Antitumor Activity and Reductive Stress by Platinum(II) N-Heterocyclic Carbenes based on Guanosine

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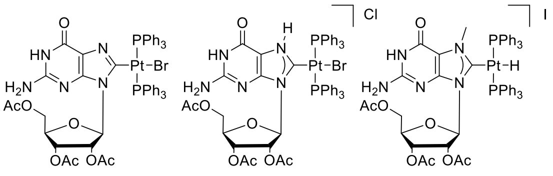
Abstract: Platinum (II) complexes bearing N-heterocyclic carbenes based guanosine and caffeine have been synthesized by unassisted C-H oxidative addition, leading to the corresponding trans-hydride complexes. Platinum guanosine derivatives bearing triflate as counterion or bromide instead of hydride as co-ligand were also synthesized to facilitate correlation between structure and activity. The hydride compounds show high antiproliferative activity against all cells lines (TC-71, MV-4-11, U-937 and A-172). Methyl Guanosine complex **3,** bearing a hydride ligand, is up to 30 times more active than compound **4**, with a bromide in the same position. Changing the counterion has no significant effect in antiproliferative activity. Increasing bulkiness at N7, with an isopropyl group (compound **6**), allows to maintain the antiproliferative activity while decreasing toxicity for healthy cells. Compound **6** leads to an increase in endoplasmic reticulum and autophagy markers on TC71 and MV-4-11 cancer cells, induces reductive stress and increases glutathione levels in cancer cells but not in healthy cell HEK-293.

Introduction

Platinum-based drugs are responsible for *circa* 50 % of all anticancer therapies worldwide, either in combination with other therapies or as standalone treatment.[1,2] The first platinum anticancer agent, cisplatin, began its clinical use more than 40 years ago and marked a milestone in the discovery and use of metallodrugs.[3] Alongside with carboplatin and oxaliplatin, cisplatin continues to play a major role in contemporary oncology.[2,3] Cisplatin arrests the cell cycle at G2/M transition, the magnitude of this cytostatic effect being dependent on the duration and concentration of the treatment.[4] Despite their effectiveness, the use of cisplatin and other metallodrugs has several limitations. Their coordination is not restricted to DNA nucleobases, and their binding to other biological substrates is at the core of severe undesired side effects.[3] Cancer cells often adapt to these drugs and become resistant, and tumour recurrence is therefore common [2][3]. Subsequently, intense research has been dedicated to the development of novel platinum drugs.[2,5] A promising strategy is the coordination of the metal moiety to naturally occurring and/or bioactive ligands, which can provide a higher degree of selectivity.[6] In this sense, modified nucleosides, such as organometallic nucleosides,[7–9] show a great potential to fill this role. Modified nucleosides are widely used in chemotherapy, acting as antimetabolites that disrupt the synthesis of nucleic acids.[10,11] The accessible incorporation of nucleoside analogues into nucleic acids by the DNA repair machinery makes them interesting candidates for combination with DNA-damaging agents, such as cisplatin.[12] Indeed, combination therapies of nucleosides and metallodrugs have proven effective for a variety of cancer treatments.[2,13]

Previous work from our lab has established new methodologies for the synthesis of guanosine complexes based on palladium and platinum.[14] In these complexes, the guanosine ligand is bound to the metal centre as an N-heterocyclic carbene, making the complex more stable.[15] These platinated nucleosides were examined for their antiproliferative activity[14] towards several human cell lines. Complex **1** (**Error! Reference source not found.**), bearing an anionic guanosine derivative, has no relevant antiproliferative activity. By contrast, protic NHC complex **2** (**Error! Reference source not found.**) is active for glioblastoma cell line U251, having a significant activity when compared to cisplatin, while showing no cytotoxic for healthy cells (HEK293 cell line).[14]

We have recently reported the synthesis of a platinum complex based on 7-methylguanosine by C–H oxidative addition leading to the formation of the hydride complex **3**[15] (**Error! Reference source not found.**). Following our results previously described for complex **2**,[14] herein we report the antiproliferative activity of compound **3**. Additionally, and motivated by the results obtained for **3,** we examine the effect of synthetic variations at the purine core on antiproliferative activity.



