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DOXORUBICIN AND PACLITAXEL CAUSE DIFFERENT CHANGES IN PLASMA MEMBRANE FLUIDITY OF MCF-7 BREAST CANCER CELLS

DOKSORUBICYNA I PAKLITAKSEL POWODUJĄ RÓŻNE ZMIANY W PŁYNNOŚCI BŁONY PLAZMATYCZNEJ KOMÓREK RAKA PIERSI MCF-7

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Streszczenie: Metodami spektroskopii fluorescencyjnej badano oddziaływanie przeciwnowotworowych leków doksorubicyny (DOX) i paklitakselu (PTX) na właściwości błony plazmatycznej komórek MCF-7 gruczolakoraka piersi. Zastosowano sondy fluorescencyjne TMA-DPH i DAUDA, umożliwiające oznaczenie płynności zewnętrznych, polarnych obszarów oraz rdzenia hydrofobowego dwuwarstwy lipidowej. Stwierdzono zróżnicowany wpływ obu leków. DOX i PTX oddziaływały w odmienny sposób na powierzchniowe i na hydrofobowe regiony błony. DOX indukowała podobne i zależne od stężenia zmiany w obu obszarach dwuwarstwy lipidowej. Niskie stężenia leku powodowały upłynnienie błony, podczas gdy wzrost stężenia wywoływał progresywne usztywnienie błony. PTX natomiast niezależnie od stężenia oddziaływał głównie na hydrofobowy obszar dwuwarstwy powodując jej usztywnienie. Jednakowe stężenia obu leków w różnym stopniu zmieniały płynność dwuwarstwy lipidowej. Może to wynikać z odmiennej interakcji PTX i DOX z komponentami błony. PTX wywoływał głębsze zmiany płynności błony i przy znacznie niższych stężeniach niż DOX, wykazując jednocześnie większą cytotoksyczność w stosunku do komórek MCF-7. Szybki, ponad 70% spadek przeżywalności komórek, któremu towarzyszył istotny spadek płynności błony, obserwowano w zakresie tych samych stężeń PTX (0,01–1 μM). Podobnie 60% spadek przeżywalności komórek MCF-7 oraz istotne zmiany płynności błony plazmatycznej – jej upłynnienie lub usztywnienie – obserwowano w zakresie tych samych stężeń DOX (0,05–5 μM). Wyniki te wskazują, że zmiany płynności błony spowodowane przez DOX lub PTX zakłócają istotnie proliferację komórek i sugerują możliwą korelację pomiędzy cytotoksycznością badanych leków i stopniem uszkodzenia błony, które one powodują. Łączny wpływ kombinacji DOX i PTX na płynność błony komórek MCF-7 był wysoce zależny od stężenia i stosunku molowego leków i znacznie różnił się od wpływu każdego z nich stosowanych pojedynczo. Nie stwierdzono synergistycznego lub addytywnego oddziaływania DOX i PTX na płynność błony plazmatycznej komórek MCF-7. Przy niektórych stężeniach leków obserwowano natomiast ich antagonizujące działanie.

Słowa kluczowe: doksorubicyna, paklitaksel, płynności błon plazmatycznych, komórki raka piersi, MCF-7.

Summary: Interaction of anticancer drugs doxorubicin (DOX) and paclitaxel (PTX) with the plasma membrane of MCF-7 human breast carcinoma cells was studied by fluorescence spectroscopy technique. TMA-DPH and DAUDA fluorescent probes were employed to examine fluidity in the upper polar and in the hydrophobic core regions of the lipid bilayer. Our data showed entirely different effects of DOX and PTX on membrane fluidity of MCF-7 cancer cells. DOX and PTX penetrated differently surficial and hydrophobic parts of the cell membrane. DOX caused similar and concentration-dependent changes in fluidity of both areas of lipid bilayer: low drug concentrations had a fluidizing effect, an increasing rigidization effect was observed with increasing drug concentrations. PTX mainly disturbed the structure of the inner part of the cell membrane and showed rigidization effect that was independent on drug concentration. The same concentrations of DOX and PTX induced different extent of alterations in the lipid bilayer, which could stem from their distinct interactions with the lipid components of the plasma membrane. Of both drugs PTX induced significantly greater changes in plasma membrane fluidity and at much lower concentrations showing at the same time considerably higher cytotoxicity towards MCF-7 cells than DOX. A rapid, over 70% decrease in cell survival with concomitant striking decrease in membrane fluidity were observed with the same concentration range of PTX (0.01–1 μM). 60% decrease in survival of MCF-7 cells treated with 0,05–5 μM concentration range of DOX, was also associated with either fluidization or rigidization of the plasma membrane. These results imply that changes in membrane fluidity occurring in the presence of DOX and PTX noticeably disturb cellular proliferation and suggest that correlation between the cytotoxicity of investigated drugs and the extent of the damage to the cell membrane they cause might exist. Combined effect of DOX and PTX on MCF-7 membrane fluidity was highly dependent on their concentration and molar ratio and was markedly different from the effects the drugs showed alone at the same concentrations. No synergistic or additive effect of DOX and PTX on the plasma membrane properties of MCF-7 human breast carcinoma cells was observed. At some of the investigated concentration ranges and molecular ratios DOX and PTX, however, showed antagonizing effect.

Key words: doxorubicin, paclitaxel, plasma membrane fluidity, MCF-7 breast cancer cells.

