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Epigenetic Modulation of the Biophysical Properties of Drug-Resistant Cell Lipids to Restore Drug Transport and Endocytic Functions

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Abstract

In our recent studies exploring the biophysical characteristics of resistant cell lipids, and the role they play in drug transport, we demonstrated the difference of drug-resistant breast cancer cells from drug-sensitive cells in lipid composition and biophysical properties, suggesting that cancer cells acquire a drug-resistant phenotype through the alteration of lipid synthesis to inhibit intracellular drug transport to protect from cytotoxic effect. In cancer cells, epigenetic changes (*e.g.*, DNA hypermethylation) are essential to maintain this drug-resistant phenotype. Thus, altered lipid synthesis may be linked to epigenetic mechanisms of drug resistance. We hypothesize that reversing DNA hypermethylation in resistant cells with an epigenetic drug could alter lipid synthesis, changing the cell membrane's biophysical properties to facilitate drug delivery to overcome drug resistance. Herein we show that treating drug-resistant breast cancer cells (MCF-7/ADR) with the epigenetic drug, 5-aza-2'-deoxycytidine (decitabine), significantly alters cell lipid composition and biophysical properties, causing the resistant cells to acquire biophysical characteristics similar to those of sensitive cell (MCF-7) lipids. Following decitabine treatment, resistant cells demonstrated increased sphingomyelinase activity, resulting in a decreased sphingomyelin level that influenced lipid domain structures, increased membrane fluidity, and reduced P-glycoprotein expression. Changes in the biophysical characteristics of resistant cell lipids facilitated doxorubicin transport and restored endocytic function for drug delivery with a lipid-encapsulated form of doxorubicin, enhancing the drug efficacy. In conclusion, we have established a new mechanism for efficacy of an epigenetic drug, mediated through changes in lipid composition and biophysical properties, in reversing cancer drug resistance.

Keywords

Cancer cell membrane; Sphingomyelin; Drug resistance; Membrane rigidity; P-glycoprotein; Cancer therapy; Epigenetic

1. Introduction

The risk that tumors may develop resistance to chemotherapeutic agents (such as doxorubicin) remains a major impediment to the successful treatment of various cancers.¹ Several factors, including genetic and epigenetic changes in cancer cells, impaired drug

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delivery to tumor, high rate of drug metabolism in resistant cells, inability of drugs to reach the target, and changes in the tumor microenvironment, have been implicated in cancer developing drug resistance.^{1, 2} In our recent study, we showed that the membrane lipids of doxorubicin-resistant breast cancer cells (MCF-7/ADR) form a more compact and rigid membrane than do those of doxorubicin-sensitive cells (MCF-7).³ Because of the hydrophobic nature of the resistant cell lipids, doxorubicin partitions into the membrane lipids, hence hindered from intracellular transport. Similarly, the rigid nature of resistant cell membrane lipids results in impaired endocytic function that inhibits intracellular drug delivery using Doxil, a liposomal formulation of doxorubicin.³

Several studies have correlated epigenetic aberrations, such as changes in DNA methylation and histone modifications, to multidrug resistance phenotype in cancer.⁴ This effect has been attributed largely to the upregulation of tumor promoter genes and/or downregulation of tumor suppressor genes.⁵ In breast cancer cells, the gene for the enzyme sphingomyelinase (SMase), which hydrolyzes the membrane phospholipid sphingomyelin (SM), has been reported to be methylation silenced.⁶ Hence, the basal activity of SMase is lower in resistant cells than in sensitive cells.⁷ In many ways, it appears that DNA hypermethylation is essential for cancer cells to acquire a drug-resistant phenotype and to maintain their malignant status.⁸

Unlike genetic mutation, DNA methylation is a reversible process. Demethylating agents such as 5-aza-2'-deoxycytidine (decitabine, also known by its trade name, Dacogen [DAC]) and 5-azacytidine can sensitize cells to anticancer drugs such as doxorubicin; the primary mechanism of action of which involves intercalation with DNA.^{9, 10} However, for such drugs to bind to DNA, they must first cross the cell membrane. Hence, drug transport across the cell membrane remains a major barrier to drug efficacy in resistant cells.

In recent years, there has been significant interest in understanding this biological phenomenon on the basis of the biophysical properties of membrane lipids. Lipids are as essential to life as proteins and nucleic acids, they are involved in several cellular activities (*e.g.*, phagocytosis, endocytosis, exocytosis, transcytosis, apoptosis, and cellular signal transduction), and they also regulate the activities of membrane-bound enzymes, proteins and receptors.^{11, 12}

In our previous studies, we have shown that doxorubicin efficacy in resistant cells is seen at almost the same intracellular drug level as in sensitive cells, but to achieve that threshold intracellular level, resistant cells required exposure to a significantly higher concentration of doxorubicin than sensitive cells.³ Therefore, modulating the cell membrane's lipidic properties can potentially overcome any obstacle to drug transport across the membrane, improving the efficacy of anticancer therapeutics aimed at intracellular targets. Our hypothesis is that reversing the hypermethylation state of the DNA in resistant cells with an epigenetic drug alters lipid synthesis, thus overcoming drug resistance by changing the cell membrane's biophysical properties and facilitating drug delivery.

2. Materials and Methods

2.1. Materials

Hydrochloric acid, sodium chloride, isopropanol, petroleum ether, glacial (water-free) acetic acid, ethanolic phosphomolybdic acid, copper sulfate pentahydrate, 98% sulfuric acid, 85% orthophosphoric acid of reagent grade, and chloroform, methanol, and isopropanol of high-performance liquid chromatography grade were purchased from Fisher Scientific (Pittsburgh, PA). Lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine, L- α -

phosphatidylinositol (PI), sphingomyelin (SM), and cardiolipin (CL) were purchased from Avanti Polar Lipid (Alabaster, AL). Ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane (Tris), ammonium hydroxide, diethyl ether, toluene and 5-aza-2'-deoxycytidine (decitabine; DAC) were purchased from Sigma-Aldrich (St. Louis, MO). Doxorubicin hydrochloride (referring to the agent in solution) was purchased from Drug Source Co. LLC (Westchester, IL) and Doxil (referring to the agent in a liposome-encapsulated form) from Ortho Biotech (Raritan, NJ).

2.2. Cell Culture

Doxorubicin-sensitive (MCF-7) and -resistant (MCF-7/ADR) breast cancer cells were grown in a 150 × 25 mm cell culture dish (BD Biosciences, San Jose, CA) at 37 °C in a 5% CO₂ atmosphere. Sensitive cells were cultured in Eagle's minimum essential medium supplemented with Earle's salts, L-glutamine, 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin. Resistant cells were cultured with 15% fetal bovine serum containing minimum essential medium. Serum concentrations used for culturing of resistant and sensitive cells were optimized. Cell culture media used to culture both cell lines were obtained from the Central Cell Services' Media Laboratory of our institution. To maintain drug resistance, cells were cultured in a medium containing 100 ng/mL of doxorubicin after every two passages.

