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Experimental Cell Research 293 (2004) 283–291
Experimental Cell Research
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Changes in biophysical parameters of plasma membranes influence cisplatin resistance of sensitive and resistant epidermal carcinoma cells

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Received 30 June 2003, revised version received 15 September 2003

Abstract

The mechanism of resistance of cancer cells to the anticancer drug cisplatin is not fully understood. Using cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells, we found that the resistant cells have higher membrane potential, as determined by membrane potential sensing oxonol dye. Electron spin resonance and fluorescence polarization studies revealed that the resistant cells have more "fluid" plasma membranes than the sensitive cells. Because of this observed difference in membrane "fluidity," we attempted modification of the plasma membrane fluidity by the incorporation of heptadecanoic acid into KB-3-1 and KCP-20 cell membranes. We found that such treatment resulted in increased heptadecanoic acid content and increased fluidity in the plasma membranes of both cell types, and also resulted in increased cisplatin resistance in the KCP-20 cells. This finding is in accord with our results, which showed that the cisplatin-resistant KCP-20 cells have more fluid membranes than the cisplatin-sensitive KB-3-1 cells. It remains to be determined whether the observed differences in biophysical status and/or fatty acid composition alone, or the secondary effect of these differences on the structure or function of some transmembrane protein(s), is the reason for increased cisplatin resistance.
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Keywords: Cisplatin resistance; Heptadecanoic acid; Plasma membrane fluidity; Membrane potential; Fluorescence polarization; Human epidermal carcinoma KB cells

Introduction

Cis-diamminodichloroplatinum II (cisplatin) is one of the most useful anticancer drugs. Treatment protocols for solid tumors of the esophagus, bladder, ovary, testes, head, and neck include this drug. As for most antitumor agents, resistance develops in cancer cells, limiting their efficacy. The reason for resistance against cisplatin is not fully understood. Several mechanisms have been suggested, including decreased cisplatin accumulation [1,2], and alteration of apoptotic signaling [3,4]. Reports also indicate that alterations in growth regulating proteins, such as c-Myc [5], inhibition of caspase-9 [6], and reduced inhibition of DNA synthesis [7] are associated with cisplatin resistance. Involvement of plasma membrane lipids in cisplatin resistance has also been implicated [8]. Britz et al. [9] succeeded in decreasing cisplatin resistance by treating monoclonal cells with a free or liposome-encapsulated bile acid derivative.

Our interest was focused on the biophysical status of plasma membranes in relation to cisplatin resistance. We investigated the biophysical differences between cisplatin-resistant and -sensitive cells and the influence of a fatty acid inserted into the plasma membranes on cisplatin sensitivity of human epidermal carcinoma KB cells.

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doi:10.1016/j.yexcr.2003.10.012

Materials and methods

Cell lines

The KB-3-1 cell line was derived from a single clone of human KB epidermal carcinoma cells (a HeLa subclone), after two subclonings, as described by Akiyama et al. [10]. The cisplatin-resistant subline of KB-3-1 cells was selected by exposure to 0.5 μg/ml cisplatin (KB-CP.5) for 42 days. After that, single clones were picked and propagated in medium containing 0.5 μg/ml cisplatin. One other cisplatin-resistant cell line, KCP-20, was obtained after 6 months' exposure of KB-3-1 cells to increasing concentrations of cisplatin, up to 20 μg/ml [11]. KCP-20 cells were maintained in medium containing 5 μg/ml cisplatin and taken out of cisplatin before making the measurements. All cell lines were grown in Dulbecco's modified Eagle medium (Invitrogen, Grand Island, NY), supplemented with L-glutamine, penicillin, streptomycin (Quality Biological, Gaithersburg, MD), and 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD).

Membrane potential measurements

Membrane potential measurements were performed by flow cytometry, using the negatively charged DiBaC4 (3) oxonol dye (Molecular Probes, Eugene, OR), essentially as described earlier [12]. Briefly, a cell suspension of 1×10^6 cells/ml was equilibrated for 1 min in PBS followed by the addition of oxonol dye, 150 M. After exactly 2 min equilibration at room temperature, histograms were collected from 10^4 cells. Reproducibility was determined by measuring membrane potentials of the cells on different days, from separate cultures, and by comparing the relative fluorescence of the oxonol-stained cell types.

Oxonol fluorescence intensity measures membrane potential when the extracellular potassium concentration is changed from 5 to 150 mM, resulting in increased fluorescence intensity of the oxonol-stained cells and thus making the cells depolarized. All measurements were made with a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), operated with a 15-mW argon ion laser tuned to 488-nm excitation wavelength. Fluorescence emission was collected at 525 nm. Results are expressed in comparative histograms of representative series.

Polarity of fluorescent membrane probes in live cells

Measurements of steady-state fluorescence polarization were done with a spectrofluorometer LS50B (Perkin Elmer, Norwalk, CT) and the lipid-soluble fluorophore, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Molecular Probes). This fluorophore is known to probe plasma membranes of cells at the surface. TMA-DPH was dissolved in tetrahydrofurane at a concentration of 2 mM and was kept in the dark at 4°C. Cells (10^6/ml) were labeled with TMA-DPH at a concentration of 2 μM in PBS. After 10 min incubation time at 4°C, the cell suspension was centrifuged and washed two times in the centrifuge with PBS. After resuspension in PBS, fluorescence anisotropy was measured at 25°C. The excitation wavelength was 355 nm and the emission was measured at 430 nm with a slit width of 5. Polarization values were calculated according to Collins and Scott [13] by the equation $P = (I_{0,0} - G * I_{0,90}) / (I_{0,0} + G * I_{0,90})$ from the measured fluorescence intensities.

