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ORIGINAL ARTICLE

Disruption of microfilaments by cytochalasin B decreases accumulation of cisplatin in human epidermal carcinoma and liver carcinoma cell lines

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Abstract
Background Although cisplatin is a frequently used cancer chemotherapeutic drug, its effectiveness is hindered by the development of resistance in cancer cells. In order to understand the reason(s) for this resistance, the mechanism of uptake of cisplatin into cells must be characterized. While several previous studies showed structural differences between cisplatin-sensitive and resistant cells, the influence of microfilaments, known to affect transport of molecules into cells, and the influence of certain biophysical characteristics of the plasma membrane needed clarification.
Results We show that resistant human epidermal carcinoma (KB-CP20) and liver carcinoma (BEL-7404-CP20) cells become relatively more resistant if their already weak microfilaments are degraded by cytochalasin B treatment (.5–2 μM). The sensitive counterparts of these cells with intact microfilaments are not significantly affected by this treatment. We also show that the "fluidity" of the plasma membrane and the membrane potential of the sensitive and resistant cells studied do not appear to influence the uptake of cisplatin into the cells.
Conclusion Our results suggest that the status of the microfilament system influences the mechanism of uptake of cisplatin into cells.

Keywords Cisplatin · Electron spin resonance · Cytochalasin B · Carcinoma cell lines · Microfilaments

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Introduction

Cisplatin is a widely used cancer chemotherapeutic drug. However, cellular resistance to this drug can cause treatment failure in cancer patients. To clarify the reason(s) for resistance to cisplatin, its modes of action have been investigated previously. It was shown that resistance could be associated with inhibition of caspase-9 [1], with decreased cisplatin accumulation in resistant cells [2–4] with increased cisplatin-DNA repair [5] and with alteration in plasma membrane lipid-composition [6]. The effect of cisplatin on microfilaments has also been investigated previously. It was observed that cisplatin treatment of mouse stomach carcinoma and human lung cells results in the collapse of microtubules and microfilaments. This indicates a second major mode of action of this drug, besides its action on DNA [7]. Furthermore, it was shown by Shen et al. that disorganization and decreased expression of actin and filamin is characteristic of cisplatin-resistant cells and may contribute to the resistance to cisplatin in at least two cell

lines [8]. In addition, there is a defect in endocytic recycling in cisplatin-resistant cells that is accompanied by alterations in microtubule function [9]. Also, mislocalization of proteins putatively associated with cisplatin binding and transport through the plasma membrane may contribute to carboplatin resistance [10]. All these cellular effects could contribute to cellular resistance to cisplatin. In other studies, plasma membrane alterations induced by cisplatin resistance were characterized by electron spin resonance, fluorescence polarization and membrane potential measurements after insertion of a long chain fatty acid into the plasma membrane of the cells [11].

It has also been shown that treatment of cells with cytochalasin B can inhibit transport of different molecules, such as deoxyglucose, uridine and guanine, into cells [12]. Cytochalasin B is known to disrupt other cellular functions as well. For example, vesicular transport of antigens is affected by cytochalasin B treatment in B lymphoblastoid cells [13]. Cytochalasin B treatment of several cell lines resulted in disruption of the contractile ring and central spindle formation in those cells [14]. Cytochalasin D, also acting on the cytoskeleton, was shown to decrease the uptake of a cisplatin derivative into MCF-7 breast cancer cells by disrupting endocytic processes [15]. Also, cytochalasin B treatment of Ltk-cells interrupted hKv1.5 currents in a concentration dependent manner [16].

We concluded from the above reports that changes in the status of the microfilaments can alter the biophysical status and permeability of the plasma membrane of cells and influence the transport of molecules, such as cisplatin, into cells. For these reasons, we investigated the effect on the microfilaments of sensitive and resistant cancer cells of a concentration of cytochalasin B which minimally affected cell proliferation. We visualized its effects on microfilament organization, determined its effect on cell proliferation, and on accumulation of cisplatin. We also report on the alteration of the membrane's physical status, plasma membrane potential after cytochalasin B treatment of the cells, and the effect of these on cisplatin uptake.