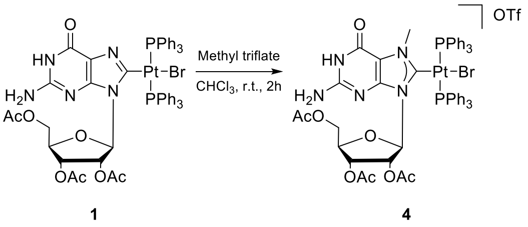
1 **2** **3**

Figure 1. Structure of complexes **1-3**.

Results and Discussion

The anticancer activity of compound **3** was evaluated via MTT assay in four different human cancer cell lines (Figure 2), namely TC-71 (Ewing's sarcoma), MV-4-11 (myelomonocytic leukaemia), U-937 (histiocytic lymphoma) and A-172 (glioblastoma). Complexes **1** and **2** were active against the MV-4-11 cell line at 10 µM (**2**) and 100 µM (**1**), showing no activity against the three other cell lines, even at the highest concentration tested. In contrast, complex **3** was active against the four cancer cell lines. Differences in the antiproliferative activity between **1**, bearing a guanosynil ligand, and **2**, with a guanosylidene ligand, were previously reported by our group,[14] with complex **2** with the NHC ligand providing a higher antiproliferative activity. Since the antiproliferative activity of **3** is considerably higher than that of **2**, we hypothesized that the presence of the methyl group and/or the presence of the hydride as co-ligands could be responsible for the increased antiproliferative activity, in addition to the beneficial effect of the NHC ligand.

To determine the main cause of the higher cytotoxicity, we prepared complex **4**. This complex bears a methyl group at N7 and a bromide instead of a hydride *trans* to the NHC. Complex **4** is easily obtained by post-functionalization of compound **1** with methyl triflate (Scheme 1).



Scheme 1. Methylation of complex 1 with methyl triflate, affording the NHC 4.

Characterization of **4** by NMR spectroscopy shows similarities with NHC **3**, as expected. The most diagnostic feature is found in the $^{13}C\{^1H\}$, with the upfield of *ca* 30 ppm of carbenic carbon (C8) with respect to **3**. In NHC **4**, the C8 resonates as a triplet at 155.3 ppm, while in NHC **3** it resonates at 181.9 ppm. This shift evidences the large differences in sigma donation of the hydride versus bromide on C8 and the resulting *trans* influence.[15] The antiproliferative activity was measured for compound **4** and the corresponding IC_{50} calculated and compared with those of **3** for the difference cell lines (Table 1).

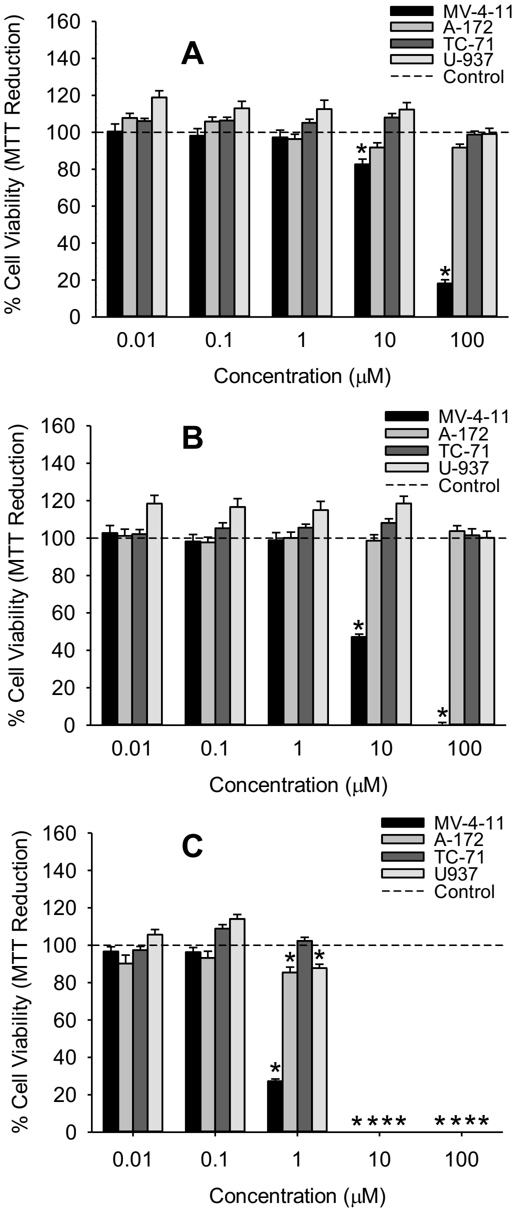
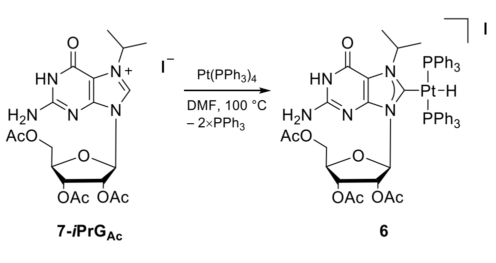


