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Mechanism of cellular accumulation of an iridium(III) pentamethylcyclopentadienyl anticancer complex containing a C,N-chelating ligand

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The effect of replacement of the N,N-chelating ligand 1,10-phenanthroline (phen) in the Ir III pentamethylcyclopentadienyl (Cp*) complex [(η^5-Cp*)(Ir)(phen)Cl] (2) with the C,N-chelating ligand 7,8-benzoquinoline (bq) to give [(η^5-Cp*)(Ir)(bq)Cl] (1) on the cytotoxicity of these Cp*Ir III complexes toward cancer cell lines was investigated. Complex 2 is inactive, similar to other Cp*Ir III complexes containing the N,N-chelating ligands. In contrast, a single atom change (C for N) in the chelating N,N ligand resulted in potency in human ovarian carcinoma cisplatin-sensitive A2780 cells, and, strikingly, 1 is active in the cisplatin-resistant human breast cancer MCF-7 and A2780/cisR cells. Replacement of the N,N-chelating ligand with the C,N-chelating ligand gives rise to an increased hydrophobicity, leading to higher cellular accumulation, higher DNA-bound iridium in cells and higher cytotoxicity. The pathways involved in cellular accumulation of 1 have been further explored and compared with conventional cisplatin. The results show that both energy-independent passive diffusion and energy-dependent transport play a role in accumulation of 1. The further results were consistent with involvement of p-glycoprotein, multidrug resistance-associated protein 1 and glutathione metabolism in the efflux of 1. In contrast, the internalization of 1 mediated by the endocytotic uptake pathway(s) seems less likely.

Understanding the factors which contribute to the mechanism of cellular accumulation of this Ir III complex can now lead to the design of structurally similar metal complexes for antitumor chemotherapy.

Introduction

Very recently, iridium(III) organometallic complexes have been gaining interest as potential anticancer agents.1-11 One group of these compounds that exhibit anticancer activity, including activity against cisplatin (cis-diaminedichloridoplatinum(II)) resistant cancer cells, comprises organoiridium half-sandwich complexes of the type [(η^5-Cp*)(Ir)(XY)Cl] where Cp = tetramethylphenyl)cyclopentadienyl or tetramethyl(biphenyl)cyclopentadienyl and XY is a chelating ligand, such as N,N-bound ethylenediamine, 2,2'-bipyridine, and 1,10-phenanthroline, or N,O-bound picolinate.12-15 It has been also shown that [(η^5-Cp*)(Ir)(XY)Cl] complexes where Cp = pentamethylcyclopentadienyl (Cp*) are all nontoxic toward A2780 human ovarian cancer cells14 although activity can be switched on by a single atom change (C for N) in the chelating N,N ligand (2,2'-bipyridine) to afford a neutral complex.13 Hence, the structure, chemical reactivity, and cancer cell cytotoxicity of the Cp*Ir III complexes can be also controlled by variation of the chelating ligands although factors responsible for this phenomenon are not completely understood. It has been suggested that the strong nucleobase binding and high hydrophobicity of the Cp*Ir III complexes with the C,N-chelating ligands probably contribute to their promising anticancer activity. The enhanced hydrophobicity is likely to result in higher cancer cell uptake which can contribute to the higher cytotoxicity of the Cp*Ir III complexes with the C,N-chelating ligands. This is so because before the active form of a metallodrug reaches its major pharmacological target in the cell, the metallodrug has to first accumulate in cells. It has been demonstrated that higher accumulation of metallodrugs generally correlates with enhanced cytotoxicity.16, 17 As the promising anticancer activity of the organometallic iridium(III) C,N-complexes provides a basis for further exploration of this new class of anticancer complexes, it is of great interest to understand the mechanisms of their cellular internalization.

