Cancer Chemother Pharmacol (2004) 54: 441–448
DOI 10.1007/s00280-004-0840-z

ORIGINAL ARTICLE

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Improved potency of cisplatin by hydrophobic ion pairing

Received: 2 September 2003 / Accepted: 7 April 2004 / Published online: 29 July 2004

Abstract Cisplatin is one of the most widely used and effective chemotherapeutic drugs ever discovered against certain forms of cancer. However, its use is limited by toxicity. A more potent form might allow lower doses to be used and would diminish the toxicity. A new analog of cisplatin has been synthesized by stoichiometric replacement of the chloride ligands with the anionic surfactant, Aerosol OT (AOT). The new compound has a very low aqueous solubility (about 2 mg/l) and a log P value of 2.17, which is more than 4 log units higher than cisplatin itself, indicating a dramatic increase in hydrophobicity. While hydrophobic cisplatin analogs have been synthesized previously, this is the first one with readily dissociable ligands replacing the chlorides. The resultant AOT complex is able to penetrate cellular membranes more efficiently, resulting in a threefold to fivefold increase in intracellular platinum levels. These increased intracellular concentrations correlate with lower IC_{50} values in a number of cancer and normal cell lines. These findings suggest that further development of the AOT complex as a chemotherapeutic agent is warranted, given its marked increase in potency over the parent compound.

Keywords Cisplatin · Ion pairing · Increased potency · Cell culture · Partition behavior

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Introduction

cis-Diamminedichloroplatinum(II) (*cis*-$[PtCl_2(NH_3)_2]$, cisplatin) is highly effective in treating testicular cancer [1] and ovarian cancer [2] and contributes to the treatment of head and neck cancer [3] and lung cancer [4]. Since this agent was approved for clinical use in 1978 [5], it has become one of the most utilized antitumor drugs. Thus far, the biggest success of cisplatin has been achieved in treating testicular cancer. It exhibits a 95% cure rate when combined with other chemotherapeutic agents, such as etoposide and bleomycin [6].

Despite the successes in clinical use, cisplatin does exhibit significant toxicity effects and acquired resistance. Clinically, cisplatin is administered by intravenous (i.v.) infusion and systemic exposure potentially leads to nephrotoxicity, neurotoxicity, and emetogenesis [7]. These adverse effects have limited any increase in dosing of cisplatin. Therefore, the maximum therapeutic effect may not have been reached. Acquired resistance makes the treatment of some cancers effective early on, but ultimately leads to long-term failure [6].

Since the introduction of cisplatin, the first platinum (Pt) based anticancer drug, there has been extensive effort to develop new Pt-based complexes that are less toxic and/or more efficacious. In the last three decades, thousands of Pt compounds have been synthesized and evaluated as potential antitumor agents. As a result of such efforts, over 28 Pt compounds have entered human clinical trials [7], but only diammine(1,1-cyclobutanedicarboxylato(2-)]-O,O-platinum(II) (carboplatin) and the related oxaliplatin have received worldwide approval and achieved routine clinical use [8, 9]. These compounds tend to be less toxic than cisplatin and can thus be given at much higher doses. Unfortunately, they are still only active in the same types of tumors as cisplatin, and are usually administered i.v., causing severe side effects, including bone marrow suppression [7].

One can hypothesize that the potency of cisplatin might be enhanced if the intracellular uptake of cisplatin

could be increased, although toxicity might increase as well. The cytotoxicity of cisplatin has been found not to be directly related to cisplatin itself but to the hydrolysis products of cisplatin, which are generated intracellularly. These products attack intracellular components such as DNA, proteins, and RNA [10]. The reaction with DNA yields monofunctional adducts, intrastrand crosslinks and interstrand crosslinks with the platinum atom coordinated to the N7 position of guanine or adenine [11]. Adduct formation results in inhibition of DNA replication and RNA transcription, cell arrest at the G_2 phase of the cell cycle, and/or programmed cell death [10, 12]. Resistance to cisplatin is due to multiple mechanisms, one of them being intracellular accumulation of cisplatin. This may arise because of decreased uptake, increased efflux, or increased inactivation of the reactive hydrolysis species by scavengers, such as glutathione. Other mechanisms may include increased repair capacity for cisplatin adducts, increased ability to replicate past cisplatin adducts, or defects in the apoptotic response pathway [6]. Therefore, if one can increase the intracellular uptake of cisplatin, the potency may be enhanced and resistance may be overcome.