INTRODUCTION

The cell membrane is a dynamic and complex interface between intracellular and external environment. It plays fundamental role in cell homeostasis and metabolism. Thus, any change in molecular architecture of the cell membrane may substantially affect its functional activities and cell functions.

Many drugs have been hypothesized to exert their pharmacological effects by influencing membrane molecular organization, mostly by disordering the membrane lipids. It is commonly accepted that the plasma membrane, besides deoxyribonucleic acid (DNA), is the most important target for activity of many anticancer drugs such as anthracycline and taxane chemotherapeutics. These drugs can induce modifications in physicochemical properties of the cellular membranes such as lipid fluidity, conformation of membrane-bound enzymes, degree of receptor exposure, changes in lipid packing density and changes in lipid-lipid and lipid-protein interactions [5,16,18,19,23,38,55]. Most of investigation concerning interaction of anthracyclines and taxanes with lipid bilayer was performed on model membranes. Little is known on interaction of these drugs with the plasma membrane of cancer cells. It is not clear how cytotoxicity of anthracyclines and taxanes is related to their effects on properties and functions of the cell membrane and how these cytostatics interact

with the lipid bilayer dependently on whether they are applied alone or in combination. Thus, in this study we aimed at investigating the effect of anticancer drugs doxorubicin (DOX) and paclitaxel (PTX), alone or in combination, on the properties of the plasma membrane of MCF-7 human breast cancer cells. Both drugs are extensively used in clinical practice in therapy of advanced and metastatic breast cancer [4]. Anthracycline doxorubicin is a topoisomerase II inhibitor with a wide range of biological activity such as damage to DNA and cell membranes [45,51]. Although intercalation into DNA is considered as a main mechanism responsible for the cytotoxic effects of doxorubicin, these effects were also observed in conditions preventing the drug from entering the cell [66,67].

Paclitaxel belongs to the class of taxanes (diterpenes) produced by the plants of the genus *Taxus* (yews). It is known as a first taxane to demonstrate activity in breast cancer [47,62,65]. Additionally paclitaxel has been shown to have antitumoral activity against ovarian carcinoma, head and non-small cell lung cancers [26,54,59]. Lately it has been also described as an efficacious chemotherapeutics against AIDS-related Kaposi's sarcoma and colon cancers [17,37].

The principal mechanism of taxane activity is the disruption of microtubule function (inhibition of microtubule depolimerization) through stabilizing GDP-bound tubulin in the microtubule. Hence, taxanes are essentially mitotic inhibitors, also named spindle or mitosis poisons [35,75]. Both doxorubicin and paclitaxel generate reactive oxygen species (ROS) that can damage lipids and proteins of plasma membrane [1,39,51,58]. Other mechanism of their action is the ability to induce apoptosis [27,36,40,50,69].

MATERIAL AND METHODS

Reagents

Doxorubicin and paclitaxel were purchased from Sequoia Research Products Limited (*Pangbourne, United Kingdom*). Powdered drugs were dissolved in absolute ethanol at concentration 2 mg/ml and stored in small 100 μl portions in sealed Eppendorf tubes at –20°C. Fluorescent probes TMA-DPH (1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene and DAUDA, 11-[5-(dimethyloamino)-1-napthalene-sulfonylamino] undecanoic acid) were purchased from Molecular Probes (*Eugene, OR, USA*) and stored in the dark at –20°C. Stock 10^{-3} M solutions were prepared by dissolving in tetrahydrofuran (TMA-DPH) or in DMSO (DAUDA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetra-zolium bromide) was from Sigma-Aldrich, *St. Louis, USA*. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin (10 U/ml) and streptomycin (100 mg/ml) were supplied by GIBCO, (*Edinburgh, Scotland*).

Breast cancer cells

The human MCF-7 cell line used in the experiments was obtained from ATCC (*ATCC HTB-22, Rockville, MD, USA*). The cells were routinely screened for

Mycoplasma contamination and maintained as a monolayer in 75 cm^2 or 150 cm^2 plastic culture flasks in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (10 U/ml) and streptomycin (50 μg/ml). Cell culture was carried out as a monolayer under the atmosphere of 5% CO_2 and 95% air at 37°C and 100% humidity. The cells were subcultured every 5 to 7 days in order to maintain their growth in a logarithmic phase.

Cytotoxicity assays

The cytotoxicity of doxorubicin and paclitaxel in MCF-7 breast cancer cells were assayed by a standard microplate MTT colorimetric method of Mossman et al. [52] in modification of Carmichael et al. [12]. 10^4 cells in 100 μl culture medium per well were seeded into 96-well microplates 24 h before drug treatment. Different concentrations of drugs in 10 μl of PBS were added to appropriate wells, and microplates were incubated in CO_2 incubator for 2 h. At the end of incubation, the medium was removed and the cells, after two washes with PBS, were grown in fresh medium for a further 72 h. Then the medium was replaced with 50 μl of MTT (5 mg/ml final concentration) and microplates were incubated in a CO_2 incubator for 4 h. Medium in each well was aspirated and the formed violet formazan crystals, a product of MTT reduction within metabolically viable cells, were dissolved with 100 μl DMSO/well. In order to facilitate complete dissolution of the formazan crystals the plates were gently shaken for 5 min at room temperature and then read at 570 nm with a microplate reader (*Awareness Technology Inc*., *USA*). Cytotoxicity of the drugs was expressed as IC50 concentration that reduces cell viability by 50% relative to the control (untreated cells) which viability was arbitrary taken as 100%.