The mode of action of antitumor metallodrugs is a multi-step process which includes cell entry or accumulation, drug activation, binding to cellular target, and cellular responses to the cellular target damage.18 In addition, decreased cellular accumulation, increased levels of glutathione, glutathione-S-transferase activity, or intracellular metallothionines, and enhanced DNA repair have been reported to be capable of producing resistance to several metallodrugs.19 Hence, efficient accumulation of metallodrugs in cancer cells is critical to the success of these agents so that an understanding of an early stage
in the metallo-drug mechanism of action, such as cellular accumulation, is required. The main routes into a cell are passive diffusion, active transport, facilitated diffusion, and endocytosis. Due to their hydrophobicity, CpIrIII complexes with the C,N-chelating ligands likely traverse the membrane in response to the membrane potential, similar to other lipophilic cations. Here, we use chemical tools to elucidate the cellular uptake mechanism of Ir analyzed by flameless atomic absorption (FAAS) or inductively coupled plasma mass spectroscopy (ICP-MS).

**In vitro growth inhibition assay**

Cell death was evaluated by using a system based on the tetrazolium compound MTT. The cells were seeded in 96-well tissue culture plates at a density of 1x10^4 A2780 or 2080 cisR cells/well or 3x10^3 MCF-7 cells/well in 100 µL of medium. After overnight incubation (16 h), the cells were treated with the compounds in a final volume of 200 µL/well. The final concentration of DMSO in cell culture medium did not exceed 0.25%. After additional 72 h 10 µL of a freshly diluted MTT solution (2.5 mg mL^{-1}) was added to each well and the plate was incubated at 37 °C in a humidified atmosphere for 4 h. At the end of the incubation period the medium was removed and the formazan product was dissolved in 100 µL of DMSO. The cell viability was evaluated by measurement of the absorbance of 570 nm, using an Absorbance Reader SUNRISE TECAN SCHÖLLER. IC_{50} values (compound concentrations that produce 50% of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (µM). All experiments were made in triplicate. The reading values were converted to the percentage of control (% cell survival). Cytotoxic effects were expressed as IC_{50}.

**Determination of partition coefficients of IrIII complexes**

Octanol-saturated water (OSW) and water-saturated octanol (WSO) were prepared using analytical grade octanol and 0.2 M aqueous NaCl solution (to suppress hydrolysis of the chlorido complexes). Aliquots of stock solutions of Ir complexes in OSW were added to equal volumes of WSO. Mixing was done by vortexing for 30 min at ambient temperature (~25 °C) to establish the partition equilibrium. To separate the phases, centrifugation was carried out at 3000 g for 5 min. The aqueous layer was carefully separated from the octanol layer for iridium analysis. Ir was quantified from aliquots taken from the octanol-saturated aqueous samples before and after partition by inductively coupled plasma mass spectrometry (ICP-MS). Partition coefficients of Ir complexes were calculated using the equation log P = log ([Ir]WSO/[Ir]OSW).

**Metal accumulation in cancer cells**

Metal accumulation studies for complexes 1, 2 and cisplatin were conducted on the A2780 ovarian carcinoma cell line. If not stated otherwise, in these experiments, 3x10^4 cells were seeded on a Petri dish; after 48 h of pre-incubation time in drug-free medium, at 37 °C in a 5% CO₂ humidified atmosphere, the test complexes were added to give final concentrations of 10 µM and then allowed a further 24 h of drug exposure under similar conditions.

**Materials**

Complexes 1 and 2 were synthesized and characterized as reported.\textsuperscript{14, 20} Cisplatin (purity was ≥ 99.9% based on elemental and ICP trace analysis), octanol (≥99%), 2-deoxy-D-glucose (≥ 98%), oligomycin A (from Streptomyces diastatichromogenes), human serum albumin (99%) (HSA), copper(II) chloride dihydrate (≥ 99% ACS reagent), progesterone (≥ 99%), hydrochloride (5-[N-(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride (≥ 99%) (verapamil), N-[3-(4-morpholinyl)propyl]-5,7-diphenylpyrazolo[1,5-alpyrimidine-3-carboxamide (≥ 98%) (reversan), L-buthionine-sulfoximine (≥ 97%) (BSO), methyl-β-cyclodextrin, and ouabain octahydrate (≥ 95%) were obtained from Sigma (Prague, Czech Republic), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Calbiochem (Darmstadt, Germany). The stock solutions of cisplatin and iridium compounds were prepared at the concentration of 1x10^{-3} M in DMSO immediately before use. RPMI 1640 medium, fetal bovine serum (FBS), and trypsin/EDTA were from PAA (Pasching, Austria). Gentamycin was from Serva (Heidelberg, Germany).