We describe here a new Pt-based complex formed using the approach of hydrophobic ion pairing (HIP). HIP is a process that involves stoichiometric replacement of polar counter-ions (i.e., chloride, acetate, nitrate, etc.) with an ionic surfactant of similar charge. Upon HIP complex formation, the drug then possesses a hydrophobic counter-ion which is much less polar than with the original counter-ion. In this case, the chloride ligands on cisplatin were replaced with a FDA-approved surfactant, dioctylsulfosuccinate, more commonly known as Aerosol OT (AOT). The aqueous solubility of the drug is thus reduced, while the lipid solubility is increased, leading to a large increase in the partition coefficient. Increased hydrophobicity has been found to be associated with enhanced intracellular uptake for many drugs [13] and enhanced cytotoxicity for Pt-based compounds [14–16]. However, unlike many of the compounds previously reported, this work involves ligands that are readily dissociated from the Pt center.

The work described here involved the synthesis and investigation of the partitioning behavior, solubility, intracellular uptake and in vitro toxicity of the AOT-cisplatin complex in comparison to cisplatin. The results from this study suggest that formation of a HIP complex of cisplatin results in a more cytotoxic compound, suggesting that further clinical evaluation is warranted.

Materials and methods

Materials

Cisplatin (*cis*-diamminedichloroplatinum) was purchased from Sigma (St Louis, Mo.), and silver hexafluorophosphate ($AgPF_6$) and sodium dioctylsulfosuccinate (AOT) were purchased from Aldrich (Milwaukee, Wis.). The stock solutions used in the ion-pairing experiment were prepared using sterilized deionized (DI) water and could be used for a week except for the $AgPF_6$ solution, which was prepared immediately before the experiment and protected from light.

Preparation of the AOT-cisplatin complex

A two-step chemical reaction was designed to prepare the hydrophobic ion-paired complex (Fig. 1). In the first step, cisplatin solution (2 mg/ml) is added with $AgPF_6$ (10 mg/ml) at a molar ratio of 1:2 (cisplatin:$AgPF_6$). After mixing, the solution was left to settle at 4°C overnight. A 10-min centrifugation at 1000 *g* followed to remove the precipitated silver chloride from the solution. The supernatant was then filtered through a 0.22 μm filter (Nalge Nunc International, Rochester, N.Y.) to further remove fine particles of silver chloride. This resulted in a water-soluble hydrolyzed *cis*-diammine platinum intermediate. In the second step, AOT (20 m*M*) solution was added into the intermediate product solution at a molar ratio of 2:1 (AOT:cisplatin) in a drop-wise manner. A light-yellow precipitate then formed and was allowed to settle for 2 h at room temperature. The precipitate was collected by centrifugation at 10,000 *g* and washed with cold DI water three times, followed by lyophilization for 24 h. The dried creamy white solid was the expected AOT-cisplatin complex, as confirmed by mass spectrometry.

Measurement of the partition coefficient

The partition coefficient was measured using a two-phase system composed of 1-octanol (HPLC grade, from Aldrich, Milwaukee, Wis.) as the organic phase, and DI water (HPLC grade, from Fischer Scientific) as the aqueous phase. To measure the partition coefficient of cisplatin, 4 mg cisplatin was dissolved in 4 ml DI water and the resulting solution was mixed with 4 ml

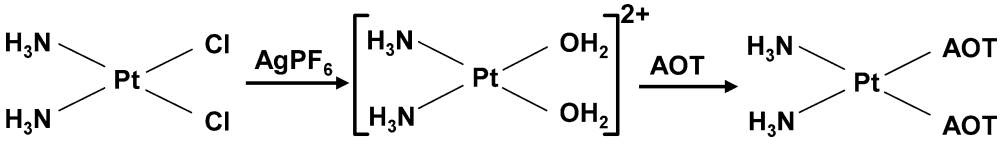

Fig. 1 Synthesis of the AOT-cisplatin complex. The two chloride ligands of cisplatin are replaced by AOT surfactant molecules of the same charge. [*AOT*: bis(3-ethylhexyl) sulfosuccinate]

1-octanol. To measure the partition coefficient of the AOT-cisplatin complex, 8 mg of the complex was dissolved in 4 ml 1-octanol and the resulting solution was mixed with 4 ml DI water. Upon mixing, the solutions were vortexed at the maximum speed (VWR Vortexer 2, Model G-560, Bohemia, NY) for 15 s every 5 min, and 30 min later, the solutions were centrifuged at 3000 *g* for 10 min. Samples from both the aqueous phase and the organic phase were then collected and analyzed for Pt concentration by atomic absorbance (AA) spectrophotometry. The partition coefficient was calculated by determining the ratio between the concentration of Pt in the organic phase and that in the aqueous phase. The partition coefficient of the AOT-cisplatin complex was also measured in two other systems, composed of 1-octanol as the organic phase and 4 m*M* NaCl solution or 100 m*M* NaCl solution (dissolved in DI water) as the aqueous phase using the same method.

