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Dinuclear Alkylamine Platinum(II) Complexes of [1,2-Bis(4-fluorophenyl)ethylenediamine]-platinum(II): Influence of Endocytosis and Copper and Organic Cation Transport Systems on Cellular Uptake

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Various possible pathways for the uptake of cationic alkylamine platinum(II) complexes into the MCF-7 breast cancer cells were studied with di[meso-1,2-bis(4-fluorophenyl)ethylenediamine]di-[sulfinylbis(methane)-S][μ-1,6-diaminohexaneN:N']diplatinum(II) disulfate (m-4F-PtDMSO-DAH) as an example. It was demonstrated that m-4F-PtDMSO-DAH competed neither for the copper transporter nor the organic cation transporters (OCT and OATP). Instead, adsorptive endocytosis by macropinocytosis played an essential role. Inhibitors of this processes such as amiloride, N-ethyl-N-isopropylamiloride (EIPA), wortmannin, and cytochalasin D decreased the intracellular uptake of m-4F-PtDMSO-DAH dramatically. These results support the understanding of the pharmacological behavior of this promising drug family, which showed no cross resistance with cisplatin.

Introduction

In recent years, polynuclear platinum complexes have been identified as a very promising class of antitumor compounds. They show a different toxicity profile from that of cisplatin[1] and a slightly different mode of action; they cross-link DNA in a 1→4 instead of a 1→2 GG pattern, as is the case with cisplatin and carboplatin. The binding to DNA occurs in two steps: a rapid electrostatic interaction with the DNA backbone due to the positive charge of the molecule followed by coordination of DNA bases at the platinum center.[2]

Prior to DNA binding, however, the complexes have to cross the cell membrane. It is assumed that cisplatin reaches the cytoplasm mainly by a passive transport process. The high chloride concentration in the blood (or in the media used for in vitro experiments) hinders hydrolysis into charged monoaqua- or diaquaplatinum(II) species, so cisplatin passes through the cell membrane as an uncharged molecule. It has recently become clear that cation transporters are also involved in the uptake of platinum complexes into tumor cells.

Most of the polynuclear complexes investigated so far can reach the cytoplasm of tumor cells despite their ionic character. The trinuclear platinum complex BBR3464 ([μ-*trans*-Pt-$(NH_3)_2$\{*trans*-PtCl$(NH_3)_2$[$NH_2(CH_2)_6NH_2$]\}$_2$]$(NO_3)_4$), for example, studied in a human osteosarcoma cell system (U2-OS), showed a higher accumulation than cisplatin. Although the transport mechanism of platinum drugs has been the subject of extensive research, the knowledge about the transfer of cationic platinum drugs into the cytoplasm is still limited.

Recently, we published investigations on the cytotoxicity, cellular distribution and DNA binding in MCF-7 cells, and protein interaction of the dinuclear di[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]di[sulfinylbis(methane)-*S*][μ-1,6-diaminohexane-*N*:*N'*]diplatinum(II) disulfate complex (*m*-4F-PtDMSO-DAH, Figure 1). Despite its ionic character, the extent of *m*-4F-PtDMSO-DAH accumulation was 10-fold higher than that of cisplatin, and was linearly dependent on extracellular concentration.[3] The nature of its transport through the cell membrane is unknown. Recently, the copper transport[4,5] and organic cation transporter[6–8] systems were discussed for their involvement in the uptake of platinum drugs. Furthermore, endocytosis was considered as an alternative possibility for cellular uptake. Therefore, we investigated the significance of these pathways for the internalization of cationic dinuclear platinum complexes in human MCF-7 breast cancer cells, using *m*-4F-PtDMSO-DAH as the example compound.

Results

In a recently published report, we demonstrated that dinuclear di[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine][μ-1,*n*-diaminoalkane-*N*:*N'*]diplatinum(II) complexes ($n = 6$, 9, 12) bind strongly to human serum albumin.[3] These inactivation reactions prevented the uptake into tumor cells and thus cytotoxic effects. In serum-free media, a high degree of accumulation in MCF-7 breast cancer cells was observed as well as high DNA binding. In continuation of this study, we tried to identify the transport-