Figure 2. Viability of four human cancer cell lines, namely MV-4-11, A-172 TC-71 and U-937, after incubation with compounds A) 1, B) 2 and C) 3, for 48h, assessed by evaluating its ability to metabolize MTT.



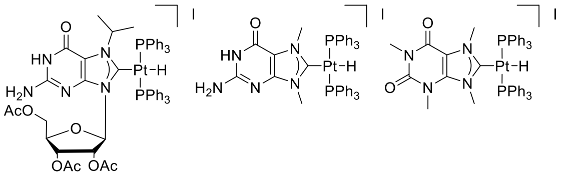
Scheme 2. Synthesis of complex 6 by C–H oxidative addition of 7-*i*PrG$_{Ac}$ to Pt^0.

Table 1. IC$_{50}$±sd values (µM) for compounds 3 and 4 towards four human cancer cell lines after incubation four 48 hours, expressed as mean of at least three separate determinations. sd – standard deviation

Cell line	**3**	**4**
TC-71	2.10±0.26	59.26±6.9
MV-4-11	1.92±0.19	32.26±6.75
U-937	1.97±0.24	58.10±9.2
A-172	2.39±0.17	23.47±2.7

Both compounds are active against all cell lines, compound **3** being far more active than **4** for all cell lines tested. For cell line U-937, from a non-solid tumour (histiocytic lymphoma), complex **3** is 30 times more active than **4**. These results indicate that the higher activity found for **3** could stem from a combination of a bulkier substituent at N7 (when compared to **2**) and the presence of a hydride as co-ligand. Complexes **3** and **4** have different counterions that could also contribute to the observed activity. Thus, we synthesized complex **5** via anion exchange of **3** in dichloromethane, using silver triflate (see SI for characterization), and evaluated the corresponding antiproliferative activity. The IC$_{50}$ values found for **5** are similar to those of **3**, without significant differences (S.I. Table S1). These results suggest that the counterion has no significant influence on the antiproliferative activity.

Complex **3** showed high cytotoxicity against four human cancer cell lines, with IC$_{50}$ values between 1.92 and 2.39 µM. Motivated by these results, we performed further modifications at the purine core to ascertain the overall effect on the activity of these compounds. Variations of the steric environment at N7 at the purine backbone and at N9 were introduced to produce complexes **6**, **7**[15], and **8** (Figure 4). Compounds **6** and **8** were synthesized by C−H oxidative addition of the corresponding ligand precursors to Pt(0), following the synthetic strategy employed for compound **3**. Complex **7** was previously reported by our group using a similar methodology.



6 **7** **8**

Figure 4. Chemical structure of complexes 6–8.

For the synthesis of compound **6**, acetate-protected guanosine was quaternized with an isopropyl group to increase steric crowding specifically at N7. **7-*i*PrG$_{Ac}$** was reacted with Pt(PPh$_3$)$_4$ in dimethylformamide at 100 °C for 7 h, yielding complex **6** in 68% isolated yield (Scheme 2). Metallation at position 8 results in a significant upfield shift of *circa* 1.1 ppm for both methyl groups from the isopropyl moiety. The hydride signal for complex **6** resonates at −6.90 ppm, a shift of 0.70 ppm with respect to **4**, which is indicative of a higher electronic donation of the isopropyl group.[16] In the $^{13}C\{^{1}H\}$ NMR, the carbenic C8 undergoes resonates at 181.4 ppm, in agreement with that of previous complexes.[14]

For the synthesis of compound **8** we employed 9-methylcaffeinium iodide (**9-MeCaff**), a more oxidized purine moiety. **9-MeCaff** was then reacted with Pt(PPh$_3$)$_4$ in dimethylformamide at 60 °C for 24 h, affording complex **8** in 42% isolated yield. Complex **8** was characterized by NMR spectroscopy in DMSO-d$_6$. In the ^{1}H NMR analysis, the platinum-bonded hydride resonates at −6.20 ppm.[15,17] In the $^{13}C\{^{1}H\}$ NMR analysis, the metallated C8 resonates at 185.0 ppm, in line with the values described for **3** and **6**.