Electron spin resonance (ESR) studies on live cells

ESR studies were conducted with 5-doxyl stearic acid (5-doxyl-SA) and with 2,2,6,6-tetramethyl piperidin-1-oxyl-4-yl-octadecenoate (T-SASL) probes (Molecular Probes). The first probe intercalates to the 5 carbon depth in the outer leaflet of the plasma membrane [14] and T-SASL at the surface of the plasma membranes [15]. Labeling the cells with the spin probes was done as follows: 5-doxyl-SA was dissolved in ethanol, 1 mg/ml, and was kept at 4°C. Then, 1×10^7 cells were mixed with 8×10^{-8} mol spin label in 0.02 ml volume of PBS. After 1 min contact time, the cell suspension was transferred into a 50-μl micropipette capillary tube and sealed at the bottom with Critoseal (Syva Co., Palo Alto, CA). The micropipette with the cells was placed into the cavity of a Varian E-9 Century series spectrometer (Syva Co.). ESR spectra were recorded at X-band, at 9.5 kHz, 100-field modulation, 4-Gous modulation amplitude, 100-Gous sweep range, and at 10 mW microwave power. The temperature of the probe was set to 24°C by the variable temperature accessory using N_2 gas flow. Evaluation of the obtained ESR spectra, when 5-doxyl-SA was used, was by the equation expressing the order parameter S:

$$S = 0.5407\ (T_{11} - T_1)/a_0,\ \text{where}\ a_0 = (T_{11} + 2T_1)/3$$

and T_{11} and T_1 are the outer and inner tensors obtained from the ESR spectra.

When the T-SASL probe was used, the same instrument parameters were applied, except that the incident microwave power was 20 mW. The spectral parameters, h_0 and h_{-1} are the spectral amplitudes and the ratio h_0/h_{-1} defines the motional freedom of the probe according to Yin et al. [15]. With both spin labels, the ESR spectra show contribution from spin labels of restricted motion with no contribution from the free-moving spin label.

Lipid packing of plasma membranes in live cells

Plasma membrane lipid packing can be studied by inserting the fluorescence probe merocyanine 540 (MRC 540) (Molecular Probes) into cell membranes and assessing the degree of insertion by fluorescence intensity measurement, using flow cytometry [16,17]. The experiment was according to Schlegel et al. [16] with some modifications. Briefly, 1×10^7 cells, suspended in 1 ml of PBS were treated with 10 μl of

a MC540 stock solution of 1 mg/ml, in 60% ethanol, 40% water. After a 10-min incubation at room temperature, cells were pelleted in a centrifuge, washed once in PBS by centrifugation, resuspended in 1 ml PBS, and fluorescence histograms were obtained. A Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used at 488-nm excitation and 575-nm emission wavelength. Histograms were collected with 10^4 cells. Results are expressed as means of fluorescence of the histograms obtained from duplicate measurements of one typical set of cells.

Preparation of cells for NMR spectrometry

KB-3-1 and KCP-20 cells were grown to 90% confluence, harvested with 0.05% trypsin, 0.53 μM EDTA, centrifuged at 4°C at 730 × *g* for 10 min, and washed twice with growth medium. To perform a long-term NMR study, the cells were restrained in an agarose thread [18,19]; 0.5 ml of (9×10^7 cells) was mixed with an equal volume of liquid agarose in phosphate-buffered saline, and immersed in a bath at 37°C for 5–7 min. The mixture was extruded under low pressure through cooled tubing (0.5 mm ID) into a 10-mm NMR tube containing growth medium. Using 0.5-mm threads ensures that there is no metabolic compromise, and the cells are viable and in stable energetic status for a long period of time, while the threads maintain their mechanical strength. The gel threads, which fill the tube, are concentrated without compression at the bottom of the NMR tube by insertion of a plastic insert with the perfusion fitting. A Teflon inflow tube (0.5 mm ID) was placed near the bottom of the tube. The gel threads were perfused with growth medium at 0.9 ml/min. Cells were continuously perfused for more than 40 h. Accumulation of data was started within 30 min after the harvest.

NMR spectrometry of live cells

The ^{31}P NMR spectra were recorded at 37°C on a Varian XL-400 machine (Varian Associates, Inc., Palo Alto, CA) at 162 MHz using RF pulse corresponding to a 72° flip angle and 2 s repetition time. The flip angle used was the Ernst angle for phosphocreatine (PCr) (T1 relaxation time, 3 s). There were more than 40 spectra obtained. Each spectrum contained 1800 transients and took 1 h. During the entire study, the system was deuterium locked with an external source (99.9% D_2O in a capillary, Sigma, St. Louis, MO) to avoid magnetic field drift.