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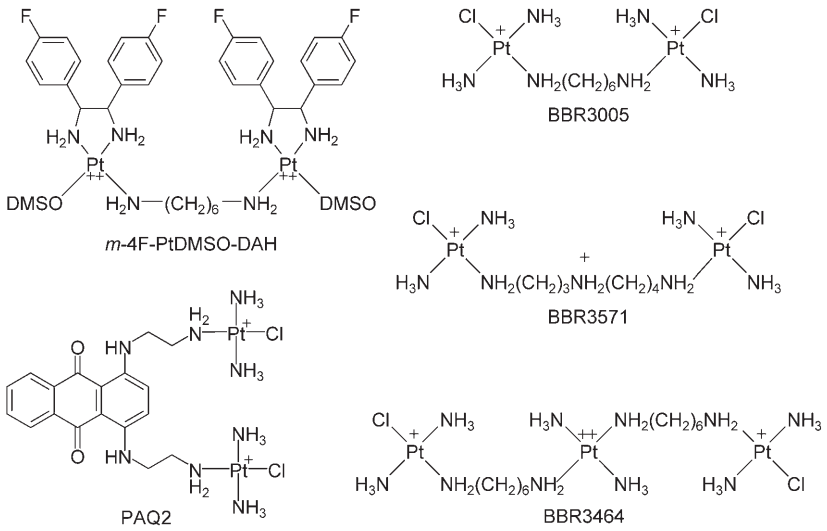

Figure 1. The structure of *m*-4F-PtDMSO-DAH and related compounds; counter ions are not shown.

ganic cation transport system is involved in the transfer of this compound, as was previously suggested for [{*trans*-PtCl-$(NH_3)_2$}$_2$($H_2N(CH_2)_6NH_2$)]$^{2+}$ (BBR3005,[8] Figure 1) and cisplatin. Cimetidine as an inhibitor or tetraethylammonium chloride (TEA) as a competing substrate decreased the accumulation of cisplatin in renal cell lines significantly.[6,7] Therefore, it was of great interest to evaluate whether the uptake of *m*-4F-PtDMSO-DAH and cisplatin in MCF-7 breast cancer cells is influenced by cimetidine or TEA. Interestingly, neither compound influenced the uptake of cisplatin into tumor cells (Figure 3 A), in contrast to the effects determined in renal cells. On the other hand, TEA decreased the intracellular platinum content caused by *m*-4F-PtDMSO-DAH by ≈20%, whereas cimetidine showed no effect (Figure 3 B).

ers which might be involved in the transfer of these cationic species through the cell membrane. We used selective inhibitors of various transporters and determined the amount of intracellular platinum with graphite furnace atomic absorption spectroscopy (GF-AAS).

We initially studied the involvement of the copper transporter, as it has been demonstrated that it is responsible for both the decreased influx[4,5] and elevated efflux of cisplatin.[9–11] The addition of 100 μM copper sulfate to the cell-culture medium strongly decreased the cellular uptake of cisplatin in yeast.[4] Thus, we incubated MCF-7 cells with a constant concentration of cisplatin (80 μM) or *m*-4F-PtDMSO-DAH (20 μM), and varied the copper concentration from 0 to 200 μM (Figure 2). The data clearly show that the copper transporter is not involved in the uptake of either compound. The intracellular platinum concentrations remained unchanged.

An intracellular copper concentration of ≈5.5 ng mL^{-1} was measured for MCF-7 cells incubated for 2 h with 200 μM $CuSO_4$. Co-incubation with enhanced concentrations of cisplatin induced an accumulation of copper and indicates that cisplatin competes with Cu^{2+} for the efflux transporter. This effect was not observed with *m*-4F-PtDMSO-DAH. Therefore, the participation of the copper transporter in the transfer of *m*-4F-PtDMSO-DAH through the cell membrane could be excluded.

The highly hydrophobic groups of *m*-4F-PtDMSO-DAH make it quite likely that the organic cation transport system is involved in the transfer of this compound, as was previously suggested for [{*trans*-PtCl-$(NH_3)_2$}$_2$($H_2N(CH_2)_6NH_2$)]$^{2+}$ (BBR3005,[8] Figure 1) and cisplatin. Cimetidine as an inhibitor or tetraethylammonium chloride (TEA) as a competing substrate decreased the accumulation of cisplatin in renal cell lines significantly.[6,7] Therefore, it was of great interest to evaluate whether the uptake of *m*-4F-PtDMSO-DAH and cisplatin in MCF-7 breast cancer cells is influenced by cimetidine or TEA. Interestingly, neither compound influenced the uptake of cisplatin into tumor cells (Figure 3 A), in contrast to the effects determined in renal cells. On the other hand, TEA decreased the intracellular platinum content caused by *m*-4F-PtDMSO-DAH by ≈20%, whereas cimetidine showed no effect (Figure 3 B).