2.3. Lipid Extraction

Lipids were extracted from resistant and sensitive breast cancer cells, either untreated or pretreated with DAC. Both resistant and sensitive cells were cultured in six plates (150 × 25 mm) at a seeding density of 7×10^6 cells/plate in 25 mL of cell culture media. Cells were cultured for ~2 days at 80–90% confluence, then the cells were harvested by scraping into 10 mL of sterile water using a Corning cell scraper (Lowell, MA). Cells were incubated with DAC (50 ng/mL) for 24 h and then scraped as above for lipid extraction. This concentration of DAC was selected because it does not cause cytotoxic effects of its own in resistant cells. Typically, cell suspensions from six plates were combined and centrifuged at 1,300 rpm and 4 °C for 7 min (Sorvall Legend RT centrifuge, Thermo Electron Corp., Waltham, MA). The resulting cell pellet was suspended in 3 mL of sterile water and lyophilized for 48 h at –48 °C, 3.5 Pa, using FreeZone 4.5 (Labconco Corp., Kansas City, MO), and the lyophilized cell mass was stored at –80 °C until used for lipid extraction. Lipids from the lyophilized cell mass were extracted by using a modified Bligh and Dyer method as described in our previous study.³ Briefly, the lyophilized cell mass was dispersed in nitrogen-purged deionized water (3 mL) to which a 10.2 mL mixture of chloroform:methanol:1 M HCl (10:23:1 v/v/v) was added, the mass was vortexed for 1 min and then the container kept in an ice bath for 15 min to obtain a monophasic cell suspension. To the above monophasic cell mass suspension, 3 mL of 0.1 M HCl and 3 mL chloroform were added and vortexed to obtain a biphasic cell mass suspension. The biphasic cell mass suspension was separated by centrifugation at 3,500 rpm at 0 °C for 5 min (Sorvall Legend RT centrifuge). The organic phase from the bottom was collected carefully using a Hamilton glass syringe (Hamilton Co., Reno, NV) and placed in a 30 mL glass vial (Fisher Scientific); this organic phase was then mixed with 3 mL of sodium chloride-Tris-ethylenediaminetetraacetic acid buffer mixture (0.1 M NaCl, 0.1 M ethylenediaminetetraacetic acid, 0.05 M Tris buffer, pH 8.2). To ensure the complete recovery of the lipids, the extraction protocol was repeated from the remaining aqueous phase. The mixture containing the organic phase from the two extractions mixed with the buffer was vortexed and then centrifuged as above to separate the organic phase containing lipids. The organic phase with lipids was mixed with isopropanol (for every 15 mL of organic phase, 1 mL of isopropanol was added) and stored at –20 °C until used for protein separation as described below.

2.4. Protein Separation from Lipid Extracts

Hydrophobic proteins from the lipid extract were removed by column chromatography as per our previously described protocol.³ Briefly, a glass column with a reservoir (10.5 mm internal diameter, 200 mL capacity; Cole-Parmer, Vernon Hills, IL) was packed with silica slurry, *i.e.*, 2 g silica (Polygosyl-60, Macherey-Nagel, Inc., Bethlehem, PA) dispersed in 15 mL of chloroform:methanol (1:1 v/v) mixture containing 1% NH₄OH. First, the column was rinsed with 45 mL of a chloroform:methanol (1:1 v/v) mixture to remove traces of HCl, then the column was saturated with egg-phosphatidylcholine (PC; Avanti Polar Lipids, Alabaster, AL) by adding 10 mL of 1.5 μ M egg-PC, followed by rinsing the column with excess egg-PC dissolved in 45 mL of the chloroform:methanol (1:1 v/v) mixture. A 10 mL aliquot of the lipid extract was added to the column, then the extract was eluted sequentially with the same volume of different solvents in the following order: chloroform alone, the chloroform:methanol mixture (1:1 v/v), and finally methanol alone. Separately collected eluents were mixed and the organic solvents from the lipids removed using a Rotavapor rotary evaporator (R-215, Buchi Corp., New Castle, DE) at 318 mbar and 50 °C. The lipid residue was dissolved in a nitrogen-purged chloroform:methanol mixture (4:1 v/v) and stored at –20 °C until used.

2.5. Phospholipid Separation from Total Lipids

Phospholipids were extracted from the total lipid mixture by means of solid phase extraction.¹³ In brief, the total lipid extract was dissolved in 1.0 mL of hexane and transferred into a preconditioned silica gel-bonded column (Supelclean LC-Si, 6 mL volume, 1 g sorbent; Supelco, Bellefonte, PA). Preconditioning of the silica gel-bonded column was carried out by rinsing the packed column with 10 mL of hexane. First, fatty acids from the lipid extracts were removed by rinsing the column with 10 mL of an n-hexane:diethyl ether mixture (4:1 v/v), then hydrocarbons, cholesterol esters and triacylglycerols by eluting the column with 18 mL of a chloroform:2-propanol mixture (2:1 v/v). Finally, phospholipids were eluted with 10 mL of methanol followed by 10 mL of a methanol:chloroform:water mixture (5:3:2 v/v/v). The phospholipid fraction collected was evaporated to dryness in a rotary evaporator as described above. The residue was dissolved in 200 μ L of chloroform for phospholipid quantification as described below.

2.6. Quantification of Phospholipids

Phospholipids extracted from different samples were quantified by high-performance liquid chromatography (HPLC) analysis using an evaporative light scattering detector (Shimadzu Scientific Instrument, Inc., Columbia, MD) and nitrogen as a nebulizing gas.¹³ The following method parameters were used: Chromolith Performance Si column (100 mm $\times$ 4.6 mm, macropore size 2.1 μ m and mesopore size 13 nm (EMD Chemical Inc., an affiliate of Merck KGaA, Darmstadt, Germany); column temperature, –25 °C; mobile phase A, chloroform/methanol/ammonia solution (80:19.5:0.5, v/v/v); mobile phase B, chloroform/methanol/triethylamine/water (69.5:25.5:0.49:4.4, v/v/v/v); flow rate, 1 mL/min; injection volume, 10 μ L; nebulizer temperature, 50 °C; detector gain, 6. The above parameters were optimized by using a mixture of phospholipids for HPLC analysis (P3817-1VL, Supelco, Bellefonte, PA). Separation of phospholipids was achieved by the following gradient elution method in which solvent B was increased from 0% to 40% in 0 to 5 min; from 5 to 7 min, B was kept constant at 40%; from 7 to 13 min, B was increased from 40% to 100%; from 13 to 20 min, B was kept constant at 100%; from 20 to 25 min, B was decreased from 100% to 0%; a post-run of 5 min was done to equilibrate the column before the next injection. The quantification for each different phospholipid class was carried out by a calibration curve for each phospholipid standard.