All the spectra were transformed and viewed separately to confirm that the spectra did not change during the experiment. There were 25,000 data points collected and zero-filled to 8k before Fourier transformation. The spectra were added and 10-Hz line broadening was applied to obtain Fig. 3. The chemical shifts were standardized to β-adenosinetriphosphate (β-ATP) set to −18.70 ppm. Many phosphorus metabolites were identified, including phosphocholine (PC, 3.57 ppm), inorganic phosphate (Pi, 2.59 ppm), glycerophosphoethanolamine (GPE, 0.81 ppm), glycerophosphocholine (GPC, 0.26 ppm), phosphocreatine (PCr, −2.69 ppm), γ-adenosine triphosphate (γ-ATP, −5.12 ppm), α-adenosine triphosphate (α-ATP, −10.19 ppm), and diphosphodiesters (dPdE, −10.86, −12.58 ppm). Chemical shifts of these molecules are also listed in the literature [18,19].

Treatment of cells with heptadecanoic acid and cell proliferation studies

Treatment of KB-3-1 and KCP-20 cells with heptadecanoic acid was carried out in 24-well plates (Corning Inc., Corning, NY), with modification of the method used by Callaghan et al. [20]. After exploratory dose selection studies for cisplatin and heptadecanoic acid, the final conditions were as follows: cells (10^6/ml) were incubated in appropriate medium, as described above for both cell types, and after 2–3 days of incubation, the medium was withdrawn and replaced by serum-free medium. Following 6 h incubation at 37°C, 5% CO_2 medium was replaced by serum-free medium containing different concentrations of heptadecanoic acid or nothing. This incubation was followed by replacement of the medium with complete medium containing cisplatin or nothing. KB-3-1 cells were treated with 0.08 μg/ml and KCP-20 cells with 5 μg/ml cisplatin from a stock solution of 500 μg/ml aqueous solution. Cells were harvested after 5–6 days' incubation and treated with heptadecanoic acid alone, with cisplatin alone, heptadecanoic acid plus cisplatin or nothing, and were counted after trypsinization by a Coulter Particle Counter (Coulter Electronics, Luton, UK). Calculation of proliferation was based on cells treated with nothing as 100%. The expected yield was: cell count of cells treated with heptadecanoic acid alone multiplied by cell count of cells treated with cisplatin alone. The cell count of cells treated with both reagents was then related to the previous cell count product and tabulated.

Preparation of plasma membranes for determination of relative fatty acid composition

For the purpose of plasma membrane preparation, 10^7 cells from each cell line were harvested at log phase and washed with ice-cold PBS. Cells were sedimented by centrifugation and then suspended in ice-cold hypotonic solution (0.5 mM KH_2PO_4, 0.1 mM EDTA containing 1% protease inhibitor aprotonin, pH 8.0) for 5 min. Cells were disrupted on ice by a tight Dounce homogenizer with constant 40 strokes. Samples were checked for complete disruption in a phase-contrast microscope. Homogenates were centrifuged at 2000 × *g* for 10 min at 4°C to discard the nuclei and then the supernatant was centrifuged at 25,000 × *g* for 25 min to pellet all other organelles. The resulting supernatant was further centrifuged for 55 min at 4°C. The membranes sedimented at the bottom and were stored at −80°C before fatty acid analysis. To determine

that the sediment contained only plasma membranes and no membranes of organelles, markers were used in connection with Western blot analyses. For a positive marker, the anti-integrin antibody anti-2/VLA-2α was used according to Emsley et al. [21].

Conversion of plasma membrane lipids to methyl esters and fatty acid analysis

Fats were extracted into organic phase by vortexing the membrane preparations obtained as described above, with minor modification of the methods of Kozar et al. [22]. In brief, 2 ml of HPLC-grade water (Fisher Scientific, Pittsburgh, PA) was added to the membranes together with 3.75 ml of 2:1 (v/v) methanol/chloroform (Fisher Scientific) and the suspension was vortexed for 15 min. Then, after addition of 1 ml chloroform and 1 min vortexing, the suspension was centrifuged for 10 min at 2000 rpm. The separated organic phase was dried in an N_2 stream and heated in 1 ml 2 M methanolic HCl [11 ml methanol with dropwise addition of 2.5 ml acetyl chloride (Sigma)] at 85°C for 18 h in a screw-capped tube. The fatty acid methyl esters which formed were then extracted into heptane for GC-MS analysis. Samples were analyzed on a Hewlett-Packard 6890 plus GC equipped with 7683 auto-injector. The injection port was held at constant 280°C with 2 μl injected in the spitless mode onto a DB-5 ms capillary column with 30 m × 0.25 mm ID × 0.25 μm film thickness (J&W Scientific, Folsom, CA). Initial oven temperature was 80°C with a ramp of 40°C/min to a final temperature of 290°C and hold for 0.75 min. Helium was used as the carrier gas at a constant velocity of 41 cm/s. Electron impact ionization at 70 eV was performed using standard autotune conditions. The source temperature was maintained at 230°C while the quadrupoles were maintained at 150°C. FAMEs were analyzed in full scan mode for qualitative identification as well as in selected ion monitoring mode for ratio determination. Location of the analyzed two fatty acids in the obtained chromatograms was ascertained by standards of hexa- and heptadecanoic acid methyl esters (Sigma). The ratio of heptadecanoic acid to hexadecanoic acid methyl esters was determined since heptadecanoic acid was used to treat the cells and the hexadecanoic acid content of cells was assumed to be constant. This ratio is defined as the area under the peak for hexadecanoic acid/area under the peak for heptadecanoic acid methyl esters.