**Cell cultures**

The human ovarian carcinoma cisplatin sensitive A2780 cells, cisplatin resistant A2780/cisR (cisplatin resistant variant of A2780 cells) and the human breast cancer MCF-7 cells were kindly supplied by Professor B. Keppler, University of Vienna (Austria). The adriamycin resistant A2780/Adr cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The A2780, A2780/cisR and A2780/Adr cell lines were grown in RPMI 1640 medium supplemented with gentamycin (50 µg mL^{-1}) and heat inactivated FBS (10%). The acquired resistance of A2780/cisR and A2780/Adr cells was maintained by supplementing the medium with cisplatin (1 µM) or adriamycin (0.1 µM), respectively every second passage. The MCF-7 cells were grown in DMEM medium (high glucose, 4.5 g L^{-1}) supplemented with gentamycin (50 µg mL^{-1}, Serva) and 10% heat inactivated FBS. The cells were cultured in a humidified incubator at 37 °C in a 5% CO₂ atmosphere and subcultured 2–3 times a week with an appropriate plating density.

![Fig. 1. Schematic representation of the metal complexes used in this work.](image-url)
unless otherwise stated. After this time, cells were treated with 0.05% trypsin, counted, and cell pellets were collected. Each pellet was digested using microwave acid (HCl) digestion system (CEM Mars®) to give a fully homogenized solution, stored at -70 °C and the amount of metal taken up by the cells was determined by ICP-MS or FAAS. Metal standards were freshly prepared before each experiment. The results of cellular metal uptake were corrected for adsorption effects.21 These experiments did not include any cell recovery time in drug-free medium. They were all carried out in triplicate in three sets of independent experiments and the standard deviations were calculated. The statistical significance of all cellular accumulation values was determined as p<0.05. Further metal accumulation experiments were carried out as described above including the following experimental variations. In all cases, 3x10^6 A2780 cells were seeded in Petri dishes, the pre-incubation time in drug-free medium at 37 °C was 24 h, and the drug concentrations used were 5 or 10 μM unless otherwise stated.

DNA metatallation in cells exposed to Ir complexes

A2780 cells grown to near confluence were exposed to 5 μM concentration of complex 1 or 2 for 24 h. After the incubation period, the cells were trypsinized and washed twice in ice-cold PBS. Cells were then lysed in DNAzol (DNAzol genomic DNA isolation reagent, MRC) supplemented with RNase A (100 μg mL^(-1)). The genomic DNA was precipitated from the lysate with ethanol, dried, and resuspended in water. The DNA content in each sample was determined by UV spectrophotometry. To avoid the effect of high DNA concentration on FAAS detection of iodine in the samples, the DNA samples were digested in the presence of hydrochloric acid (11 M) using a high pressure microwave mineralization system (MARS5, CEM). Experiments were performed in triplicate, and the values are the means ± SD.

Temperature dependence

Experiments were carried out using 2 h of drug exposure (30 μM) with no recovery time in drug-free medium. The chosen temperatures for incubation with the drugs were: 4 °C, 20 °C and 37 °C. It was verified that the viability of A2780 cells did not decrease below 98% at all temperatures tested.

Role of Na^+/K^+ pump in cellular metal accumulation, as a facilitated diffusion endocytosis pathway

These experiments involved 24 h of drug exposure (10 μM) at 37 °C, co-administration of the drug with 200 μM of ouabain. No recovery time in drug-free medium was allowed.

