Metallodrugs for therapy and imaging: investigation of their mechanism of action
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Chapter B4

Investigation of the Molecular Accumulation Mechanisms of an Au(III) Cyclometallated Compound Compared to Cisplatin in vitro: Are OCT2 and CTR1 involved?

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1. Abstract

The molecular mechanisms of toxicity and cellular transport of anticancer metallodrugs, including platinum-based agents, have not yet been fully elucidated. Here, we studied the toxic effects and metal content (by ICP-MS) of a novel Au(III) compound of the (C^N) theme compared to cisplatin in a panel of cancer cell lines as well as one healthy cell line. A correlation between the concentration dependent toxicity and metal content could be observed. The most sensitive cell lines, A2780 and A2780 cisR, were selected for further transporter studies in vitro. The involvement of OCTs and MATE transporters as well as the copper transporters CTR1 and ATP7A/B in the accumulation of both metal compounds in A2780 cells was evaluated by co-incubating the cells with cimetidine or CuCl₂, respectively. The Au(III) compound 1 seems to be more potent compared to cisplatin, when evaluated after 24h and 72h incubation. In addition, an increase in toxicity was seen after 72h for both compounds co-incubated with CuCl₂. After 72h, also cimetidine had an effect on the accumulation of both compounds. Uptake studies between 0-120 min incubation showed no effect of either cimetidine or CuCl₂ on metal compound accumulation. To conclude, the Au(III) compound 1 and cisplatin might have different mechanisms of transport. The uptake transporter OCT2 and CTR1 are most likely not involved in both accumulations mechanisms, but the efflux transporter MATE and ATP7A/B should be investigated further.
2. Introduction

In the field of metallodrugs, platinum(II) compounds (cisplatin, carboplatin, oxaliplatin and nedaplatin) are still the gold-standard for numerous types of cancer,\(^1\) mostly applied in combination with other chemotherapeutics. Unfortunately, their use is limited due to intrinsic or acquired resistance and severe side-effects (eg. nephrotoxicity in the case of cisplatin). Consequently, to develop a potent and specific drug that limits the number of disadvantages described above, new metallodrugs are designed as anticancer agents that incorporate different metals. Many of them are in preclinical studies, but only a very small number reached clinical trials, including NAMI-A and KP1019, which are ruthenium(III) based compounds (Figure 1).\(^2\)

![Chemical structures of Cisplatin, NAMI-A, and KP1019](image)

**Figure 1.** Metallodrug used in the clinic (cisplatin) and metallodrugs containing ruthenium that reached clinical trials (NAMI-A and KP1019).

Among other metal-based complexes, gold(I) and gold(III) compounds raised interest in the last years showing promising anticancer properties *in vitro*, which prompted the investigation of their molecular mechanisms of biological action.\(^3,4\) In Figure 2, representative examples of cytotoxic gold(I) complexes (Auranofin) and gold(III) complexes are presented. Auranofin is currently used as an antirheumatic drug and bears Au(I) as the reactive metal species, stabilized by a thioglucose and a phosphine ligand coordinated in a linear fashion. The complex is highly cytotoxic *in vitro* against different
types of cancer cell lines. Nitrogen donor atoms are found to greatly stabilize the Au(III) ions compared to softer donor atoms like sulphur, the latter preferring Au(I). Thus, a vast number of Au(III) complexes with \( N \)-donor ligands have been reported so far, including polypyridyl ligands such as \([\text{Au(bipy)(OH)}_2][\text{PF}_6]\) (Figure 2). In medicinal inorganic chemistry, the use of Au(I) and Au(III) based organometallic complexes is advantageous due to their redox and thermodynamic stability. Additionally, by modification of the ancillary ligands or steric and electronic properties, the lipophilic character of the anionic cyclometallated ligands can easily be tuned.

![Figure 2. Au(I) (Auranofin) and Au(III) compounds discussed in this work.](image)

\([\text{Au(py}^{\text{b-H}}\text{)}\text{Cl}_2]\) represents an organometallic cyclometallated \( \text{C}^\text{N} \) Au(III) compound, with an even greater stability in aqueous solution compared to \([\text{Au(bipy)(OH)}_2][\text{PF}_6]\), due to the direct metal to carbon bond. This Au(III) compound was used as the precursor for the synthesis of the **Au(III) compound 1** investigated in this chapter. In general, Au(III) compounds exhibit relevant reactivity with several biomolecules, including DNA and proteins, consisting of either ligand exchange processes or redox processes. Depending on the specific nature of the various Au(III) complexes, their electrochemical profile, and the type of reacting species in the target molecule, these reactions may lead either to the formation of tight Au(III)-biomolecule coordinative bonds or to the oxidation and damage of the involved biomolecule itself. Protein targets which are relevant for Au(III) complexes are thioredoxin reductase (TrxR), cathepsin cysteine
protease and deubiquitinases. Au(III) complexes can both oxidize TrxR at various amino acid sites or form a coordination bond directly with the Seleno-Cys residue and sometimes also with Cys residues in the active site. Au(I) complexes can only form a coordination bond directly with the seleno-cys residue. For this reactions it is essential that both Au(III) or Au(I) complexes undergo ligand exchange reactions to bind to the Seleno-Cys.\(^3\)

Overall, the interactions of the gold complexes with different biological targets induce direct DNA damage, modification of the cell cycle, mitochondrial damage, proteasome inhibition, modulation of specific kinases, and other cellular processes, which eventually trigger apoptosis. These processes seem to play a major role in the mechanism of the cytotoxic action of gold compounds.

However, very little is known about the mechanisms of cellular accumulation (uptake and efflux) of the gold complexes in cancer cells or in healthy tissue. Only one \textit{in vitro} study has been published thus far, evaluating the role of hOCT1-2 (the human organic cation transporter 1 and 2), hCTR1 (the human copper transporter 1) and endocytotic processes in the uptake of Au(I) NHC (\(N\)-heterocyclic carbene) complexes by the 518A2 melanoma cell line, using the MTT assay for toxicity determination as surrogate marker for uptake.\(^6\) This clearly shows the lack of extensive mechanistic cellular accumulation studies.\(^7\)

In general, the differential toxicity of different anticancer drugs in certain organs, such as the nephrotoxicity of cisplatin, may be related to a different Pt accumulation in the cells of these tissues. It is worth mentioning that a higher drug accumulation in a particular cell type might be caused by either a higher uptake or a lower efflux\(^8,9\), which emphasizes the need for mechanistic information on the transport mechanisms involved. Even for the anticancer platinum drugs, knowledge about their mechanism of accumulation in cancer cells as well as in healthy cells is incomplete. The OCT2 and CTR1 have been postulated to be involved in the uptake of cisplatin, whereas multi
extrusion protein (MATE) and ATP7A/B seem to be involved in its efflux, as recently reviewed by Spreckelmeyer et al.\(^7\)