Visualization of distribution of K^+ channels in cells by immunofluorescence microscopy

For visualization of distribution of K^+ channels in cells, cells were grown on 189-mm glass coverslips in petri dishes. Cells were fixed with 3.5% formaldehyde in PBS for 10 min, followed by 0.1% Triton X-100 treatment for 5 min for permeabilization. After washing, cells were treated with 3% BSA in PBS for 30 min and subsequently treated with the primary antibody (Ab) for 1 h. The Ab was TW1K-2 (P-19) goat polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). After five washings, cells were incubated with Cy^{TM}3-conjugated affinity pure donkey anti-goat, secondary Ab (1:100 dilution) (Jackson Immuno-research Laboratory). Cells were washed extensively after the secondary Ab treatment. The slides with the treated cells were mounted on microscope slides with fluorescence mounting medium (Dako, Carpinteria, CA). Background fluorescence was determined from cells treated only with the secondary Ab, but otherwise treated the same way as described with the primary Ab. Fluorescent images were collected with a Bio-Rad 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60× planapochromat lens. Excitation at 568 nm was provided by a krypton–argon gas laser. An emission filter of 598/40 was used for collecting red fluorescence in channel one and phase contrast images of the same cell were collected in another channel using a transmitted light detector.

Results

Membrane potential of cisplatin-sensitive and -resistant cells

Membrane potential was measured using several series of independently grown cell cultures. For each measurement at different times and with the different cell lines, we observed the same pattern of membrane potentials. Fig. 1 shows one typical result of several measurements with the series of the cells. The KCP-20 cells, which are highly resistant to cisplatin, had lower fluorescence intensities and thus are hyperpolarized as compared to cisplatin sensitive and single-step, low-level resistant cells (KB-CP.5). Oxonol is a negatively charged membrane potential sensing dye and therefore less dye diffuses into membranes of cells which are more negative, and thus are hyperpolarized.

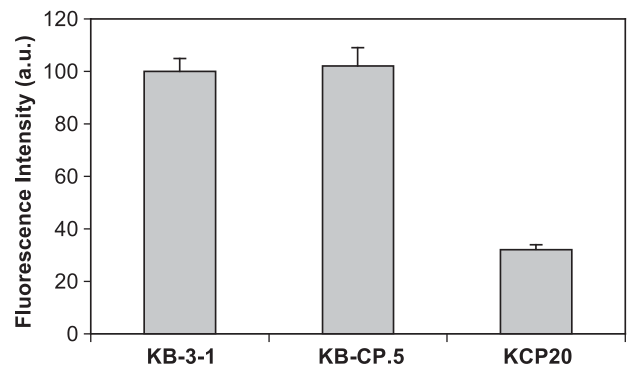

Fig. 1. Relative membrane potential of cisplatin-sensitive KB-3-1 and -resistant cells is indicated by the fluorescence of oxonol (150 mM)-stained cells. Oxonol is a negatively charged membrane potential sensing dye and was used as detailed in Materials and methods. One typical series of measurement of cells were grown simultaneously in culture (n = 2–3). $P <$ 0.05 between KB-3-1 and KCP-20 cells. No statistical difference exists between KB-3-1 and KB-CP.5 cells.

Polarity of the fluorescent TMA-DPH molecule in the plasma membrane of live cells

The TMA-DPH fluorescence probe was used to measure the fluidity of plasma membranes because it has been shown that this probe does not penetrate into the cells and probes at the upper leaflet of the membrane [13]. Table 1 shows the results obtained with sensitive KB-3-1 and resistant KB-20 cells treated or not treated with heptadecanoic acid. Cisplatin-resistant KCP-20 cells were found to have membranes which were more fluid; the calculated polarization number, *P*, was of lower value. These results parallel those obtained with ESR measurements (see below). Also, polarization numbers obtained with the TMA-DPH probe indicate that heptadecanoic acid treatment of the cells results in lower polarization numbers. Therefore, the plasma membranes become more fluid. These results also parallel those obtained by ESR measurements (see below).

ESR studies on the motional freedom of ESR probes, 5-doxyl-SA and T-SASL, in the plasma membranes of cells

Two ESR probes were used to measure membrane fluidity in cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells. 5-doxyl-SA probes at 5 carbon depth in the outer leaflet, while the T-SASL probes at the surface of the plasma membrane [14,15]. Table 2 shows both results. The calculated order parameters, *S*, for the 5-doxyl-SA yielded lower numbers for the cisplatin-resistant KCP-20 cells, indicating more "fluid" membranes of these cells at 24°C. The calculated h_0/h_{-1} parameters also indicate a more fluid membrane for the cisplatin-resistant KCP-20 cells. These results are in line with those of the polarization experiments.

To measure membrane fluidity changes of heptadecanoic acid treated KB-3-1 and KCP-20 cells, we employed the 5-doxyl-SA ESR probe. Fig. 2 shows the results and indicates that both types of cells became more fluid (had lower *S* values) after heptadecanoic acid treatment. The heptadecanoic acid-treated KCP-20 cells were shown to be more resistant than the nontreated KCP-20 cells. This result is consistent with the fact that the cisplatin-resistant KCP-20 cells were found to have more fluid plasma membranes than the sensitive KB-3-1 cells by both ESR and polarization techniques (Tables 1 and 2). Contrary to this, the cisplatin-sensitive KB-3-1 cells became even more sensitive after heptadecanoic acid treatment, despite the fact that their plasma membranes became more fluid after this treatment (data not shown).