Materials and methods

Cell lines

The KB-3-1 cell line was selected from a single clone of human KB epidermal carcinoma cells after several subclonings, as detailed by Akiyama et al. [17]. The cisplatin-resistant subline of KB-3-1 cells, KB-CP20, was obtained by further exposure of KB-3-1 cells to increasing concentrations of cisplatin, up to 20 μg/ml [18]. These KB-CP20 cells were cultured in the presence of 5 μg/ml cisplatin. The BEL-7404 (7404) is a human liver carcinoma cell line. The highly resistant BEL-7404-CP20 (7404-CP20) cell line was obtained by growing the 7404 cells in increasing concentrations of cisplatin, up to 20 μg/ml and then further grown in the presence of 5 μg/ml cisplatin. The less resistant, single-step KB-CP.5 cell line was also obtained by selection and grown in the presence of 0.5 μg/ml cisplatin [9]. All cell lines were cultured in Dulbeccos's modified Eagle medium (Invitrogen, Grand Island, NY), supplemented with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), and with L-glutamine, penicillin, streptomycin, and in an atmosphere of 5% CO_2 at 37°C.

Cell proliferation studies

Cells were seeded in 24 well plates at conditions as described above for the cell lines and at a density of 2×10^4 cells/ml. After 2 days of incubation, cells were treated with cytochalasin B or not treated and incubated for 3 days. The cytochalasin B concentration varied between 0.5 and 1.0 μM for KB-3-1, 7404 and 7404-CP20 cells and between 1.0 and 2.0 μM for KB-CP20 cells. At harvest time cells had not reached 80–90% confluency. Medium was withdrawn and the cells were trypsinized with 0.2 ml trypsin. Cells were than transferred into isotonic solution and counted on a Coulter Particle Counter (Coulter Electronics, Luton, UK). Relative numbers of cells as compared to the non-treated cells were graphed for comparison. Each result was obtained from 3 to 4 different cell types, each in triplicate. Statistical evaluation was done with the paired Student's *t*-test.

Visualization of the effect of cytochalasin B and cisplatin on the microfilaments of cells

Visualization of the effect of Cytochalasin B on the microfilaments of cells was investigated by confocal microscopy. For this purpose, cells were seeded on microscope cover slides and two days after attachment of the cells to the slides the medium was withdrawn, and the cells were washed with PBS. They were then treated with 70% ethanol for 15 min for permabilization of the cell membrane. Cells were washed with PBS and then incubated with rhodamine phalloidin (Molecular Probes, Eugene, OR) 5 units/ml in 0.5% bovine serum albumin/PBS, for 30 min at room temperature. Phalloidin binds to polymerized but not to depolymerized F-actin [19]. The incubation was followed by three washings with PBS and then confocal microscopy. At least ten images were scanned, each image containing several cells. Average fluorescence intensity of the scanned cells was plotted on graphs for each of the cell lines.

Confocal microscopy was performed using a Zeiss LSM 510 NLO confocal system (Carl Zeiss Inc., Thornwood, NY) with an Axiovert 200M inverted microscope and a 1 mW HeNe laser tuned to 543 nm as well as the 2-photon

laser tuned to 760 nm. Cells were imaged with a 63 × 1.4 NA Zeiss Plan-Apochromat oil immersion objective. Using Zeiss AIM software, cells were scanned using a multi-track configuration where the Rhodamine and DAPI signals were sequentially collected with a BP 565–615 nm filter and with a BP 390–465 nm filter after sequential excitation with the 543 and 760 nm laser lines, respectively.

In another experiment, cells were treated with 20 μg/ml cisplatin for 90 min and stained with rhodamin–phalloidin as above for determination of the effect of cisplatin on the microfilaments in the KB and 7404 cells.

Cisplatin determination, as measured by platinum content, in cytochalasin B-treated and non-treated cells

For platinum (Pt) determinations, cells were grown as described above and treated or not treated with a concentration of cytochalasin B, as detailed for each cell line in the section above on proliferation studies. After 24 h of cytochalasin B treatment, cells were exposed to 50 μg/ml cisplatin for 4 h in the culture flasks. After washing three times with cold PBS, cells were trypsinized and counted using a hemacytometer. Cells were then pelleted in a microfuge and then immediately frozen to avoid efflux of cisplatin from the cells. Known numbers of cells were then submitted for Pt determination (Galbraith Laboratories Inc., Knoxville, TN), done essentially as described by Johnson et al. [5], except that Pt content of whole cells was measured and not of isolated DNA.

