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Synthesis and Antiproliferative Activity of Cisplatin-3–Chloropiperidine Conjugates

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We report the synthesis and characterization of two novel cisplatin- alkylating agents conjugates. Combining a platinum based cytostatic agent with a sterically demanding alkylating agent could potentially induce further DNA damage, block cell repair mechanisms and keep the substrate active against resistant tumor cell lines. The 3-chloropiperidines utilized as ligands in this work are cyclic representatives of the *N*-mustard family and were not able to coordinate platinum on their own. The introduction of a second coordination site, in form of a pyridine moiety, led to the isolation of the desired conjugates. They were characterized with HRMS, CHN-analyses and XRD. We concluded this work by examining the cytotoxicity of the ligands and the obtained complexes with MTT assays in human cancer cell lines. While the ligands showed hardly any activity, the novel conjugates both displayed a high antiproliferative and cytotoxic potency in a panel of three cell lines. Moreover, both complexes were able to largely circumvent the acquired cisplatin resistance of A2780cisR ovarian cancer cells, both in the MTT assay and a flow-cytometric apoptosis assay.

Introduction

Cisplatin (Figure 1a) was first synthesized by *Michele Peyrone* in 1844 and became known as Peyrones chloride.[1] Over hundred years later in the 1960s *Barnett Rosenberg* accidently discovered its cytotoxic potential during electrochemical experiments on bacterial cells by utilizing platinum electrodes. Cisplatin dramatically changed cancer treatment after its worldwide approval in 1978.[2,3] Together with its analogs carboplatin (Figure 1b) and oxaliplatin (Figure 1c) it forms an indispensable class of drugs administered in at least half of all cancer chemotherapies, with excellent cure rates of up to 90% for testicular cancer.[4–6]

The mechanism of action is believed to involve a hydrolysis leading to a cationic complex, which quickly forms crosslinks between two adjacent nucleobases. The N^7-position of the guanine nucleobase appears to be the main target, forming predominantly guanosine guanosine crosslinks.[7,8] These platinum nucleobase adducts interfere with different enzymes such as DNA and RNA polymerases and can ultimately lead to apoptosis.[9] In spite of the therapeutic success it became apparent that the application of cisplatin leads to a variety of adverse effects, such as nephrotoxicity, peripheral neurotoxicity and hematological toxicity.[10,11] In an effort to reduce these side effects the aforementioned carboplatin, often referred to as second generation platinum drug, was developed. While showing greatly reduced toxicity, it also shows a lower anticancer potency, generally requiring much higher dosage which is limited by the still occurring myelosuppression.[5,11,12] Another drawback of carboplatin is the cross resistance with cisplatin that many cancer types exhibit. Consequently, oxaliplatin, the third generation platinum drug, has been developed and was first approved in 1996. The bulky (1*R*, 2*R*)-1,2-diaminocyclohexane (DACH) ligand introduced in this compound hinders DNA repair mechanisms and the complex remains effective against many cisplatin resistant tumor cells.[13–15] Besides DNA repair mechanisms other factors, such as lesion tolerance or platinum deactivation by glutathione have been shown to contribute towards cisplatin resistance.[3,16] Consequently a plethora of other platinum-based drug candidates has been synthesized and evaluated over the past decades,[17,18] with some compounds even obtaining national approvals.[6] Today cisplatin and its analogs are frequently administered in combination with other chemotherapeutic agents such as 5-fluorouracil or

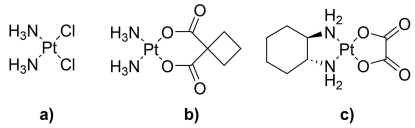

Figure 1. Cisplatin (a), Carboplatin (b) and Oxaliplatin (c).