Estimation of membrane lipid fluidity

A fluorescence spectroscopy technique and measurement of fluorescence anisotropy of hydrophobic fluorescent probes TMA-DPH and DAUDA were employed to investigate the type of alterations caused in lipid bilayer biophysical properties by investigated drugs. Measurement of fluorescence anisotropy of hydrophobic fluorescent probes TMA-DPH and DAUDA enables monitoring changes in membrane fluidity/rigidity induced by DOX and PTX as a function of depths within lipid bilayer of plasma membrane of drug treated cancer cells. TMA-DPH and DAUDA fluorescent probes were chosen because of their specific localization within the plasma membrane. TMA-DPH locates in the polar head-group region, while DAUDA is mainly placed in the hydrophobic core region. The degree of fluorescence polarization of the probes depends on the rotation of the fluorophore relative to the directions of its emission transition moment [44].

Trypsinized drug-treated and control MCF-7 cells were suspended in Tris-KCl buffer, pH 7.4, at a concentration of 350,000 cells/cm^3 and labeled with 10^{-6} M (final concentration) of TMA-DPH or DAUDA. Samples were incubated with the probes at 20°C for 4 min (TMA-DPH) and 10 min (DAUDA), i.e. the requisite time to obtain stationary fluorescence equilibrium. Fluorescence intensities were measured with a

Perkin-Elmer LS-5B luminescence spectrometer with the excitation/emission wavelengths of 360/425 nm for TMA-DPH and 335/471 nm for DAUDA. Each sample was illuminated with the linear (vertically – *v* or horizontally – *h*) polarized monochromatic light (λex) and the emitted fluorescence intensities (*I* – in arbitrary units) parallel or perpendicular to the direction of the excitation beam were recorded. The anisotropy (*r*) of the fluorescent probes in the samples was automatically calculated by the computer program on the basis of the following equation:

$$r = 2P/(3–P),$$

$$P = (Ivv–IvhIhv/Ihh)/(Ivv+IvhIhv/Ihh),$$

where *Ivv* and *Ivh* are the components of emitted light intensity (in arbitrary units), which are parallel and perpendicular, respectively, with reference to the direction of polarization (*P*) of the excitation light. *Ihv/Ihh* ratio describes the correction factor for the optical system given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction [68]. The degree of fluorescence polarization depends on the rotation of the fluorophore relative to the direction of its emission transient moment [44]. Since the fluorescence anisotropy values are inversely proportional to cell membrane fluidity a high degree of fluorescence anisotropy represents a high structural order or low cell membrane fluidity [61].

Statistical analysis

Statistical analysis was performed with a statistical program STATISTICA (*StatSoft, Tulsa, OK, USA*). All data are expressed as a mean ± S.D. For statistical evaluation and multiple comparisons an analysis of variance with a Tuckey post hoc test were used. A P value of < 0.05 was considered significant.

RESULTS

Cytotoxicity of doxorubicin and paclitaxel in MCF-7 cells

Survival curves of MCF-7 human carcinoma cells treated with doxorubicin or paclitaxel are shown in Fig. 1. In each of the treatments a marked decrease in cell viability with increasing drug concentrations was observed, however, survival curves for DOX and PTX differed notably in terms of their shape and course suggesting distinct mechanisms of action. Of both cytostatics paclitaxel was considerably more cytotoxic toward MCF-7 cells. Its IC50 concentration (0.4 μM) was lower by an order of magnitude than that of doxorubicin (3 μM) (Fig. 2). Rapid fall of survival curve, reflecting a substantial, over 70% decrease in survival of PTX-treated MCF-7 cells, was observed with the lowest concentrations of the drug (0.01–0.1 μM). For comparison, no visible toxicity has been seen with ten-fold higher concentrations of DOX (0.1–1 μM) under the same conditions. Presence of a short shoulder in a DOX survival curve only confirmed lack of significant cytotoxicity of

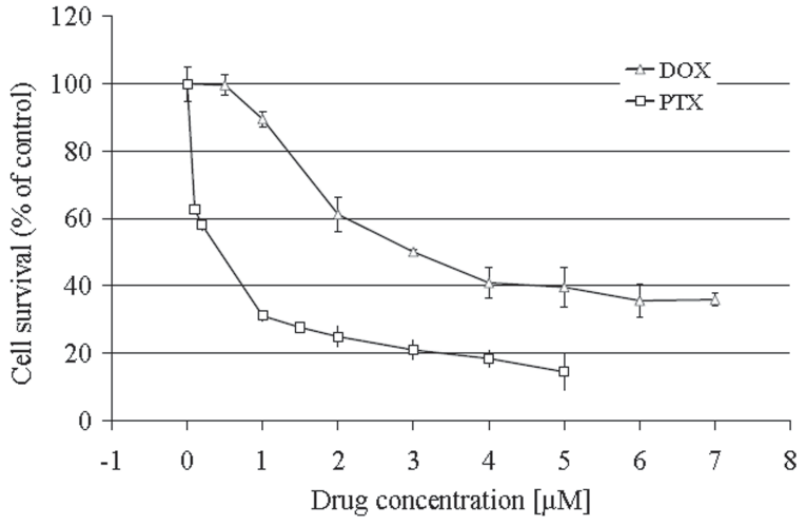

FIGURE 1. Survival curves obtained for MCF-7 human breast cancer cells treated with anticancer drugs doxorubicin or paclitaxel. Each point represents the mean ± SD from 5 independent experiments in 8 repeats each

this drug toward MCF-7 cells at concentrations lower than 1 μM. 90% of MCF-7 cells survived treatment with 1 μM DOX, while only one-third of them endured incubation with 1 μM PTX. Thus, on the basis of these results, we chose different ranges of concentrations of these drugs for our study on plasma membrane fluidity: 0.5–20 μM (DOX) and 0.05–20 μM (PTX), i.e. an arrays compromising drug concentrations used in cytotoxicity assay. Chosen concentration ranges also referred to the therapeutic concentrations and maximal daily doses achieved by DOX and PTX *in vivo* during chemotherapy [13].