In this work, we present the synthesis and characterization of a novel Au(III) cyclometallated compound featuring a coumarin ligand endowed with fluorescence properties for imaging in cells by fluorescence microscopy and to evaluate cell accumulation. The evaluation of the toxic effects of the Au(III) complex compared to cisplatin was performed using a panel of cancer cell lines as well as non-cancer cells \textit{in vitro}. Moreover, the involvement of OCT2, MATE, ATP7A/B and CTR1 in the uptake and excretion of the gold compound was studied via transporter inhibition experiments \textit{in vitro} and the accumulation of the compounds was further analyzed via ICP-MS in two A2780 cell lines, one sensitive and one resistant to cisplatin.

3. Results and Discussion

3.1. Synthesis and Characterization

A novel cyclometallated Au(III) compound \textbf{1} was synthesized adapting already established procedures for similar complexes (Figure 3).\(^10\) The C\(\equiv\)N precursor \([\text{Au(py}_{\text{H}}\text{-H})\text{Cl}_2]\)\(^11\) was reacted with a phosphine, adding \(3\text{-}[4\text{-}(\text{diphenylphosphino})\text{phenyl}]\text{-7-methoxy-2H-chromen-2-one}\) (PPh\(_2\)Arcoum) to yield the fluorescent Au(III) compound \textbf{1}.\(^12\)

\[\text{Au}^{\text{III}}\text{ compound } 1\]

\textbf{Figure 3.} Synthesis route of Au(III) compound 1.
The complexation of the phosphine ligand with [Au(py-H)Cl2] as well as the isomeric purity of Au(III) compound 1 was analyzed by 31P(1H) NMR spectroscopy. The NMR spectrum shows a singlet at 31.5 ppm of the coordinated phosphorous shifted downfield by 35 ppm with respect to the corresponding precursor. Moreover, the 1H NMR spectrum of Au(III) compound 1 shows a downfield shift of the signal of the pyridine proton in position 6 by 0.05 ppm associated with a morphological change of the pyridine moiety. A splitting of the signals corresponding to the carbons of the two phenyl rings was observed due to their diastereotopic feature induced by the fixed geometry of the cyclometallated ligand as depicted in Figure 4. Moreover, another splitting of the signal is observed due to the coupling of the phosphorous atom with carbons and hydrogens.

![Figure 4. General scheme of the two possible boat-like stereoisomers of [Au(py-H)ClP].](image)

### 3.2. Fluorescence

Au(III) compound 1 in dichloromethane exhibits a typical absorption at 381 nm and a fluorescence emission band of the coumarin chromophore at 429 nm with ΦF = 38 % (Figure 5). Interestingly, this value is higher than the one of the free phosphine ligand (ΦF = 20 %). We already noticed this phenomenon when we previously complexed Au(I) on the same coumarin-phosphine ligand. Moreover, the emission wavelength is not sensitive to complexation of the chromophore to [Au(py-H)Cl2] (λem = 426 nm for the free phosphine ligand in methanol).
Figure 5. Absorption, excitation and emission spectra of Au(III) compound 1 (dichloromethane, 2.5 x 10^{-5} M).

3.3. Antiproliferative effects

The antiproliferative effects of Au(III) compound 1 were studied in different human cancer cell lines in comparison to cisplatin, via a classical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after 72 h incubation. Specifically, the selected human cancer cell lines were A2780 (ovarian carcinoma), HCT116 p53 +/- and -/- (colon carcinoma), MCF 7 (breast carcinoma) and A549 (lung carcinoma), as these cancer types are the most common ones. The tumour protein p53 is a tumour suppressor gene and known to be downregulated in cisplatin resistant cancer tissues. The obtained IC_{50} values are summarized in Table 1. Au(III) compound 1 exhibits a toxicity profile in all tested cell lines which was also found for other Au(III) cyclometallated compounds from the same class with a (C^N) theme. However, compared to cisplatin, Au(III) compound 1 shows a higher toxic effect against HCT116 p53 -/- and a significant higher toxic effect against MCF7, while it has a lower toxicity in HCT p53+/- and a significant lower toxicity in A549 cells. No difference was observed for the toxicity of Au(III) compound 1 in A2780 and against the non-tumorigenic HEK-293T cells with respect to cisplatin. Overall,
the observed activity profiles might indicate different mechanisms of action compared to cisplatin as well as different accumulation mechanisms. Due to the highest toxicity against the ovarian cancer A2780 cells, these were selected for further experiments to investigate the transport mechanisms for the **Au(III) compound 1** and cisplatin.

**Table 1.** Toxicity of **Au(III) compound 1** (expressed as IC$_{50}$ values$^a$) compared to cisplatin against different human cancer cell lines and against non-tumorigenic HEK-293T cells, after 72 h of incubation. For statistical analysis, the t-test was used. * (p ≤ 0.05) indicates the difference is statistically significant when compared to cisplatin treated samples

<table>
<thead>
<tr>
<th>Compound</th>
<th>A2780</th>
<th>HCT116 p53 +/+</th>
<th>HCT116 p53 -/-</th>
<th>MCF7</th>
<th>A549</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4 ± 0.3</td>
<td>9.8 ± 0.9</td>
<td>18.4 ± 1.1</td>
<td>12.6 ±</td>
<td>25.9 ±</td>
<td>10.1 ± 3</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.3 ± 0.5</td>
<td>5.3 ± 0.2</td>
<td>22.9 ± 2.3</td>
<td>20.0 ± 3</td>
<td>12.1 ± 1</td>
<td>8.6 ± 1.3</td>
</tr>
</tbody>
</table>

$^a$ The reported values are the mean ± SD of three independent experiments.