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gemcitabine, in order to enhance efficacy and minimize side effects.[3,8,13,14]

Taking this combination approach one step further, various groups have explored the idea of linking an *N*–mustard moiety to platinum in order to induce further DNA damage (Figure 2).[19–21] *N*-Mustard, often cited as the first cancer chemotherapeutic drugs,[23] also target the N^7-position of guanine to covalently transfer an alkyl unit and form DNA crosslinks.[23,24] Contrary to platinum induced lesions these alkylation products can lead to depurination, which in turn cause DNA strand breaks, ultimately leading to apoptosis.[25] While the conjugates reported by *Siddik et al.*[19] and *Noji et al.*[20] coordinate the metal center directly with the nitrogen of the mustard moiety (Figure 2b), *Carell* reasoned that this would drastically diminish the reactivity of the *N*-mustard. Due to the coordination towards the metal center the lone pair of the nitrogen is no longer available and therefore the critical aziridinium intermediate cannot be formed. Consequently *Carell et al.* opted for an indirect coordination (Figure 2b) through the use of an ethylene glycol spacer. With this approach both moieties, the platinum as well as the alkylating agent, remain active as demonstrated via LC-ESI-HRMS.[21] Recently, *Mukherjee et al.* introduced a cisplatin conjugate featuring *bis*-(2-chloroethyl) pyridylmethylamine as a ligand,[22] along with derivatives incorporating carboxylic acids as leaving groups.[26] (Figure 2b) These conjugates exhibited markedly reduced susceptibility to deactivation by thiol-containing molecules, such as glutathione, a tripeptide linked to the development of cisplatin resistance. Furthermore, they demonstrated a delayed release of the alkylating moiety even after Pt(II) was sequestered by other cellular nucleophiles, leading to promising cytotoxicity against various cancer cell lines.[22,26]

In our previous publications we have reported cyclic *N*-mustard analogs.[27–29] These 3-chloropiperidines react with nucleophiles in a similar mode of action[30] (Figure 3) and have shown good activity in DNA cleavage assays as well as high potency against pancreatic tumor cell lines.[31] Their cyclic nature allows for an easy adjustment of their reactivity through the exploitation of ring strain and the *Thorpe-Ingold* effect.[32] The aforementioned works by *Siddik, Noji* and *Carell*[19–21] inspired us to investigate possible 3-chloropiridine platinum conjugates.

Results and Discussion

As a consequence of the high reactivity of the 3-chloropiperidine compared to other *N*-mustards, like chlorambucil (Figure 2a), and the resulting susceptibility towards hydrolysation[31] we reasoned that a direct coordination, without the use of a linker unit, might be beneficial in order to keep the alkylating moiety intact. The sterically demanding 3-chloropiperidine could also function similar to the DACH ligand in oxaliplatin, blocking potential DNA repair mechanisms.[13,14] From preliminary experiments with various metals, only being able to obtain complexes from the likes of copper and cobalt (see supporting information), we concluded that the 3-chloropiperidine unit only poorly coordinates to metal centers. We hypothesized that a weakly coordinating ligand is more readily replaced, releasing the active alkylating moiety in the process. With these theoretical considerations we set our focus on finding suitable 3-chloropiperidine ligands. Noticing that most published novel platinum analogs used primary or secondary amines to coordinate,[19,20,33] we started from our recently published secondary 3-chloropiperidines.[28] Similar to *Siddik et al.* we tried to conjugate potassium tetrachloroplatinate with two separate monofunctional 3-chloropiridines **A** (Figure 4a) but were unable to observe any complex formation. Attributing this to lack of any chelating effect we switched our efforts to bifunctional ligand **B** (Figure 4a). The synthesis along the already established route of chlorinating and TBAI mediated cyclization of a suitable diamine[29] proved to be unexpectedly challenging, resulting in low yield of only 25% over 2 steps. The low yield might be explained by one piperidine ring forming the bicyclic aziridinium ion and subsequently getting attacked by the second