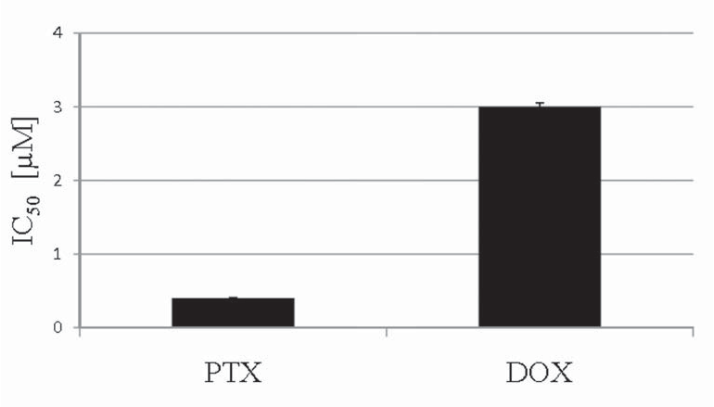

FIGURE 2. Cytotoxicity od doxorubicin and paclitaxel in MCF-7 human breast cancer cells. Values of IC50 parameter represents concentration of drugs reducing viability of treated cells by 50% compared to control (untreated cells), which viability is taken as 100%

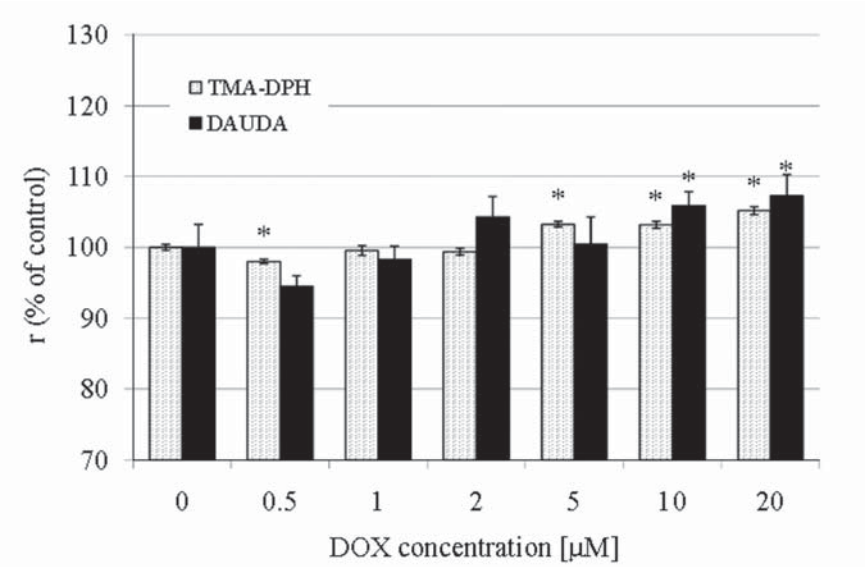

FIGURE 3. Changes in fluidity of the plasma membrane of MCF-7 human breast cancer cells after their treatment with doxorubicin. Values of the anisotropy parameter for TMA-DPH and DAUDA probes reflect changes at surface and hydrophobic regions of the lipid bilayer, respectively. The anisotropy parameter r is presented as a function of molar concentrations of doxorubicin and expressed as a percent of anisotropy parameter r for control taken as 100%. Each point represents the mean ± SD from at least 3 independent experiments in 6 repeats each. The symbol (*) indicates values statistically significant in comparison to the control (untreated) cells. $p < 0.5$ was considered as significant

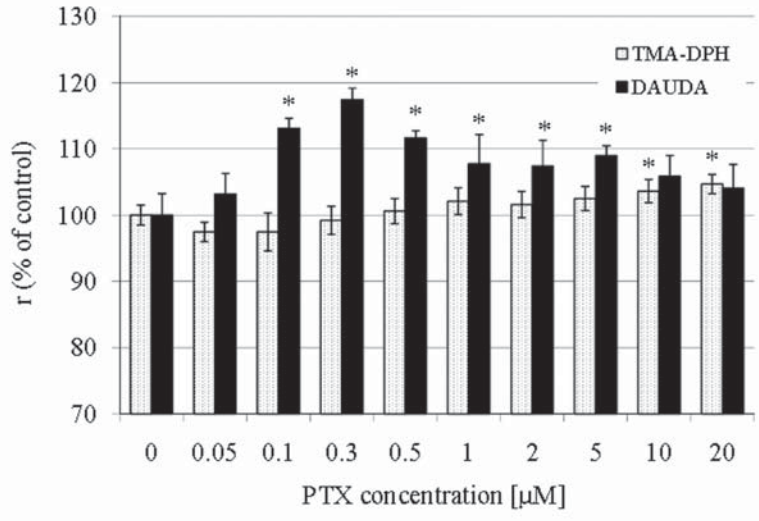

FIGURE 4. The effect of paclitaxel on fluidity of the plasma membrane of MCF-7 human breast cancer cells. Fluorescence anisotropy parameter r for TMA-DPH probe shows the effect of the drug on surface regions of the lipid bilayer, while fluorescence anisotropy parameter r for DAUDA probe – the effect on hydrophobic regions of the lipid bilayer. The anisotropy parameter r is presented as a function of molar concentrations of paclitaxel and expressed as a percent of anisotropy parameter r for control taken as 100%. Each point represents the mean ± SD from at least 3 independent experiments in 6 repeats each. The symbol (*) indicates values statistically significant in comparison to the control (untreated) cells. $p < 0.5$ was considered as significant