2.7. Lipid Analysis

Separation of phospholipids, neutral lipids, and ceramides was achieved by using the high-performance thin layer chromatography (HPTLC) technique. In a typical experiment, HPTLC plates (10 cm × 10 cm, Sigma-Aldrich) were dried at 140 °C for 30 min, and a 5 μ L aliquot of lipid extract solution (5 mg/mL) was added at a distance of 1 cm from the bottom of the plate. The mobile phase was allowed to run in a trough chamber containing 50 mL of the mobile phase for a distance of 8 cm from the point at which lipid samples were added. Post run, plates were dried under nitrogen gas for 15 min. Different mobile phases and staining procedures were used to separate and identify phospholipids, neutral lipids, and ceramides from the lipid extracts. For instance, phospholipids were separated by using a mobile phase consisting of a mixture of chloroform:methanol:water:ammonia (120:75:6:2 v/v/v) and identified by immersing them in a copper sulfate solution for 5 s, followed by heating at 140 °C for 30 min. Neutral lipids were separated by a mobile phase consisting of petroleum ether:diethylether:glacial acetic acid (80:20:1 v/v/v) and marked by using 5% ethanolic phosphomolybdic acid solution, followed by heating at 140 °C for 30 min. Ceramides were separated by a mobile phase consisting of toluene:methanol (7:3 v/v) and stained with iodine vapor.

2.8. Lipid Isotherms

Lipid isotherms were created using a Langmuir balance (Minimicro, KSV Instruments, Helsinki, Finland). A complete SP-area (π -A) isotherm was obtained by adding 2.5 μ L of a chloroform:methanol (4:1 v/v) solution containing a lipid mixture (5 mg/mL) onto the subphase; following a 10-min waiting time, the barriers were then compressed at 5 mm/min.

2.9. Analysis of Lipid Membranes

Langmuir-Blodgett (LB) films were transferred onto a glass substrate at SPs of 30 mN/m as per our previously described protocol.³ This SP was chosen as it is known to equal the lateral pressure in the cell membrane bilayer.¹⁴ To prepare LB films, a clean glass substrate (24 × 55 mm) was immersed into the subphase, prior to the addition of lipids to the subphase, then the lipids were compressed until the SP 30 mN/m was reached; LB films were then transferred onto the glass substrate by lifting vertically at the rate of 5 mm/min through the monolayer. The transfer ratio for all LB films ranged from 1.1 to 1.35. The LB films were allowed to dry in a vacuum desiccator at room temperature for 24 h. The dried films were then analyzed for surface morphology using a BioScope atomic force microscope (Veeco Metrology, Inc., Santa Barbara, CA) in tapping mode using a 125 μ m long silicon probe with a resonance frequency of approximately 300 Hz and a tip radius of <10 nm (Ted Pella, Inc., Redding, CA).

2.10. Doxorubicin Interaction with Lipids

A 6.5 μ L aliquot of the lipid solution (5 mg/mL) was added onto the subphase and compressed until the SP 30 mN/m was reached. After 5 min, an aliquot of 10 μ L (1 mg/mL) of dox solution in an ethanol:water (1:1 v/v) mixture was injected into the subphase using a Hamilton glass syringe. The change in SP of the lipid membrane was monitored for 20 min following the injection. Ten microliters of ethanol:water (1:1 v/v) only (without doxorubicin) and 50 μ L of deionized water was injected as a control for dox and Doxil, respectively.

2.11. Sphingomyelinase Assay

Resistant cells were seeded in 24-well plates at the density of 30×10^4 cells/well/mL and allowed to attach for 24 h. Prior to experiments, cells were washed twice with phosphate-buffered saline (PBS). Subsequently, cells were incubated with cell culture media containing

DAC (50 ng/mL) for 6 h or 24 h. Untreated cells were used as controls. Cells were scraped into 1 mL PBS using a Corning cell scraper and collected in an Eppendorf tube kept on ice. Cells were spun down and reconstituted in either 1x reaction buffer (0.1 M Tris-HCl, 10 mM MgCl₂, pH 7.4) or acidic buffer (50 mM sodium acetate, pH 5.0) and sonicated for 20 s. The resultant homogenate was used for the assay of neutral vs. acid SMase. Enzymatic activity was assessed by formation of H₂O₂, which forms resorufin when it reacts with Amplex Red reagent, and the enzyme activity was assessed by absorbance measurement using a plate reader. The assay was performed according to the Amplex Red Sphingomyelinase Assay Kit from Invitrogen (Grand Island, NY).

2.12. Western Blotting for P-Glycoprotein

Cell lysates were made by lysing 1×10^6 treated or untreated cells with radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing 1x protease inhibitor cocktail (Calbiochem, Gibbstown, NJ). Lysates were collected by centrifugation at 14,000 rpm for 15 min. Protein concentration was determined by a bicinchoninic acid assay kit (Pierce, Rockford, IL). Fifty to 100 μ g of proteins from the cell lysates were electrophoresed through a 4%–15 % linear pre-cast polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride membranes (GE Healthcare Bio-sciences, Corp., Piscataway, NJ). The blots were probed for mouse monoclonal P-gp (Calbiochem) and mouse monoclonal anti-actin (Sigma-Aldrich). To detection bound antibody, the polyvinylidene difluoride membrane was incubated with horseradish-tagged goat anti-mouse antibody. After the incubation membrane was washed with Tris-buffered saline with 0.5% Tween 20 (TBST), it was stained with enhanced chemiluminescence reagent or ECL-Plus reagent (GE Healthcare Bio-Sciences) according to the manufacturer's protocol.

2.13. Cellular uptake of doxorubicin and Doxil

Resistant and sensitive cells were seeded at a density of 1.2×10^5 cells/mL/well in 24-well plates. Cells were allowed to attach and grow for 48 h, then were treated with DAC (50 ng/mL) for 24 h. The cells were then washed with 1x DPBS and incubated with 1 mL of cell culture media containing doxorubicin (1 μ g/mL) or Doxil (1 μ g/mL doxorubicin equivalent) for different lengths of time. In a second set of experiments, drug uptake was determined at different doses of doxorubicin and Doxil following incubation for 8 h using the identical protocol. At the end of each drug incubation time, cells were washed twice with 1x DPBS, and then 100 μ L of radioimmunoprecipitation assay buffer was added to each well and cells were scraped using a cell scraper. Cells from each well were collected in separate Eppendorf tubes and sonicated for three 5-s bursts at an energy output of 25 W (Sonicator XL, Misonix, Inc., Farmingdale, NY). An aliquot of 80 μ L of cell lysate from each tube was lyophilized as described above. Also, 20 μ L of cell lysate from each tube was used to determine the total protein content using a Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL). To each lyophilized cell lysate, 1 mL of methanol was added, and the samples were kept in a LabRoller rotator (Denville Scientific, Inc., Metuchen, NJ) for 18 h in a cold room. The samples were then centrifuged in a microcentrifuge (Eppendorf 5417R, Eppendorf North America, Inc., Hauppauge, NY) at 14,000 rpm for 10 min at 4 °C. The supernatant from each sample was collected and analyzed for doxorubicin levels using HPLC. The chromatographic analysis of doxorubicin was performed using a mobile phase (acetonitrile:water:triethylamine [25:75:0.1, v/v/v] at pH 3) on a Nova-Pak C8 column (4 μ m, 2.1 $\times$ 150 mm; Millipore-Waters, Milford, MA) as a stationary phase. Analytes were separated in the isocratic elution mode for 6 min at a flow rate of 1.0 mL/min and were detected using a Shimadzu RF-10 AXL fluorescence detector. The detector was set at 480 nm excitation and 560 nm emission wavelengths with gain 4 and high sensitivity. A standard

plot of dox vs. Doxil (1–25 ng/mL) was prepared for the cell lysates under identical conditions.