### 3.4. Studies on the mechanisms of transport

#### 3.4.1. Competition experiments

The toxicity of the new **Au(III) compound 1** and cisplatin were evaluated in human ovarian cancer cells sensitive (A2780) and resistant (A2780cisR) to cisplatin. Both A2780 and A2780cisR cells were recently evaluated from Sorensen et al.\textsuperscript{15} for the CTR1, OCT2 and ATP7A/B transporters expression levels, as summarized in Figure 6 (reproduced with permission from reference\textsuperscript{15}). The organic cation transporter 2 (OCT2) is an uptake transporter for many substrates, mainly small cationic compounds, and the copper transporter 1 (CTR1) is an uptake transporter for mainly Cu$^{2+}$ cations. Instead, ATP7A and ATP7B are pumps known to be involved in the efflux of Cu$^{2+}$. Notably, A2780cisR cells show a significantly higher expression of the copper efflux transporters ATP7A (1.3 fold) and ATP7B (5-fold) as well as the
cation uptake transporter OCT2 (1.3 fold). The copper uptake transporter CTR1 is on the other hand lower expressed in A2780cisR cells compared to the A2780 wild type (0.4 fold). These data suggest that the resistance observed in the A2780cisR cells is at least partly caused by a different balance between uptake and efflux of cisplatin resulting from a decreased influx via CTR1 and increased efflux of cisplatin by ATP7B, thereby decreasing the intracellular exposure of the cells to cisplatin.

![Graph showing relative protein expression of ATP7A, OCT2, CTR1, and ATP7B in WT and CisR cells.](image)

**Figure 6.** Relative drug transporter expression levels in A2780 and A2780cisR cells, reproduced with permission from reference.15

The IC$_{50}$ values of **Au(III) compound 1** and cisplatin were determined after 24h and 72h incubation time in order to observe the development of the toxicity over time (Table 2). Overall, **Au(III) compound 1** is more toxic in the A2780 cells, than in the resistant A2780cisR cells. In addition, after 24h incubation, **Au(III) compound 1** is 5-fold more toxic than cisplatin in A2780 cells, and 7-fold more toxic in A2780cisR cells. However, after 72h incubation, **Au(III) compound 1** and cisplatin show the same toxicity in A2780, but the **Au(III) compound 1** is still ca. 3-fold more toxic in A2780cisR than cisplatin. In addition, there is no significant difference between the IC$_{50}$s at 24h and 72h treatment of A2780cisR cells with **Au(III) compound 1**, leading to
the assumption that the Au(III) compound does not show accumulation of toxic effects during subchronic exposure. In A2780 cells, the Au(III) compound 1 shows a 2-fold decrease of the IC₅₀ values from 24h to 72h incubation, indicating accumulation of toxicity during chronic exposure.

Table 2. IC₅₀ values of Au(III) compound 1 and cisplatin against A2780 and A2780cisR cells, incubated in absence and presence of CuCl₂ or cimetidin (Cim), recorded after 24h and 72h incubation. The reported values are the mean ± SD of three independent experiments. For statistical analysis, the Two-way ANOVA was used. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to samples treated with the metallodrugs only (control).

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 h</th>
<th>72 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A2780</td>
<td>A2780cisR</td>
<td>A2780</td>
<td>A2780cisR</td>
</tr>
<tr>
<td>1</td>
<td>5.8 ± 1.5</td>
<td>15 ± 4</td>
<td>2.4 ± 0.3</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>1 + Cim</td>
<td>6.6 ± 0.3</td>
<td>18.2 ± 6.1</td>
<td>0.7 ± 0.2**</td>
<td>8.1 ± 0.5*</td>
</tr>
<tr>
<td>1 + CuCl₂</td>
<td>4.1 ± 0.3</td>
<td>6.1 ± 1.9*</td>
<td>0.2 ± 0.1**</td>
<td>3.1 ± 1.0**</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>26 ± 2</td>
<td>103 ± 3</td>
<td>2.3 ± 0.5</td>
<td>30 ± 1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>1.0 ± 0.1**</td>
<td>21.3 ± 1.1**</td>
</tr>
<tr>
<td>Cim</td>
<td></td>
<td></td>
<td>22.0 ± 2.1</td>
<td>91.7 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>22.2 ± 1.3</td>
<td>51.0 ± 4.9**</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td></td>
<td>0.5 ± 0.2**</td>
<td>18.0 ± 0.6**</td>
</tr>
<tr>
<td>CuCl₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In order to evaluate the involvement of OCT2 and CTR1 uptake transporters in the accumulation of Au(III) compound 1 and cisplatin, the compounds’ toxicity was further tested in the presence of transporter inhibitors or competitor substrates, respectively. If these transporters are involved in the drug uptake, their inhibition should lead to a reduced intracellular accumulation of the metal complexes and to a decrease in cytotoxic effects and thus an increase in IC₅₀. Cimetidine (300 µM) was selected as inhibitor for OCTs and MATE, and CuCl₂ (30 µM) as competitive substrate of CTR1 and ATP7A/B. Experiments were performed in A2780 and A2780cisR cells after 24h and 72h
incubation with the compounds. CuCl$_2$ and cimetidine do not show a toxic effect on A2780 or A2780cisR cell lines at the used concentrations. The obtained results are summarized in Table 2 and in Figures 7 and 8.

After 24h incubation, neither cimetidine nor CuCl$_2$ showed an effect on the toxicity of Au(III) compound 1 or cisplatin in A2780 cells (Figure 7A). However, in A2780cisR cells (Figure 7B), both cisplatin and Au(III) compound 1 showed a significantly increased toxicity when co-incubated with CuCl$_2$. For cisplatin the resistance was only partly reduced, but for Au(III) compound 1 the resistance was fully compensated resulting in a similar toxicity in both cell lines. These results support the hypothesis that CuCl$_2$ is inhibiting de-toxification mechanisms, possibly the copper efflux transporters (ATP7A/B), which are expressed at much higher levels in the A2780cisR cells than in the wild type A2780 cells (Figure 6). Apparently, these efflux transporters play a minor role in the wild type A2780 cells. The lack of effect of cimetidine on the toxicity of cisplatin or Au(III) compound 1 in both cell lines might indicate that the uptake by OCTs and efflux by MATE is not limiting for the toxicity.

![Figure 7](image-url)

**Figure 7.** IC$_{50}$ values of Au(III) compound 1 (black) and cisplatin (grey) on (A) A2780 and (B) A2780cisR after 24h incubation. Data are
expressed as mean ± SD (n=3). For statistical analysis, the Two-way ANOVA was used. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to samples treated with the metallodrugs only (control).

After 72 h incubation of the A2780 cells, both cimetidine and CuCl₂ increased the toxicity of Au(III) compound 1 significantly, 3.5-fold and 12-fold, respectively. For cisplatin, a 2.5-fold and 4.5-fold increased toxicity could also be observed with cimetidine and CuCl₂, respectively (Figure 8A). The same effect was observed in A2780cisR cells, but to a lower extent (Fig 8B). This supports the hypothesis, that for both compounds, at 72 h, incubation efflux mechanisms are inhibited by CuCl₂ and cimetidine, possibly via inhibition of ATP7A/B and MATE, respectively.