As a further transport system, the organic anion transporting polypeptides (OATP) may be involved in the uptake of platinum complexes, because they also modulate the uptake of bulky amphipathic cations in several tumor cell lines.[12,13] Hence we incubated MCF-7 cells with *m*-4F-PtDMSO-DAH and co-administered the OATP inhibitors glycocholate or ouabain.[12] In this experiment (Figure 3 B), glycocholate did not influence the cellular platinum content, whereas ouabain decreased the cellular accumulation of *m*-4F-PtDMSO-DAH by ≈40%. However, it cannot be excluded that the effect observed with ouabain may be independent of the OATP path-

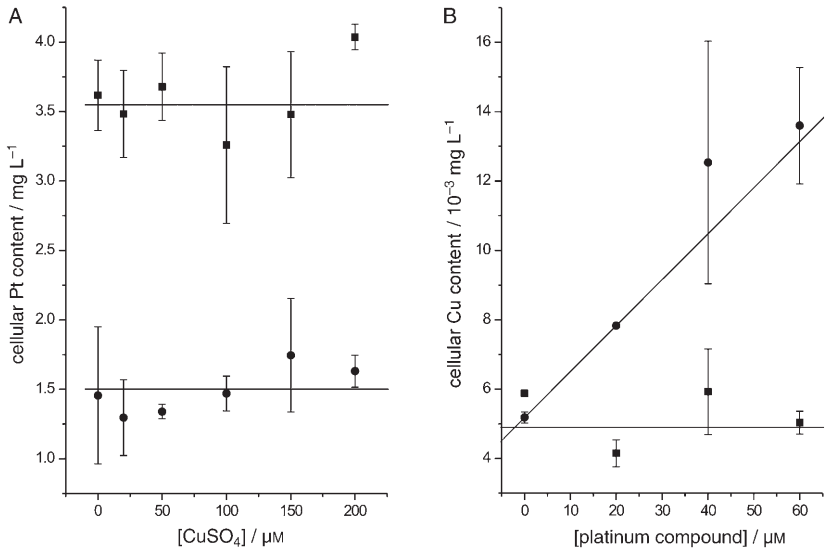

Figure 2. A) The influence of medium copper concentration on the cellular uptake of cisplatin (●) and *m*-4F-PtDMSO-DAH (■) into MCF-7 cells; B) the influence of the concentration of cisplatin (●) and *m*-4F-PtDMSO-DAH (■) in the medium on the copper content in MCF-7 cells ($CuSO_4$ concentration in medium: 200 μM).

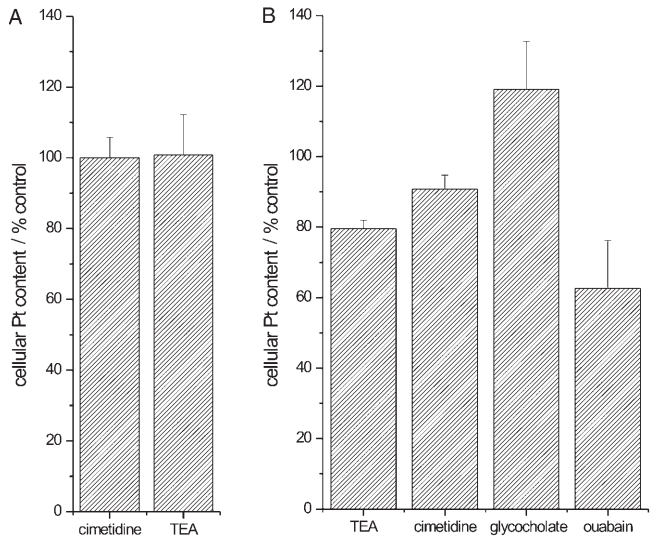

Figure 3. The influence of the organic cation transport system on the cellular uptake of A) cisplatin and B) *m*-4F-PtDMSO-DAH in MCF-7 cells.

way. It has been reported[14,15] that some endocytotic processes require a sodium gradient, and ouabain is also an inhibitor of the Na–K ATPase. Therefore, ouabain can also act through this pathway.