Stock solutions of cisplatin and the AOT-cisplatin complex

A stock solution of cisplatin was prepared by dissolving cisplatin in sterilized 0.9% saline (NaCl injection USP, from Baxter Healthcare, Toronto, Canada) at 1 mg/ml (3.33 m*M*). This solution was stable for 1 week after preparation. The stock solution of AOT-cisplatin complex was prepared by dissolving the complex in ethyl alcohol (200 proof, USP grade; Pharmaco Products, Brookfield, Ct.) at 10 m*M*. The concentration of the AOT-cisplatin complex was expressed as the molar concentration of platinum, which was determined by AA spectrophotometry. Immediately prior to the experiment, the stock solutions of cisplatin or the AOT-cisplatin complex were diluted as described below.

Intracellular uptake of cisplatin and the AOT-cisplatin complex

Solutions of cisplatin were prepared by diluting the stock solution of cisplatin with 0.9% saline and solutions of the AOT-cisplatin complex were prepared by diluting the stock solution of the AOT-cisplatin complex with 100% ethanol. Chinese hamster ovary (CHO) cells were trypsinized and resuspended at 1×10^6/ml in DMEM/F-12 medium (50:50) with 10% fetal bovine serum (FBS). All cell culture media and supplements were purchased from Invitrogen (Grand Island, NY). Suspended CHO cells (5 ml) were then transferred to a Petri dish (60×15 mm; Corning, Corning, NY). After 1-h of incubation at 37°C in an atmosphere of 95% air/5% CO_2, the CHO cells were treated with either cisplatin or the AOT complex at 25 or 50 μ*M* (in triplicate). The volume of the drug solution added took 1% of the cell culture medium volume, except for that exposed to cisplatin at 50 μ*M*, in which case, the cisplatin solution took 1.5% of the whole volume. After a 1-h incubation, cells were collected and centrifuged at 1000 rpm for 5 min. Cells were washed with 0.9% NaCl solution followed by a centrifugation. The wash and centrifugation cycle was repeated twice, then 0.5 ml of DI water was added to the cells. The cells were then lysed using a sonicator (Sonic 300 Dismembrator, Artek Systems, Farmingdale, NY). Solutions were analyzed for total Pt by AA spectrophotometry.

Cytotoxicity by clonogenic assay

There were two control groups and two treatment groups in this experiment. The control groups comprised a negative control and an ethanol vehicle control. One two treatment group received cisplatin and the other received the AOT-cisplatin complex. A series of solutions of cisplatin was obtained by diluting the stock solution with sterilized 0.9% NaCl solution. A series of solutions of the AOT-cisplatin complex was prepared by diluting the stock solution with 100% ethanol. CHO cells were used to determine cytotoxicity. Incubations and exposures were conducted at 37°C in an atmosphere of 95% air/5% CO_2. Cells were grown in DMEM/F12 (50:50) supplemented with 10% FBS, penicillin G (10 U/ml), and streptomycin sulfate (10 μg/ml). Cells were passaged twice a week using trypsin to remove the cells from the plates. CHO cells were harvested and resuspended in Petri dishes at a density of 1×10^6/ml in the culture medium followed by a 1-h incubation.

In the treatment groups, cisplatin or AOT-cisplatin was added into the cell culture medium to reach certain exposure concentrations of 0.5, 1, 5, 10, 50, and 100 μ*M* of Pt in triplicate. After a 1-h incubation, the cells from all four groups were collected and washed twice with Hank's balanced salt solution (Invitrogen). The cells were then plated into a cell culture dish (60×15 mm; Corning). After an 8-day incubation, the plates from all four groups were fixed with freshly prepared fixative composed of acetic acid and methanol (1:3) and then stained with crystal violet. The colonies that had more than 50 single cells were counted. The plating efficiency and survival rate of the cells were then determined.

