Effect of free fatty acids and lysolipids on cellular uptake of doxorubicin in human breast cancer cell lines

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Several fatty acids and lysolipids have been shown earlier to increase the permeability of membranes of artificial liposomes, thereby increasing the release of drugs such as doxorubicin (Dox) contained within them. Free fatty acids can also inhibit cancer cell growth *in vitro*, and it has been suggested that this inhibition results from increased membrane permeability. Clearly, therefore, increased membrane permeability could be used in the design of liposomes for targeted drug delivery. For example, as free fatty acids and lysolipids are released upon phospholipase degradation of the liposome, the liposome could deliver membrane permeability enhancers in addition to the drug to increase the targeted anticancer effect. In this study, we examined the effect on Dox uptake in the breast cancer cell lines MDA-MB-231, MCF7, and MCF7-MDR when incubated with a large panel of different free fatty acids and lysolipids. Dox uptake was quantified by flow cytometry and fluorescence microscopy. We observed no increased Dox uptake in any of the breast cancer cell lines, suggesting that growth inhibitory effects observed earlier subsequent to the addition of free fatty acids to cancer cells are not caused by increased cell membrane permeability.

Anti-Cancer Drugs 2010, **21**:674–677

Keywords: breast cancer, doxorubicin, free fatty acids

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Received 25 March 2010 **Revised form accepted** 16 May 2010

Introduction

The plasma membrane is composed of a large number of different lipids that make up the physical boundary separating the interior and exterior of a cell. The plasma membrane also harbours a vast number of different proteins that are involved in many different cellular functions [1]. Administration of fatty acids to tumour cells has been shown to sensitize these cells to the effects of anticancer drugs [2,3]. Several mechanisms have been proposed to explain this effect, including increased uptake of drugs, and a few studies have shown increased uptake of doxorubicin (Dox) in MCF7 and K562 MDR cells after incubation with γ-linolenic acid (GLA) [4,5] and in MGH-U1 cells after incubation with meglumine γ-linolenic acid (MeGLA) [6]. Conversely, another study using MCF7/MDR and MGH-U1/R showed no increased uptake of Dox, epirubicin, mitoxantrone or idarubicin after incubation with GLA [7]. In other studies, the addition of fatty acids and lysolipids to artificial liposomes led to a significant increase in liposome permeability, as shown by the addition of palmitic acid (PA) or 1-hexadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysoPPC) to liposomes, which were constructed from 1,2-*O*-octadecyl-*sn*-glycero-3-phosphocholine (1,2-di-*O*-SPC). Interestingly, PA and lysoPPC had a synergistic effect on permeability for lipid bilayers in the gel phase [8]. Similar results have been obtained using ether lipids or free fatty acids [9]. In particular, in-vitro studies on HT-29 and Colo 205 cell lines, and in-vivo studies on an MT-3 xenograft breast cancer model [10], showed, in the case of Dox and cisplatin, that these drugs in liposomal formulations limited cell growth more effectively than the free drugs, presumably because secretory phospholipase A2 action, induced by upregulation in the cancer cells, liberated free fatty acids and lysolipids at the target. Cumulatively, these studies support earlier findings that the addition of free fatty acids and lysolipids results in increased permeability of lipid bilayers and may facilitate increased uptake of cytotoxic drugs. This could be an important aspect of targeted drug delivery using liposomes as they can be designed to release specific combinations of free fatty acids and lysolipids upon phospholipase degradation.

We systematically investigated the effect on Dox uptake upon addition of several different fatty acids and lysolipids to breast cancer cells using flow cytometry and fluorescence microscopy to determine whether the permeability enhancement observed for artificial liposomes could also be observed in more complex biological membranes. This is, to our knowledge, the first study examining such an extensive panel of free fatty acids and lysolipids to determine their effect on membrane permeability of cancer cells using a precise quantitative technique.