Adsorptive endocytosis had to be considered as a third possible transport mechanism for polynuclear compounds. Indeed, the adsorption of positively charged molecules at the negatively charged surface of the cell membrane seems very likely.[16] Such electrostatic cell contacts can be assumed for *m*-4F-PtDMSO-DAH because such interactions were already demonstrated with human serum albumin in our previous studies.[3] In this case, the decreased cellular platinum content caused by the co-administration of TEA (see above) could be explained by competition for binding sites at the membrane. To verify this assumption, we incubated MCF-7 cells with *m*-4F-PtDMSO-DAH (20 μM) for 1 min and 120 min at 37 °C and observed a very fast electrostatic binding at the cell surface. After a drug–cell contact time of 1 min the cells contained ≈0.50 mg L^{-1} platinum, presumably electrostatically bound.

Subsequent internalization into the cells was strongly energy dependent, because a decrease in the incubation temperature from 37 to 4 °C was accompanied by a lowering of the cellular platinum concentration from 4.9 to 1.87 mg L^{-1} (incubation time, 120 min).

For the subsequent internalization, various endocytotic pathways have to be discussed. It is well known[17,18] that macropinocytotic processes, a distinct form of endocytosis, depend on a sodium gradient and can be blocked by amiloride and *N*-ethyl-*N*-isopropylamiloride (EIPA) as inhibitors of a Na^+–H^+ exchanger.[14,18] Furthermore, as macropinocytosis is dependent on F-actin microfilament rearrangement, the inhibition of F-actin elongation by cytochalasin D, and the wortmannin-mediated inhibition of phosphoinositide 3-kinase (a key enzyme in the downstream signaling of macropinocytosis) blocked macropinocytosis efficiently.[15,17,19,20]

We therefore pre-incubated MCF-7 cells with these inhibitors for 15 min and subsequently co-incubated the cells with *m*-4F-PtDMSO-DAH and the inhibitor for 2 h. The cellular platinum content of the inhibitor-treated groups were then compared with an inhibitor-free control. As depicted in Figure 4, the inhibitors influenced the uptake of *m*-4F-PtDMSO-DAH. Amiloride decreased cellular platinum content by ≈70%, whereas EIPA (decrease: 40%), cytochalasin D (decrease: 46%), and wortmannin (decrease: 37%) were less efficient. This demonstrates the strong influence of macropinocytosis on the cellular uptake of polynuclear platinum drugs.

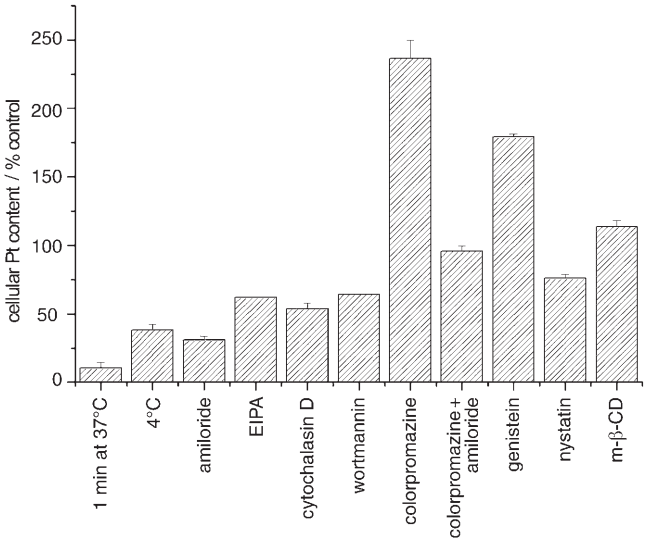

Figure 4. The effect of the inhibition of endocytosis by various inhibitors on the uptake of *m*-4F-PtDMSO-DAH in MCF-7 cells.

In a further experiment, we tried to block clathrin-mediated endocytosis with chlorpromazine.[17,21,22] However, a strong increase of cellular platinum content (230%) was observed in experiments with chlorpromazine. This could be a consequence of the upregulation of clathrin-independent pathways.[23] Co-incubation of *m*-4F-PtDMSO-DAH with chlorpromazine and amiloride led to a strong decrease in platinum accumulation (230→92% of control). This indicates that macropinocytosis is the primary upregulated endocytotic pathway after clathrin-dependent endocytosis has been blocked.