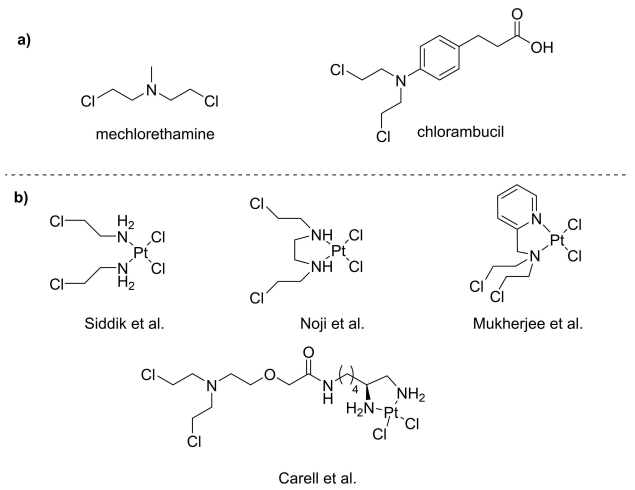

Figure 2. a) Approved *N*-mustards mechlorethamine and chlorambucil; b) cisplatin *N*-mustard conjugates by Z. H. Siddik,[19] M. Noji,[20] T. Carell[21] and Muhkerjee.[22]

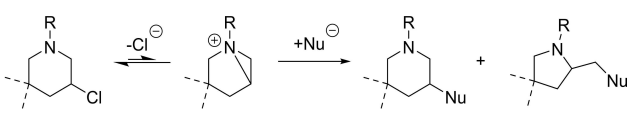

Figure 3. 3-Chloropiridenes: cyclic *N*-mustard analogues utilizing a bicyclic aziridinium intermediate.

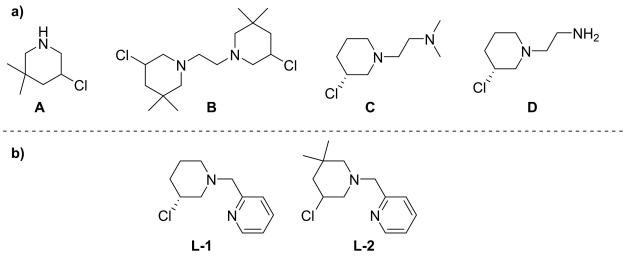

Figure 4. a) Synthesized unsuccessful ligands A–D; b) successful ligands L-1 and L-2.

piperidine moiety, leading to an inherent instability. With chelating ligand **B** at hand we tried to form the corresponding platinum complex, but yet again could not observe any formation, which might be due to steric hindrance. We reasoned that substituting one of the 3-chloropiperidine units with a less sterically demanding and therefore better coordinating group might be beneficial.

The synthesis of ligand **C** (Figure 4a) starting from *S*-prolinol via functionalization of the nitrogen, followed by chlorination with thionylchlorid was plagued with problems similar to ligand **B**, resulting in 22% yield. To prevent this instability and further side reactions with thionylchlorid the primary amine in ligand **D** (Figure 4a) was protected with tert-butyloxycarbonyl during the synthesis and stored as an HCl salt after deprotection, resulting in a total yield of 54% over 5 steps. Attempting to convert ligands **C** and **D** to their respective platinum complexes led to no isolable product, but the reaction of the HCl salt from 3-chloropiperidine **D** with potassium tetrachloroplatinate and 2 equivalents of NaOH (or NaOD in D_2O as reported by *Carell et al.*)[21] resulted only in a short term color change to yellow, indicating the temporary formation of the desired product. Reinforced by this observation we introduced pyridine as a coordinating unit, similar to the ligands utilized by *Muhkerjee et al.*[22,26] Ligands **L-1** and **L–2** (Figure 4b) could be synthesized in good yields, starting from *S*–prolinol and 2,2-dimethyl-4-pentenal, respectively. Finally reacting **L-1** and **L-2** with equimolar amounts of potassium tetrachloroplatinate in H_2O/MeOH gave the desired complex as a yellow precipitate (Figure 5).

Platinum conjugates **Pt-C-1** and **Pt-C-2** (Figure 5) were purified by repeated precipitation from DMF/H_2O and characterized by ESI-MS, elemental analysis and single crystal X-ray diffraction (Figure 6).