Cytotoxicity by MTS test

A series of stock solutions of cisplatin was obtained by diluting the stock solution of cisplatin with Hank's buffer. A series of solutions of the AOT-cisplatin complex were prepared by diluting the stock solution with 100% ethanol. Incubation and exposures were conducted at 37°C in an atmosphere of 95% air/5% CO_2. Different cell lines were used in the study, each requiring somewhat different culture media. The cell lines used are listed in Table 1.

Cells were harvested, resuspended at certain densities, and inoculated into 96-well plates (Corning) at a volume of 200 μl in each well. After a 24-h incubation, cells

Table 1 Cell lines, culture media, and inoculated number of cells in the MTS assay

Cell lines	Origin	Culture medium[a]	Cell number per well	MTS incubation time (h)
CHO	Chinese hamster ovarian epithelium	DMEM/F12 (50:50)	6,000	3
HeLa	Human cervix epithelial carcinoma	DMEM/F12 (50:50)	6,000	3
Gollin-B	Mouse mammary carcinoma	DMEM/F12 (50:50)	10,000	3
MCF7	Human breast adenocarcinoma	DMEM/F12 (50:50)	10,000	3
MCF10A	Immortalized human normal breast epithelial cells	DMEM/F12 (50:50)	10,000	3
A2780	Human ovarian adenocarcinoma (epithelial cell)	RPMI1640	10,000	3
Ivan	Immortalized human normal ovarian epithelial cell	M199/MCDB 105	5,000	8

[a]All cell culture media were supplemented with 10% fetal bovine serum, penicillin G (10 U/ml) and streptomycin sulfate (10 μg/ml).

received different treatments. The exposure concentrations were 0.5, 1, 5, 10 and 50 μ*M* (cisplatin equivalent) for both cisplatin and the AOT-cisplatin complex. After a 24-h exposure, 25 μl CellTiter 96 AQueous One Solution for cell proliferation assay (MTS assay from Promega, Madison, Wis.) was added directly to each well for all the four groups. After a 3-h or 8-h incubation, the absorbance at 490 nm (A_{490}) was measured using a MCC/340 microplate reader (Labsystems, Finland) supported by Genesis Lite (version 3.03; Life Sciences International, Basingstoke, UK) software. The percentage survival fraction was determined by calculating the ratio between ΔA_{490} for the treatment groups and ΔA_{490} for the controls. IC_{50} values were then determined based on the curve of survival fraction verse concentration.

Atomic absorbance spectrophotometry

The Pt concentration was analyzed using flameless atomic absorbance spectrophotometry. The instrument was a SpectrAA 220 with a model GTA 110 graphite tube atomizer from Varian Analytical Instruments (Sugar Land, Tx.). During detection, the wavelength was set at 265.9 nm. Calibration standards were prepared by diluting a primary standard solution of Pt powder dissolved in hydrochloric acid (Spex Certiprep, Metuchen, NJ).

Results

Preparation of the AOT-cisplatin complex

A two-step reaction was designed to prepare the AOT-cisplatin complex, as shown in Fig. 1. The chloride ligands are removed by addition of silver ion. After the resultant silver chloride had been removed by filtration, the labile PF_6^- ligands were replaced by the anionic surfactant, AOT. Analysis of the resulting AOT-cisplatin complex, using nuclear magnetic resonance (NMR) spectroscopy, indicated that there were two carbonyl signals in the final product, consistent with replacement of both chlorides with AOT. However, the Pt NMR signal was not detected, possibly due to line broadening. Characterization using electrospray ionization (ESI) mass spectrometry indicated the molecular weight of the product to be *m*/*z* 1094, 22 Da higher than the formula weight of the AOT-cisplatin complex (*m*/*z* 1072). This was likely due to a sodium adduct ($M + Na^+$) of the AOT-cisplatin complex at the electrospray source. For hydrophobic drugs, sodium adducts are commonly found and are not easily dissociated. Chemical-induced dissociation reduced the intensity of the peak, verifying that the peak was due to adduct formation.