Electron spin resonance determination of motional freedom of a lipid probe in plasma membranes of cells

A lipid probe, 5-doxyl stearic acid (5-dox-SA) was used to determine the motional freedom ("fluidity") of this molecule in plasma membranes. The free radical of 5-dox-SA indicates the movement of the probe at five-carbon depth in the membranes. Labeling the cells with the probe was done as detailed earlier [20]. In brief, a cell suspension of 10^7 cells in 40 μl PBS was mixed with 8×10^{-8} mol spin label of ethanolic solution and incubated for 1 min. The cell suspension was aspirated into a 50 μl micropipette (Syva Co., Palo Alto, CA) and was placed into the cavity of a Varian E-9 Century series spectrometer (Paulo Alto, CA). The temperature of the cavity was set to 24°C by a variable temperature accessory, using N_2 gas. ESR spectra were recorded at X-band, at 9.5 kHz, 4 G modulation amplitude, 100 G sweep range and 15 mW microwave power. Evaluation of the recorded ESR spectra was by the equation expressing the Order Parameter (S):

$S = 0.5407\ (T_{11} - T_1)/a_0$, where $a_0 = (T_{11} + T_1)/3$, and T_{11} and T_1 are the outer and inner tensors obtained from the ESR spectra. The spin label in the ESR spectra showed contribution from the restricted motion of the spin label, with no contribution from any free moving spin label. In these experiments, all cells were treated with 1 μM cytochalasin B, 24 h before harvest and ESR measurements. These cytochalasin B concentrations are the same as in the cell proliferation studies. Statistical evaluation was done with the paired Student's *t*-test.

Membrane potential measurements of cytochalasin B treated and non-treated cells

Membrane potential measurements were carried out essentially as described earlier by Aleman et al. [21]. In brief, 10^6 cells in 0.5 ml PBS were equilibrated for 1 min followed by equilibration with 150 nM $DiBaC_4$ (3) oxonol dye (Molecular Probes, Eugene, OR) for 2 min at room temperature. Fluorescence data were collected from 10^4 cells on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The cytometer was operated with a 15-mW argon ion laser tuned to 488 nm excitation wavelength and emission was collected at 530 nm. Results are expressed as median fluorescent intensities of three measurements each with individual cultures. Statistical evaluation was done with the paired Student's *t*-test.

Results

Cell proliferation studies

Cell proliferation studies were performed as described in Materials and Methods. Results indicated that KB-3-1 and 7404 cells, the cisplatin-sensitive cells, are not significantly affected by treatment with various concentrations of cytochalasin B and cisplatin (Fig. 1a,c). Similarly, the slightly resistant KB-CP.5 cells did not show synergy with a combined treatment of cytochalasin B and cisplatin (not shown). In contrast, the level of resistance of the highly resistant KB-CP20 and 7404-CP20 cells was affected by treatment with the various concentrations and combinations of cytochalasin B and the appropriate elevated concentrations of cisplatin (Fig. 1b,d). Both cell lines grew better than predicted after cytochalasin B treatment. These cell proliferation results correlate with the decreased amount of microfilaments in the highly resistant but not in the sensitive cells, as detected by confocal microscopy (Fig. 2).

Confocal microscopic visualization of the effect of cytochalasin B and cisplatin on the microfilaments of cells

Cells were treated or not treated with the same concentration of cytochalsin B as applied in the cell proliferation