To evaluate the biological activity of the novel complexes we first conducted some preliminary experiments to assess their DNA-damaging properties. Compound **Pt-C-1** was dissolved in DMF and incubated at 37 °C with 10 eq of 2′–deoxyguanosine. After 72 h the solution was analyzed with HRMS and mono- and

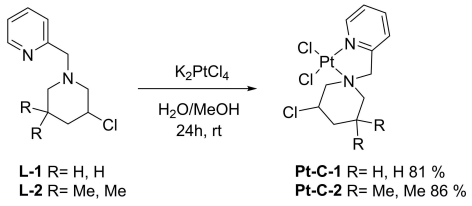

Figure 5. Synthesis of novel platinum 3-chloropiperidine conjugates Pt–C-1 and Pt–C-2.

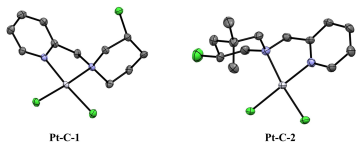

Figure 6. Molecular structures of the novel cisplatin analogues Pt–C-1 and Pt–C-2.

di-2′–deoxyguanosine adducts where confirmed by matching mass values and isotope patterns. Having established the reactivity towards DNA-bases we turned our attention to study their antiproliferative activity, which was assessed over 96 h by the MTT assay in monolayer cultures of three human malignant tumor cell lines. The ligands **L-1** and **L-2** alone hardly showed any inhibitory effects on the growth of cancer cell lines below a concentration of 100 μM (Figure 7) and yielded IC_{50} values below 200 μM only in the broadly chemosensitive CH1/PA-1 cells (Table 1). Previous studies have shown that 3-chloropiperidines, particularly aromatic-functionalized *bis*-3-piperidine derivatives, exhibit high efficacy against BxPC-3 pancreatic cancer cell lines, surpassing the potency of the reference compound chlorambucil.[31] However, their potency was markedly reduced against other cancer cell lines and for mono-3-chloropiperidines. Additionally, chirality has been demonstrated to significantly influence cytotoxicity.[27] Together with premature hydro-

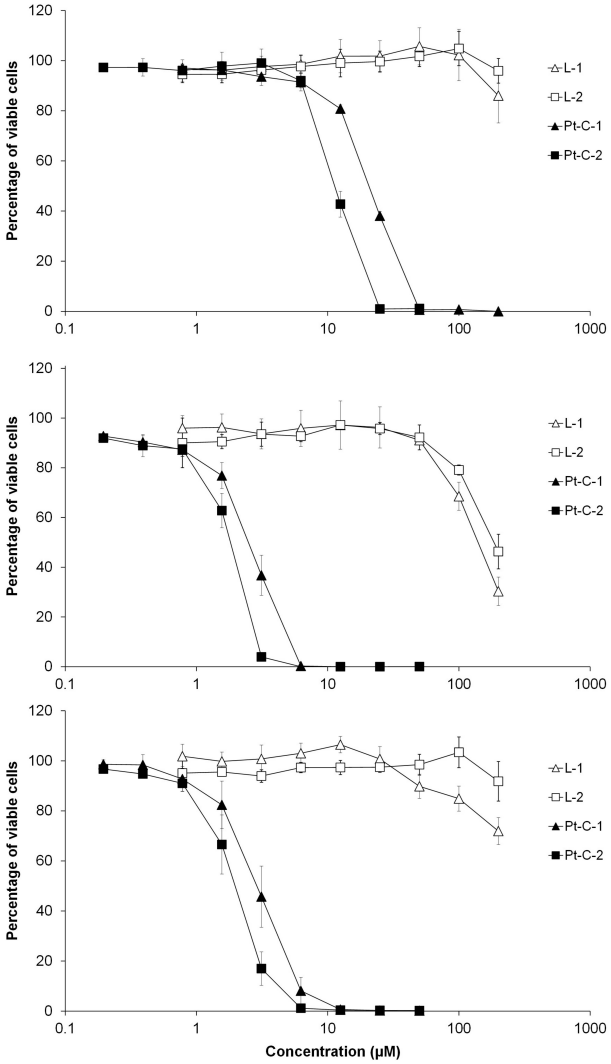

Figure 7. Concentration-effect curves in A549 (top), CH1/PA-1 (center) and SW480 cells (bottom) relative to untreated controls (100%). Values are means ± standard deviations from at least three independent MTT assays (exposure time: 96 h).