Crystals of **8** were obtained from a saturated solution of **8** in DMSO-*d*$_6$, allowing for their characterization by single-crystal X-ray analysis ( ). The crystal structure confirms the *trans* orientation for the two phosphines. The N7−C8−N9 angle is 106.7(5)°, identical to other metal NHCs based on caffeine.[17–19] The Pt−C8 bond length is 2.064(5) Å, longer than in the related systems having chlorine and bromine as co-ligands,[20,21] probably due to the *trans* influence of the hydride.[15]

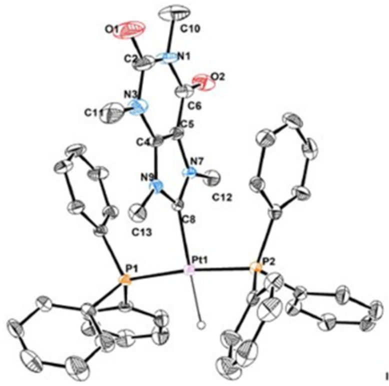


Figure 3 Molecular structure of complex 8 obtained by X-Ray crystallography (H atoms omitted for clarity).

Anticancer activity

The anticancer activity of these compounds was then evaluated in the same four cancer cell lines and in the healthy cell line HEK-293. The IC$_{50}$ values obtained for complexes **3** and **6**–**8** are indicated in Table 2. All the variations introduced for complexes **6**–**8** improved the activity in relation to complex **3**. Specifically, the introduction of a bulkier group at N7 (the isopropyl in **6**, instead of a methyl in **3**) provides a beneficial effect in the anticancer activity. Compound **6** is more active against all cancer cell lines: although it is still cytotoxic for HEK-293 cells, the safety index values are more favourable for complex **6** than for complex **3**.n Replacing the sugar with a the methyl group at N9 (complex **7**), leads to an antiproliferative activity of *circa* two to three times higher in the four cell lines. However, the effect is even more prominent in healthy HEK-293 cells, since complex **7** is slightly more toxic for this cell line than for cancer lines TC-71 and A-172. Finally, a more oxidized purine ligand (complex **8**) had no relevant benefit. Compound **8** is more active than complex **3**, but it is also more toxic for the healthy cell line HEK-293, in a similar degree to that of complex **7**.

Table 2. IC_{50}±sd values (μM) for compounds **3** and **6**–**8** towards four human cancer cell lines after incubation four 48 hours, expressed as mean of at least three separate determinations. sd – standard deviation

Cell line	3	6	7	8
TC-71	2.10±0.26	1.12±0.13	1.23±0.15	0.83±0.11
MV-4-11	1.92±0.19	1.24±0.19	0.79±0.10	1.21±0.23
U-937	1.97±0.24	0.61±0.09	0.61±0.08	0.73±0.08
A-172	2.39±0.17	1.66±0.30	1.49±0.25	1.48±0.16
HEK-293	4.10±0.15	2.98±0.32	1.10±0.22	0.99±0.10

Thus, when comparing guanine- and caffeine-derived NHCs, all IC_{50} values are very similar for each cell line. Compound **6**, bearing an isopropyl group at N7, is slightly less active than compounds **7** and **8** for all cancer cell lines. However, complex **6** is approximately three times less toxic for the non-tumour cell line HEK-293 with an IC_{50} of 2.98 μM (while **7** and **8** showed an IC_{50} of 1.10 μM and 0.99 μM against HEK-293, respectively). Although all compounds are toxic for non-tumour cells, the safety index is above 1 in most cases (Table 3), indicating that they are more toxic for cancer cells than normal proliferating cells. At the concentrations required for this compound to inhibit the proliferation of cancer cells, there should be a reduced effect on the healthy ones, showing its potential as an anticancer agent. In particular, the presence of a bulkier group at N7 resulted in a more favourable ratio between anticancer activity and a lower toxicity for healthy cells, a characteristic that warrants further development.

Table 3. Safety index values of Pt−H compounds **3** and **6**–**8** for four cancer cell lines.[a]

Cell line	3	6	7	8
TC-71	1.95	2.66	0.89	1.19
MV-4-11	2.14	2.40	1.39	0.82
U-937	2.08	4.89	1.80	1.36
A-172	1.73	1.80	0.74	0.67

[a] range of the ratios between the IC_{50} for the healthy cell line (HEK-293) and the IC_{50} value for each cancer cell line presented in Table 2.