A further well-characterized pathway is caveolae-mediated endocytosis. It is inhibited by methyl-β-cyclodextrin (m-β-CD), nystatin, and genistein.[15,20–22] The use of these inhibitors in our experiments led to contradictory results (Figure 4). Whereas genistein-mediated blockade of tyrosine kinases necessary for this type of endocytosis resulted in an increase in cellular platinum content (180% of control), nystatin, which depletes cholesterol from the membranes, decreased the platinum content slightly (80% of control). No effect was observed with m-β-CD, which extracts cholesterol from membranes. These results indicate that caveolae-mediated endocytosis plays only a minor role, if any role at all, in the cellular uptake of *m*-4F-PtDMSO-DAH. The high accumulation induced by genistein

can be explained again by the upregulation of other endocytosis routes.

Discussion

Dinuclear and polynuclear cationic platinum complexes represent a new and promising class of anticancer drugs. Good examples of this are complexes with the polyamines spermine and spermidine and the trinuclear complex BBR3464, which are currently in clinical trials. It is believed that the ability of these compounds to overcome cisplatin resistance is the result of the structurally different DNA adducts that they generate. However, uptake and intracellular transport also play an important role in the mechanism of action of platinum-based drugs and in the mechanism of resistance.

Dinuclear platinum complexes modified with a fluorescent or fluorogenic reporter were used to get further insight into cellular distribution. The dinuclear platinum complex PAQ2 (Figure 1) was shown to quickly enter cisplatin-sensitive A2780 and cisplatin-resistant A2780cisR ovarian carcinoma cells. In the A2780cisR cell line, the platinum complex is sequestered into lysosomes, which is not the case in A2780 cells.[24] Lysosomes of A2780cisR cells are less acidic than those of the sensitive A2780 cells. As lysosomal acidification is essential for regular endocytosis,[25] its failure might indicate a general defect in endocytosis. It is possible that this defect partly confers the cross-resistance of these complexes with cisplatin in the A2780cisR cell line. Furthermore, the lysosomal localization of PAQ2 was also documented for U2-OS human osteosarcoma cells.[26] Rapid internalization was also observed in the same cell line with 1,1/c,c/CFDA, a complex with the same geometry as *m*-4F-PtDMSO-DAH, but equipped with a fluorescein fluorophore in the ethylenediamine ligand. The fluorescence was quickly concentrated in the golgi complex. Similar results were also obtained with complexes with different geometries at the platinum center, and with other fluorophores.[27] Therefore, we suggested this class of compound undergoes endosomal intracellular uptake.

To verify this assumption, we included the endocytotic pathway in this study on the mode of internalization of *m*-4F-PtDMSO-DAH into MCF-7 cells. Results of initial experiments excluded the participation of the copper transporter and the organic cation transporters (OCT and OATP) in this process. We then inhibited endocytosis by using amiloride, EIPA, wortmannin, and cytochalasin D and observed a decrease in the intracellular uptake of *m*-4F-PtDMSO-DAH. An exact analysis of the data presented in Figure 4 indicates that particularly macropinocytotic processes are involved in the transport of *m*-4F-PtDMSO-DAH and related complexes into tumor cells.

Although the results presented herein were verified only for di[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine][μ-1,*n*-diaminoalkane-*N*:*N*′]diplatinum(II) complexes, endocytosis might also be involved in the mode of action of other alkylamine platinum(II) complexes such as BBR3464, BBR3571, and BBR3005 (Figure 1). These compounds showed charge-dependent accumulation in L1210 murine leukemia cells.[8,28] BBR3464 behaved similarly to *m*-4F-PtDMSO-DAH in cell-culture experiments. It showed a strong initial binding to the cell surface[29] and a linear, non-saturable accumulation in tumor cells.[30] However, further experiments are necessary to confirm the participation of adsorptive macropinocytosis on the internalization of this kind of complex.

Conclusions

The study presented herein shows that endocytotic processes are strongly involved in the uptake of polynuclear alkylamine platinum(II) complexes. Macropinocytosis was the main uptake route for *m*-4F-PtDMSO-DAH. This type of endocytosis is only expressed for a short time in normal cells, but in cancer cells it is responsible for increased motility and metastasis.[19] Therefore, cancer cells may be targeted more selectively by polynuclear platinum complexes than by conventional drugs such as cisplatin.

