 ± 0.19*
500 μg/ml	−54.22 ± 1.13*	−11.23 ± 0.22*	−47.63 ± 0.12*	−59.71 ± 0.36*
1000 μg/ml	15.76 ± 2.19*	29.18 ± 0.27*	12.37 ± 0.07*	20.81 ± 0.21*

*Statistically reliable differences in experiment in comparison with control at $p < 0.05$

Discussion

Drug resistance of tumour cell is defined by the properties of its membrane as well as molecular weight of a drug which is crucial for development of drug resistance (Rauch 2008). We detected doxorubicin-resistant leukemic cell clones of lympho- and myeloblastic types of proliferation. The most resistant to the drug are myeloblastic forms. Among them ca. 40% of tumour cells preserve their viability even after 24-h incubation in the medium with the highest drug concentration.

At the relatively low concentrations of doxorubicin quite high percentage (50–95%) of surviving cells maintains high values of stiffness and negative charge of membrane. This indicates that primarily the drug enters a cell by means of diffusion which was modelled earlier (Dalmark and Hoffmann 1983; Skovsgaard and Nissen 1982). Drug passage through a membrane leads to death of less than 4–6% of leukemic clones partly differentiated in cell cycle.

However, on increasing both drug concentration and incubation time the cell membrane becomes softer, and the charge of cell surface increases gradually, reaching positive values at the highest concentrations of doxorubicin. This is due to the high affinity of doxorubicin to a lipid bilayer of membrane. According to Bell et al. (2013) used model cell lines, doxorubicin decreases the difference of surface tension between lipid layers in the membrane, but does not change surface potential of cell. Our experimental results show the change of surface potential of leukemic clones.

At the high drug concentrations, cells maintained their therapy-resistance and acquired both positive charge of cell surface and a very soft cell membrane. The mechanism of this survival, probably, lies in changing the mechanical properties of membranes and/or the work of membrane transporters under the influence of high doses of doxorubicin. According to Hurwitz et al. (1997) the drug molecules are accumulated in lysosomes which are involved in the formation of drug resistance of myeloid cells. In turn, the formation of lysosomes can be related to the change of membrane curvature (Rauch and Farge 2000). Thus, we explain the change of the elastic properties of cell membranes by the existence of additional bends caused by formation of lysosomes to accumulate doxorubicin.

Another explanation is that doxorubicin molecule changes flow properties of cell membrane being highly affined to its lipids and thereby affecting the work of membrane transporters. It is known that drug molecule can influence membrane transporters via changing the membrane stiffness (Rauch et al. 2013). Possibly, the change of both cell membrane charge and its elastic properties under the influence of doxorubicin relate to the drug uptake by membrane. Despite its hydrophobicity, doxorubicin facilitates

Table 2 The elastic properties of the doxorubicin-resistance cells (μPa)

Samples	Acute lymphoblastic leucosis		Acute myeloblastic leucosis	
	1 h	24 h	1 h	24 h
Control	1.783 ± 0.001		1.854 ± 0.001	
1 μg/ml	1.842 ± 0.002	1.712 ± 0.001	1.872 ± 0.001	1.835 ± 0.001
10 μg/ml	2.245 ± 0.001*	1.385 ± 0.002*	2.620 ± 0.001*	2.057 ± 0.001*
100 μg/ml	3.472 ± 0.002*	1.210 ± 0.002*	3.212 ± 0.001*	2.240 ± 0.002*
500 μg/ml	5.140 ± 0.001*	0.905 ± 0.001*	3.585 ± 0.001*	1.847 ± 0.002
1000 μg/ml	0.582 ± 0.001*	0.397 ± 0.001*	1.027 ± 0.001*	0.252 ± 0.001*

*Statistically reliable differences in experiment in comparison with control at $p < 0.05$

water transport into a cell through polar groups of phospholipids in biomembrane. Particularly, doxorubicin molecule contributes to the distortion of a membrane and, therefore, water penetration into a cell while attracting dipalmitoyl phosphatidylcholine headgroups (Yacoub et al. 2011). The analysis of literature suggests that multidrug cell resistance of patients with acute forms of leucosis is associated with expression and mutation of ATP binding cassettes in cell membrane. The functional activity of ATP binding cassettes changes after the use of anthracyclines in the therapeutic schemes (Sehgal et al. 2015). A number of studies show the chemotherapeutic resistance of tumour cells being related to the existence of transport pump P-glycoprotein in the membrane which actively removes the drug from cell (Aller et al. 2009; Rauch et al. 2013).

Conclusions

Tumour cells resistance to doxorubicin is initially formed at membrane level. The stiffness and the charge of cell surface are the objective criteria indicating the resistance of tumour cells to a therapy. The positive charge of membrane and 'soft' cell surface are the characteristic properties of the membranes of antibiotic-resistant leukemic cell clones. These biomechanical properties allow cells to maintain a high degree of malignancy, and proliferative and invasive potentials. In circulation system blood cells are able to attract to negatively charged vascular endothelium and quickly migrate into tissues thereby providing there metastasis and formation of foci of proliferation. This is due to the detected unique membrane properties possessed under the influence of doxorubicin.

The data obtained have important clinical and diagnostic value in the therapeutic treatment and the disease prognosis. The electrical and mechanical properties of the surface of tumour cells are of paramount importance in the prediction of drug resistance and can be used as the objective markers of functional state of leukemic clones.

Acknowledgements We thank the Russian Science Foundation (project No. 14-14-00328) for financial support and also appreciate the participation of anonymous donors.

Compliance with ethical standards

Conflict of interest The local ethic committee at the Medical Institute of Belgorod State University approved the present study. The consciously agreements were obtained from all the patients according to the recommendations of the Declaration of Helsinki (The International Response to Helsinki VI, The WMA's Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, as adopted by the 52nd WMA General Assembly, Edinburgh, October, 2000).

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