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833c2cf7

Materials and methods

Cell lines

The breast cancer cell lines, MDA-MB-231 and MCF7, were obtained from ATCC (Manassas, Virginia, USA). The doxorubicin-resistant MCF7/MDR was a gift from LiPlasome Pharma (Copenhagen, Denmark). All cells were grown in Dulbecco's modified Eagle's medium (Sigma, Glostrup, Denmark) at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, cholesterol, 1-capryl-2-hydroxy-*sn*-glycerol-3-phosphocholine [10:0 LysoPC (LPC)]), 1-palmitoyl-2-hydroxy-*sn*-glycerol-3-phosphocholine [16:0 LysoPC (LPPC)] and 1-behenoyl-2-hydroxy-*sn*-glycerol-3-phosphocholine (22:0 LysoPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Capric acid (CA), behenic acid, PA, steric acid, oleic acid, GLA and α-linolenic acid (ALA) were purchased from Sigma (Glostrup, Denmark). Dox was purchased from MEDA (Solna, Sweden).

Fluorescence-activated cell sorter analyses

The cells were harvested by trypsination and 2.5×10^5 cells were used for each sample. The cells were resuspended in 5 ml Dulbecco's modified Eagle's medium, and 1 μg/ml Dox, fatty acids and lysolipids were added at different concentrations and allowed to incubate for different times. The cells were then washed twice and analysed on a Becton-Dickinson (BD, Franklin Lakes, New Jersey, USA) FACScan. All data was analysed using the FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

Fluorescence microscopy

Cells were prepared as described for FACS analyses; however, after incubation with Dox, fatty acids and lysolipids and subsequent washing, the cells were resuspended in 25 μl Prolong Gold antifade (Invitrogen, Carlsbad, California, USA), mounted on a glass slide and visualised on an Leica fluorescence microscope (Leica Microsystems A/S, Ballerup, Denmark).

Results

To study whether the addition of fatty acids and lysolipids increases the uptake of DOX in breast cancer cells, Dox, combined with a panel of different fatty acids or lysolipids (PA, LPPC, CA, LPC, ALA, GLA), was incubated with the cell lines MDA-MB-231, MCF7 and MCF7/MDR. As Dox is capable of emitting light at 570 nm when excited at 470 nm, cellular uptake was evaluated by flow cytometry without the need for secondary staining for the drug. To ensure that a potential increase in Dox uptake could be observed in our experimental setup, we investigated the uptake of Dox at several different concentrations and incubation times (Fig. 1a and b). We found that the cellular uptake of Dox increased concomitantly with increasing incubation time and increasing Dox concentration. From these data, we chose a Dox concentration of 1 μg/ml and an incubation time of 1 h to assure that the cells were not saturated with Dox. These conditions assured an unequivocal determination of whether the tested fatty acids or lysolipid compounds showed a membrane permeability-enhancing effect proved by an increase in Dox uptake.

Next, the cells were incubated with Dox along with one of the fatty acids or lysolipids in the panel, testing the effect of all the fatty acids or lysolipids individually. Representative data are shown in Fig. 1c. No increased cellular uptake of Dox was observed after 1-h incubation at the test concentrations (10 and 20 μmol/l for PA, LPPC, CA and LPC and 10, 20 and 40 μmol/l for GLA and ALA) compared with Dox alone. The highest concentrations of free fatty acids and lysolipids used were based on the their solubility properties, that is, 40 μmol/l was the highest concentration that could be solubilized. It should be noted that the difference observed between the untreated MCF7 cells in Fig. 1b and c is a result of daily instrument variation. We also incubated the cells with GLA for 24 h, but this also failed to increase Dox uptake in any of the cell lines tested (data not shown).