Table 1
Polarity[a] of TMA-DPH fluorescence probe, inserted into plasma membranes of heptadecanoic acid[b]-treated and untreated cisplatin-sensitive (KB-3-1) and -resistant (KCP-20) cells

Cells/treatment	Polarity	±SD
KB-3-1 untreated	0.3668	0.0018
KB-3-1 treated	0.3590	0.0013
KCP-20 untreated	0.3612	0.0018
KCP-20 treated	0.3556	0.0016

[a] Mean polarization numbers are calculated from six independent measurements.
[b] Heptadecanoic acid treatment is detailed in Materials and methods. Heptadecanoic acid (40 μM) was used in each experiment. $P < 0.05$ between treated and untreated cells as well as between KB-3-1 and KCP-20 cells.

Table 2
Motional freedom[a] of 5-doxyl-SA and T-SASL ESR probes inserted into the plasma membranes of cisplatin-sensitive and -resistant cells

Cells	Temp, °C	5-doxyl-SA, order parameter, *S*		T-SASL, h_0/h_{-1}	
		S	±SD	h_0/h_{-1}	±SD
KB-3-1	24	0.6443	0.0038	2.423	0.093
KCP-20	24	0.6208	0.0111	1.820	0.147

[a] Order parameter, *S*, and h_0/h_{-1} were calculated as described in Materials and methods. Experiments were done with several cultures ($n = 2-4$), and ESR measurements were in triplicate; $P < 0.05$.

Membrane packing as determined by fluorescence intensity of merocyanine 540-stained cells

Merocyanine (MRC) 540 staining was found to be indicative of the lipid packing density of cell plasma membranes [16]. We applied this measurement to cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells as detailed in Materials and methods, and found that cisplatin resistant cells had lower fluorescence intensity than the sensitive KB-3-1 cells. The fluorescence intensity of merocyanine540-stained KB-3-1 cells was 95 ± 10 while that of the resistant KCP-20 cells was 58 ± 12 in a typical cell preparation (n = 3). We interpret these results to mean that in KB-3-1 cell membrane lipids are more tightly packed and intercalate MRC 540 more tightly than in KCP-20 cells. These results parallel those of membrane fluidity measurements by the polarization and ESR methods (Tables 1 and 2).

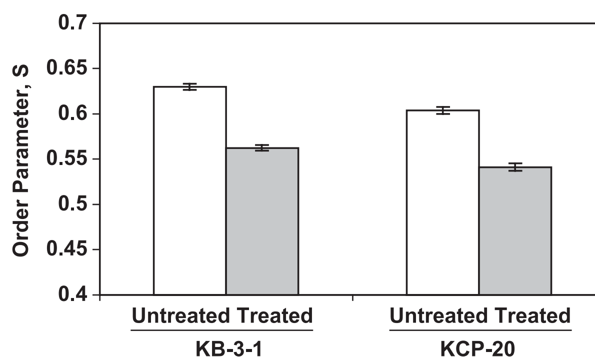

Fig. 2. Membrane "fluidity" as expressed by the order parameter, *S*, of cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells, treated or not treated with heptadecanoic acid. Order parameters, *S*, were calculated from ESR spectra obtained as described in Materials and methods. Heptadecanoic acid treatment is also described in Materials and methods. Average of three to four measurements are shown with SDs. There is a statistically significant difference between *S* values of heptadecanoic acid treated and untreated cells for both types of cells ($P < 0.05$).

Assessment of phospholipids in cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells by ^{31}P NMR spectrometry

Spectra obtained by 400-MHz NMR spectrometry were compared to detect any difference in phospholipid composition between cisplatin-sensitive KB-3-1 and -resistant KCP-20 cells. The two cell line types were grown in the same media, harvested before confluence, and spectra were obtained as described in Materials and methods. Similar experiments have been performed for the detection of such differences between P-glycoprotein expressing and nonexpressing cells [23,24]. Spectra from both cells detected phospho-ethanolamine, 4.11 ppm; -choline, 3.6 ppm; -creatine, −2.69 ppm; glycerophospoethanolamine, 0.7 ppm, and choline, 0.1 ppm, besides inorganic phosphate and different adenosintriphosphates (Fig. 3). No significant differences could be detected between the two cell lines in the above listed phospholipid signals with the applied NMR technique.

Cell proliferation of heptadecanoic acid-treated and untreated cells

Cell proliferation studies were done as described in Materials and methods. Relative cell counts are shown in Table 3. Results indicate that heptadecanoic acid treatment increased resistance of the KCP-20 cells to cisplatin, since the cells grew better in the combination of cisplatin and heptadecanoic acid than would have been mediated from the growth of these cells in either agent alone. In contrast, heptadecanoic acid treatment of the sensitive KB-3-1 cells increased their sensitivity to cisplatin.