Table 1. Antiproliferative activity in three human cancer cell lines. Values are means ± standard deviations from at least three independent MTT assays (exposure time: 96 h).

	IC_{50} value (μM)		
Compound	A549	CH1/PA-1	SW480
L-1	> 200	140 ± 15	> 200
L-2	> 200	≈ 200	> 200
Pt–C-1	20.6 ± 0.4	2.5 ± 0.3	2.9 ± 0.6
Pt–C-2	11.4 ± 0.7	1.8 ± 0.1	2.0 ± 0.3
Cisplatin[a]	3.8 ± 1.0	0.073 ± 0.001	2.3 ± 0.2

[a] taken from ref.[34]

lysation of the alkylating unit these factors could account for the lower-than-expected activity observed for **L-1** and **L-2**. Their respective platinum(II) complexes **Pt-C-1** and **Pt-C-2** on the other hand yielded IC_{50} values in the low micromolar range in CH1/PA-1 ovarian teratocarcinoma as well as SW480 colon carcinoma cells and in the low two-digit micromolar range in the intrinsically multidrug-resistant lung adenocarcinoma cell line A549 (Table 1). **Pt-C-2** seems to be very slightly (1.4–1.8 times at the IC_{50}) but consistently more potent than **Pt-C-1**, contrary to the tendency shown by the corresponding ligands at concentrations higher than 100 μM. Concentration-effect curves of the complexes consistently approached the zero line at concentrations just about three times the respective IC_{50} in all three cell lines, indicating a high cell-killing potency (Figure 7).

In an isogenic pair of ovarian cancer cell lines, consisting of cisplatin-sensitive parental A2780 cells and a cell subline with acquired cisplatin resistance, A2780cisR, **Pt-C-1** and **Pt-C-2** were able to largely overcome cisplatin resistance, as reflected by resistance factors as low as 1.7 and 1.1, respectively (compared to 13.8 for cisplatin). In the resistant subline, **Pt-C-1** came close to the absolute potency of cisplatin and **Pt-C-2** even surpassed it by 1.7 times in terms of IC_{50} values (Table 2, Figure 8).

Finally, complexes **Pt-C-1** and **Pt-C-2** were tested for their potency to induce apoptotic cell death in the isogenic cell pair following 24 h exposure. In good agreement with cytotoxicity results, novel conjugates and cisplatin showed a tendency to induce programmed cell death in a dose-dependent manner (Figure 9A–C, Table S1). The cisplatin-resistant subline demonstrated a lower sensitivity towards all three compounds, however, the resistance towards cisplatin remained the largest (with almost 80% viable cells after exposure to 25 μM, Figure 9C). The concentrations higher than IC_{50} values had to be applied to compensate for relatively short exposure time (24 h vs. 96 h in MTT assay).

Table 2. Antiproliferative activity in A2780 and A2780cisR cancer cell lines. Values are means ± standard deviations from three independent MTT assays (exposure time: 96 h).

	IC_{50} value (μM)		Resistance
Compound	A2780	A2780cisR	factor
L-1	173 ± 9	> 200	(> 1.2)
L-2	*n.d.*	*n.d.*	*n.d.*
Pt-C-1	4.8 ± 0.6	8.3 ± 0.9	1.7
Pt-C-2	3.4 ± 0.5	3.9 ± 0.2	1.1
Cisplatin	0.47 ± 0.08	6.5 ± 0.1	13.8

n.d. not determined.