Experimental Section

General: Chemicals were purchased from Sigma and Fluka. Drugs were freshly prepared as stock solutions in *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), or H_2O, and diluted with cell-culture media before use in biochemical experiments (final solvent concentration: 0.1% *v*/*v*). Platinum levels were determined by graphite furnace atomic absorption spectroscopy (GFAAS) with deuterium background correction (AAS vario 6; AnalytikJena AG) using a detection wavelength of $\lambda = 265.9$ nm. With the same instrumentation, the copper levels were determined with a detection wavelength of $\lambda = 324.8$ nm. The temperature program used for platinum consisted of drying at 105 °C for 30 s (ramp rate = 10 °C s^{-1}) and at 120 °C for 20 s (ramp rate = 15 °C s^{-1}), ashing at 500 °C for 30 s (ramp rate = 45 °C s^{-1}), at 1000 °C for 10 s (ramp rate = 100 °C s^{-1}), and at 1700 °C for 10 s (ramp rate = 300 °C s^{-1}), as well as atomizing at 2400 °C for 4 s (ramp rate = 1500 °C s^{-1}). The temperature program for copper involved a drying stage (90 °C for 5 s, ramp rate 10 °C s^{-1}; 105 °C for 30 s, ramp rate 7 °C s^{-1}; 120 °C for 10 s, ramp rate 15 °C s^{-1}), an ashing stage 700 °C for 15 s, ramp rate 400 °C s^{-1}; 1100 °C for 15 s, ramp rate 400 °C s^{-1}), and an atomization stage (2000 °C for 4 s, ramp rate 1500 °C s^{-1}). Purge gas (Ar) flow rate was 2 L min^{-1} except at the atomization, at which point it was stopped. The limit of detection was approximately 5 ng L^{-1} for platinum and 0.3 ng L^{-1} for copper (injection volume: 20 μL).

Cell culture: The human MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Eagle's minimal essential medium (EMEM) containing L-glutamine supplemented with $NaHCO_3$ (2.2 g L^{-1}), sodium pyruvate (110 mg L^{-1}), gentamycin (50 mg L^{-1}), and 10% fetal calf serum (FCS, Gibco, Eggenheim, Germany) using culture flasks (75 cm^2, Nunc) in a water-saturated atmosphere (5% CO_2) at 37 °C. The cells were serially passaged weekly following trypsin treatment using a solution of trypsin (0.05%) and EDTA (0.02%). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

Cellular uptake studies: MCF-7 cells were seeded in 6-well plates (Nunc). When the cells had reached 50–60% confluency (after ≈5 days of incubation) the medium was exchanged for serum-free EMEM containing the inhibitors ($CuSO_4$ up to 200 μM; ouabain, glycocholate, cimetidine, TEA, 0.5 mM; chlorpromazine, 10 μg mL^{-1}; amiloride, 1 mM; EIPA, 0.1 mM; m-β-CD, 10 mM; nystatin, 25 μg L^{-1};

genistein, 0.4 mM; cytochalasin D, 10 μM; wortmannin, 0.5 μM) and preincubated for 15 min. The medium was then removed, and medium containing platinum compound (cisplatin, 80 μM; *m*-4F-PtDMSO-DAH, 20 μM) and inhibitor were added. The control groups were not preincubated, and only platinum compound was added. The medium was removed after 2 h, and the cells were washed with ice-cold PBS. After trypsin treatment, the cells were harvested, washed twice with ice-cold PBS, and centrifuged (2000 *g*, 4 °C, 5 min) for storage at −18 °C until analysis.

The cell pellet was homogenized by sonication in a solution of Triton X-100 (1% *w*/*w*) and was adequately diluted for protein determination[31] and for platinum and copper analysis (GFAAS). Calibrations were carried out under identical conditions with standard solutions of K_2PtCl_4 and $Cu(NO_3)_2$. The results were calculated as the average of six experiments. The cellular concentrations of the platinum compounds and copper in the MCF-7 cells were calculated as previously described.[32]

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Keywords: antitumor agents · copper transport · dinuclear platinum complexes · endocytosis · organic cation transport

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