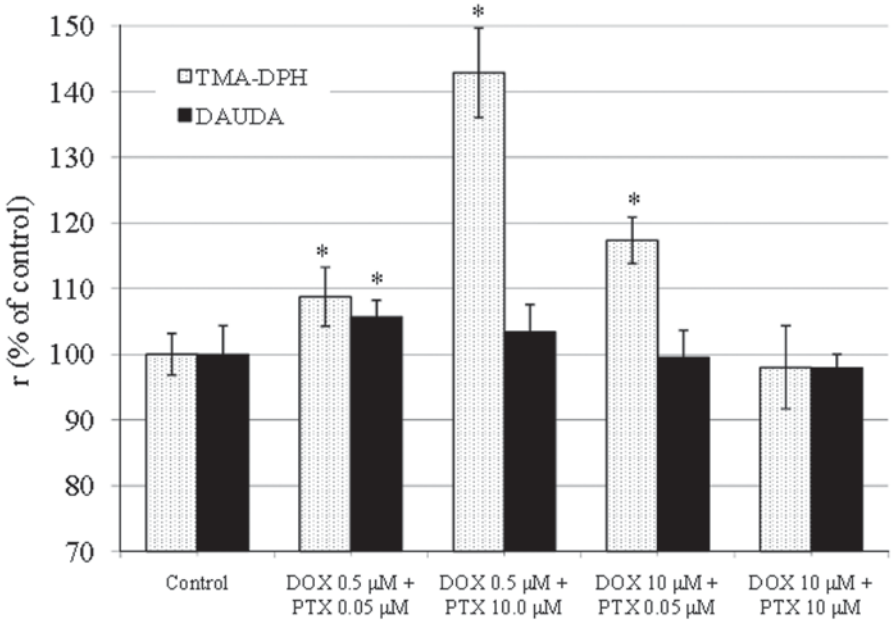

FIGURE 5. Changes in fluorescence anisotropy parameter r for TMA-DPH and DAUDA probes incorporated into the plasma membrane of MCF-7 human breast cells treated simultaneously with doxorubicin and paclitaxel. The anisotropy parameter r is presented as a function of molar concentrations of both drugs and expressed as a percent of anisotropy parameter r for control taken as 100%. Each point represents the mean ± SD from at least 3 independent experiments in 6 repeats each. The symbol (*) indicates values statistically significant in comparison to the control (untreated) cells. $p < 0.5$ was considered as significant

Effect of doxorubicin and paclitaxel on membrane fluidity

We applied spectroscopic technique and fluorescence anisotropy measurements to monitor lipid dynamics in the plasma membrane of MCF-7 human breast carcinoma cells under pharmacological treatment with anticancer cytostatics doxorubicin and paclitaxel. Two types of fluorescent probes, TMA-DPH and DAUDA, were employed to examine fluidity in the upper polar and in the hydrophobic core regions of the lipid bilayer. TMA-DPH and DAUDA differently localize within lipid bilayer. TMA-DPH is a cationic fluorescent aromatic hydrocarbon, which polar part mainly anchors at the lipid-water interface of the lipid bilayer and thus provides information on fluidity of the polar head group region of the plasma membrane. The probe is located among the headgroups of the phospholipids and does not reflect the fluidity in the lipid core of the membranes where the limiting step of drug permeation, namely drug flip-flop, occurs. DAUDA, like 12-AS (12-(9-anthroyloxy)-stearic acid), another lipid probe from the same family, incorporates relatively deeply in the hydrocarbon interior of the lipid bilayer and thus can be used for detection of fluidity gradients across the plasma membrane [43,46,49].

Fluorescence anisotropy parameters r of TMA-DPH and DAUDA incorporated into the plasma membrane of nontreated (control) and drug-treated MCF-7 cells are shown in Fig. 3 (DOX), Fig. 4 (PTX) and Fig. 5 (combination of DOX and PTX). Fluorescence anisotropy values of drug-treated cells were recalculated and presented as a percentage of anisotropy of corresponding control cells (100%). Values of fluorescence anisotropy of TMA-DPH and DAUDA revealed that DOX and PTX differently affected membrane fluidity of MCF-7 cells. Treatment with doxorubicin equally changed fluidity of both superficial and hydrophobic parts of the lipid bilayer. Paclitaxel, in contrast to doxorubicin, mainly interacted with the hydrophobic regions of lipid bilayer. The drug enhanced anisotropy of DAUDA, which indicated an increased lipid density and rigidization of the lipid core region of the plasma membrane.