Effect of ATP depletion on cellular metal accumulation

The A2780 cells were pre-treated with 50 mM 2-deoxy-D-glucose + 5 μM oligomycin in PBS for 1 h at 37 °C. The solutions also contained 2.5 mg mL^(-1) HSA. After pre-treatment, the cells were suspended in either PBS with 10% HSA for the metabolically inhibited cells or PBS with 10% HSA and 10 mM glucose for the control cells and treated with 30 μM complex 1 or cisplatin for 30, 60, 120 and 180 min at 37 °C in the humidified atmosphere of 5% CO_2. No recovery time in drug-free medium was allowed.

Extent of efflux

These experiments involved 24 h of drug exposure (5 μM) at 37 °C. After 24 h the drugs were removed and fresh drug-free medium was added to the cells in the Petri dishes. Cells were incubated again in drug-free medium for 24 h to allow for recovery before being treated with trypsin to collect the cell pellets.

Inhibition of efflux

Experiments were done using 24 h of drug exposure (5 μM) of A2780/Adr cells at 37 °C and 24 h of recovery time using drug-free fresh medium with 10 μM or 20 μM of verapamil. In other experiments, A2780 cells were incubated with reversan (10 μM) or BSO (1 mM) as single agents or a cocktail combining reversan (10 μM) and BSO (1 mM) for 24 h before addition of cisplatin (5 μM) or complex 1 (5 μM) for additional 24 h. In the latter experiments, no recovery time in drug-free medium was allowed.

Role of CTR1 in cellular metal accumulation

Experiments were carried out using 24 h of drug exposure time at 37 °C and co-administration of the drug (10 μM) with of copper(II) chloride (0.1 mM). No recovery time in drug-free medium was allowed.

Role of the endocytosis pathways in cellular metal accumulation

These experiments involved 24 h of drug exposure (10 μM) and co-administration of the drug with methyl β-cyclodextrin (0.2 mM). No recovery time in drug-free medium was allowed.

Other physical methods

Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out with an Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. The analysis with the aid of ICP-MS was performed using Agilent 7500 (Agilent, Japan). Statistical evaluation of the untreated control cells and drug-treated cells was carried out using Student’s t-test. If not stated otherwise, a probability of 0.05 or less was deemed statistically significant.

Results

Cytotoxicity

The cytotoxicity of Cp*Ir^{III} complexes containing an anionic C,N bound 7,8-benzoquinoline (bq) chelating ligand (1) or a neutral N,N-bound 1,10-phenanthroline (phen) chelating ligand (2) towards cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines A2780 and A2780/cisR, respectively, and the MCF-7 breast cancer cell line was investigated (Table 1).

| Table 1 | Cytotoxicity [IC_{50} mean values (μM)] for complexes 1, 2 and cisplatin
<table>
<thead>
<tr>
<th>Compound</th>
<th>A2780</th>
<th>A2780/cisR</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.31 ± 0.20</td>
<td>1.33 ± 0.04 (0.4)</td>
<td>1.59 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>cisplatin</td>
<td>3.12 ± 0.71</td>
<td>14.98 ± 1.08 (4.8)</td>
<td>22.93 ± 2.92</td>
</tr>
</tbody>
</table>