Solubility of the AOT-cisplatin complex

The AOT-cisplatin complex was poorly soluble in water (about 0.002 mg/ml or about 2 mg/l). It did dissolve in normal saline at about 0.8 mg/ml (cisplatin equivalent), although much would be converted to cisplatin in the presence of high concentrations of chloride. The AOT-cisplatin also dissolved in ethanol, dimethyl sulfoxide (DMSO) (to levels as high as 50 m*M*), and chloroform. By comparison, the aqueous solubility of cisplatin itself is about 1 mg/ml. Based on the analytical data and the solubility behavior, it was estimated that the AOT-

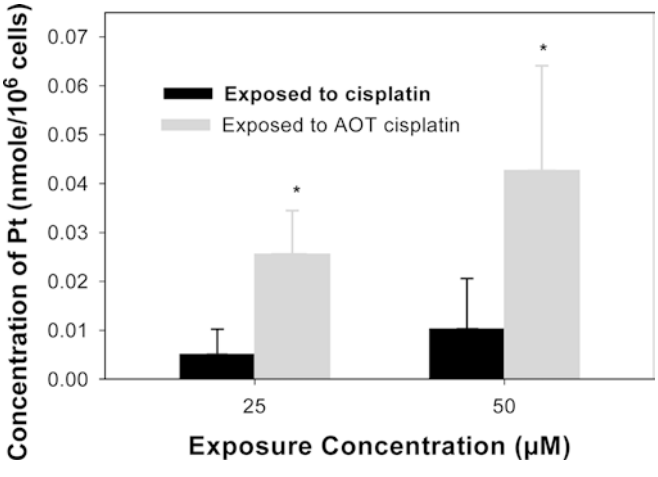

Fig. 2 Levels of platinum in CHO cells after a 1-h exposure to either cisplatin or the AOT-cisplatin complex. $*P < 0.05$, Student's *t*-test

Table 2 Partition coefficient of cisplatin and the AOT-cisplatin complex. The two-phase systems were composed of 1-octanol and DI water, 4 m*M* NaCl solution or 100 m*M* NaCl solution. Concentrations of Pt were measured by AA spectrophotometry

Chemicals	$\log_{10}$ P (average ± SD)		
	1-Oct/DI water	1-Oct/4 m*M* NaCl	1-Oct/100 m*M* NaCl
Cisplatin	−2.35 ± 0.10		
AOT complex of cisplatin	2.17 ± 0.19	0.095 ± 0.026	−0.80 ± 0.046

cisplatin was greater than 95% pure, with the amount of cisplatin present being less than 5%.

Partition coefficient of cisplatin and the AOT-cisplatin complex

The partition coefficient was measured for both cisplatin and the AOT-cisplatin complex in a conventional two-phase system composed of 1-octanol (1-Oct) and DI water (Table 2). The average log P value for cisplatin was −2.34, while the average log P for the AOT-cisplatin complex was 2.17. The difference between the partition coefficients of the two drugs was over 10,000-fold. The partition coefficient of the AOT-cisplatin complex in the two-phase system composed of 1-octanol and a 4 m*M* NaCl solution or 1-octanol and a 100 m*M* NaCl solution was also measured. The log P was 0.095 in the system composed of 1-octanol/4 m*M* NaCl solution and −0.80 in the system composed of 1-octanol/100 m*M* NaCl solution.

Cellular Pt levels after exposure

Platinum levels were measured in CHO cells after a 1-h exposure to either cisplatin or the AOT-cisplatin complex at two concentrations (25 and 50 μ*M* Pt). As shown in Fig. 2, cells exposed to cisplatin at 25 and 50 μ*M* had cellular levels of Pt averaging 0.005 and 0.010 nmol/10^6 cells, respectively. Cells exposed to the AOT complex had cellular levels of Pt of 0.026 and 0.043 nmol/10^6 cells, respectively. Levels of Pt in cells exposed to the AOT-cisplatin complex were approximately fivefold higher ($P < 0.05$, Student's *t*-test) than in those exposed to cisplatin.

Cytotoxicity assessed by clonogenic assay

A clonogenic assay was conducted to test the cytotoxicity of both cisplatin and the AOT-cisplatin complex in CHO cells with 1-h drug exposure. As shown in Fig. 3, concentration-dependent cytotoxicity was observed for both cisplatin and the AOT-cisplatin complex. The IC_{50} of cisplatin was about 22 μ*M*, and of the AOT-cisplatin complex was 4.5 μ*M*. There was an approximately fivefold difference between the IC_{50} values of the two drugs.