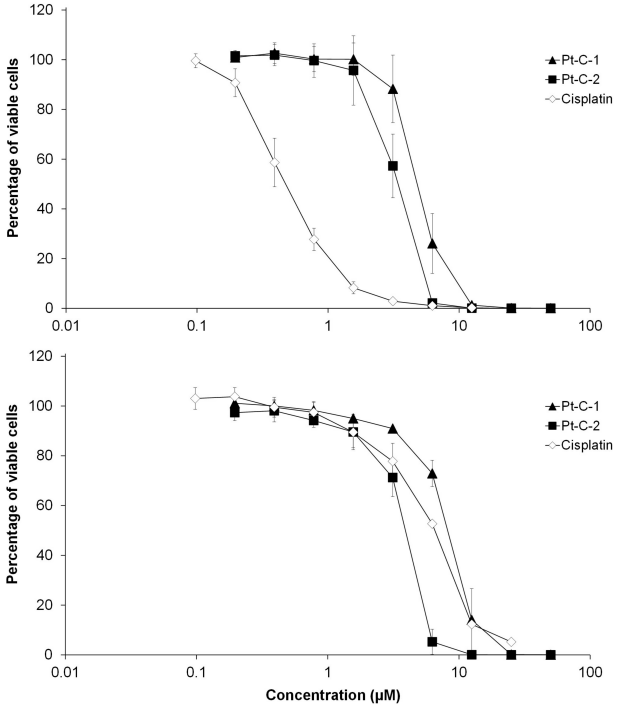

Figure 8. Concentration-effect curves in A2780 (top) and A2780cisR cells (bottom) relative to untreated controls (100%). Values are means ± standard deviations from three independent MTT assays (exposure time: 96 h).

Conclusions

3-Chloropiperidines have already been shown to effectively alkylate DNA and inhibit cell growth of pancreatic cancer cells. In this work we strived to combine cisplatin, a well-known cytotoxic drug, with these cyclic alkylating agents. Their sterically demanding structure seems to be only poorly coordinating to metal centers, which could result in a cleavage of the coordination and a release of the highly reactive alkylating moiety in the cell, but also made the synthesis very challenging. Secondary 3-chloropiperidines as well as a suitable *bis*-3-chloropiperidine have not been forming any conjugation with platinum whatsoever. Introducing a second coordination site in the form of a pyridine unit finally led us to the path of success. Resulting in the formation of two novel platinum alkylating agent conjugates, which have been characterized by HRMS and CHN-analysis and their structure confirmed by single crystal XRD. We concluded this work by assessing the DNA damaging potential of said compounds, by MTT assays of three different tumor cell lines (A549, CH1/PA-1 and SW480). Both complexes showed a high antiproliferative and cell killing potential in all

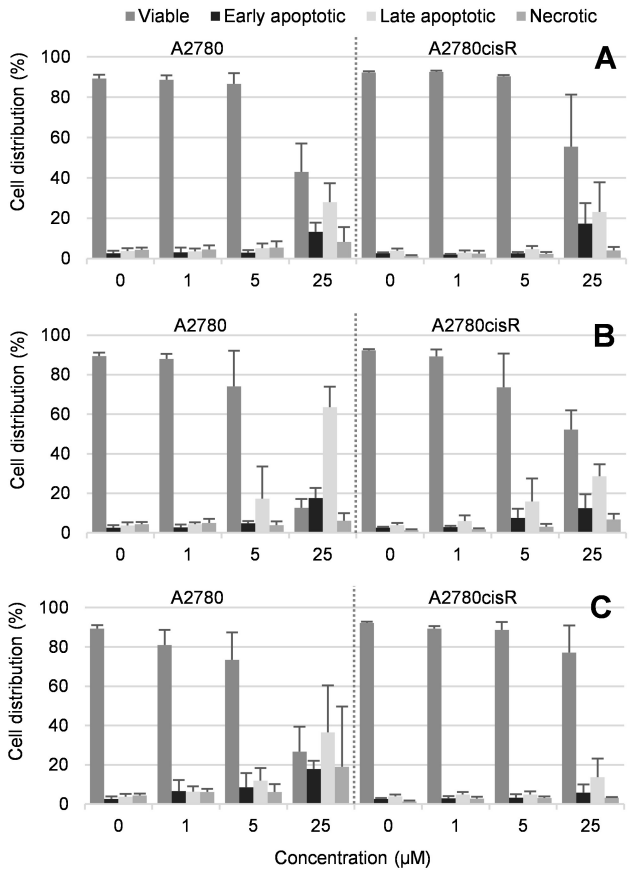

Figure 9. Induction of apoptosis/necrosis in A2780 and A2780cisR cells. **Pt-C-1** (**A**), **Pt-C-2** (**B**) and cisplatin (**C**) induced cell death detected by means of flow cytometry upon double annexin-FITC/propidium iodide staining. Values are means ± standard deviations from three independent experiments (exposure time: 24 h).