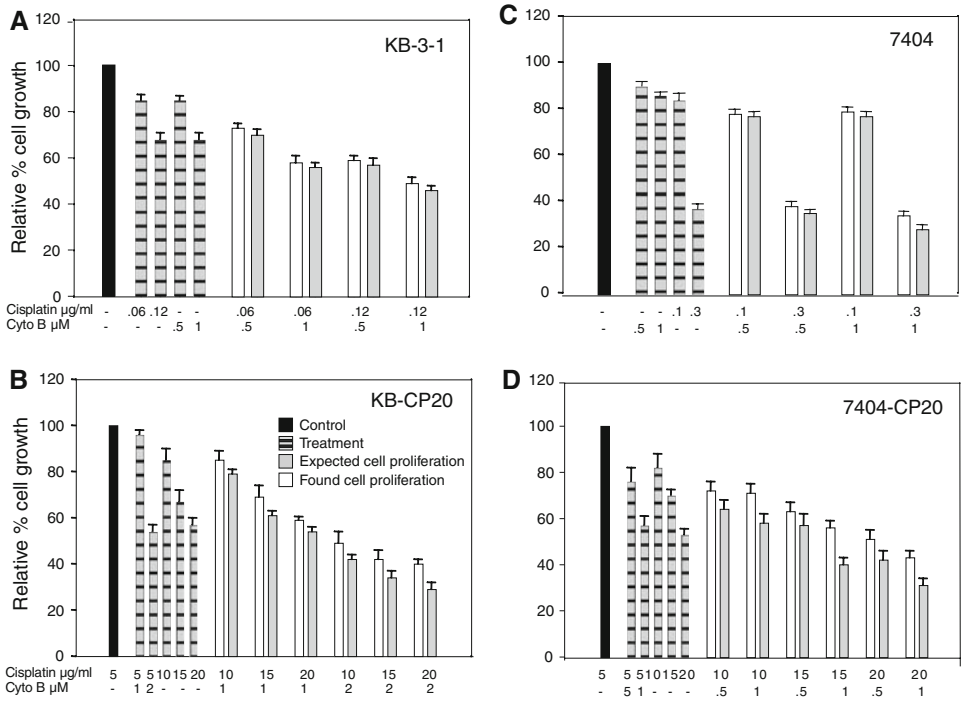

Fig. 1 Cell proliferation studies. Done as described in Materials and Methods. In each panel, cells without treatment (*black bars*), controls with individual treatments (*striped bars*), expected cell proliferation (*gray bars*) and found cell proliferation (*white bars*). Expected cell proliferation: cell count of cells treated with cisplatin alone × cell count treated with cytochalasin B alone. Each proliferation result was calculated from 3 to 4 independent cultures, each in triplicate. Differences are significant for the 7404-CP20 and the KB-CP20 cells ($P < 0.05$) (**b**, **d**), but not significant for the sensitive, KB-3-1 and 7404 cells ($P > 0.05$) (**a**, **c**)

studies and incubated with rhodamine–phalloidin, as described in Materials and Methods. Fig. 2a shows confocal images (presented in black and white) comparing cytochalasin B-treated and non-treated cells. Cytochalasin B-treated KB-CP20 and 7404-CP20 cells showed significantly less cytoskeleton staining than the non-treated ones. In contrast, the staining intensity of the cytoskeleton is about the same in the sensitive KB-3-1 and 7404 cells. The average fluorescence intensities of 8–12 fields, each with several cells and stained are shown in Fig. 3.

Because of a previous report that cisplatin itself depolymerizes the cytoskeleton [7], we determined the effect of a high concentration of cisplatin, 20 µg/ml, on the microfilaments by confocal microscopy. This amount of cisplatin was higher than that used by Kopf–Maier and Mulhausen (0.75–30 µg/l). Figure 2b shows the confocal images, presented in black and white. Figure 4 shows the average observed intensities, which quantitate that cisplatin treatment had no effect on the microfilaments in these cells.

Determination of cisplatin as measured by Pt content in cytochalasin B treated and non-treated cells

Determination of Pt in cells was done essentially as described previously [5] and in Materials and Methods. Results are shown in Table 1, and indicate that cisplatin-resistant cells accumulated less cisplatin after cytochalasin B treatment than the cytochalasin B non-treated resistant cells. Results also show that cisplatin accumulates 4–5 times more in sensitive cells than in the resistant ones. There is a relatively large average difference (41.2%) in cisplatin accumulation in resistant cells after cytochalasin B treatment, while in sensitive cells the average relative difference is less (9.2%).

These results are consistent with the notion that cytochalasin B treatment affects the rate of cisplatin accumulation, which in turn influences cell proliferation.

Motional freedom of 5-dox-SA spin-labeled probe in the plasma membrane of cytochalasin B-treated and non-treated cells

Membrane "fluidity" is affected in all four cell lines by cytochalasin B treatment. Results in Table 2 indicate that the Order Parameter, *S*, calculated as described in Materials and Methods, is significantly lowered for all cell lines. They became more "fluid". A lower *S* value indicates more "fluidity" in membranes, on a scale of 0–1.0.