Cytotoxicity assessed by a MTS assay

The potency of the AOT complex was assessed in cancer cell lines and in immortalized normal tissue cell lines. Figure 4 shows the cytotoxicity in CHO cells, as determined by the MTS assay. The IC_{50} of the AOT complex

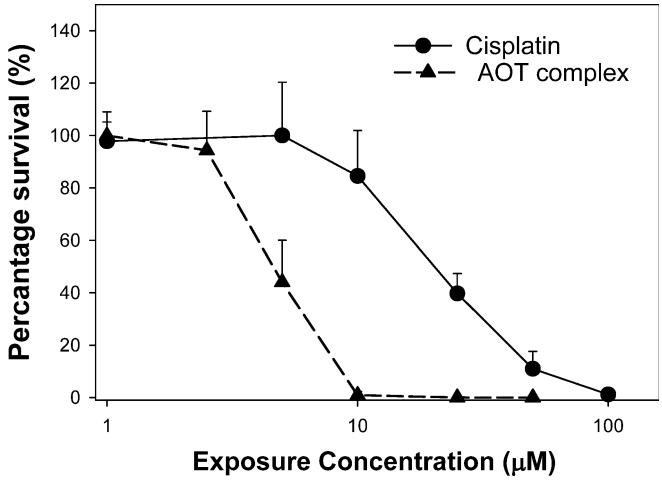

Fig. 3 CHO cell survival rate after a 1-h exposure to cisplatin or the AOT-cisplatin complex as determined by clonogenic assay. The IC_{50} of cisplatin was 22 μ*M* as determined in the curve, and of the AOT-cisplatin complex was 4.5 μ*M*

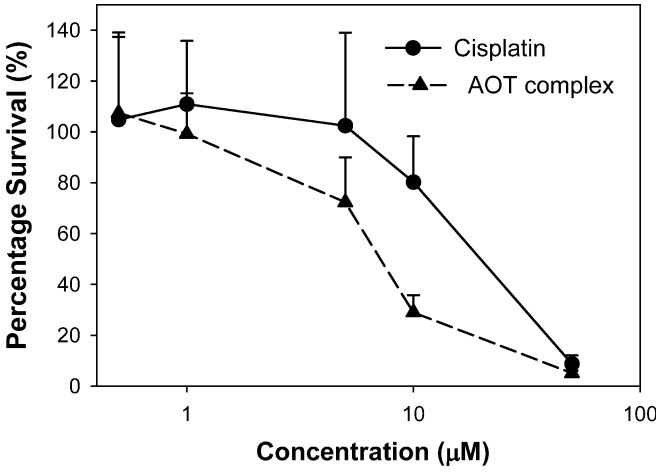

Fig. 4 Concentration-dependent survival rate of CHO cells after exposure to cisplatin or the AOT-cisplatin complex as determined by MTS assay

was 7 μ*M* and of cisplatin was 27 μ*M*. The difference in potency was approximately 3.7-fold.

The IC_{50} for both cisplatin and the AOT-cisplatin complex in other cell lines were determined using the MTS assay as listed in Table 3. The potency of the AOT complex in all the cell lines tested was higher than that of cisplatin with differences ranging from 1.7-fold to greater than 5-fold.

Discussion

The AOT-cisplatin complex synthesized in this study had significantly increased hydrophobicity compared to that of cisplatin itself, as evidenced by a 10,000-fold enhancement in the 1-octanol/water partition coefficient. The logarithm of the partition coefficient of cisplatin was measured to be −2.35, similar to the value of −2.19 reported by Souchard et al. [17]. The low value of the partition coefficient indicates that cisplatin highly favored the aqueous phase over the organic or lipid phase. In contrast, the logarithm of the partition coefficient of the AOT complex was 2.19, indicating that the AOT-cisplatin complex highly favored the lipid phase. The 10,000-fold increase in the partition coefficient is comparable to the increases reported for other HIP complexes [18, 19]. The increase in the partition coefficient can be attributed to both an increase in solubility in the organic solvent and a reduced solubility in aqueous medium. The water solubility for cisplatin is about 1 mg/ml, while the water solubility for the AOT-cisplatin complex is only about 0.002 mg/ml (cisplatin equivalent).