2.14. Cytotoxicity of Doxorubicin and Doxil

The relative cytotoxicity of doxorubicin and Doxil in both doxorubicin-sensitive and -resistant cancer cells, with and without DAC pretreatment (50 ng/mL), over 24 h was determined. This concentration of DAC was optimized for pre-treatment so that it has insignificant cytotoxic effect of its own. In a typical experiment, cells were seeded at a density of 3,000 cells/well in 96-well plates (Microtest Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to attach for 24 h. Post attachment, cell culture media were replaced with fresh media with or without DAC (50 ng/mL) and incubated for another 24 h, then washed with 1x DPBS. Media containing doxorubicin or Doxil at different concentrations were added and incubated in CO₂ incubator for additional 48 h. Cell viability was determined at the end of the incubation period using a standard MTS colorimetric assay (CellTiter 96 Aqueous, Promega, Madison, WI). To each well, 20 μ L of reagent was added, then plates were incubated for 2 h at 37 °C in a cell culture incubator. Color intensity was measured at 490 nm using a microplate reader (Bio-Tek Instrument, Winooski, VT). The effect of the drug on cell proliferation was calculated as the percentage cell growth vs. growth of control cells that received no drug treatment.

The synergistic cytotoxic action of DAC plus either doxorubicin or Doxil in both resistant and sensitive cells was determined using a CalcuSyn software program (Biosoft, Ferguson, MO). The program is based on the Chou-Talalay method, which calculates the Combination Index (CI) of drugs. From the CI values, the synergistic action of drugs can be analyzed. A CI = 1 indicates an additive effect; a CI < 1 and a CI > 1 indicate synergistic and antagonistic activity, respectively.¹⁵

2.15. Statistical Analysis

Data are expressed as mean $\pm$ standard error of mean (s.e.m.). Statistical analyses were performed using Student's *t* test. Differences were considered significant for *p* < 0.05.

3. Results

3.1. Lipid analysis

We compared changes in phospholipid vs. neutral lipid content with respect to total lipids following DAC treatment in drug-resistant and drug-sensitive cells. Resistant cells showed a lower ratio of phospholipids:total lipids but a higher ratio of neutral lipids:total lipids than sensitive cells did. Following treatment with DAC, resistant cells reversed these ratios: the treatment increased the phospholipid:total lipid ratio and decreased the neutral lipid:total lipid ratio. However, treating sensitive cells with DAC did not change their phospholipid:total lipid or neutral lipid:total lipid ratios. The most significant observation was that the ratios of phospholipids:total lipids and neutral lipids:total lipids of DAC-treated resistant cells were almost the same as those of untreated sensitive cell lipids (Figure 1).

Further analysis of lipids demonstrated significant changes in phospholipid and neutral lipid composition following DAC treatment in both resistant and sensitive cells (Figure 2). Certain changes in the lipid composition were seen specifically in resistant cell lipids but not in sensitive cell lipids, but some changes seen were either the same in both sensitive and resistant cells or, surprisingly, the opposite. For instance, the spots for sphingomyelin (SM) and phosphatidylinositol (PI) were darker in the lipids of untreated resistant cells than in DAC-treated resistant cell lipids, suggesting a decrease in their levels following treatment. However, the spot for SM was relatively darker in DAC-treated sensitive cell lipids than in

untreated sensitive cell lipids, suggesting an increase in SM levels following treatment. Analysis of neutral lipids shows that the triglyceride spot, which was present in untreated sensitive cell lipids, was not visible in the DAC-treated sensitive cell lipids.

Further quantitative analysis of lipids showed changes in the relative concentrations of different phospholipids in sensitive and resistant cell lipids following DAC treatment (Figure 3). The most noticeable changes were a three-fold reduction in SM and PI content in resistant cell lipids following DAC treatment. The level of SM in treated resistant cell lipids appears to be similar to that seen in untreated sensitive cell lipids. Interestingly, DAC treatment reduced SM and PI levels in resistant cell lipids but increased them in sensitive cell lipids. In both sensitive and resistant cell lipids compared with untreated cell lipids, DAC pretreatment reduced phosphatidylcholine (PC) and phosphatidylserine (PS) levels but increased phosphatidylethanolamine (PE) levels (see supplemental data).

3.2. Biophysical Characterization

In general, DAC treatment significantly changed resistant cell lipids; they acquired biophysical characteristics similar to those of untreated sensitive cell lipids. The compression isotherm of DAC-treated resistant cell lipids shifted towards a higher trough area compared with the untreated cell lipid isotherm (Figure 4a). The isotherm of untreated resistant cell lipids began at the 70% trough area, that of treated resistant cell lipids at the 85% trough area. The isotherms of both treated and untreated resistant cell lipids showed a gradual increase in surface pressure (SP) until collapse (42 mN/m vs. 44 mN/m, respectively); however, the collapse occurred at a significantly lower trough area for treated cell lipids (25%) than for untreated cell lipids (45%). DAC treatment had opposite effects on the isotherms of sensitive cell and resistant cell lipids. The isotherm of resistant cell lipids shifted to the right following DAC treatment, whereas that of the sensitive cell lipids shifted to the left. The interesting observation was that the isotherm of treated resistant cell lipids began at the 85% trough area, which was almost the same as that for untreated sensitive cell lipids.

3.3. Surface compression modulus

The surface compression modulus, which characterizes the lipid monolayer's resistance to compression at the interface, was calculated from the SP-area (π -A) isotherm data as described in our previous studies.³ After DAC treatment, the lipids of both sensitive and resistant cells showed a lower compression modulus over the entire SP range vs. the respective untreated cell lipids (Figure 4b). However, regardless of whether the cells had DAC treatment or not, the difference in the compression modulus was significantly greater for resistant than for sensitive cell lipids (Figure 4b). For instance, at a biologically equivalent SP (30 mN/m), treated resistant cell membrane lipids showed a significantly lower compression modulus than untreated resistant cell membrane lipids (60 mN/m vs. 93 mN/m, respectively; Figure 4b); this difference was significantly lower for treated vs. untreated sensitive cell lipids (45 mN/m vs. 63 mN/m). At a similar SP, the compression modulus for treated resistant cell lipids was almost the same as that for untreated sensitive cell lipids (60 mN/m vs. 63 mN/m). The compression-expansion isotherm of both control and treated sensitive/resistant cell lipids showed the reversibility of the lipid monolayers on the buffer surface (Figure 4c).