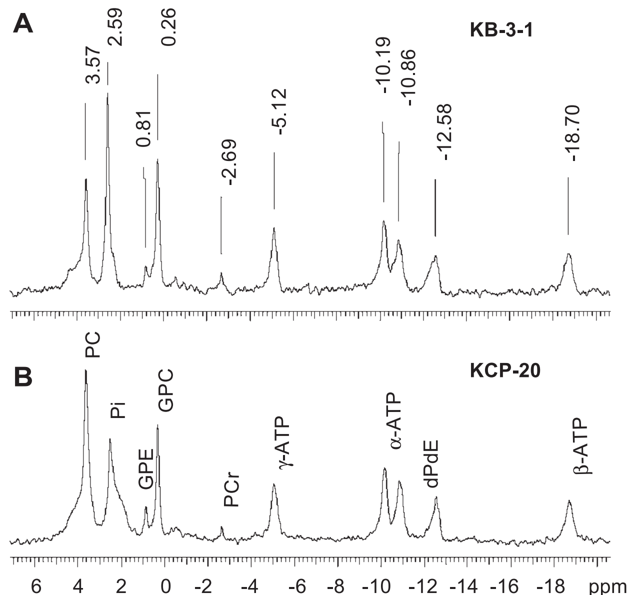

Fig. 3. ^{31}P NMR spectra (400 MHz) of cisplatin-sensitive KB-3-l (A) and -resistant KCP-20 (B) cells. Cells (9 × 10^{7}) from each culture were embedded in agarose and packed in 10-mm NMR tubes. Many water-soluble phosphates were identified, including phosphocholine (PC, 3.57 ppm), inorganic phosphate (Pi, 2.59 ppm), glycerophosphoethanolamine (GPE, 0.81 ppm), glycerophosphocholine (GPC, 0.26 ppm), phosphocreatine (PCr, −2.69 ppm), γ-adenosine triphosphate (γ-ATP, −5.12 ppm), α-adenosine triphosphate (α-ATP, −10.19 ppm), diphosphodiesters (dPdE, −10.86 ppm, −12.58 ppm), and β-adenosinetriphosphate (β-ATP, −18.70 ppm).

Table 3
Cell proliferation as expressed in percentage ± SD cell growth relative to nontreated KB-3-1 and KCP-20 cells in the presence and absence of treatment with heptadecanoic acid or cisplatin or the combination of both

Cells	Treatment	% Proliferation	
		Expected	Found
KB-3-1	[a] cisplatin (0.8 μg/ml)	–	85 ± 6
	[b] HAD[a] (20 μM)	–	97 ± 3
	[c] HDA (40 μM)	–	94 ± 4
	[a] × [b]	82 ± 8	49 ± 15
	[a] × [c]	80 ± 10	68 ± 12
KCP-20[b]	[d] cisplatin (5 μg/ml)	–	67 ± 10
	[e] HDA (40 μM)	–	68 ± 5
	[f] HDA (50 μM)	–	41 ± 11
	[d] × [e]	45 ± 14	62 ± 6
	[d] × [f]	27 ± 20	46 ± 5
	[g] cisplatin (6 μg/ml)	–	51 ± 8
	[h] HDA (40 μM)	–	68 ± 13
	[i] HDA (50 μM)	–	41 ± 7
	[g] × [h]	34 ± 21	47 ± 5
	[g] × [i]	19 ± 19	23 ± 7

[a] HDA: heptadecanoic acid.
[b] KCP-20 cells: Each measurement of cell growth was done in triplicate wells and each dose was used in multiple experiments with separately grown cell cultures (n = 3–5). $P < 0.05$ for the difference between expected growth and actual growth for all experiments, except for KCP20 cells treated with cisplatin, 6 μg/ml and 50 μM HDA.

Fatty acid analysis in heptadecanoic acid-treated and nontreated plasma membranes

Results of the fatty acid analysis are shown in Table 4. Two sets of membrane preparations were made from

Table 4
Area[a] and ratios of area of hexadecanoic and heptadecanoic acids as measured by GC-MS in the membranes of heptadecanoic acid-treated and untreated KB-3-1 and KCP-20 cells

Cells/treatment	Area at m/z 270 heptadecanoic acid[a]	Area at m/z 284 m/z 270/m/z 284	Ratio of hexadecanoic acid
KB-3-1	5,243,334	65,763	79.73
KB-3-1 + treatment	14,684,547	1,998,304	7.35
KB-3-1	13,859,715	260,720	53.16
KB-3-1 + treatment	15,527,957	1,789,737	8.68
KCP-20	17,554,572	321,183	54.66
KCP-20 + treatment	11,695,325	2,260,349	5.17
KCP-20	6,596,479	122,458	53.87
KCP-20 + treatment	7,656,383	1,332,033	5.75

[a] Area under peaks obtained by GCMS represents fatty acid methyl esters analyzed in full scan mode with the instrument software.

treated and untreated sensitive and resistant cells. For each preparation, Western blot analyses indicated that only plasma membranes were collected (not shown). Results indicated that heptadecanoic acid treatment increased this fatty acid relative concentration to hexadecanoic acid in both cell lines. The hexadecanoic acid content of cell membranes was used to normalize the relative concentration of heptadecanoic acid.

Fluorescence visualization of K^+ channels in human epidermal carcinoma KB cells

Fig. 4 shows fluorescence images of the KB-3-1 and KCP-20 cells stained with the Cy^{MB}3-conjugated affinity pure donkey anti-goat Ab after incubation of the cells with the primary TW1K-2 (P-19) goat polyclonal Ab. The red fluorescence intensities indicate that there are substantially more K^+ channels on the plasma membranes of KCP-20 cells than of KB-3-1 cells.