**Figure 8.** IC₅₀ values of Au(III) compound 1 (black) and cisplatin (grey) on (A) A2780 and (B) A2780cisR after 72h incubation. Data are expressed as mean ± SD (n=3). For statistical analysis (Two-way ANOVA) * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to the metallodrugs treated samples (control).
3.4.2. Metal content determination

After evaluating the toxic effects of both compounds in cancer cells in the presence and absence of transport inhibitors/competitors, the metal content was determined by inductively coupled plasma mass spectrometry (ICP-MS) to gain further insights into the drug accumulation mechanisms. For these experiments two concentrations of the metal compounds were chosen based on their IC_{50} and the IC_{50} times two (IC_{50} x 2). Interestingly, no differences in Au content could be observed between the two cell lines treated with the same concentrations of Au(III) compound 1 (5 µM or 10 µM at 24 h or 3 µM and 6 µM at 72 h) (Table 3). However, increased accumulation of Au was observed in both cell lines treated with 10 µM of Au(III) compound 1 for 24 h and co-incubated with CuCl_{2} (Figure 9A, B). The effect of the CuCl_{2} co-incubation was most marked in the A2780 cells with a 5-fold increase in Au uptake compared to the controls. A 2-fold increase was observed at 5 µM, but this increase was not statistically significant.
Table 3. Metal content of Au(III) compound 1 and cisplatin in A2780 and A2780cisR cells, measured after 24h and 72h. The reported values are the mean ± SD of three independent determinations. nd = not determined

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pt or Au content /µg protein</th>
<th>24 h</th>
<th>72 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A2780</td>
<td>A2780cisR</td>
<td>A2780</td>
<td>A2780cisR</td>
</tr>
<tr>
<td>1 (5 µM)</td>
<td></td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>1 (10 µM)</td>
<td></td>
<td>0.12 ± 0.03</td>
<td>0.10 ± 0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>1 (3 µM)</td>
<td>nd</td>
<td>nd</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>1 (6 µM)</td>
<td>nd</td>
<td>nd</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>(15 µM)</td>
<td></td>
<td>0.17 ± 0.04</td>
<td>0.08 ± 0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.04 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>(20 µM)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.04 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>(5 µM)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>(10 µM)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

Co-incubation of Au(III) compound 1 with 300 µM cimetidine resulted in an increased accumulation of Au only in the A2780 wild type cells at 10 µM of Au(III) compound 1, but not at 5 µM and no effect was seen in the A2780cisR cells (Figure 9A, B).
Figure 9. Au content after 24h incubation of (A) A2780 (B) A2780cisR cells treated with 5 µM (black) and 10 µM (grey) of Au(III) compound 1. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was applied. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to the metallodrugs treated samples (control).

The same type of experiments was repeated after 72 h treatment of cells with Au(III) compound 1 at 3 and 6 µM. After 72 h incubation, in the presence of 30 µM CuCl$_2$ a ca. 2-fold increase in Au content was observed both in A2780 cells and in A2780cisR cells at both concentrations of Au(III) compound 1, except for the co-incubation of 6 µM Au(III) compound 1 in the wild type cells (Figure 10 A, B). This may be explained by the fact that after 72 h, 6 µM is ca. 30 fold the IC$_{50}$ value for Au(III) compound 1, which may induce extensive cell death, and cell membrane integrity may be altered and the Au compound may have leaked out of the cells during washing of the samples. Cimetidine did not have any significant effect on the Au content of the slices after 72 h of incubation.
Subsequently, we measured the Au content at different time points during the first 120 min to monitor metallodrug uptake in the cancer cells. Samples were taken after 10, 20, 30, 60 and 120 min in the case of A2780 cells (Figure 11). With cimetidine (Figure 11A) no effect could be observed on Au uptake. With CuCl₂ (Figure 11B) only after 120 min, a significantly higher Au content could be observed. In the case of A2780cisR cells (Figure 12), no significant effect of cimetidine or CuCl₂ on the Au content could be observed up to 120 min of incubation. Between 10-20 min incubation, a drop in Au content can be observed in A2780 cells. We don’t have an explanation for that thus far, but it should be further investigated in additional experiments.
Figure 11. Au content in 3 µM treated A2780 with/without (A) cimetidine (B) CuCl₂. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was applied. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to its control (treatment with 3 µM Au(III) compound 1 taken at the same time).

Figure 12. Au content in 3 µM treated A2780cisR cells with/without (A) cimetidine (B) CuCl₂. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was applied. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to its control (treatment with 3 µM Au(III) compound 1 taken at the same time).
When evaluating the Pt content in A2780 and A2780cisR cells treated with cisplatin after 24h (Figure 13A-B) or 72h incubation (Figure 14A-B) by ICP-MS, no significant difference could be observed after co-incubation with cimetidine or CuCl₂. However, a marked increase in Pt uptake was observed in A2780 cells treated for 24h with the higher concentration of cisplatin (20 µM) compared to cells treated with the lower concentration (15 µM). This concentration dependent effect could not be observed in the case of the A2780cisR cells at either 24 or 72 h incubation, supporting the idea that higher detoxification and efflux mechanisms may be in place. In Figure 15 and Figure 16, the Pt content within the first 120 min of cisplatin incubation is shown co-incubated with cimetidine or CuCl₂. No significant differences between cisplatin alone and co-incubated with the transporter inhibitors are shown, besides for cisplatin at 5 µM (Figure 15A), where cimetidine increased the Pt content at the 120 min time point.

Figure 13. Pt content after 24h incubation of (A) A2780 and (B) A2780cisR with 15 µM (black) and 20 µM (grey) cisplatin. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was used. No sign differences were found between the controls and the inhibitor-treated cells.
Figure 14. Pt content after 72h incubation in (A) A2780 (B) A2780cisR cells, treated with 5 µM (black) and 10 µM (grey) cisplatin. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was used. No sign differences were observed between the cells incubated with the inhibitors and their respective controls.

Figure 15. Pt content in 5 µM treated A2780 cells with/without (A) cimetidine (B) CuCl₂. Data are expressed as mean ± SD (n=3). For statistical analysis (Two-way ANOVA) * (p ≤ 0.05), ** (p ≤ 0.01) indicate
the difference is significant when compared to its control (5 µM cisplatin).