Membrane potentials of cytochalasin B-treated and non-treated cells

Figure 5 shows that cells treated with cytochalasin B, for 1 or 24 h before measurements, have higher plasma membrane potential than non-treated cells. The fluorescence values of the treated cells are lower than those of the untreated cells. The higher the membrane potential (cells are more negative) the less negatively charged oxonol dye diffuses into the cells. Cytochalasin B treatment was at the same doses as used in the cell proliferation studies, i.e., 1 µM for

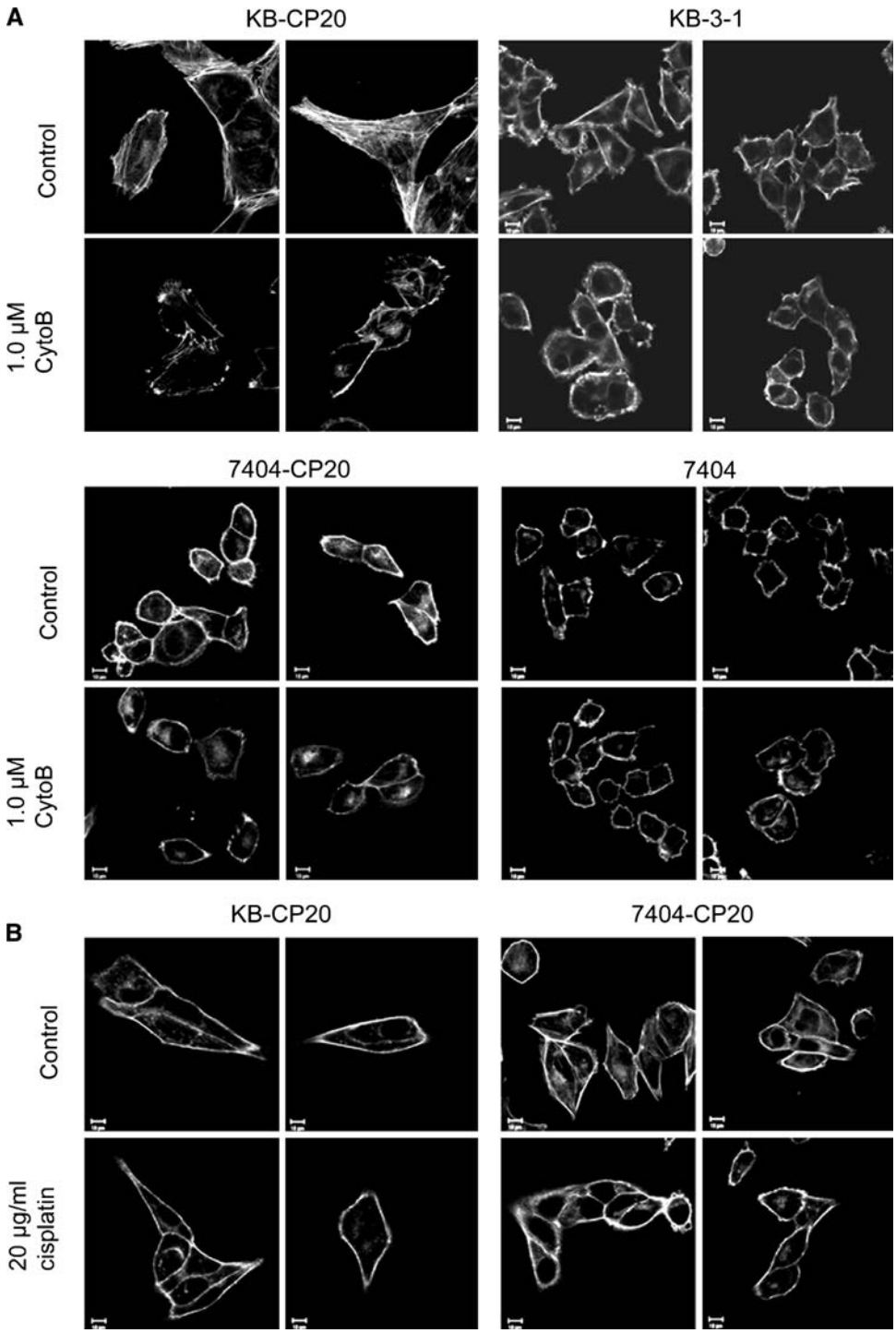

Fig. 2 Confocal images (*presented in black and white*). **a** Cells treated or not treated with 1 µM cytochalasin B and grown as described in Materials and Methods. This concentration was chosen in order to apply one of the same concentrations as was applied in the cell proliferation assays. **b** KB-CP20 and 7404-CP20 cells treated or not treated with 20 µg/ml cisplatin. Treatment of cells with cisplatin for 4 h and subsequent staining with rhodamin–phalloidin are described in Materials and Methods

KB-3-1, 7404 and 7404-CP20 cells and 2.0 µM for KB-CP20 cells.