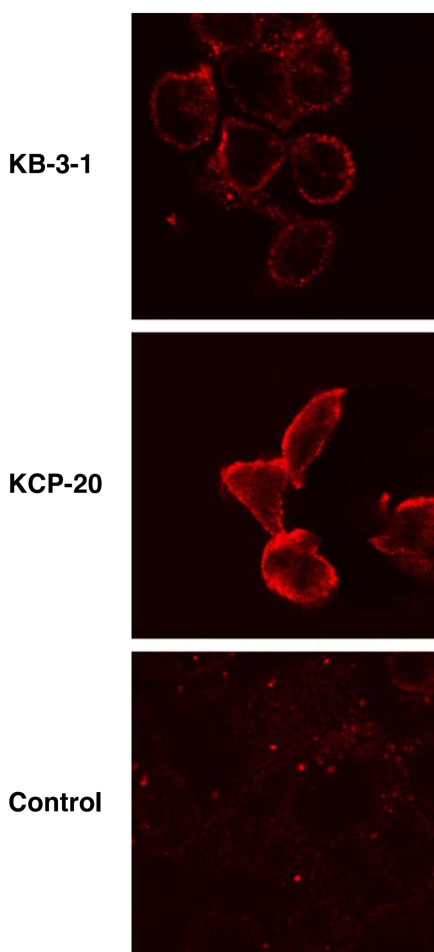

Fig. 4. Confocal visualization of K^+ channels as detected by TW1K-2 primary and Cy^{TM}3-conjugated donkey anti-goat secondary Ab. Ab treatment and confocal microscopy are detailed in Materials and methods. Several fields of cells were captured and one representative field of each is shown (n = 5–8). Control: KCP-20 cells treated only with secondary Ab.

Discussion

We used five different methods to study biophysical differences in the plasma membranes of cisplatin-sensitive KB-3-1 adenocarcinoma cells and their cisplatin-resistant counterparts. We also influenced these biophysical differences in the plasma membranes of KB cells by inserting heptadecanoic acid into the cell membranes. The alteration of lipid composition by addition of heptadecanoic acid resulted in changes in some biophysical parameters of the membranes along with changes in the cells' resistance to cisplatin.

First, we showed that the highly resistant KCP-20 cells have higher membrane potential, and are therefore more hyperpolarized than the sensitive, parental KB-3-1 cells and the low level resistant KCP.5 cells (Fig. 1). We have attributed this increased membrane potential of KCP-20 cells to the increased expression of K^+ channels on their plasma membranes (Fig. 4). Our results are in line with the observation of Thomson et al. [25] that cisplatin treatment of cells influences K^+ channel activity and that of Mahaswari et al. [26] that cisplatin can change ion conductivity in bilayer lipid membranes. Efflux of K^+ from cells can elevate membrane potential. Second, polarization studied with the TMA-DPH fluorescence probe, which probes at the outer leaflet of plasma membranes, indicates that the resistant KCP-20 cells have lower polarization values, and thus have more fluid plasma membranes than the sensitive KB-3-1 cells (Table 1). Third, similar conclusions could be drawn from the ESR studies, performed with two types of ESR probes. Results indicate that the order parameter, S, and parameters of the measurements with the second probe, h_0/h_{-1}, are lower for the resistant cells, indicating more fluid membranes for these cells as compared to the sensitive cells (Table 2).

Fourth, more MRC 540 fluorescent dye is packed into the plasma membranes of the sensitive KB-3-1 cells than into the plasma membranes of the resistant KCP-20 cells (see Results). We have interpreted this difference by assuming that more "rigid" membranes can bind more of this dye tightly than the more loosely packed membranes. Both cells are equal in size, so more dye incorporation into a cell cannot be interpreted from different cell sizes. Our interpretation, based on our polarization and ESR measurements done with the same cells with which we performed our MRC 540 lipid packing experiment, is seemingly contrary to the interpretation of Schlegel et al. [16] and Stillwell et al. [17]. However, Schlegel's experiments with lymphocytes could not be interpreted in terms of the ability of MRC 540 to distinguish between loosely or tightly packed membranes. Stillwell found that more MRC 540 dye intercalates into loosely packed membranes of phospholipid vesicles, but he made no such comparison with live cells. His experiments with the T27A leukemia cells demonstrated only that MRC 540 intercalates into docosahexanoic acid modified cells differently than into the nonmodified cells and no interpre-

tation was given for the relationship between membrane fluidity and dye packing. In a previous study [27], MRC 540 intercalated into cisplatin-sensitive and -resistant lung adenocarcinoma cells with the same relative dye ratio as in our study. Unfortunately, no correlation was made between dye packing and membrane fluidity in that study.

We have not detected any significant difference between the 400 MHz ^{31}P NMR spectra of KB-3-1 and KCP-20 cells (Fig. 3), suggesting that there are no major differences in measured water-soluble phosphates, including phospholipid precursors in the cisplatin-sensitive and -resistant cell lines. Our results reflect relative peak intensities of individual phosphates which depend on their T1 relaxation times and the repetition time (2 s) of the applied NMR technique. Spellman [8] found that phosphatidylserine binds cisplatin in vitro, but formation of a phosphatidylserine–cisplatin complex could not be found when experiments were conducted with intact cells [28]. Kaplan et al. [23] found that there are differences in the glycerophosphocholine and glycerophosphoethanolamine ratio in some MCF-7 wild type and P-glycoprotein expressing cell lines by their ^{31}P NMR studies. However, these differences proved not consistently present in all such cell lines.