three tested cell lines, while the 3-chloropiperdine ligands alone hardly showed any activity. Furthermore, both complexes were able to overcome the acquired cisplatin resistance of A2780cisR ovarian cancer cells, yielding IC_{50} values in a range of low micromolar concentrations similar to cisplatin in this cell subline. Finally, the complexes were demonstrated to induce programmed cell death in both the parental A2780 cell line and the resistant subline, clearly surpassing the potency of cisplatin in the latter. Several factors must be considered and thoroughly investigated in future studies to understand the mechanisms by which these structures circumvent cisplatin resistance. Potential explanations include the steric bulk of the piperidine moiety and the delayed release of the alkylating unit, which may lead to additional DNA damage. Additionally, given the structural similarity to the conjugates reported by *Mukherjee et al.* (Figure 2b), the enhanced stability against hydrolysis and deactivation by glutathione should also be taken into account. We believe that further optimization of the ligand, such as utilizing bifunctional bis-3-chloropiperidines or exploiting chirality to more effectively induce DNA damage, along with a careful investigation into the activity of the coordinated ligand, could lead to the development of a potent new anticancer drug.

Materials and Methods

Pt-C-1

207 mg (0.50 mmol, 1 eq) potassium tetrachloroplatinate were dissolved in 2 ml of water and 105 mg (0.5 mmol, 1 eq) of **L-1** in 1 ml of methanol were added. The solution was stirred at room temperature for 24 h. The yellow precipitate was filtered of and washed with acetone. The crude product was finely dispersed in an ultrasonic bath and washed with water, before redissolving in DMF and precipitating it with water. The latter was repeated two times. The product was obtained as a yellow powder in 81% yield (193 mg, 0.41 mmol). HRMS (ESI): *m/z* calcd for $C_{11}H_{15}Cl_3N_2PtNa^+$: 497.9841; found 497.9849 [M + Na^+]; *m/z* calcd for $C_{11}H_{15}Cl_3N_2PtK^+$: 513.9580; found 513.9560 [M + K^+], elemental analysis calcd (%) for $C_{11}H_{15}Cl_3N_2Pt$: C 27.72, H 3.17, N 5.88; found: C 27.78, H 3.14, N 5.78.

Pt-C-2

299 mg (0.72 mmol, 1 eq) potassium tetrachloroplatinate were dissolved in 3 ml of water. 172 mg (0.72 mmol, 1 eq) of **L-2** dissolved in 1.5 ml methanol were added and the solution was stirred at room temperature for 24 h. The yellow precipitate was filtered of, finely dispersed in an ultrasonic bath and washed with water. The crude product was redissolved in DMF and precipitated with water. The latter was repeated two times. The product was obtained as a yellow powder in 86% yield (312 mg, 0.62 mmol) HRMS (ESI): *m/z* calcd for $C_{13}H_{19}Cl_3N_2PtNa^+$: 526.0154; found 526.0156 [M + Na^+]; *m/z* calcd for $C_{13}H_{19}Cl_3N_2PtK^+$: 541.9893; found 541.9898 [M + K^+], elemental analysis calcd (%) for $C_{13}H_{15}Cl_3N_2Pt$: C 30.94, H 3.79, N 5.55; found: C 31.31, H 3.84, N 5.36.

Ligand **A** was prepared according to literature.[28] The synthesis of ligands **B**-**D** as well as Ligands **L-1** and **L-2** can be found in the supporting information.