^a The results are expressed as mean ± SD of three independent experiments. Resistance factor, defined as IC_{50} (resistant)/IC_{50} (sensitive), is given in parentheses.
The IC$_{50}$ value (concentration at which 50% of the cell growth is inhibited) for the 1,10-phenanthroline complex 2 was >100 µM in all three cell lines. Hence, complex 2 is deemed inactive, similar to other Cp*Ir$^{II}$ complexes containing the N,N-bound chelating ligands, such as ethylenediamine or 2,2'-bipyridine. However, the Cp*Ir$^{III}$ complex 1, exhibits potency in cisplatin-sensitive A2780 cells very similar to that of cisplatin (IC$_{50}$ values found for 1 and cisplatin were 3.3 and 3.1 µM, respectively, Table 1). Even more strikingly, 1 is markedly more active than cisplatin in the cisplatin-resistant line A2780/cisR (with acquired cisplatin resistance$^{22,23}$) (Table 1). Thus, the cytotoxicity of 1 in the cisplatin-resistant line A2780/cisR is characterized by considerably lower value of resistance factor compared to cisplatin (Table 1). In addition, 1 exhibits a promising potency also in the MCF-7 breast cancer cell line (with inherent cisplatin resistance).$^{24}$ The IC$_{50}$ values for 1 and cisplatin were 1.6 and 22.9 µM, respectively, Table 1.

**Partition coefficients (log P)**

log P values for octanol/water partition provide a measure of hydrophobicity that is often a factor relevant for cell uptake and anticancer activity. For several classes of metallo-anticancer complexes, a correlation between increased hydrophobicity and increased cytotoxic activity has been reported.$^{13,25-28}$ The log P values for complexes 1 and 2 were 0.07 ± 0.02 and -0.95 ± 0.03, respectively (means of three independent experiments, expressed as means ± standard deviations).

The neutral complex 1 is slightly hydrophobic while positively-charged complex 2 is hydrophilic (partitions preferentially into water ca. 10-fold).

The hydrophobicity and cancer cell activity (Table 1) of complexes 1 and 2 correlate significantly in this study. Complex 2 is hydrophilic and inactive. Complex 1 displays hydrophobicity and is cytotoxic. This hydrophobicity difference appears to contribute to the higher cytotoxicity of complex 1 and is very likely responsible for inactivity of complex 2. In addition, it seems reasonable to suggest that complex 1 (contrary to complex 2) is hydrophobic enough to partition efficiently into cells so that it is very likely that this difference results in efficient cancer cell uptake of complex 1 and reduced uptake of complex 2.

Interestingly, a similar result was obtained when the N,N-chelating ligand 2,2'-bipyridine in the Cp*Ir$^{III}$ complex was replaced with the $C,N$-chelating ligand 2-phenylpyridine.$^{13}$ Hence, an interesting generalization of these results might be that replacement of N,N-chelating ligand with $C,N$-chelating ligand in the Cp*Ir$^{III}$ complexes enhances markedly hydrophobicity and consequently also toxicity in tumor cells of this class of Ir$^{III}$ complexes.

**Effect of temperature, ATP depletion with metabolic inhibitors and inhibition of Na$^+/K^+$-ATPase**

Cellular accumulation of metallodrugs may involve multiple mechanisms, including passive diffusion, entry via ion channels, and active or receptor-mediated transport. For instance, a role for passive diffusion in addition to transporters or gated channels has been postulated for the mechanism of cell accumulation of conventional cisplatin.$^{19}$ These mechanisms of cell accumulation can be energy-independent, as for channels and passive carriers, or be energy-dependent, as for ATP-powered pumps.$^{29}$ Different mechanisms of cellular accumulation can be distinguished on the basis of whether the mechanism of accumulation requires energy, as for endocytosis and active transport proteins, or is energy-independent, as is the case for passive diffusion through the membrane and diffusion facilitated by channels and carriers. Processes that require energy can be blocked by incubating cells at low temperature (4 °C) or by ATP depletion with metabolic inhibitors, such as 2-deoxy-D-glucose (competitively inhibits glycolysis) and oligomycin (blocks oxidative phosphorylation).$^{29}$

The temperature dependence of cell accumulation of Ir from complex 1 and Pt from cisplatin was explored in A2780 cells. These cells were incubated with the metal complexes at 4 °C, 20 °C, and 37 °C. The temperature dependence of cell accumulation of Pt from cisplatin (2.1 ± 0.3 Pt/10$^6$ cells) is consistent with the previous observations,$^{30}$ which indicates the active nature of its accumulation. In contrast, Ir from complex 1 accumulated in A2780 cells even at this low temperature (30 ± 3 Ir/10$^6$ cells), which suggests that energy-independent passive diffusion plays at least a partial role in its accumulation. The mean levels of iridium or platinum increased with incubation temperature 4-fold or 6-fold, respectively, if the temperature increased from 4 °C to 37 °C (Fig. 2). Hence, the latter observation implies that energy-dependent transport of complex 1 also plays a role in its cellular accumulation.