**Figure 16.** Pt content in 5 µM treated A2780cisR cells with/without (A) cimetidine (B) CuCl₂. Data are expressed as mean ± SD (n=3). For statistical analysis the Two-way ANOVA was applied. * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to its control (5 µM cisplatin).

Finally, it is also worth noting that metal accumulation is more efficient in the case of Au(III) compound 1 compared to cisplatin in both cancer cell lines over time (Table 3). This result is in line with the observed toxic effects which are more pronounced for Au(III) compound 1 compared to cisplatin at either 24h or 72h incubation (Table 2), suggesting that a more efficient uptake of compound 1 could explain at least a part of the difference in toxicity.

### 3.4.3. Passive/active mechanisms

To investigate if carrier-mediated or passive molecular mechanisms are responsible for metal accumulation, cells were incubated with the Au(III) compound 1 or cisplatin at either 37°C or 4°C for 10 min, 20 min, 30 min, 60 min and 120 min. This experiment was performed only once (n=1) as a pilot experiment. At 4°C, carrier-mediated transport, including active mechanisms like ATP dependent
transporters, is not functional, resulting in complete reduction of carrier-mediated uptake of the substrate, leaving only passive transport. Although at 4°C the viscosity of the cell membrane increases, leading to a decreased fluidity, which may also result in decreased passive diffusion, the reduction in uptake of by passive diffusion is much less than for transporter-mediated uptake.\(^{16}\) In Figure 17, the Au content in A2780 and A2780cisR cells treated with 3 µM Au(III) compound 1 is shown. The Au content is markedly higher at 37°C compared to 4°C in A2780 cells after 60 min incubation, suggesting that active mechanisms are important for Au(III) compound 1 uptake into A2780 cells. In A2780cisR cells, an effect temperature can only be seen after 120 min incubation. These experiments were only performed once, thus the results have to be interpreted with caution.

**Figure 17.** Au content in (A) A2780 and (B) A2780cisR cells treated with 3 µM Au(III) compound 1; red bar: 37°C, blue bar: 4°C (n=1).

Concerning cisplatin, the Pt content does not change at 37°C compared to 4°C, leading to the assumption that active mechanisms of metal accumulation do not play a crucial role within the first 120 min of incubation.
Figure 18. Pt content in 5 µM cisplatin treated (A) A2780 WT and (B) A280 Res cells; red bar: 37°C, blue bar: 4°C (n=1)

3.5. Copper accumulation

The viability results of cisplatin treated cells showed a decreased IC$_{50}$ value after co-incubation with CuCl$_2$. However, the ICP-MS data did not show an increased Pt content that could be responsible for such an effect. As inhibition of the copper transporters by cisplatin or by the Au(III) complex cannot be excluded, we investigated whether the Cu$^{2+}$ content was increased due to the co-incubation with each metallodrug. Concerning copper uptake, CTR1 affinity to Cu is between 0.6 µM in fibroblasts and 13 µM in murine hepatocytes. ATP7B affinity to Cu is $2.5 \times 10^{-17}$ M. Both transporters are not only selective for Cu, but also for example for Ag(I), Cd(II) and Fe(III).

The copper content was evaluated in both the wild-type A2780 and cisplatin resistant A2780cisR cells since the different CTR1/ATP7A/B transporter expression levels (see Figure 6) may have an effect in its accumulation. After 24h, the Cu content in the control cells is the same ($0.056 \pm 0.02$ ng Cu in A2780 and $0.045 \pm 0.01$ ng Cu in A2780cisR). Overall, it could be observed that the copper content strongly increased in both cell lines upon treatment with the Au(III) compound 1 for 24 and 72h (Figure 19). The co-incubation of Au(III) compound 1 with CuCl$_2$
showed an increase in toxicity compared to cells incubated with **Au(III)** compound 1 alone. With ICP-MS we showed an increased Au and Cu content after 24h and 72h incubation, that may be responsible for the observed increased toxicity. The effect of the increased Cu-content is not fully understood yet, but an additive or even synergic toxic effect with Au may be possible. To note, a lower Cu content was observed in the A2780 cells treated with 30µM CuCl₂ after 24h incubation as well as after 72h incubation. In order to prevent cell death, the cells might have downregulated the CTR1 uptake transporter due to an excess of CuCl₂ in the medium.

![Graph showing Cu content vs. treatment conditions](image)

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**Figure 19.** Cu content in A2780 (WT) and A2780cisR (Res) cells after (A) 24h and (B) 72h incubation with 30 µM CuCl₂ and different concentrations of Au(III) compound 1. Data are expressed as mean ± SD (n=3). For statistical analysis (Two-way ANOVA) * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to its control (compared to incubation with Cu alone).
Also for cisplatin treated A2780 cells (Figure 20), we could observe that, after 24h and 72h incubation, the Cu content increases at both concentrations of cisplatin. However this effect was absent in the A2780cisR cells incubated for 72h.

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Figure 20. Cu content after (A) 24h and (B) 72h incubation with 30 µM CuCl₂ and cisplatin. Data are expressed as mean ± SD (n=3). For statistical analysis (Two-way ANOVA) * (p ≤ 0.05), ** (p ≤ 0.01) indicate the difference is significant when compared to its control (incubation with 30 µM CuCl₂ alone).

Overall, both metal compounds seem to have an effect on Cu accumulation in A2780 cells.

3.6. Fluorescence Microscopy

In order to investigate the sub-cellular localization of the Au complex, we performed fluorescence microscopy experiments on A2780 cells treated with Au(III) compound 1 and the two inhibitors cimetidine and CuCl₂. Propidium iodide (PI) staining was used to stain for nucleic acids and shows the nuclei. The pictures show the A2780 cells after 2h incubation with Au(III) compound 1 at IC₅₀ x 2 concentration (Figure 21). The compound itself does not co-localize with PI in the nuclei, but clearly enters the cells and is localized in the cytoplasm. This observation points towards a different mechanism of action compared
to cisplatin, and suggests that the DNA damage may not play a pivotal role in the toxicity of the Au compound. Co-incubation with either cimetidine or CuCl₂ did not change the intracellular accumulation pattern.

**Figure 21.** Fluorescence microscopy of A2780 cells treated for 2 h with Au(III) compound 1 with/without 300 µM cimetidine or with/without 30 µM CuCl₂.
4. Conclusions

In the past years, several studies have been carried out to elucidate the mechanisms of uptake and efflux of cisplatin in cancer cells and kidney slices (eg. Chapter B3). Nonetheless, such transport mechanisms are not yet fully understood. Moreover, the mechanisms leading to toxicity and accumulation of new generation anticancer gold complexes have not been fully elucidated. Therefore, we investigated and compared the toxicity and the accumulation of cisplatin and a cytotoxic experimental organometallic Au(III) complex in a panel of cancer cell lines. Additionally, we evaluated the involvement of OCTs, MATEs, CTR1 and ATP7A/B using either their inhibitor cimetidine or competitor CuCl2.