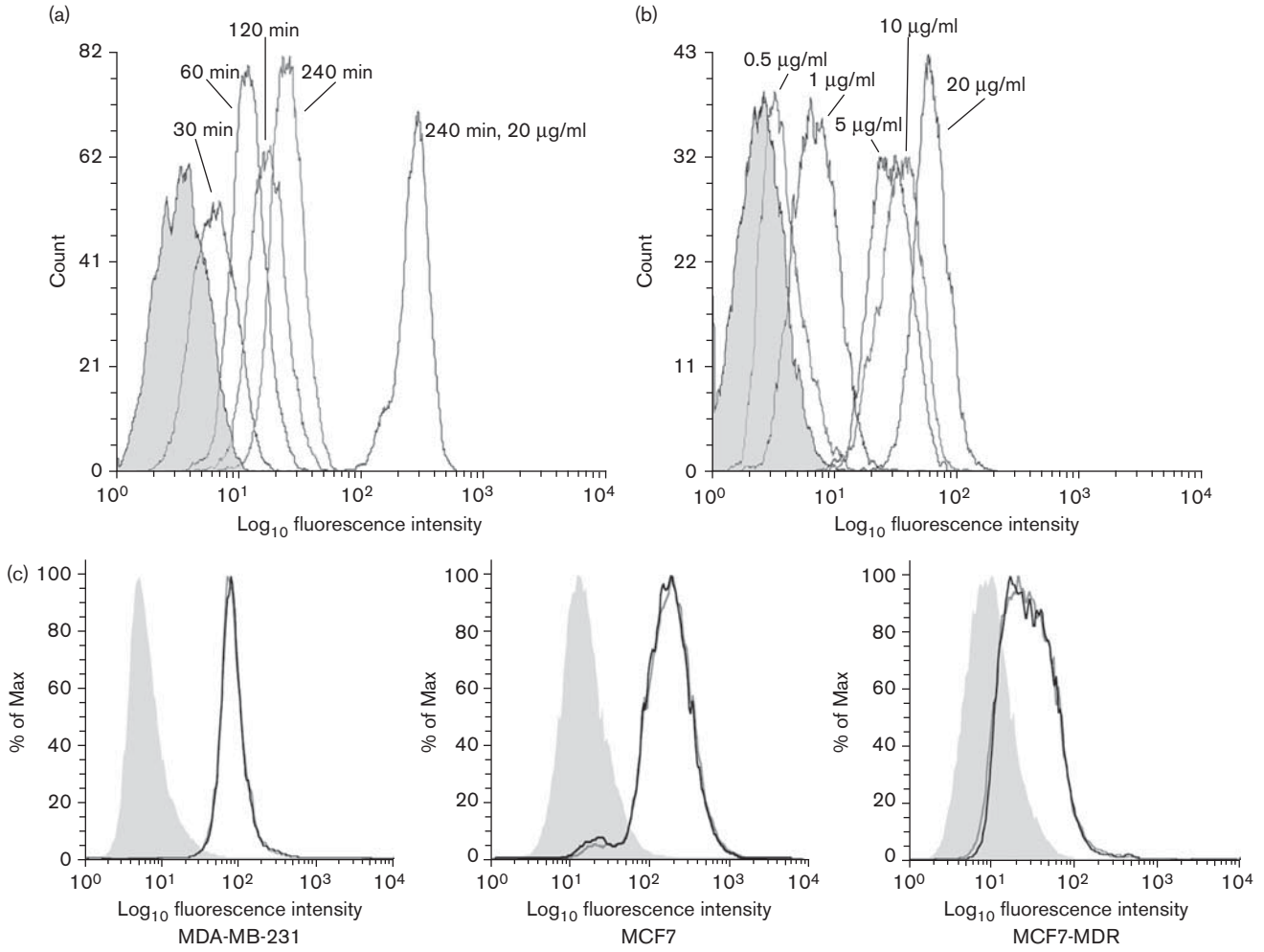
In addition, we performed fluorescence microscopy to visualize whether the cellular localization of Dox was altered as a result of incubation with free fatty acids or lysolipids. We observed no differences in the cellular localization of Dox after incubation with any of the free fatty acids or lysolipids (data not shown).