3.4. Surface morphology of lipid films

Atomic force microscopic images of lipid Langmuir-Blodgett (LB) films showed differences in the domain structures of both resistant and sensitive cell lipids; however, the most significant changes were seen in DAC-treated cell lipids, both in terms of domain dimensions and arrangements (Figure 5a). For untreated cells, the LB films of both resistant

and sensitive cell lipids showed domains with bright and thick regions surrounded by dark liquid-expanded regions. However, following DAC treatment of cells, the LB films of both resistant and sensitive cell lipids showed crystal-like structures, suggesting phase separation of lipids (Figure 5a). On these LB films, lipids were seen as clearly separated from crystal structures in resistant cells but surrounded by crystal structures in sensitive cells.

Section analysis of the height images showed that domain structures of sensitive cell lipids are smaller and uniform in size (width, $\sim 10\ \mu\text{m}$; height, $\sim 100\ \text{nm}$), whereas those in resistant cell lipids are larger and heterogeneous in size (width, $20\ \mu\text{m}$; height, $\sim 200\ \text{nm}$) (Figure 5b). The domain structures of treated resistant and sensitive cell lipids also showed differences. In treated sensitive cell lipids, domain structures were relatively uniform in width (width, $3\text{--}4\ \mu\text{m}$) but greater in height (height, $20\text{--}30\ \text{nm}$) compared with domain structures of treated resistant cell lipids, which were markedly heterogeneous in width ($1\text{--}6\ \mu\text{m}$) but uniform in height and smaller ($<10\ \text{nm}$) (Figure 5b). Crystal structures seen in treated sensitive and resistant cell lipids also showed differences. Section analysis showed that crystals in treated resistant cell lipids were smaller (width, $<1\ \mu\text{m}$; height, $\sim 100\ \text{nm}$) than in treated sensitive cell lipids (width, $1\text{--}2\ \mu\text{m}$; height, $\sim 150\ \text{nm}$).

3.5. Expression of SMase

In drug resistant cells, acidic SMase was found to have a higher basal activity than neutral SMase. DAC treatment increased the activity of both SMase in a time dependent manner, with acidic SMase showing a more drastic change (~ 2 fold increases at its peak). Neutral SMase, however, showed a ~ 1.3 -fold increase in activity at its peak expression. Expression of both enzymes peaked within 6 hours post treatment, with a nonsignificant decrease in activity post 24 h (Figure 6).

3.6. P-Glycoprotein expression

Western blot analysis showed a 45% reduction in P-glycoprotein (P-gp) expression in cell lysates of DAC-treated resistant cells collected at 24 h post treatment; however, there was no difference in P-gp expression between treated and untreated resistant cells in cell lysates collected at 72 h post treatment (Figure 7).

3.7. Doxorubicin interactions with cell membrane lipids

Resistant and sensitive cell lipids showed different patterns of interaction with doxorubicin. The resistant cell lipid membrane showed a steady increase in SP over time, whereas sensitive cell lipids, after an initial increase, showed a gradual decrease over time. Treated and untreated sensitive cell lipids did show a significant difference in interaction with doxorubicin; however, treated resistant cell lipids showed a different pattern of interaction than with untreated resistant cell lipids. The initial increase in SP was followed by a gradual decrease, similar to that seen with sensitive cells lipids (Figure 8).

3.8. Cellular uptake of doxorubicin

Resistant cells first treated with DAC then with doxorubicin, either as drug in solution or Doxil, showed increased drug uptake with incubation time (Figure 9). In untreated resistant cells, doxorubicin uptake reached almost a plateau level at 4 h with no significant increase thereafter with incubation time. However, in DAC-pretreated cells, doxorubicin uptake continued to increase with incubation time (Figure 9a). Doxorubicin uptake in DAC-treated resistant cells was 4-fold higher than that in untreated cells at 12 h post incubation (36 ± 1.1 vs. $9 \pm 0.3\ \text{ng doxorubicin/mg protein}$). A similar effect was seen in resistant cells that had been pretreated with DAC, then with Doxil (Figure 9b). At 12 h, the drug uptake was 2.2-fold higher in pretreated cells than in untreated resistant cells (Figure 9b). In untreated

resistant cells, the drug uptake with Doxil was 4.6-fold greater than the uptake with doxorubicin (41 ± 2.0 ng vs. 9 ± 0.3 ng doxorubicin/mg protein); however, in treated resistant cells, this difference was ~ 2.5 -fold (90 ± 2.7 ng vs. 36 ± 1.1 ng doxorubicin/mg protein). Dose-response studies also demonstrated increased drug uptake in DAC-pretreated cells than in untreated resistant cells (Figure 9c and d). In sensitive cells, pretreatment with DAC did not show as significant a level of enhancement in drug uptake, with doxorubicin or Doxil, as compared with that seen in resistant cells (Figure 9a vs. e or Figure 9b vs. 9f). It is interesting to note that pretreatment of resistant cells with DAC increased drug uptake, but those levels remained about 10-fold lower than the uptake levels seen at 12 h in untreated sensitive cells with doxorubicin (36 ± 1.1 ng vs. 406 ± 16.8 ng doxorubicin/mg protein; Figure 9a vs. e) and 5-fold lower with Doxil (90 ± 2.7 ng vs. 427 ± 9.9 ng doxorubicin/mg protein; Figure 9b vs. f).

3.9. Cytotoxicity of doxorubicin and Doxil

Pretreatment of resistant cells with DAC significantly reduced the IC_{50} of doxorubicin, both with doxorubicin and Doxil, and the sequential treatment showed a highly synergistic effect, as evident from low combination index (CI) values. Sensitive cells also showed lower IC_{50} values and synergistic effect, but the CI value was higher than that for resistant cells, indicating that less of a synergistic effect occurred with sequential treatment in sensitive cells (Figure 10, Table 1). Resistant cells treated simultaneously with DAC and doxorubicin did not show this synergistic effect; in these cells, the effect was additive (CI = 1). Although the effect was highly synergistic with sequential treatment in resistant cells, the dose-response study shows that some resistant cells do not respond to doxorubicin treatment (Figure 10a,c).

4. Discussion

Drug resistance is a multifactorial phenomenon,¹ but in this study we focused on understanding the role of epigenetic mechanisms of drug resistance on the biophysical characteristics of cell membrane lipids and how these membrane lipid characteristics can be modulated with an epigenetic drug to overcome drug resistance. The conventional mechanism by which epigenetic drugs are known to overcome drug resistance in cancer is via resuming the expression of certain tumor suppressor genes, which are otherwise silenced due to hypermethylation of DNA or de-acetylation of chromatin.⁵ As we have shown in our previous studies, membrane lipids are altered in drug-resistant cells, such that cells can maintain a resistant phenotype by inhibiting drug transport.³ It is conceivable that epigenetic changes in resistant cells are intended to alter lipid synthesis to influence the trafficking of drugs into cells as an adaptive protective mechanism against the cytotoxic effect of drugs. In this study, we explored the mechanism of action of one epigenetic drug, decitabine as a membrane modulator in doxorubicin-resistant breast cancer cells and tested efficacy of doxorubicin, either in solution or in liposome-encapsulated form. We correlated its effects to changes in lipid synthesis and biophysical properties that influenced doxorubicin uptake and its cytotoxicity.