Moreover, we also examined the contribution of Na$^+/K^+$-ATPase to energy-dependent accumulation of Ir from complex 1 in A2780 cells using the specific inhibitor of Na$^+/K^+$-ATPase ouabain.$^{31}$ The cells were co-incubated with ouabain (200 µM) and complex 1 or cisplatin at the concentration of 10 µM for 24 h.

In both cases cellular metal accumulation decreased as a consequence of co-incubation with ouabain (Fig. 2). In the case of cisplatin, Pt accumulation decreased to ca. one half of its original value when co-incubated (to 5.2 ± 0.9 from 12.1 ± 0.7 ng from 111 ± 2 to 70 ± 6 ng of Ir per 10$^6$ cells at the same ouabain...
However, quite surprisingly it increased approximately 2-fold significantly affected by depletion of intracellular ATP (Fig. 2).

For other details, see the text.

To examine further how Ir from complex 1 accumulates in cells also by an energy-dependent process, A2780 cells were co-treated with 2-deoxy-D-glucose (50 mM) and oligomycin (5 µM) 60 minutes before treatment with complex 1 or cisplatin at the concentration of 30 µM for 120 minutes at 37 °C. This co-treatment results in depletion of intracellular ATP. The cellular accumulation of Ir from complex 1 or Pt from cisplatin was significantly affected by depletion of intracellular ATP (Fig. 2).

However, quite surprisingly it increased approximately 2-fold when cells were under metabolic inhibition, with a mean level of iridium or platinum of 217 ± 3 or 28.6 ± 0.8 ng of Ir or Pt/10^6 cells, respectively compared to 115 ± 5 or 12 ± 1 ng of Ir or Pt/10^6 cells, respectively, for the cells not pre-treated with 2-deoxy-D-glucose and oligomycin. The effect of intracellular ATP depletion on Ir accumulation increase was also clearly evident if the cells were co-treated with 2-deoxy-D-glucose (50 mM) plus oligomycin (5 µM) for 60 minutes and subsequently treated with complex 1 (30 µM) or cisplatin (30 µM) for various time points in the range of 30 – 180 minutes (Fig. S1).

Extent of efflux

Cellular accumulation of Ir or Pt arises as the result of two important processes: cellular influx and efflux. The latter is especially important in antiproliferative activity determinations that involve a cell recovery period in drug-free medium. The extent of the efflux of Ir from complex 1 was investigated. A2780/Adr ovarian cells were exposed to 5 µM complex 1 and for comparison also to 5 µM cisplatin for 24 h and then left to recover for 24 h. The results show a significant efflux during 24 h, but even after 24 h in drug-free media, Ir from complex 1 was not completely excreted from the cells (Fig. 3), being retained to more than 67% of the original uptake. Impaired cellular accumulation due to an increased extent of efflux represents one of the important mechanisms of resistance to anticancer agents. Studies of the mechanism underlying efflux of antitumor drugs may be useful for understanding factors responsible for resistance to these agents.