The cyclometallated Au(III) compound 1 is more toxic than cisplatin in a number of cell lines, including HCT16 p53 -/-, MCF7 and A2780cisR cells. However, it has a similar or lower toxicity for several other cancer cell lines. The compound is also moderately toxic towards the non-tumorigenic HEK cells. The A2780cisR cell line is not only resistant to cisplatin but also to the Au compound, and this may be based on the differential expression of transporters like OCTs and ATP7A/B with respect to the A2780 cells.

The intracellular accumulation of the Au(III) compound 1 is more efficient than that of cisplatin, as judged from the accumulation of Au and Pt respectively, in both A2780 and A2780cisR cells as demonstrated by ICP-MS. Overall, after 24h incubation with Au(III) compound 1, co-incubation with CuCl2 but not with cimetidine, showed a significant higher toxicity in A2780cisR cells, whereas in A2780 cells no effect was seen of either CuCl2 or cimetidine. However, after 72h, co-incubation of Au(III) compound 1 with either cimetidine or CuCl2 showed a higher toxicity. After 72h incubation, co-incubation of Au(III) compound 1 with CuCl2 resulted in an increase in toxicity and increase in Au and Cu content in both cell lines as demonstrated by ICP-MS. Based on these results two explanations can be suggested for these effects: Au(III) compound 1 is a substrate for ATP7A/B and inhibition of
the efflux transporter ATP7A/B by Cu ions results in a higher Au accumulation and higher toxicity, and/or the increased toxicity is a result of additive or synergistic toxicity of Au and Cu accumulation. A direct involvement of the CTR1 or OCT2 in the uptake of the drug could not be shown by inhibition by CuCl₂ or cimetidine. The increased toxicity of **Au(III) compound 1** in the presence of cimetidine after 72 h of incubation, but not after 24h, is difficult to explain, as no concomitant increased accumulation of Au was observed by ICP-MS. Studies with other, more selective, inhibitors for each single drug transporter are needed to confirm these results. **Au(III) compound 1** localizes mainly in the cytoplasm in A2780 cells (in the presence or not of CuCl₂ and cimetidine) as demonstrated by fluorescence microscopy.

Similarly to **Au(III) compound 1**, 24h incubation of cisplatin with CuCl₂ shows a significant decrease in viability in A2780cisR cells, although not as strong as for **Au(III) compound 1**. After 72h, co-incubation of cisplatin with either cimetidine or CuCl₂ leads to an increase in toxicity, but no increase in Pt content could be observed in both cell lines as demonstrated by ICP-MS. This result, together with the evidence for increased Cu content in A2780 cells, leads to the hypothesis that copper accumulation is the reason for the increased toxicity in this cell lines. A direct involvement of OCTs or CTR1 in the uptake of cisplatin could not be concluded from our results. If this would be the case then inhibition by cimetidine should have resulted in lower toxicity and lower accumulation in the cells. Holzer et al. showed that, as a consequence of cisplatin incubation, the CTR1 transporter might be sequestered away from the membrane, leading to a decreased Pt uptake into cells. However, a decreased Pt uptake was not confirmed by our results.²⁰ Similarly, a direct involvement of MATE or ATP7A/B in the accumulation of cisplatin could not be confirmed. Concerning passive vs. active uptake for **Au(III) compound 1** and cisplatin within the first 120 min of incubation, very preliminary results suggest that active mechanisms might play a role for **Au(III) compound 1** uptake into A2780 cells, whereas in A2780cisR this might not be the case. For cisplatin, no difference between 37°C and 4°C incubated cells
could be observed, leading to the assumption, that active transport mechanisms might not play a crucial role in these cell lines.

5. Experimental section

Synthesis

General Remarks

All reactions were carried out under an atmosphere of purified argon using Schlenk techniques. Solvents were dried and distilled under argon before use. The precursor \([\text{Au(py}^\text{b-H})\text{Cl}_2]\) has been synthesized according to literature procedure. All other reagents were commercially available and used as received. All the analyses were performed at the “Plateforme d’Analyses Chimiques et de Synthèse Moléculaire de l’Université de Bourgogne”. The identity and purity (≥ 95%) of the complexes were unambiguously established using high-resolution mass spectrometry and NMR. Exact mass of the synthesized complexes were obtained on a Thermo LTQ Orbitrap XL. \(^1\text{H}\) (300.13, 500.13 or 600.23 MHz), \(^{13}\text{C}\) (125.77 or 150.90 MHz) and \(^{31}\text{P}\) (121.49, 202.45 or 242.94 MHz) NMR spectra were recorded on Bruker 300 Avance III, 500 Avance III or 600 Avance II spectrometers. Chemical shifts are quoted in ppm (δ) relative to TMS (\(^1\text{H}\) and \(^{13}\text{C}\)) using the residual protonated solvent (\(^1\text{H}\)) or the deuterated solvent (\(^{13}\text{C}\)) as internal standards. 85% H\(_3\)PO\(_4\) (\(^{31}\text{P}\)) was used as an external standard. Infrared spectra were recorded on a Bruker Vector 22 FT-IR spectrophotometer (Golden Gate ATR) and far infrared spectra were recorded on a Bruker Vertex 70v FT-IR spectrophotometer (Diamant ATR). X-ray diffraction data for the \textbf{Au(III) compound 1} were collected on a Bruker Nonius Kappa CCD APEX II at 115 K.

\textbf{Au(III) compound 1, [Au(py}^{\text{b-H}})(\text{PPh}_2\text{Archrom})\text{Cl]}\text{PF}_6

A round-bottom flask was charged with the precursor \([\text{Au(py}^{\text{b-H}})\text{Cl}_2]\) (50 mg, 0.115 mmol), KPF\(_6\) (106 mg, 0.573 mmol, 5 eq.) and 3-[4-
(diphenylphosphino)phenyl]-7-methoxy-2H-chromen-2-one (PPh$_2$Ar) (50 mg, 0.115 mmol, 1 eq.) in suspension into 5 mL of distilled acetone under argon atmosphere. Starting Au complex was solubilized after some minutes. The reaction was maintained at room temperature for 1.5 h; afterward 10 mL of dichloromethane were added and the yellow solution was filtrated through Celite® and concentrated under vacuum. The pure product was obtained after recrystallization from a dichloromethane/pentane mixture as a yellow powder (91 mg, 80 % yield).