Cell Culture

CH1/PA-1 cells were provided by L. R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK, and confirmed by STR profiling as PA-1 ovarian teratocarcinoma cells at Multiplexion, Heidelberg, Germany. SW480 colon carcinoma and A549 non-small cell lung cancer cells as well as A2780 and A2780cisR ovarian cancer cells were obtained from the Institute of Cancer Research, Department of Medicine I, Medical University of Vienna. All media and supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. All cell lines were grown as adherent cultures in 75 cm^2 culture flasks (Starlab, Hamburg, Germany) under a humidified atmosphere with 5% CO_2 in air at 37 °C. CH1/PA-1, SW480 and A549 cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWest, Nuaillé, France), 1 mM sodium pyruvate, 4 mM L-glutamine and 1% (v/v) nonessential amino acids (from a 100× stock solution), A2780 and A2780cisR cells in RPMI 1640 medium supplemented with 4 mM L-glutamine and 10% heat-inactivated FBS.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess the antiproliferative activity of the compounds. For this purpose, cells were harvested from culture flasks by using trypsin/EDTA (Sigma-Aldrich), and the following cell numbers were seeded into 96-well tissue culture plates (Starlab, Hamburg, Germany): 1×10^3 (CH1/PA-1), 1.5×10^3

(A2780), 2×10^3 (SW480) 2.5×10^3 (A2780cisR) and 3×10^3 (A549) cells per well, each in 100 μL of the appropriate supplemented medium (as specified in the section above). Cells were incubated for 24 h to re-adhere and resume exponential growth and then treated with concentration series of the test compounds. Compounds were dissolved in DMF (Fisher Scientific, Hampton, NH, USA), diluted in the appropriate medium, and 100 μL of each dilution were added to the respective wells in triplicates. After 96 h, the medium was replaced with 100 μL per well of an MTT/medium mixture, i.e., 5 mg/mL MTT (Acros Organics, Geel, Belgium) in phosphate-buffered saline (PBS), diluted 1:7 in RPMI 1640 medium (supplemented with 4 mM L-glutamine and 10% heat-inactivated FBS). Upon conversion of MTT into the formazan product by viable cells for 4 h, 150 μL DMSO were added per well, and absorbance at 550 nm (and at 690 nm as a reference) was measured with a microplate reader (ELx808) and Gen5 software, version 3.08 (both from BioTek, Winoosky, VT, USA). Blank-corrected optical densities were graphically evaluated, and IC_{50} values were interpolated from concentration-effect curves and each result averaged from at least three independent experiments.

Apoptosis Assay

The potency to induce apoptotic cell death was quantitatively analyzed via flow cytometry using double staining with FITC-conjugated annexin V (eBioscience, San Diego, CA, USA) and propidium iodide (Sigma-Aldrich). A2780 and A2780cisR cells were seeded into 12-well plates (8×10^4 cells/well) in 1 mL of supplemented RPMI 1640 medium per well and allowed to settle for 24 h. Consecutively, cells were exposed to a range of concentration (1-5-25 μM) of the compounds prepared from DMF stocks not to exceed 0.25% DMF on cells. Following treatment, the supernatant media were separately collected, and cells were washed once with 37 °C warm PBS and harvested via trypsinization. The corresponding cell suspensions were added to the pre-collected media and probes were centrifuged (300 g, 3 min). The supernatant was discarded, and the cell pellets were resuspended in 150 μL of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 2.5 mM CaCl2) supplemented with 1.5 μL FITC-conjugated annexin V per probe. Samples were stained for 15 min in the dark at 37 °C. An amount of 1 μg of propidium iodide dissolved in 150 μL of binding buffer (for each probe) was added shortly before the measurement. The flow-cytometric analysis was conducted using a Guava easyCyte 8HT instrument (Merck Millipore, Burlington, MA, USA) with InCyte software. Results were quantified by means of FlowJo software (TreeStar). At least three independent experiments were analyzed.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Medicinal Chemistry · Antitumor Agents · Platinum · Cytoxicity · 3-Chloropiperidines

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