Inhibition of efflux by verapamil. Verapamil, an L-type calcium channel blocker capable of reversing multi-drug resistance, effectively abrogates efflux of anticancer drugs mediated by p-glycoprotein, including several ruthenium arene complexes in A2780/Adr cells by competitive inhibition of drug transport. The A2780/Adr cell line utilized in the present study displays the classic multi-drug resistance phenotype also mediated via over-expression of the p-glycoprotein. Therefore, complex 1 was used to investigate the extent of Ir efflux when cells were allowed to recover in drug-free medium that contains verapamil. The efflux of Ir from complex 1 was impaired by increasing the concentration of verapamil (Fig. 3). Interestingly, Ir from complex 1 was retained by more than ~90% in the presence of 20 µM of this calcium channel blocker. In contrast, verapamil does not restore cisplatin sensitivity (Fig. 3) consistent with its known lack of recognition by p-glycoprotein.

Inhibition of efflux by reversan and buthionine sulfoximine. Accumulating evidence also suggests a critical role of intracellular glutathione (GSH) in tumor cell resistance to transition metal-based drugs which are inactivated by this and other sulfur-containing compounds. GSH-conjugated drugs must be exported from the cells in which are formed before they can be eliminated from the body. A significant role in this process is played by multidrug resistance-associated protein 1 (MRP1) acting as an ATP-dependent efflux pump. MRPI gene
encodes ATP-dependent glutathione S-conjugate export pump, ‘GS-X pump’ that plays an important role in several physiological processes including antitumor drug resistance. In addition, MRP1/GS-X pump plays a role in the elimination of the GS-platinum complex from several tumor cells treated with cisplatin. Thus, these facts suggest that the MRP/GS-X pump and GSH biosynthesis together may affect efflux of metals including Ir from complex 1 as well.

To investigate whether conjugates of GSH with Ir from complex 1 cross the membrane into extracellular space using the MRP1/GS-X pump, accumulation of Ir from complex 1 in the presence of reversan, a potent MRP1 inhibitor and buthionine sulfoximine (BSO), a potent inhibitor inhibitor of GSH synthesis was analyzed. A2780 cells, in which expression of MRP1 has been confirmed, were incubated with reversan (10 µM) or BSO (1 mM) as single agents or a cocktail combining reversan (10 µM) and BSO (1 mM) for 24 h before addition of cisplatin (5 µM) or complex 1 (5 µM) for additional 24 h. Cellular Ir accumulation slightly increased or remained almost unchanged as a consequence of the incubation with reversan or BSO alone, respectively (Fig. 4). In contrast, pretreatment with the mixture of reversan plus BSO increased cellular Ir accumulation to 48 ± 1 from 38 ± 1 ng of Ir per 10^6 cells (the values represent mean ± standard deviations for three independent samples). An asterisk denotes a significant difference (p<0.05) from the untreated control.

**Fig. 4.** Accumulation of Pt/Ir in A2780 cells after co-incubation with cisplatin (5 µM) or complex 1 (5 µM) and 10 µM reversan, 1 mM buthionine sulfoximine or a cocktail combining reversan (10 µM) and BSO (1 mM) at 37 °C. Results are expressed as ng of metal per 10^6 cells.

**Role of CTR1 in cellular metal accumulation**

A2780 cells were co-incubated with complex 1 or cisplatin at a concentration of 10 µM and copper(II) chloride (0.1 mM) to study the effect of Cu on Ir accumulation, and therefore the involvement of the copper transporter CTR1 in the cellular uptake. Results indicate that Ir accumulation from complex 1 is reduced by ca. 10% and Pt accumulation from cisplatin by ca. 20% (Fig. 2). These data suggest that the CTR1 pathway may also be involved in the uptake of complex 1. In addition, these results are consistent with previous reports which indicate that Ctrl mediates cytotoxicity of cisplatin by regulating its cellular accumulation.