To understand the role of any epigenetic drug in relation to the biophysical mechanisms by which cell membrane lipids overcome drug resistance, we isolated and analyzed the lipids from DAC-treated and untreated sensitive and resistant cells. Our results demonstrated that after DAC treatment, both sensitive and resistant cells indeed showed altered lipid composition and biophysical characteristics compared with the respective untreated cells. There were noticeable specific changes in resistant cell lipid composition and biophysical properties following DAC treatment that suggest that a transformation of resistant cell membrane occurs, making it become more like sensitive cell membrane. A particularly noticeable difference was the significant increase in concentration of total phospholipids and

the decrease in concentration of total neutral lipids in treated resistant cells (Figure 1). The phospholipid composition of cell membranes has been reported to play a significant role in drug transport by modulating drug diffusion across the cell membrane¹⁶ as well as in sorting mechanisms in endocytosis.¹⁷ Since drug transport to intracellular targets is a major limiting factor to the success of anticancer agents in drug-resistant cells, even a small change in the membrane lipid composition could significantly affect the efficacy of drug delivery.

The increase in levels of phosphatidylethanolamine (PE) (Figures 2 and 3) in both treated sensitive and resistant cell lipids than in the respective untreated cell lipids indicate a possible common effect of DAC on phospholipid biosynthesis in both types of cells. Based on our lipid analysis, we attribute this PE increase to a decrease in PC and PS in DAC-treated cells. We speculate that DNA hypomethylation might have decreased or inhibited PC synthesis via a PE methylation pathway or increased PE synthesis via PS decarboxylation, or both (see Figure 11).¹⁸

Biophysical characterization data indicate that DAC-treated resistant cell lipids are arranged in a less condensed manner than untreated resistant cell lipids (Figure 4a). Since lipid condensation at the interface is related to the lipid packing density in cell membrane bilayers,¹⁶ lower condensations of treated resistant cell lipids suggest that treating these cells with DAC decreases lipid packing density. The compression modulus of a Langmuir monolayer is related to membrane fluidity,³ a critical biophysical parameter of the cell membrane bilayer. The compression modulus reflects the flexibility of acyl chains within the membrane, lateral diffusion of molecules in the plane of the membrane, and transverse diffusion of molecules across the membrane. The lower compression modulus of treated cell lipids compared with untreated cell lipids (Figure 4b) further confirms that treating cells with DAC increases membrane fluidity.

Since lipid packing density and membrane fluidity depend on lipid composition, these changes in DAC-treated cells can be attributed to altered lipid composition. In particular, the changes could be explained on the basis of the Israelachvili's lipid molecular shape hypothesis.¹⁹ The major membrane lipids such as PC, PE, PS, SM, and cholesterol have distinct molecular shapes due to differences in the relative size of each one's head group and tail. For instance, PC and PS have a cylindrical shape, as they have head groups and tails with similar cross-sectional area, PE and cholesterol display a cone shape because of their small head groups, and SM has an inverted cone shape due to its large head group.^{20–22} Because of these differences in molecular shapes, PC, PS and SM preferentially adopt the lamellar bilayers, whereas PE and cholesterol prefer nonlamellar bilayers such as spherical vesicles.^{20–22} In addition, phospholipids show different degrees of affinity to cholesterol, which is known to increase the structural order of acyl chains. Phospholipids have an affinity for cholesterol in the order SM > PS > PC > PE; this hierarchy means that higher levels of SM are associated with higher cholesterol, whereas an increase in PE results in a lowering of cholesterol.²³

Based on the above information, the effect of DAC on the biophysical properties of membranes could be attributed to altered phospholipid composition in cell membrane lipids (Figure 11). In particular, the lower packing density and higher fluidity seen in treated vs. untreated cell phospholipids could be attributed to an increase in PE levels and a corresponding decrease in SM levels (Figure 3). SM, a phospholipid and cholesterol, a major fraction of neutral membrane lipids, are known to increase structural order and packing density²⁴ within lipid domains, the low packing density seen in treated cell lipids could be attributed to a greater reduction in the levels of SM in treated vs. untreated cell lipids. In summary, the increase in membrane fluidity of treated resistant cell membrane

could be attributed to an increase in nonlamellar lipid (PE) content and a decrease in lamellar bilayer-forming lipids (SM, PC, and PS).

Significant changes in the lipid domain structures of treated vs. untreated cell lipids confirm the biophysical changes seen in cell lipids following DAC treatment (Figure 5a and b). The presence of crystal structures in treated cell lipids suggests a phase separation of lipids. We speculate that these crystals are composed of cholesterol, since other neutral lipids (cholesterol esters ceramides, diacylglycerol, triglycerides) are significantly low in concentration in cell lipids. It is known that cholesterol can form crystal structures when the phospholipid and cholesterol mixing ratio exceeds the cholesterol solubility threshold.²⁵ In our high-performance liquid chromatographic (HPLC) analysis, we found that treated resistant cell lipids have significantly higher levels of PE and lower levels of SM than untreated cell lipids (Figure 2). Due to its unique molecular shape, PE accommodates fewer cholesterol molecules than SM or PC. Therefore, any increase in PE levels in treated resistant cell lipids reduces the membrane cholesterol level. In addition, SM and cholesterol levels in cell membrane complement each other; therefore, if either one of them is depleted by any means, the other follows.²⁶ The decrease in SM levels that we saw in DAC-treated resistant cells could be due to increased SMase activity in treated resistant cells (Figure 6) that indirectly influenced the cholesterol level. Slotte and Bierman²⁷ have shown that the degradation of SM increases the translocation of plasma membrane cholesterol to the endoplasmic reticulum, signifying the relationship between SM and cholesterol.

The membrane cholesterol level is also critical for the proper functioning of P-gp.^{28–30} P-gp was reported to be more active when localized in lipid rafts rich in SM and cholesterol than outside the rafts.³¹ In addition to its efflux activity, P-gp also functions as a flippase to transport a variety of lipids from the inner leaflet of the plasma membrane to its outer leaflets.³² It is known that cholesterol, SM and P-gp activity are interdependent. Thus, the reduced P-gp in DAC-treated resistant cells could be attributed to depletion of cholesterol and SM from membrane lipids (Figure 7).