$^1$H NMR (Acetone-d$_6$, 500.13 MHz, 298 K): 3.97 (s, 3 H, OCH$_3$), 4.49 (d, 1 H, $^2$J$_{HH}$ = 15.6 Hz, CH$_2$-PyrBz), 5.05 (d, 1 H, $^2$J$_{HH}$ = 15.6 Hz, CH$_2$-PyrBz), 6.52 (dt, 1 H, $^3$J$_{HH}$ = 8.5 Hz, $^4$J$_{HH}$ = 1.5 Hz, H$^6$), 6.88 (dd, 1 H, $^3$J$_{HH}$ = 7.5 Hz, $^4$J$_{HH}$ = 3.0 Hz, H$^6$), 6.98 (d, 1 H, $^4$J$_{HH}$ = 2.5 Hz, H$^3$), 7.00 (d, 3 J$_{HH}$ = 0.5 Hz, H$^4$), 7.33 (dd, 1 H, $^3$J$_{HH}$ = 8.5 Hz, $^4$J$_{HH}$ = 1.5 Hz, H$^3$), 7.60-7.66 (m, 4 H, Hortho-pC$_6$H$_4$), 7.70 (d, 3 J$_{HH}$ = 0.5 Hz, H$^4$), 8.27 (s, 1 H, H$^4$), 8.31 (dt, 1 H, $^3$J$_{HH}$ = 8.5 Hz, $^4$J$_{HH}$ = 1.5 Hz, H$^4$), 9.25 (broad s, 1 H, H$^6$).

$^{13}$C($^1$H) NMR (Acetone-d$_6$, 125.77 MHz, 300 K): 47.9 (s, CH$_2$-PyrBz), 56.5 (s, O -CH$_3$), 101.2 (s, CH$^5$), 113.8 (s, CH$^3$), 114.0 (s, Cquat-coum), 122.9 (s, Cquat-p-C$_6$H$_4$), 124.0 (d, $^1$J$_{PC}$ = 84.3 Hz, Cipso-Ph), 124.5 (d, $^1$J$_{PC}$ = 83.0 Hz, Cipso-Ph), 124.8 (d, $^1$J$_{PC}$ = 70.4 Hz, Cipso-pC$_6$H$_4$), 125.4 (d, $^4$J$_{PC}$ = 3.8 Hz, CH$^5$), 127.3 (d, $^4$J$_{PC}$ = 3.8 Hz, CH$^3$), 128.8 (s, CH$^4$), 128.9 (d, $^4$J$_{PC}$ = 2.5 Hz, CH$^5$), 129.8 (s, CHpara-Ph), 129.9 (s, CH$^3$), 130.2 (d, $^2$J$_{PC}$ = 10.1 Hz, CHortho-Ph), 130.3 (d, $^2$J$_{PC}$ = 10.1 Hz, CHortho-Ph), 131.0 (s, CH$^5$), 133.7 (d, $^3$J$_{PC}$ = 7.5 Hz, CH$^6$), 134.5 (d, $^3$J$_{PC}$ = 2.5 Hz, CHortho-pC$_6$H$_4$), 134.6 (s + d, $^3$J$_{PC}$ = 2.5 Hz, Cquat-Bz + CHortho-pC$_6$H$_4$), 136.1 (s, CHmeta-Ph), 136.2 (s, Cmeta-Ph + Cmeta-pC$_6$H$_4$), 136.4 (s, CHmeta-pC$_6$H$_4$), 141.6 (d, $^2$J$_{PC}$ = 2.5 Hz, C-Au), 143.1 (s, CH$^4$), 144.2 (s, CH$^4$), 150.8 (s, Cquat-coum), 152.4 (s, CH$^5$), 156.8 (s, Cquat-pyr), 157.9 (s, Cquat-coum), 160.4 (s, Cquat-coum), 164.6 (s, Cquat-coum).

$^{31}$P($^1$H) NMR (Acetone-d$_6$, 202.45 MHz, 300 K): 31.5 (s, 1 P, PPh$_3$-Coum), -144.2 (h, 1 P, PF$_6$).
ESI-MS (DMSO-MeOH), positive mode exact mass for [C\textsubscript{40}H\textsubscript{31}NO\textsubscript{3}PAuCl]\textsuperscript{+}
(836.13901): measured m/z 836.13656 [M-PF\textsubscript{6}]\textsuperscript{+}.

IR (ATR & FIR, cm\textsuperscript{-1}): 1725, 1613, 1569, 1437, 1362, 1025, 836, 751, 311, 229. Anal. Calc. for C\textsubscript{40}H\textsubscript{31}NO\textsubscript{3}P\textsubscript{2}F\textsubscript{6}AuCl: C, 48.92, H, 3.18, N, 1.43 %. Found: C, 48.40, H, 2.70, N, 1.52 %.

Cell experiments

Cell viability assay

The human breast cancer cell line MCF7, human lung cancer cell line A549, human colon cancer cell lines HCT116 p53+/+ and human ovarian cancer cell lines A2780 and A2780cisR (obtained from the European Centre of Cell Cultures ECACC, Salisbury, UK) were cultured in DMEM (Dulbecco’s Modified Eagle Medium) and the A2780 cells in RPMI containing GlutaMax supplemented with 10 % FBS and 1 % penicillin/streptomycin (all from Invitrogen), at 37°C in a humidified atmosphere of 95 % of air and 5 % CO\textsubscript{2} in an incubator (Heraeus, Germany). Non-tumoral human embryonic kidney cells HEK-293T were kindly provided by Dr. Maria Pia Rigobello (CNRS, Padova, Italy) and were cultivated in DMEM medium, added with GlutaMax (containing 10 % FBS and 1 % penicillin/streptomycin (all from Invitrogen) and incubated at 37°C and 5 % CO\textsubscript{2}. For evaluation of toxicity, cells were seeded in 96-well plates (Costar, Integra Biosciences, Cambridge, MA) at a concentration of 10.000 cells/well (A2780, MCF-7, HEK-293T) or 6000 cells/well (HCT116 p53 +/-, A549) and grown for 24 h in the appropriate medium mentioned above. Solutions of the compounds were prepared by diluting a freshly prepared stock solution (10\textsuperscript{-2} M in DMSO) of the corresponding compound in aqueous media (RPMI or DMEM for the A2780 or A549, MCF-7, HCT116 p53+/- and HEK-293T). Afterwards, 200 µL of these dilutions of the compounds were added to the wells to obtain a final concentration ranging from 0 to 120 µM, and the cells were incubated for 72 h. Following 24 or 72 h drug exposure,
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg ml\(^{-1}\) and incubated for 2 h, then the culture medium was removed and the violet formazan (artificial chromogenic precipitate of the reduction of tetrazolium salts by dehydrogenases and reductases) dissolved in how much ml? DMSO. The optical density of each well (96-well plates) was quantified in triplicates at 550 nm using a multi-well plate reader, and the percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC\(_{50}\) value was calculated as the concentration reducing the proliferation of the cells by 50 % using which calculation program? and it is presented as a mean (± SE) of at least three independent experiments.