The AOT-cisplatin complex can be dissolved in aqueous electrolyte solutions. The aqueous solubility limit in 0.9% saline was 0.8 mg/ml (cisplatin equivalent) compared to 0.002 mg/ml in DI water. Mostly likely the increase is the result of reverse ion pairing as the chloride ion substitutes the hydrophobic organic ion, i.e., the anionic AOT ion. As found in this study, the rate for reverse ion pairing is strongly affected by the concentration of the electrolyte. The partition coefficients of the AOT complex in 1-octanol/4 m*M* NaCl solution and 1-octanol/100 m*M* NaCl solution were measured. These salt concentrations were chosen because 4 m*M* is accepted as the typical intracellular NaCl concentration and 100 m*M* is typical of the extracellular NaCl concentration [20]. The logarithm of the partition coefficient of the AOT-cisplatin complex measured in these two systems was 0.095 and −0.80, respectively. If calculated based on the partition coefficient, the fraction of the AOT complex distributed in the aqueous phase of the two-phase system composed of 1-octanol/DI water was about 0.5%. It increased to 45% when NaCl at 4 m*M* replaced DI water as the aqueous phase, and to 86% when NaCl at 100 m*M* replaced DI water. Hence, in 30 min, the time used to allow the drug to distribute between the organic and aqueous phases, the fraction of the drug distributed in the aqueous phase significantly increased. This strongly suggests that reverse ion pairing of the AOT-cisplatin complex is a relatively rapid process in physiological solutions.

Table 3 IC_{50} of cisplatin and the AOT-cisplatin complex in different cell lines tested by MTS assay

Cell line	IC_{50} (μ*M*)		Potency improvement
	Cisplatin	AOT-cisplatin	
CHO	27	7	3.8
HeLa	> 50	9.5	> 5.3
Gollin-B	13	7.5	1.7
MCF-7	> 50	24	> 2.0
MCF-10A	> 50	15	> 3.3
A2780	24	9	2.7
Ivan	9	4.5	2.0

Intracellular uptake of cisplatin is enhanced upon HIP. Such a finding is similar to those found with many other ionizable drugs, when the hydrophobicity of these agents is enhanced [13]. Passive diffusion is accepted as the major pathway for cisplatin to cross the cell membrane [21]. Since the molecular size of the AOT-cisplatin complex is much larger than that of cisplatin (the molecular weight of the AOT complex is 1072, and that of cisplatin is 300), we can expect that the AOT-cisplatin complex also enters cell via passive diffusion. Even though the molecular weight is increased threefold, the level of cellular penetration increases, primarily due to increased hydrophobicity. This has been found for other organic analogs of cisplatin [15]. In this case, the intracellular levels increased much more dramatically in this study. Whether this was due to the nature of the cell line, the time of exposure or the molecular structure of these compounds is uncertain. While many hydrophobic cisplatin analogs have been synthesized previously [14, 15, 17], this is the first one with readily dissociable ligands replacing the chlorides. Therefore, this may be a more general approach towards new highly active forms of cisplatin. What is clear is that achieving a logarithm of the partition coefficient greater than zero results in significant increases in intracellular Pt levels [15], as was found with the AOT complex.

It is important to note that there are two parts to the partitioning of a drug entering a cell via passive diffusion. The first phase is partitioning between the extracellular fluid and the lipid cell membrane. The second phase is partitioning between the cell membrane and the intracellular fluid. Enhanced partitioning into the organic phase suggests increased drug distribution into the cellular membrane. Enhanced distribution into the membrane assists the drug's transport from the extracellular fluid to the intracellular fluid (via a larger concentration gradient). One might expect that if the logarithm of the partition coefficient were too large, some of the drug would remain sequestered in the membrane and cytotoxicity might actually decrease. This type of behavior has been observed for Pt compounds, where increased hydrophobicity does not

correlate with increased cytotoxicity [16]. This may be due to inefficient transport from the membrane into the cell or it might be due to the steric hindrance of the bulky aromatic rings used in that study, as the drug attempts to bind to DNA.

The potency of the AOT-cisplatin complex in CHO cells was enhanced, as indicated by the results of the clonogenic assay. The enhancement factor for cytotoxicity was about fivefold, consistent with increased intracellular uptake. This strongly suggests that the enhancement of cytotoxicity was associated with increased intracellular levels of Pt. Again, two aspects of cytotoxicity must be considered: intracellular levels and the ability of the new compound to bind to DNA. The use of bulky groups may not be able to bind efficiently or they may alter the DNA structure enough to enhance repair mechanisms, thereby decreasing cytotoxicity [11, 14, 22]. Apparently, the reactivity of the AOT complex was similar enough to that of cisplatin itself that there was a good correlation between increased levels in the cell and the IC_{50}.