Discussion

Targeted delivery of drugs to tumours has been the focus of intense investigation to abrogate the severe side effects of systemic cytotoxic drugs. The specific delivery of drugs using artificial liposomes have yielded promising results, and it has been suggested that the free fatty acids and lysolipids, which can be generated by the breakdown of these liposomes in the tumour tissues, can have a positive effect on the uptake of cytostatic drugs [8,9]. The possible molecular mechanism behind a permeability-lowering effect on lipid membranes because of free fatty acids and lysolipids is the destabilization of bilayers caused by these compounds because of their average conical molecular shape and propensity for forming nonlamellar structures [11].

In this study, we investigated whether the addition of fatty acids and lysolipids to the media of breast cancer cells increased membrane permeability and, as a consequence, accumulation of intracellular Dox. We tested several fatty acids and lysolipids in different concentrations and incubation times, but found no increase in intracellular Dox accumulation, and no changes in the

Fig. 1



Cellular uptake of Dox at different incubation times and using different Dox concentrations as measured by flow cytometry. (a) The uptake of Dox in MDA-MB-231 cells was measured for different incubation times with a fixed Dox concentration (1 μg/ml) unless other is indicated, and (b) at different Dox concentrations using a fixed incubation time (1 h). At a Dox concentration of 1 μg/ml and an incubation time of 1 h the cells were not saturated with Dox. Shaded chromatogram represents data obtained from cells without Dox. (c) Representative chromatograms of Dox uptake in MDA-MB-231, MCF7 and MCF7-MDR cells after incubation with Dox and one of the investigated fatty acids (α-linolenic acid, ALA). Shaded chromatogram represents data obtained from cells without Dox and ALA. Grey line: cells incubated with 1 μg/ml Dox without ALA. Black line: cells incubated with 1 μg/ml Dox and 40 μmol/l ALA. No increased uptake of Dox was observed after incubation with ALA or any of the other fatty acids and lysolipids investigated.

cellular localization of Dox after fatty acid/lysolipid treatment. This suggests that the earlier observation of antitumour effects of some fatty acids is likely not caused by increased Dox accumulation. Our results are somewhat surprising as artificial liposomes can exhibit highly altered permeability when free fatty acids and/or lysolipids are added [8,9]. We have recently found (Jespersen *et al.* unpublished) that a range of saturated and unsaturated free fatty acids are capable of enhancing the permeability of lipid bilayers when they are in a fluid-ordered or liquid-ordered phase, which biological membranes are believed to be in at physiological temperatures [8]. In addition, artificial liposomes exhibit very low permeability of Dox, whereas whole cells rapidly accumulate Dox. This could indicate that increased membrane permeability of whole cells will have a modest effect as Dox readily enters the cell, and highlights the challenges in using artificial liposomes as model systems for cell membranes.

Multidrug resistant (MDR) cancer cells are often more sensitive to free fatty acids than non-MDR cancer cells, and as shown for the MDR leukaemic cell line K562, addition of GLA results in decreased expression of P-gp, leading to increased accumulation of Dox [5], indicating that increased membrane permeability is not the primary consequence of adding GLA to this cell line. In contrast, Sagar and Das [4] and Kong *et al.* [5] reported increased uptake of Dox in MDR cancer cells after incubation with

GLA, although in these studies Dox uptake was quantified by fluorescence microscopy, an imprecise technique for quantifying fluorescence.

In conclusion, our study shows that the addition of free fatty acids and lysolipids has no significant effect on Dox uptake in any of the cancer cell lines examined.

Acknowledgements

The authors thank M.K. Occhipinti-Bender for editorial assistance. This study was supported in part by the Danish Cancer Society, Danish Research Council, A Race against Breast Cancer, Danish Centre for Translational Breast Cancer Research, Centre of excellence: Sino-Danish Breast Cancer Research Centre, the National Danish Graduate School of Molecular Biophysics, and Lundbeck Research Center NanoCAN.

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