**ICP-MS**

The concentrations of platinum or gold were measured by inductively coupled plasma mass spectrometry (ICP-MS) using an Elan 6000 spectrometer (Perkin Elmer Sciex, Concord, ON, Canada) and Micromist Nebulizer and a cyclonic spraychamber (Glas Expansion Pocasset, MA, U.S.). Nebulizer gas, lens voltage and RF power were optimized daily using a 10 µg/L Pt standard in 0,1% (V/V) HCl with 0.65% (V) HNO\(_3\), normal settings were: Rf power 1350 W, nebuliser gas flow rate 0.95 L min\(^{-1}\), and lens volgate 10 V. Acquisition parameters were 50 ms dwell time, 1 sweeps reading, 5 reading replicates and 25 readings monitoring all or a subset of \(^{195}\text{Pt}\), \(^{196}\text{Pt}\), \(^{197}\text{Au}\), and \(^{63}\text{Cu}\). The instrument was tuned at the beginning of each analysis to ensure optimal operation.

Samples were dried by vacuum centrifugation (eppendorf, concentrator plus) at 60°C for 2 hours. Cell samples were digested by 200 µL 65% HNO\(_3\) and 50 µL 30% H\(_2\)O\(_2\) overnight until the solution is clear. Samples were prepared for analysis by dilution to 5 mL with 0.65% (V/V) HNO\(_3\) and 0,1% (V/V) HCl. The external standards were prepared from a 10 mg/L Au andPt stock solution (CPI International, Peak Performance, 4400-120213WG01, Lot# 12B214) or Cu stock solution (Plasma CAL, SCL SCIENCE, Q.C. no 4, Cat# 140-102-045, Lot SC5322198) with 0.65%
(V/V) HNO$_3$ and 0,1% (V/V) HCl. All reagents were of the highest available purity.

**Cell culture**

Human ovarian sensitive (A2780) and cisplatin resistant (A2780cisR) cells were grown in 75 cm$^2$ culture flasks (CellStar, Grenier Bio, Germany) in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L148 glutamine. Both cell lines were kept at 37°C, 5% CO$_2$, and 100% humidity. Cells were passed on every 3-4 days using 0.25% trypsin in phosphate-buffered saline (PBS, pH 7.4). To maintain resistance, cisplatin-resistant A2780cisR cells were treated with 1 μM cisplatin between every third passage.

**Au/ Pt content after 24h/72h incubation with metal compound +/- inhibitor**

A2780 or A2780cisR cells were plated into 25cm$^2$ culture flasks (CellStar, Grenier Bio, Germany) with RPMI 1640 medium up to a volume of 5 mL per flask. The next day, the cells were treated with 15 μM/ 20 μM cisplatin for 24 hours and 5 μM/ 10 μM cisplatin for 72 hours +/- cimetidine or +/- CuCl$_2$. Cimetidine was used in a nontoxic concentration of 300 μM and CuCl$_2$ was used at 30 μM. The same set-up is used for Au(III) compound 1, but with 5 μM/ 10 μM for 24 hours incubation and 3 μM/ 6 μM for 72 hours incubation.

After 24 hours or 72 hours, the medium was removed and the cells were washed two times with 3 mL ice-cold PBS. 500 μL lysis buffer was added and the samples were stored on ice for 20 minutes. Afterwards, cells were detached by using a scrubber policemen and the cell suspension was transferred to a 1.5 mL eppendorf tube. 200 μL of the sample was transferred to a new eppendorf tube for ICP-MS analysis. 5 μL of cell suspension was used for protein determination.
Au/ Pt accumulation during 120 min incubation with metal compound +/- inhibitor

A2780 or A2780cisR cells were plated into 25 cm² culture flasks (CellStar, Grenier Bio, Germany) with RPMI 1640 medium up to a volume of 5 mL per flask. The next day, the cells were treated with 5 µM cisplatin (+/- cimetidine or +/- CuCl₂) for 10’, 20’, 30’, 60’ and 120’ min. For compound 1, we used a concentration of 3 µM. The samples were processed as described above.

Protein assay

Protein determination was performed using BioRad DC™ Protein Assay reagents A, B and S (Bio-Rad, ). Using a 96-well-plate, 5 µL of standard (0, 5, 10, 15, 20, 25 and 30 mg/ml bovine serum albumin) and samples were analyzed in duplicate. First, 25.5 µL reagent-mix A+S (25 µL A + 0.5 µL S) was added and then 200 µL reagent B. The absorbance at 610 nm was measured after 15 minutes (stored in dark) using a PlateReader (Perkin Elmer, EnVision, 2104 Multilabel Reader).

Fluorescence Microscopy

A2780 cells were seeded (5 x 10⁵ for each sample) and grown on 8 well microscope plates, coated with Poly-L-lysine hydrobromide (Sigma-Aldrich, P6516) with RPMI medium. After 24h, cells were incubated with various concentrations of Au(III) compound 1 in RPMI, without FCS for 1h at 37 °C. At the end of incubation, cells were rapidly washed with cold PBS and then fixed with 2 % paraformaldehyde for 30 min at 4°C. For visualization of the nuclei with PI, cells were permeabilized with 0.2% Triton X-100 for 20 min at 4°C and treated with 1 µg/µl of PI for 10 min at room temperature. Cells were washed once with PBS and then analyzed by confocal microscopy. As preparation for visualization, the plate wells were removed from the slide and glycerol was used to cover the slide with a glass cover slip. The fluorescence was analysed using a Leica DM4000 B Automated Upright Microscope, equipped with the appropriate filters. PI was excited at 547 nm (emission wavelength 572 nm) and compound 1 at 358 nm (emission wavelength 461 nm,
DAPI filter). The acquired images were obtained using the individual filters and a combined image, overlaying the fluorescence was acquired using the Leica microscope software.

6. References