**Role of the endocytosis pathways in cellular metal accumulation**

Many molecules of biological significance are internalized into cells by vesicles formed by the invagination of the cell membrane. It has been shown that cholesterol-depletion can inhibit the formation of these invaginations of the plasma membrane. Therefore, the role of endocytosis via pathways involving the formation of cholesterol-enriched invaginated vesicles in cellular accumulation of complex 1 or cisplatin was explored as well. A2780 cells were co-incubated for 24 h with complex 1 or cisplatin at a concentration of 10 µM and methyl-β-cyclodextrin (0.2 mM), a cholesterol extracting agent, and changes in metal accumulation were determined (Fig. 2). In both cases, the presence of methyl-β-cyclodextrin caused no significant change in Ir or Pt accumulation in cells. In the case of complex 1, Ir increased accumulation insignificantly (to 122 ± 7 from 115 ± 8 ng of Ir per 10^6 cells) and accumulation of Pt from cisplatin slightly decreased (to 9.4 ± 0.8 from 11.5 ± 0.5 ng of Pt per 10^6 cells) at the same methyl-β-cyclodextrin concentration. Thus, there are no significant changes in cellular metal concentrations in the presence of a relatively high concentration of methyl-β-cyclodextrin.

**Discussion**

It has been shown that the Cp*IrIII complex containing an anionic C,N bound 7,8-benzoquinoline chelating ligand (I) exhibits very promising potency in ovarian and breast cancer cell lines. In contrast the isoelectronic Cp*IrIII complex containing a neutral N,N-bound 1,10-phenanthroline chelating ligand (2) is deemed inactive (Table 1). Hence, the class of iridium(III) complexes containing both Cp* and a C,N chelating ligand appears to be attractive for development as new anticancer agents, which provides a basis for further exploration of this new class of anticancer complexes.

One of the important early phases of the mechanism by which anticancer metallo-drugs exert their biological activity is cellular uptake and accumulation. Cellular accumulation of metallo-drugs may involve multiple mechanisms which can be energy-independent or be energy-dependent. The temperature dependence of cellular accumulation of Ir from complex 1 (Fig. 2) and diminished Ir accumulation as a consequence of treatment of A2780 cells with ouabain (the specific inhibitor of Na+/K+-ATPase) (Fig. 2A) indicate that energy-independent passive diffusion plays at least partial role in its accumulation. On the other hand, the cellular accumulation of Ir from complex 1 or Pt from cisplatin was significantly enhanced by depletion of intracellular ATP due to co-treatment of ovarian tumor cells with 2-deoxy-D-glucose and oligomycin (Fig. 2 and S1). This observation is consistent with the thesis that energy-dependent transport of complex 1, in particular out of the cell, plays a role in its cellular accumulation as well.

Efflux systems function via an energy-dependent mechanism to pump out toxic substances outside the cell through specific
In conclusion, the results of the present work enhance the rich diversity of cellular accumulation pathways of transition metal-based antitumor compounds and some discrete accumulation mechanisms further differentiate the potential of non-platinum agents over the conventional antitumor platinum drugs.

Acknowledgments

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Notes and references

A new antitumor iridium complex \([\eta^5\text{Cp}^*](\text{Ir})(\text{bq})\text{Cl}\) (Cp*=pentamethylcyclopentadienyl, bq=7,8-benzoquinoline) and conventional cisplatin have contrasting mechanisms of accumulation in ovarian cancer cells.
[Image of a bar graph showing Pt/Ir accumulation in ng/10^6 cells for different conditions: No recovery, 24h recovery, 10μM verapamil, and 20μM verapamil. The graph compares accumulation for cisplatin and complex 1.]
Supplementary Information

Mechanism of cellular accumulation of an iridium(III) pentamethylcyclopentadienyl anticancer complex containing a C,N-chelating ligand

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Figure S1
Fig. S1: Time dependence of accumulation of Ir from complex 1 (A) or Pt from cisplatin (B) in A2780 cells. Symbols: open squares, accumulation after incubation with complex 1 (30 µM) or cisplatin (30 µM) alone; filled squares, accumulation after 1 h pre-incubation of the cells with 2-deoxy-D-glucose (50 mM) plus oligomycin (5 µM) and subsequent incubation with complex 1 (30 µM) or cisplatin (30 µM). The values represent mean ± standard deviations for three independent samples.