Treatment with low concentration of DOX (0.5 μM) decreased anisotropy of TMA-DPH and DAUDA, which is consistent with a decrease in lipid order and associated with an increase in membrane fluidity. Instead, a concentration-dependent enhancement of anisotropy of fluorescent probes was observed with high doses of this anthracycline (5–20 μM) pointing out the rigidization of the cell membrane within this range of drug concentrations (Fig. 3).

Changes in anisotropy of TMA-DPH fluorescence in cells incubated with PTX treatment showed tendency of following the pattern seen with doxorubicin treatment. Most of the changes with PTX, however, were not statistically significant. A comparable to 0.5 μM of DOX decrease in TMA-DPH anisotropy emerged at 10-times lower dose of paclitaxel (0.05 μM) and maintained up to drug concentration of 0.1 μM. Further changes in the anisotropy of TMA-DPH showed a gradual increase in anisotropy parameter r with increasing PTX concentrations. Highest doses of paclitaxel (10 and 20 μM) caused slight membrane rigidization. This was indicated by a statistically significant increase in anisotropy of TMA-DPH at these drug concentrations compared to the control cells (Fig. 4).

An enhancement in anisotropy of DAUDA fluorescence was evident within the entire range of PTX concentrations (0.05–20 μM). Maximal increase by about 20% compared to anisotropy of the control cells was found in cells exposed to the lowest range of drug doses (0.1–1 μM). Less significant changes were observed with higher concentrations of PTX (2–20 μM). Even though, the anisotropy parameter r for DAUDA fluorescence in treated cells was greater than the anisotropy of the control cells (Fig. 4).

Doxorubicin and paclitaxel used in combination caused membrane rigidization, predominantly in the surface regions of the lipid bilayer (Fig. 5). Changes in TMA-DPH anisotropy were considerably greater than changes cased by each of the drugs alone. As can be seen in Fig. 4 this effect seems to be dependent on both concentrations and molar ratio of the drugs. Addition of high concentration of PTX (10 μM) to low concentration of DOX (0.5 μM) enlarged by about 40–50% TMA-DPH anisotropy compared to the same doses of DOX and PTX used alone. Considerably lesser changes (about 15% increase in TMA-DPH anisotropy) were

seen for both drugs combined in inverse ratio: low concentration of PTX (0.05 μM) and high concentration of DOX (10 μM) (Figs. 3, 4 and 5). Interestingly that depending on concentrations, the drugs combined in equimolar ratio displayed entirely different effect. Separately, low doses of DOX (0.5 μM) and PTX (0.05 μM) decreased anisotropy of TMA-DPH, which reflected fluidization of the surficial regions of the membrane. Combined at the same concentrations, the drugs caused membrane rigidization (an increase in TMA-DPH anisotropy). No significant changes were seen for the combination of high concentrations (10 μM) of DOX and PTX (Fig. 5). It is worth mentioning that each of the drugs applied at 10 μM-concentrations caused rigidization of the plasma membrane. Despite the notable effect of PTX on DAUDA anisotropy addition of DOX, irrespective of its concentration, attenuated PTX effect and the combined effect of both drugs on fluidity of hydrophobic core of lipid bilayer was negligible.

DISSCUSION

The interaction between anticancer drugs and the cell membrane is essential for their pharmacokinetics and penetration to the site of action at an appropriate concentration [60,63]. Inadequate drug delivery to tumors is now recognized as a key factor that limits the efficacy of anticancer drugs in clinical practice. Since most of the anticancer drugs are hydrophobic their therapeutic effects are highly dependent on molecular interactions with lipid membranes [22]. Binding and partitioning of the drugs within the cell membrane are significantly influenced by composition of the fatty acids, fluidity of the lipid bilayer and its penetration [33,53]. What is more, membrane interactions could be involved in drug retention in resistant tumor cells. A preferential decrease in the content of DOX in the lipid fraction of the membrane, as compared to the whole cell between sensitive and resistant cells without over-expression of P-gp has been found [3,5,34]. It has been also shown that intracellular concentration of DOX is highly dependent on drug movement across the plasma membrane. Membrane potential, pH gradient, the composition of membrane lipids and membrane fluidity can significantly affect DOX diffusion rate [20,24]. Membrane fluidity is the critical factor in the potency of anthracycline drugs as it determinates the partitioning of anthracycline aminoglycosides into cell membrane [30]. Moreover, association with membrane proteins that determines the free drug gradient between extra- and intracellular spaces might be important factor for the uptake of drug by the cells.

Doxorubicin and paclitaxel belong to chemically different groups of compounds and possess distinct mechanisms of action in tumor cells. The drugs significantly vary in their size, electrical charge of the molecule and affinity to lipids. As hydrophobic molecules DOX and PTX enter cells by diffusion through the membrane without the requirement for a specific transporter. Doxorubicin, like other anthracyclines, is positively charged amphipathic molecule, and as such is located at the

surface of membrane among the headgroups of the phospholipids. The drug is embedded within the lipid bilayer and was shown to bind, with high-affinity constant, to negatively charged phospholipids, e.g. cardiolipin and phosphatidylserine [28,31,32]. It is believed that DOX transport across membranes occurs by a passive „flip-flop" mechanism between two membrane leaflets rather than by diffusion down a continuous concentration gradient located in the lipid core of the membrane. The rate of DOX flux across membranes is determined by both the massive binding to the membranes and the slow „flip-flop" across the membrane. Compared with other anthracyclines DOX exhibits relatively low partition coefficient. However, no direct correlation between the lipophilicity of anthracyclines and their lipid phase/aqueous medium partition coefficient or their flip-flop rate has been found. The kinetics of doxorubicin transport demonstrated the presence of two similar sized drug pools located in the two leaflets of the membrane Doxorubicin like other anthracyclines can also interact electrostatically with the cellular membranes [25,56,57].