MTT assays detect a decrease in cell number, but do not allow to determine whether this decrease is due to antiproliferative or cytotoxic effects. Trypan blue exclusion assay allows to determine the number of viable cells, which possess intact cell membranes and exclude the trypan blue dye, whereas dead cells do not. Thus, we used trypan blue exclusion assay to confirm that there was a reduction in the number of cells (Figure 5A) and that this reduction was due, at least in part, to induction of cell death (Figure 5B). Consistently, the compound was not cytotoxic for HEK-293 cells at 1 μM. We next explored the mechanism of cytotoxicity of compound **6**. We evaluated oxidative stress, apoptosis, endoplasmic reticulum stress and autophagy, four common mechanisms of action of antitumor drugs. Surprisingly, compound **6** did not induce oxidative stress. Instead, **6** reduced significantly the intracellular levels of reactive oxygen species in cancer cell lines, but not in normal HEK-293 cells (Figure 5C). This decrease was accompanied by an increase in the mitochondrial membrane potential (Figure 5D). This difference between healthy and cancer cells is not unusual. Cancer cells often show higher basal levels of ROS, due to an altered mitochondrial function.[22,23] What is more uncommon is the co-existence of a reductive intracellular environment and a higher mitochondrial potential, which we found only once in the literature in conditions of reductive stress.[24,25] Reductive stress is defined as a condition characterized by excessive and/or sustained accumulation of reducing equivalents that jeopardizes cellular survival. Recent reports have highlighted the potential of reductive stress as a mode of action for the development of anticancer drugs, making use of the so-called catalytic anticancer compounds. Reductive stress can be promoted in cancer cells by selenium-containing metabolites[26–28] or ruthenium complexes [29] and examples with other metals have also been reported.[30–33]

Glutathione (GSH) is the most abundant intracellular antioxidant, and its levels are often increased in reductive stress.[34] Consistently, we found increased intracellular GSH levels in two selected cancer cell lines upon incubation with compound **6**, but not in HEK293 cells (Figure 5E). Moreover, Reductive stress is often associated with other forms of stress. In TC71 and MV-4-11 cancer cells, compound **6** increased the levels of CHOP and LC3-II, two endoplasmic reticulum stress and autophagy markers, respectively (Figure 5F). In contrast, HEK293 cells showed a substantially lower increase in CHOP expression and no change in LC3-II levels. The type of cancer cell death induced by compound **6** was not apoptotic, as indicated by the absence of caspase-3 cleavage, necessary to execute apoptosis.

Conclusions

In summary, the combination of steric hindrance at N7 and a hydride *trans* to the NHC improves cytotoxicity against four different cancer cell lines, with the best outcome found for compound 6, bearing an isopropyl group at N7. The trypan blue exclusion assay shows that for that the reduction in the number of cells is due to cell death. Compound **6** induces reductive stress on cancer cells, with an increase in GSH levels and