We have previously suggested that residence time of doxorubicin within the membrane lipids influences its efflux via P-gp.³ The longer the drug's residence time in the membrane, the more it remains available to the efflux action of P-gp. In this study, we show that doxorubicin has only transient interaction with treated resistant cell lipids compared with untreated cell lipids (Figure 8); hence the drug may not be available long enough when interacting with resistant cell lipids for the efflux action of P-gp to work. There may be multiple reasons for the increased doxorubicin uptake that we noted in treated resistant cells (Figure 9): reduced drug interaction with membrane lipids, hence facilitating drug transport; decreased P-gp expression and activity, thus reducing efflux; and increased membrane fluidity, which also favors doxorubicin influx into the cells. However, all these pathways for enhanced drug uptake are linked to changes in the lipid composition of resistant cells following DAC treatment.

The increase in doxorubicin uptake using the Doxil (encapsulated) formulation in DAC-treated cells (Figure 9) indicates increased endocytosis, which otherwise is limited in untreated resistant cells.³ Membrane phospholipid composition, membrane fluidity, and lipid packing density membrane influence the process of endocytosis.³³ For instance, well-ordered, densely packed lipids (such as PC and SM) are resistant to the bending required for budding or for the formation of highly curved membranes during endocytosis.³³ Alternatively, a high concentration of lipids (such as PE) that show a preference for formation of membrane curvature can facilitate budding, thereby increasing endocytosis.³⁴ In DAC-treated resistant cells, a decrease in SM density (a major phospholipid in the outer leaflet) and an increase in PE density (a major phospholipid in the inner leaflet of the lipid

bilayer) could facilitate the biomechanical torque needed to generate membrane budding, thereby increasing endocytosis.³⁴

The cytotoxic effect of doxorubicin significantly increased in resistant cells pretreated with DAC (Table 1). This effect could be the direct result of enhanced drug delivery in DAC-treated vs. untreated resistant cells, making more drug available for diffusion into the nucleus for DNA intercalation and thereby inducing cell death. Although pretreatment of resistant cells with DAC changed the lipid composition and biophysical membrane properties to improve drug transport and efficacy, it did not completely reverse the drug resistance of the cells. The amount of drug delivered even in treated resistant cells is still lower than that seen in sensitive cells. Similarly, the cytotoxic effect of doxorubicin, although significantly enhanced in DAC-treated resistant cells than in untreated cells, did not achieve the same level of efficacy as in sensitive cells. It is also worth noting that a fraction of resistant cells following DAC treatment did not respond to the cytotoxic effect of doxorubicin at lower doses (Figure 10). There could be multiple reasons for this, but one explanation could be the instability of DAC in cell culture medium (half-life ~17.5 h³⁵). Hence, the 24 h DAC treatment time used in our study may not have been long enough for all cells to change their membrane properties to match the lipid characteristics of sensitive cells. Furthermore, DAC is a cytosine analog, so it is recognized as a nucleotide by cellular machinery and incorporated in DNA during the synthesis (S)-phase of the cell cycle.³⁶

Therefore, it is possible that a fraction of the cells may not have had a chance to interact with DAC because they were not at S-phase prior to drug degradation. The relatively short-lived effect of DAC is evident from its transient effect in suppressing P-gp as well from the transient increase in SMase activity in resistant cells. Yet another possibility cannot be ruled out, *i.e.*, it may be that a fraction of the cells that were not responsive to sequential treatment with DAC and doxorubicin may have a different phenotype for drug resistance, one that is not dependent on membrane lipid composition. Nonetheless, the line of reasoning outlined above provides a good rationale for further investigating the long-term effects of DAC treatment on changes in the biophysical properties of resistant cell membrane and the cytotoxic effects of doxorubicin. It will also be interesting to study the membrane-modulating action of DAC using other drug-resistant cells and different anticancer drugs.

One aspect of our study is intriguing but cannot be explained at this stage. Treating resistant cells with DAC increased membrane lipid fluidity but had an opposite effect on sensitive cell lipids (Figure 4a), although both resistant and sensitive cells showed greater doxorubicin delivery in treated cells than in untreated cells (Figure 9) and a synergistic effect following sequential treatment with DAC and doxorubicin (Figure 10, Table 1). Our analysis of the cells' biophysical characterization is based on changes in major lipids generally present in cell membranes. It is known that the cell membrane contains a complex combination of several lipids. Some of them may be present in quite small fractions, enough to contribute to changes in biophysical properties but not enough to significantly influence drug transport properties. Nonetheless, our data clearly demonstrate the role of lipid composition and biophysical properties of resistant cell membrane lipids in drug transport and modulation of lipid composition to facilitate resumption of endocytic function following their treatment with an epigenetic drug.

An important point is that nanocarriers designed to treat sensitive cell tumors may not be effective in drug-resistant tumors because of impaired endocytic functions in those resistant cells. Although our study was carried out using the encapsulated formulation Doxil, the issue of endocytic transport in resistant cells would remain with other similar PEGylated nanocarrier systems. Therefore, our approach of modulating membrane lipid properties to regain endocytic function could also enhance the efficacy of other nanocarrier systems for

drug delivery in drug-resistant cells. Our study highlights the role of membrane lipids in drug delivery and signifies the importance of biophysical interaction studies with drugs/nanocarrier systems to enhance therapeutic outcome.

5. Conclusions

Our study demonstrates that pretreatment with an epigenetic drug (DAC) modulates cell membrane lipid composition in resistant cells; this in turn influences the biophysical properties of membrane lipids to facilitate drug transport and endocytic function, ultimately reversing drug resistance to a significant extent in resistant breast cancer cells. Further studies with a stabilized formulation of DAC could potentially be explored for translation of our approach *in vivo* in treating drug-resistant tumors. In conclusion, we show a new mechanism via which epigenetic drugs could reverse drug resistance in cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

CE	Cholesterol esters
CI	Combination Index (CI)
CL	Cardiolipin
CHOL	Cholesterol
DAC	Decitabine (trade name Dacogen)
DAG	Diacylglycerol
Dox	Doxorubicin in solution
Doxil	Trade name of a liposome form of doxorubicin
DPBS	Dulbecco's phosphate-buffered saline
ECL	Electrochemiluminescence
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
LB	Langmuir-Blodgett
NL	Neutral lipid
PA	Phosphatic acid
P-gp	P-glycoprotein
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol

PL	Phospholipids
PS	Phosphatidylserine
SM	Sphingomyelin
SMase	Sphingomyelinase
SP	Surface pressure
SPE	Solid phase extraction
STE	Sodium chloride-Tris-ethylenediaminetetraacetic acid
TG	Triglycerides
TL	Total lipids

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Figure 1. Effect of decitabine (DAC) on phospholipid (PL) and neutral lipid (NL) fractions of the total lipid (TL) extracts of resistant and sensitive breast cancer cells. PLs and NLs were separated from the lipid extract by solid phase extraction. The ratio represents the weight fraction of PLs or NLs in the lipid extract added to the solid phase extraction column. Data from four different lipid extracts for each cell line. Data as mean $\pm$ s.e.m., $n=4$. Resistant cells PL/NL and NL/TL treated vs. untreated, $p < 0.05$

Figure 2.