Discussion

Our investigation aimed to determine the effect of disruption of the microfilaments by cytochalasin B on cisplatin accumulation in cells. We studied the cisplatin sensitivity of the cells after cytochalasin B treatment, accumulation of cisplatin in the cells, as well as the affected membrane biophysical changes.

First we determined the effect of a certain concentrations of cytochalasin B which minimally affected cell proliferation in both sensitive and resistant cells (0.5, 1.0 and 2.0 µM). Upon application of these concentrations, resistant cells became relatively more resistant, while not much change could be observed with the sensitive cells (Fig. 1).

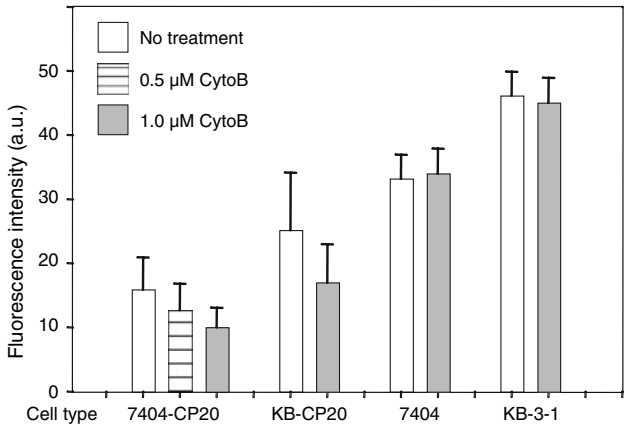

Fig. 3 Average fluorescence intensities of cytoskeletons of cells in 8–12 images, each with several cells, treated or not treated with cytochalasin B and stained with rhodamin–phalloidin and scanned by confocal microscopy. Treatment of cells with cytochalasin B and the subsequent staining with rhodamine–phalloidine is described in Materials and Methods. There is no significant difference in the fluorescence intensities between 1 μM cytochalasin B-treated and non-treated KB-3-1 and 7404 cells ($P > 0.05$). The difference is significant between 1.0 μM cytochalasin B-treated and non-treated 7404-CP20 and KB-CP20 cells ($P < 0.05$)

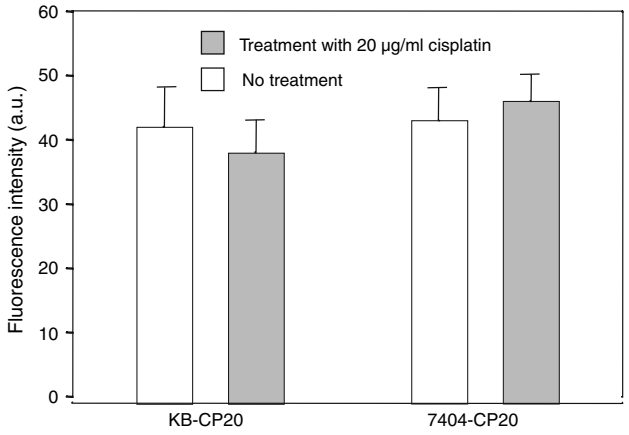

Fig. 4 Relative average fluorescence intensities of cytoskeletons by confocal microscopy of cells treated or not treated with cisplatin and stained with rhodamin–phalloidin. Treatment of cells with cisplain for four hours and subsequent staining with rhodamine–phalloidin are described in Materials and Methods. Fluorescence intensities were collected from 8 to 10 cells and average fluorescence was expressed as described in Materials and Methods. Differences are not significant between cisplatin-treated and non-treated cells for both types of cells, $P > 0.05$