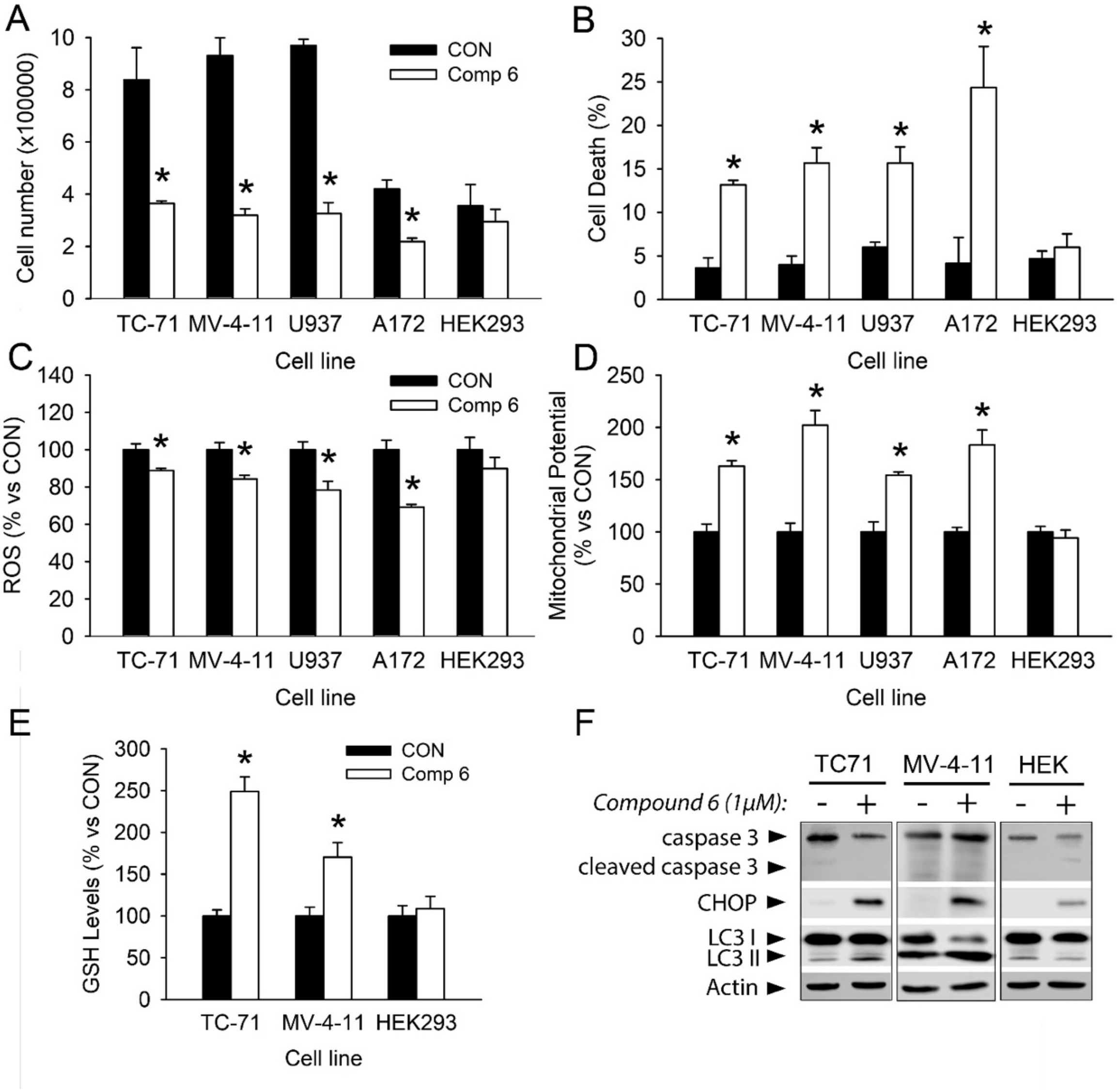


Figure 5. Compound 6 induces reductive stress and cell death in cancer cells. Human cancer cell lines TC-71, MV-4-11, U937 and A172, and normal HEK293 cells were incubated with compound 6 (1 µM, white bars) or the vehicle (DMSO 0.1% v/v, black bars). Incubation of cancer cells with compound 6 induced a decrease in cell number (A) partially explained by cell death (B), as determined by trypan blue assays; a decrease in the levels of intracellular reactive oxygen species (ROS), as determined by DCFHDA staining (C); an increase in mitochondrial membrane potential, as determined by 123-rhodamine staining (D); and an increase in intracellular glutathione (GSH)(E), CHOP and LC3-II levels (F); but not caspase activation. HEK293 cells did not respond to compound 6 as cancer cells, with the exception of a slight increase in CHOP levels. Results are the mean ± SEM of 3 independent experiments. *, significant vs CON, $p<0.05$ (*t*-student test).

endoplasmic reticulum and autophagy markers on TC71 and MV-4-11 cancer cells (Ewing's sarcoma and myelomonocytic leukaemia, respectively). In contrast, healthy cells showed neither signs of reductive stress, nor of autophagy, and the putative increase in endoplasmic reticulum stress was lower than in cancer cells. These platinated nucleosides thus show anticancer activity with a different mode of action than that of cisplatin, hence showing great potential to develop novel metallodrugs able to circumvent the well know resistance associated with cisplatin.

Experimental Section

All experimental procedures, NMR and mass spectra, as well as the crystallographic data, are included in the Supporting Information.

Data Availability Statement: X-ray crystallographic data in CIF format are available from the Cambridge Crystallographic Data Centre (Deposition Number 2217136).

Keywords: platinum complexes • organometallic nucleosides • C–H activation • anticancer agents • reductive stress

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