Analysis of the HPTLC separated phospholipids and neutral lipids from total lipid extract from untreated and DAC-treated resistant and sensitive cells. Cells were treated with DAC (50 ng/mL) for 24 h. Representative data from four different lipid extracts of each cell line are shown. NL, neutral lipids; CL, cardiolipin; PA, phosphatic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; CE, cholesterol esters; TG, triglycerides; 1,3 DAG, diacylglycerol; CHOL, cholesterol. Data as mean $\pm$ s.e.m., n=4.

Figure 3.

Quantification of the phospholipids from untreated and DAC-treated resistant and sensitive cells by HPLC. Cells were treated with DAC (50 ng/mL) for 24 h. CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. Major changes were seen in SM, PI and PE levels following treatment. Data as mean $\pm$ s.e.m., n=4. (see supplemental data for detail statistical analysis of lipid composition of resistant and sensitive cell lipids and changes following treatment with DAC).

Figure 4.

Biophysical characterization of untreated and DAC-treated resistant and sensitive cell lipids. Cells were treated with DAC (50 ng/mL) for 24 h. **a)** In resistant cells, compression isotherms (π -A) demonstrate that DAC treatment decreases the lipid packing density at the interface. The lipids were spread at different SPs, and then lipids were compressed at 5 mm/min. Different parts of the isotherms were collected in two experiments and merged at the overlapping region to show a complete isotherm in one graph. **b)** Compression modulus of Langmuir-Blodgett (LB) films. The modulus was calculated from π -A isotherm data using $C_s^{-1} = -A * (d\pi/dA)$. **c)** A compression-expansion isotherm demonstrates the reversibility of LB films at the interface. Representative data from four different lipid extracts from each cell line are shown.

Figure 5.

Morphological analysis of lipids extracted from untreated and DAC-treated sensitive and resistant cells. **a)** Analysis of lipid domains and crystal structures of both control and treated sensitive/resistant cell lipids. The LB films were transferred at SP 30 mN/m and analyzed by atomic force microscopy. **b)** Section profiles of the height images clearly demonstrate that the treated cell lipids form very small lipid domains for both sensitive/resistant cell membrane lipids compared with domains for control sensitive/resistant cell membrane lipids.

Figure 6. Change in sphingomyelinase (SMase) activity following DAC treatment. Drug-resistant breast cancer cells treated with DAC for 6 h or 24 h showed a marked increase in SMase activity. Data are shown as mean $\pm$ s.e.m., $n=4$. * $p < 0.05$ with respect to untreated cells.

Figure 7. Change in P-gp expression in resistant cells treated with DAC. **a)** Expression of P-gp protein analyzed by western blot in drug-resistant cells treated with DAC for 1 day or 3 days. **b)** Quantification of the western blot data using Image J analysis software. Representative results from two separate experiments.

Figure 8.

Drug interaction with lipid membranes. Changes in surface pressure (SP) of DAC-treated and -untreated sensitive/resistant membrane lipids following interaction with doxorubicin in solution. Ten μL of doxorubicin solution (1 mg/mL) in ethanol:water (1:1 v/v) was injected into the subphase, and the change in SP over time was recorded.

Figure 9.

Time- and dose-dependent cellular uptake of doxorubicin (dox, *i.e.*, drug in solution) or Doxil (encapsulated form of drug) in resistant and sensitive cells, without and with pretreatment with DAC (50 ng/mL) for 24 h. **a and b:** Doxorubicin uptake over time, with and without pretreatment with DAC, in resistant (MCF7/ADR) cells with doxorubicin vs. Doxil. Cells pretreated with DAC showed increased drug uptake compared with untreated cells. **c and d:** Dose-dependent uptake of doxorubicin in resistant (MCF-7) cells, with or without pretreatment with DAC, comparing doxorubicin with Doxil. Drug uptake was higher in DAC-pretreated cells than in untreated cells regardless of the drug formulation used. **e and f:** Doxorubicin uptake over time, with and without pretreatment with DAC, in sensitive (MCF7) cells, comparing doxorubicin vs. Doxil. Cells pretreated with DAC showed increased drug uptake compared with untreated cells. For time course uptake experiments, cells were pretreated with DAC (50 ng/mL; 24 h) and then incubated with a fixed concentration of doxorubicin or Doxil (1 μ g/mL) for the indicated time points. For dose-dependent uptake experiments, cells were pretreated with DAC for 24 h and then treated with varying concentrations of doxorubicin/Doxil for 8 h. Data are shown as mean $\pm$ s.e.m., $n = 4$. ** $p < 0.0005$, * $p < 0.005$ doxorubicin/Doxil alone vs. DAC + doxorubicin/Doxil.

Figure 10.

Cytotoxicity of doxorubicin in resistant and sensitive cells pretreated with and without DAC. **a–d**; Cytotoxicity of doxorubicin/Doxil (drug in solution vs. encapsulated form) in resistant and sensitive breast cancer cells, with and without DAC pretreatment (50 ng/mL) for 24 h. At this concentration DAC has insignificant cytotoxicity of its own. DAC pretreatment enhanced the cytotoxicity of both doxorubicin and Doxil in the breast cancer cells mentioned. Cytotoxicity was assessed by standard MTS colorimetric assay. Data are expressed as mean $\pm$ s.e.m., $n = 6$. Error bars are not visible for certain data points because they are smaller than the symbol. Data obtained from resistant cells was plotted on log scale (x-axis) whereas for sensitive cells it was plotted on a linear scale for better representation of the cytotoxic effect of doxorubicin in the respective cells.

Figure 11.

Schematic representation of the effect of DAC pretreatment on the change in biophysical characteristics of resistant cell lipid membrane and its proposed mechanism of action. DAC increases the intracellular SMase concentration, which in turn degrades the membrane SM. Because of SM's high affinity to cholesterol, any decrease in SM also results in a decrease in cholesterol. Similarly, an increase in PE reduces the lipid cholesterol level because of the reduced ability of PE to accommodate cholesterol. The overall mechanism of increased membrane fluidity of treated resistant cell lipids involves an increase in PE level and a decrease in SM and cholesterol levels, resulting in reduced lipid packing density. An increase in PE levels increases the propensity of the treated cell membrane to form nonlamellar structures, which facilitate the vesicle formation required for budding during endocytosis. Furthermore, because of its conical shape when aligned with cylinder-shaped PC and PS, PE results in defective membrane lipid packing, which increases drug permeability.

Table 1

Effect of decitabine treatment on cytotoxicity of doxorubicin in sensitive and resistant breast cancer cells.

Breast cancer cells	Doxorubicin		Doxil	
	IC ₅₀ values (ng/mL)	IC ₅₀ values (ng/mL)	CI	CI
Drug resistant (MCF-7/ADR)	5,703	< 1	0.13	0.14
Sensitive (MCF-7)	8	5	0.51	0.28

Combination index (CI) values: < 1 Synergistic; = 1 Additive; and > 1 Antagonistic.