Our data show that DOX and PTX penetrate differently surficial and hydrophobic parts of the cell membrane. Doxorubicin caused similar changes in both areas. Paclitaxel mainly disturbed the structure of the inner part of the cell membrane. The surface regions of the membrane seem to be much more stable. Our results also revealed that the same concentrations of DOX and PTX induced different extent of alterations in the membrane fluidity. These differences could stem from the distinct interactions of these drugs with the lipid components of cellular membranes.

Data on the effects of antracyclines on fluidity of the cell membrane are inconsistent. Both an increased and decreased fluidity in the plasma membrane of different types of cells exposed to anthracyclines have been reported [46,48,53,55]. In our previous studies using TMA-DPH and 12-AS (12-(9-antroiloxy)-stearic acid) fluorescent probes we have found similar to MCF-7 cells effect of DOX and other anthracyclines (daunorubicin and aclarubicin) on plasma membrane fluidity of several cell lines of immortalized rodent fibroblasts, cardiomyocytes, normal and trisomic human fibroblasts: a decrease in membrane fluidity at low drug concentrations (0.5–2 μM) and its increase at high drug concentrations (5–20 μM) [38,41]. We observed more profound changes in hydrophobic core of the cell membrane. Our results obtained in the above studies and in the present work with living cells are in an accord with the results reported with the model membrane systems, which showed that DOX is intercalated into the hydrocarbon part of the bilayer with deeper penetration into fluid phase than into solid phase vesicles [14]. The interaction of adriamycin with lipids was studied in model (monolayers, small unilamellar vesicles, large multilamellar vesicles) and natural (Chinese hamster ovary cell) membranes by measurement of fluorescence energy transfer and fluorescence quenching. The results showed that around 40% of the adriamycin molecules were deeply embedded in the model lipid bilayer with the aminoglycosyl group interacting with the lipid phosphate groups and the dihydroanthraquinone residue in contact with the lipid fatty acid chains. The drug, however, penetrated the plasma membrane of CHO cells to a much more limited extent than in the model membrane systems. Analyzing these

experiments the authors conclude that the penetration of adriamycin into lipid bilayers strongly depends on the molecular packing of the lipid [21].

In our experiments the effects of DOX and PTX on membrane fluidity were entirely different and an increasing rigidization of the lipid bilayer with increasing drug concentrations was observed in the cells exposed to DOX only. PTX effect on membrane fluidity was independent on drug concentration. Relatively low drug dose (0.1 μM) was sufficient to induce considerable changes in membrane fluidity, while insignificant effect was observed at 100-fold higher concentrations (10 μM). Intercalation of paclitaxel into the hydrophobic core of the plasma membrane of MCF-7 cancer cells caused more striking perturbation in membrane fluidity and considerably greater membrane rigidization than DOX, which probably reflects the larger size and complexity of the PTX molecule compared to that of DOX. Paclitaxel is an extremely hydrophobic compound with low aqueous solubility. The drug possesses no charge and its hydrophobic character rather endorses portioning and location within the lipid bilayer. Profound rigidization of the deeper regions of the lipid bilayer caused by PTX indicates that the drug intercalates mainly into the hydrophobic core of the cell membrane. At high concentrations (10 and 20 μM), paclitaxel also caused a decrease in fluidity of the surficial part of the plasma membrane.

Studies on interaction of PTX with model lipid bilayers and liposomes employing various techniques, such as fluorescence polarization, circular dichroism (CD), differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), atomic force microscopy (AFM) and 31-phosphorus nuclear magnetic resonance (^{31}P-NMR) showed that the drug depending on concentrations can exhibit both fluidizing and rigidization effects [2,8,16,71]. It was also demonstrated that the molecular structure of phospholipids, such as lipid chain length, chain unsaturation and head group type have a profound effect on the paclitaxel-biomembrane interactions [23, 72–74]. Paclitaxel was found to partition into the lipid membrane, perturbing the hydrocarbon chain conformation and inducing a broadening of the lipid phase transition. Incorporation of paclitaxel into the lipid bilayer also affects other physical properties of the bilayer such as the lipid order parameter [2,7–9,70].

Another important factor for drug/membrane interactions is the degree of lipid chain saturation. Amount of membrane cholesterol and the degree of unsaturation of the membrane phospholipid fatty acids are the two main membrane components affecting its fluidity. Thus lipid composition of the membrane may influence binding and partitioning of the drug in the cell membrane. Incorporation of paclitaxel into the saturated bilayers reduces the lipid order parameter in the gel phase of the lipid bilayers (fluidizing effect). In contrast, partitioning of paclitaxel into the unsaturated bilayers in the liquid phase has a slight rigidization effect. Fluorescence anisotropy measurements of three probes: 12-AS, DPH (1,6-diphenyl-1,3,5-hexatriene) and ANS (8-anilino-1-naphthalene sulfonate) monitored as a function of paclitaxel concentration in the unsaturated bilayers of liposomes indicated that paclitaxel has a fluidizing effect in the upper region of the bilayer whereas the hydrophobic core is slightly rigidized.