We attribute these differences to the reported fact that resistant KB-CP20 and 7404-CP20 cells have disorganized and decreased expression of actin and filamin. Our current results reinforce those findings [8]. The applied cytochalasin B concentration had a greater effect on the resistant cells than the sensitive ones. Sensitive cells have more intact microfilaments even after treatment with such concentrations of cytochalasin B. It should be noted that cytochalasin

Table 1 Cisplatin uptake, as determined by Pt content of cytochalasin B-treated and non-treated cells

Cells	Treatment with cytochalasin B	Pt content[a], μg/10^6 cells	
		Exp. 1	Exp. 2
KB-3-1	None	106	107
	CytoB, 1 μM	89	104
KB-CP20	None	31.2	22.7
	CytoB, 2 μM	26.4	16.0
7404	None	90	–
	CytoB, 1 μM	82	–
7404-CP20	None	31	54
	CytoB, 1 μM	15	20

[a] The error limit in determination of Pt content in cells was usually 10% of the lowest Pt value in any group of samples. Numbers in each column represent treated and untreated samples from the same culture of cells and at the same time

Table 2 Motional freedom, represented by the order parameter, S[a], of 5-doxyl-SA ESR probe inserted in the plasma membranes of the sensitive KB-3-1 and cisplatin-resistant KB-CP20 cells

Cell line	S[a]	±SD
KB-3-1	0.63081	0.0011
KB-3-1+CytoB	0.60280	0.0008
KB-CP20	0.63165	0.0012
KB-CP20+CytoB	0.59210	0.0013
7404	0.62944	0.0007
7404+CytoB	0.60077	0.0010
7404-CP20	0.62804	0.0009
7404-CP20+CytoB	0.61497	0.0014

[a] Calculations of the order parameter S, and treatment of cells with cytochalasin B are given in Materials and Methods. Each S value represents average of three independent measurements. ESR measurements were made at 22°C. Cells were treated with cytochalasin B, as in the cell proliferation assays. $P < 0.05$ between the S values of cytochalasin B-treated and non-treated cells

B treatment (0.5 μM) of KB-CP.5 cells, resistant only to 0.5 μg/ml cisplatin, did not result in increased resistance after 0.8 or 0.9 μg/ml cisplatin exposure (not shown).

The proliferation results are supported by confocal images. Images and summarized fluorescence data of the rhodamine–phalloidin stained cells are shown in Figs. 2 and 3, respectively. Fluorescence images indicate that the applied cytochalasin B concentration disrupted the microfilaments in resistant cells but had a minimal effect on the microfilaments of the sensitive cells. Since it has been reported that transport even of small molecules can be affected by cytochalasin B treatment of cells [12,13], disruption of the weak microfilaments in resistant KB-CP20 and 7404-CP20 cells could reduce the accumulation of cisplatin, perhaps by aggravating the protein mislocalization defect in these cell lines [22].

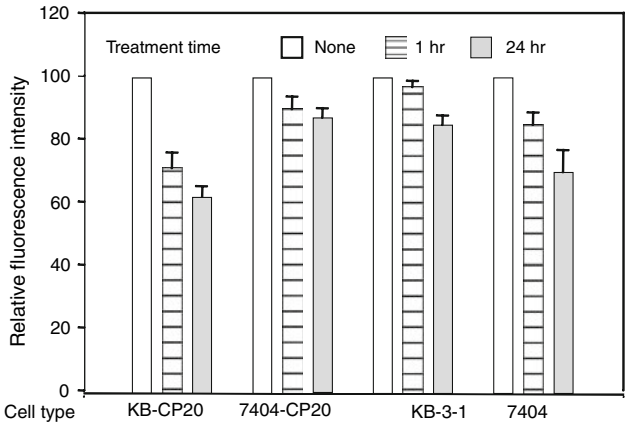

Fig. 5 Relative membrane potential of cytochalasin B-treated and non-treated cells as expressed by the relative fluorescence intensity. Relative membrane potentials were determined as described in Materials and Methods. Lower fluorescence is due to less oxonol dye in cells, indicating more negative cells, hyperpolarization. Results are the average of three measurements. $P < 0.05$ is between cytochalasin-treated and non-treated cells. Cytochalasin B treatment of the cells was 1.0 μM for KB-3-1, 7404 and 7404-CP20 cells and 2.0 μM for KB-CP20 cells, as in cell proliferation studies. Statistical evaluation was with the paired Student's *t*-test