We found differences in biophysical parameters between the KB-3-1-sensitive and the KB-20-resistant cells, as described above. Therefore, our next experiment focused on the introduction of biophysical changes to the plasma membrane of the sensitive and the resistant KB cells and determining the cisplatin sensitivity of the altered cells. In a previous study, Callaghan et al. [20] found that the incorporation of heptadecanoic acid into plasma membranes can hinder the function of a transmembrane protein, P-glycoprotein, possibly by altering the biophysical milieu of this transmembrane protein. Therefore, we incorporated this fatty acid into the plasma membrane of KB-3-1 and KCP-20 cells. We demonstrated that during a short treatment period of the cells with this fatty acid, other components of the plasma membrane, such as the cholesterol content, did not change significantly.

After heptadecanoic acid treatment of our cells, we determined changes in membrane biophysical status, relative heptadecanoic acid content, and sensitivity of the treated cells to cisplatin. We found that heptadecanoic acid treatment of the cells increases the relative content of this fatty acid in cell membranes (Table 4). Simultaneously, this treatment increased the fluidity of plasma membranes of both KB-3-1 and KCP-20 cells as measured by polarization (Table 1) and by ESR (Fig. 2). We also measured the influence of heptadecanoic acid treatment on cell proliferation (Table 3). After heptadecanoic acid treatment of KCP-20 cells, their resistance to cisplatin increased (Table 3) and their plasma membrane fluidity increased (Fig. 2). This finding is in accord with our results that the resistant KCP-20 cells have more fluid plasma membranes than the sensitive KB-3-1 cells, as discussed above. That a bile acid derivative increased sensitivity of monoclonal cells to cisplatin, as reported by Briz et al. [9], is not a direct contradiction to our findings. First, it was not shown by Briz et al. whether the bile acid derivative was incorporated into plasma membranes, and second, no determination was made on the biophysical status of the membranes of the cells before and after the treatment.

Contrary to the results with the KCP-20 cells, KB-3-1 cells became more sensitive to cisplatin after heptadecanoic acid treatment (Table 3). In this case, increasing the fluidity of the plasma membrane did not result in higher resistance to cisplatin, as it did with the KCP-20 cells. Nevertheless, cisplatin sensitivity was altered by heptadecanoic acid treatment in these cells also. Further experiments with cell lines of different plasma membrane fluidity and sensitivity to cisplatin may explain these results. Results detailed above, that incorporation of heptadecanoic acid into plasma membranes of KB-3-1 and KCP-20 cells resulted in opposite sensitivity to cisplatin, indicate that this fatty acid per se is not involved in cisplatin binding. Somewhat different results were obtained by Timmer-Bosscha et al. [29] who found that incorporation of docosahexaenonoic acid into the membranes of a human small cell lung carcinoma line, GLC4 and its resistant subline, GLC4-CP, decreased resistance of the resistant cells but had no influence on the parent cell line. However, their experimental results suggested that DNA-related effects, and not alteration in the plasma membrane, are the reasons for changes in resistance. Interestingly, these authors also found that their treatment does not cause the same change in cisplatin resistance in the parental as in the resistant cells. Our results parallel this different effect on sensitive and resistant cells. In both cases, this difference suggests that increased fluidity per se may not be responsible for cisplatin resistance, but may facilitate a mechanism of resistance found only in the selected cell line.

Recent physiological studies on the mechanism of resistance of KCP-20 cells to cisplatin (and cross-resistance to other compounds such as methotrexate) have revealed the following phenotype: (1) decreased drug accumulation for many drugs associated with decreased expression on the cell membrane of many different transporters, carriers, and channels [11]; (2) neutralization of the usual acidic pH of lysosomes and endosomes [30]; (3) hypermethylation of genes whose expression is decreased in KCP-20 cells (Shen, D.-W., Liang, X.-J., Pai-Panandiker, A., and Gottesman, M.M., unpublished data); and (4) mislocalization of membrane proteins with accumulation of certain transporters in the cytoplasm [31]. Although a single molecular defect is unlikely to account for all of these changes in cells selected in multiple steps, alteration of the biophysical properties of plasma membranes in cisplatin-resistant cells could facilitate defects in membrane protein trafficking which might underlie cisplatin resistance due to decreased accumulation. If increased membrane fluidity amplifies the effect of another defect in KB-CP20 cell membranes, rather than independently causing resistance to cisplatin, this could explain why

measured membrane fluidity in the KB-3-1 parental, drug-sensitive cells does not result in resistance.

To summarize, we have determined that there are differences in biophysical parameters, membrane potential, motional freedom of polarization and ESR probes, and MRC 540 dye packing between cisplatin-sensitive and -resistant human epidermal carcinoma cells in vitro. ^{31}P NMR studies indicated no essential differences in water-soluble phosphates. Modification of the plasma membrane fluidity of these cells by incorporation of heptadecanoic acid resulted in changes in their sensitivity to cisplatin. Whether changes in membrane fluidity transmitted to some membrane molecules in sensitive and resistant cells cause alterations in cisplatin sensitivity remains to be determined.

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