These studies collectively ascertained that paclitaxel mainly occupies the cooperativity region, interacts with the interfacial region of unsaturated bilayers and induces fluidity in the headgroup region of bilayer. Results obtained in these studies indicated that paclitaxel induced perturbation of bilayers to disruption of hydrogen bonding at lipid-water interface, which pointed to the localization of paclitaxel at the membrane-water interface. So far, experimental evidence on the type of interaction and paclitaxel-induced perturbation of interfacial region has not been substantial.

Lipid peroxidation is an important process that could count for the effect of investigated drugs on membrane fluidity. Our previous studies indicated that 10 μM concentration of DOX increased the formation of thiobarbituric acid-reactive substances (TBARS) in immortalized B14 fibroblasts [15] and caused rigidization of the plasma membrane [38,41]. An increase in the TBARS concentration in DOX treated cells reflects the enhancement of peroxidative events in lipidic membranes. Membrane lipid peroxidation is known to increase the order of the phospholipid acyl chains, resulting in rearrangements in membrane physical state, i.e. decreased fluidity [10,11]. Doxorubicin is a potent inducer of oxidative stress, which is believed to be mainly responsible for the high cardiotoxicity of this anthracycline. Prooxidant activity of DOX comes up from its redox cycling inside the cell and is mediated by a complex oxyradical cascade involving superoxide, hydroxyl radical and iron. Hydroxyl radical is particularly capable of inducing oxidative damage to cells by proteins, DNA and lipid peroxidation. The oxyradicals cause damage to the membrane structures of cells through peroxidation of proteins and phospholipids [29,42]. Thus, lipid peroxidation generated by the redox cycle of doxorubicin can be of great importance for the interaction of this drug with the plasma membrane. Recently paclitaxel has also been shown to generate ROS and peroxidation of mitochondrial membrane. In our study (manuscript in preparation) we have found that both drugs, DOX and PTX, generated oxidative stress in MCF-7 cells, but the amount of ROS produced by PTX treatment was about 3-fold lesser compared to the amount of ROS induced by doxorubicin under the same conditions.

Other biochemical changes induced by lipid peroxidation that can be important for interaction of DOX with biological membranes include changes in protein conformation, aggregation of membrane-bound cytoskeletal proteins, changes in lipid-lipid and protein-lipid interactions. Carbonylation of protein as a result of oxidative stress may lead in turn, to changes in plasma membrane protein conformation or lateral organization of membrane proteins and the subsequent perturbation of the lipid-protein interaction. All these events may affect to a different degree physical state of the plasma membrane.

In our study fluorescence spectroscopy method did not reveal any changes in membrane fluidity of MCF-7 cells under simultaneous treatment with 10 μM of both drugs. It may suggest antagonizing effect of DOX and PTX since used separately at this concentration each of the drugs caused an increase in fluorescence anisotropy of TMA-DPH and DAUDA.

We were also interested whether there is any relation between cytotoxicity of DOX and PTX and their effects on the properties of the plasma membrane of MCF-7 breast cancer cells. We found out that of both drugs PTX caused significantly greater changes in membrane fluidity of MCF-7 cells showing at the same time considerably higher cytotoxicity than DOX. High cytotoxicity of PTX was reflected by its IC50 dose (0.05 μM), which was about 60-fold lower than that of DOX (3 μM). Compared with doxorubicin, PTX also caused significantly greater changes in membrane fluidity and at much lower concentrations. We observed rapid, over 70% decrease in survival of cells exposed to 0.01–1 μM PTX, the concentration range at which the drug caused the most striking decrease in membrane fluidity. Similarly, about 60% decrease in survival of MCF-7 cells noticed at the concentration range of 0.05–5 μM DOX was associated with either fluidization or rigidification of the plasma membrane. These results imply that the damage to the plasma membrane by PTX and DOX have significant impact on viability of MCF-7 breast carcinoma cells and their capability to divide. Changes in membrane fluidity occurring in the presence of DOX and PTX noticeably disturbed cellular proliferation, which suggest that correlation between the cytotoxicity of investigated drugs and the extent of the damage to the cell membrane they cause might exist.

The biochemical basis for cytotoxicity of DOX and PTX to cells is still not fully understood. Although in most of the studies intercalation with DNA has been proposed as a main mechanism responsible for cytotoxicity of DOX in cancer cells, other studies have suggested that the cytotoxicity of this anthracycline may be due to the inhibition of the plasma membrane redox system, which is involved in the control of cellular growth. It has been shown that 10^{-6}–10^{-7} M concentrations of doxorubicin inhibit plasma membrane redox reactions by over 50%. Some of the forms of DOX e.g. AD32, which do not intercalate with DNA, are cytotoxic and inhibit the plasma membrane redox system. Hence, it has been concluded that the cytotoxic effects of DOX may be based on the inhibition of a membrane dehydrogenase involved in a plasma membrane redox system [64].

Summarizing, on the basis of the presented results we can conclude that the plasma membrane of MCF-7 human breast carcinoma cells is more susceptible to paclitaxel than to doxorubicin. Paxlitaxel caused more profound changes in the membrane fluidity and at considerably lower doses. Even relatively low concentrations of this drug (0.1–0.3 μM) were sufficient to cause a marked increase in DAUDA anisotropy indicating rigidization of the hydrophobic core of the lipid bilayer. The inner region of the plasma membrane of MCF-7 cells appeared to be more susceptible to PTX, while DOX equally influenced fluidity of surficial and hydrophobic parts of the cell membrane. No synergistic or additive effect of both drugs on the plasma membrane of MCF-7 human breast carcinoma cells was found.

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