It was also reported previously that cisplatin could disrupt the cytoskeleton of tumor cells [7]. Therefore, we sought to determine whether the maximal amount of cisplatin used in our study would affect the microfilaments in the resistant cells studied, which showed diminished microfilaments in the presence of cytochalasin B. The results shown in Figs. 2b and 4 indicate that 20 μg/ml cisplatin did not result in disruption of the microfilaments, as determined by rhodamine–phalloidin staining and confocal microscopy. We can conclude that disruption of the microfilaments in the resistant cells studied is due to the applied cytochalasin B treatment.

Our above arguments on reduced cisplatin accumulation in resistant cells after cytochalasin B treatment are supported by Pt determinations. The sensitive cells, KB-3-1 and 7404, accumulate more cisplatin, determined as Pt, than resistant cells. Furthermore, the cytochalasin B treatment of resistant KB-CP20 and 7404-CP20 cells led to decreased cisplatin accumulation, and this decrease is consistent with an increase in relative resistance in these cells (Table 1, Fig. 1).

There are several reports indicating that cisplatin is transferred into cells by a transport mechanism and not by diffusion or charge related uptake. Shen at al. [23] reported that carboplatin is taken up into cells by an energy dependent mechanism, and Liang et al. [10] reported that a defect in the endocytic apparatus in KB cells could be part of the reason for cisplatin resistance. Furthermore, association between the actin cytoskeleton and endocytosis was shown to exist by Qualmann et al. [24] and by Schafer [25]. It is also interesting that it was shown by Liang et al. [11] that resistant KB-CP20 cells have more "fluid" membranes than the sensitive KB-3-1 cells, as determined by ESR spectrometry and by the fluorescence polarization technique. These reports stimulated us to investigate how treatment with cytochalasin B influences these biophysical parameters in our studied cells. A question arose concerning certain changes in these parameters. Would these changes influence cisplatin uptake into the cells and therefore influence cell resistance?

Based on the above background, we investigated cytochalasin B-affected biophysical changes, membrane fluidity and membrane potential by ESR spectrometry and by mesasuring the relative membrane potential in treated and non-treated cells. We used the ESR probe 5-doxyl-SA, which reports at the 5 carbon level in the membrane-inserted SA, and found that the sensitive and resistant cells showed significantly more fluid membrane after cytochalasin B treatment (Table 2). Since Pt accumulation assays indicate significantly less cisplatin accumulation in the resistant cells than in sensitive cells after cytochalasin B treatment, but both types of cells became more fluid, we concluded that the biophysical changes in the plasma membranes appear not to influence cisplatin accumulation in cells. This is in accordance with the findings of Shen et al. [23] as discussed above.

Similar conclusions could be drawn from membrane potential measurements. Cytochalasin B-treated cells have more negative membrane potential than the non-treated ones (Fig. 5). This finding is supported by that of Choi et al. [16], who showed that cytochalasins could block ion channels. We should mention that the membrane potential was higher for the resistant KB-CP20 and 7404-CP20 cells than that of their sensitive counterparts (not shown). This is in agreement with previous finding that drug-resistant phenotypes have higher membrane potential than drug-sensitive cells [21,26]. Cisplatin has a slight positive charge on its N atoms. Despite this fact, less cisplatin accumulates in the resistant KB-CP20 and 7404-CP20 cells. We conclude that plasma membrane potential does not appear to significantly influence cisplatin accumulation in cells, in agreement with the finding of Liang et al. [11].

Taken together, these results show that treatment with the applied concentrations of cytochalasin B affects the resistance of the cisplatin-resistant KB-CP20 and 7404-CP20 cells by reduced accumulation of cisplatin in these cells. The same level of cytochalasin B treatment does not significantly affect the sensitive KB-3-1 and 7404 cells, which have a more intact cytoskeleton structure. We could also show that alterations in biophysical parameters, membrane "fluidity" and plasma membrane potential do not noticeably affect cisplatin accumulation in cells, in agreement with previous studies. Our results augment the find-

ings of Shen [23], Plageman et al. [12] and Liang et al. [9] and indicate that the conditions of the microfilaments play a role in determining cisplatin accumulation in resistant cells with an already altered microtubule system.

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