The cytotoxicity of cisplatin is due to its hydrolysis products [20]. In the extracellular fluids, where the chloride concentration is high (about 100 m*M*) [20], the Pt(II) remains coordinated by its chloride ligands. Once it enters a cell, in which the chloride concentration is low (about 4 m*M*) [20], chloride ligands are replaced by water molecules, generating a positively charged, aquated species that can react with nucleophilic sites on intracellular macromolecules, forming protein adducts, RNA adducts, and DNA adducts [10]. Reaction with DNA yields monofunctional adducts, and intrastrand and interstrand crosslinks with the platinum atom coordinated to the N7 position of guanine or adenine [11]. Adduct formation results in inhibition of DNA replication and RNA transcription, cell arrest at the G_2 phase of the cell cycle, and/or programmed cell death [10, 12]. Apparently, the AOT complex can dissociate in the intracellular matrix, generating the same reactive Pt species. Control experiments were performed using AOT alone (data not shown). The surfactant alone showed none of the toxicity exhibited by the AOT-cisplatin complex.

Others have correlated the increased reactivity to aqueous solubility rather than the logarithm of the partition coefficient [14]. In this case, the most reactive species had an aqueous solubility of 1 mg/l or less. The best estimate of the solubility of the AOT complex in water is about 2 mg/l, consistent with increased cytotoxicity. Similar measures of cytotoxicity were observed using the MTS assay and the clonogenic assay. In all of the tested cell lines, the cytotoxicity of the AOT complex was higher than that of cisplatin itself, as indicated by an improvement in potency from 1.7-fold to more than fivefold (Table 3). The exact error in these measurements is uncertain. However, the fact that every cell line exhibited a similar increase in potency is compelling evidence that the AOT complex does possess greater activity than cisplatin.

Based on the in vitro cytotoxicity data, it appears that the sensitivities of the normal tissue cell lines to cisplatin and the AOT complex were similar to those of the cancer cell lines that originated from the same types of tissue. Two pairs of cell lines derived from the same tissue were used to compare sensitivity. One pair was MCF7 and MCF10A; the other pair was A2780 and the Ivan cell line. MCF7 is a human breast adenocarcinoma cell line while MCF10A is from normal human breast tissue. In both cell lines, the IC_{50} of cisplatin was greater than 50 μ*M*. The IC_{50} of the AOT-cisplatin complex was 24 μ*M* in MCF7 cells and 15 μ*M* in MCF10A cells. A2780 is a human ovarian adenocarcinoma (epithelial cell) cell line while Ivan is an immortalized normal tissue cell line obtained from the human ovary. The IC_{50} of cisplatin was 24 μ*M* in A2780 cells and 9 μ*M* in Ivan cells. The IC_{50} values of the AOT-cisplatin complex were 9 and 4.5 μ*M* in the two cell lines, respectively. No specific sensitivity or protection of normal cell lines to the AOT complex was indicated.

The results of this study suggest that there is a potential clinical use for the AOT complex. Currently, cisplatin is administered via i.v. infusion and is associated with adverse effects such as nephrotoxicity and nausea. A more potent compound would require lower doses and should be associated with less-severe side effects. However, the AOT complex has extremely limited aqueous solubility (about 2 mg/l), which is almost three orders of magnitude less than cisplatin itself, making systemic delivery very difficult. The AOT-cisplatin complex can be dissolved in organic solvents at high concentrations (> 50 m*M*). The question is whether these solvents would affect the bioactivity. For example, although cisplatin can be dissolved in DMSO, this solvent has been found to suppress the cytotoxicity of cisplatin [23] due to formation of DMSO adducts, so one must use nucleophilic nonaqueous solvents with care.

If the AOT complex could be delivered locally or regionally in a controlled manner, the therapy may display improved bioavailability at the local site, while avoiding or reducing systemic toxicity. The increased potency of the AOT complex over cisplatin in all cell lines tested makes it a candidate for further development.

In conclusion, the HIP approach can be used to prepare a more hydrophobic form of cisplatin. Upon formation of the AOT-cisplatin complex, the intracellular permeability of cisplatin is significantly improved. Increased intracellular levels correlate with enhancement in the potency of this drug. Given that the activity is greater than that of cisplatin in all cell lines tested, further investigation of the AOT-cisplatin complex as a chemotherapeutic agent is warranted, whether for systemic or local delivery.

Acknowledgements The authors would like to thank the Animal Cancer Center for financial support and the McKee Foundation for a generous gift in support of pharmacokinetic studies at the Veterinary Teaching Hospital.

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