Dinuclear Alkyldiamine Platinum Antitumor Compounds: A Structure−Activity Relationship Study

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Six related dinuclear *trans*-platinum complexes, with the formula [{*trans*-$PtCl_2(NH_3)(L)$}$_2$(μ-$H_2N(CH_2)_nNH_2$)]$^{2+}$ (L = pyridine, 2-picoline, 4-picoline; n = 4, 6) and chloride or nitrate anions, are compared with known cytotoxic dinuclear compounds (L = NH_3; n = 4, 6) that overcome cisplatin resistance. The cytotoxicity of the compounds was determined in L1210 murine leukemia and L1210/2, a cisplatin-resistant derivative. Unlike the L = NH_3 compounds, the substituted n = 4 compounds are more susceptible toward the resistance mechanisms in L1201/2. The n = 6 compounds, however, have comparable IC_{50} values in both cell lines. In general, the substituted compounds are less cytotoxic than their NH_3 counterparts. After incubation with equimolar concentrations, the amount of platinum bound to cellular DNA was determined. The compounds show comparable binding, except for the sterically hindered 2-picoline compounds that bind significantly less. The amounts of platinum bound to DNA do not correlate with the cytotoxicity data. As DNA is considered to be the cellular target of platinum antitumor drugs, structural details of the DNA adducts probably account for the differences in cytotoxic activity.

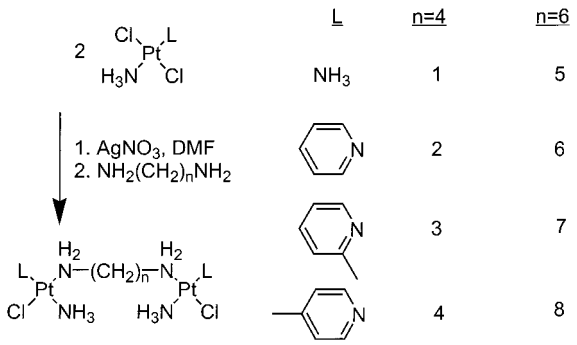
Introduction

Cisplatin [*cis*-$PtCl_2(NH_3)_2$] is one of the most widely used antitumor drugs.[1−3] Nuclear DNA is the cellular target commonly associated with the therapeutic action of platinum drugs.[4] In recent years research has provided insight in the molecular pathways that occur after binding of cisplatin.[1−4] In affected cells, binding to DNA results in complex events that eventually lead to apoptosis.[5]

Resistance, either developed or intrinsic, remains one of the major drawbacks of platinum antitumor drugs.[6,7] Compounds structurally different from cisplatin may behave differently with respect to resistance mechanisms. In this respect, cationic dinuclear compounds of the 1,1/t,t type (see Scheme 1), as developed by Farrell, are among the most promising new platinum compounds.[3,8,9] The DNA adducts formed by these compounds are structurally different from those of cisplatin,[10−12] which may explain their ability to overcome cisplatin resistance. New variations have been investigated, such as dinuclear compounds using spermine and spermidine as linkers[13] and trinuclear[14−17] and tetranuclear[18,19] compounds. The most successful member of the family of polynuclear platinum compounds is the bifunctional trinuclear compound BBR3464, which has recently passed phase I clinical trials.[20,21]

In the present study, an attempt is made to sort out relationships between the chemical structure and the cytotoxic activity of cationic dinuclear platinum complexes bearing one planar ligand on each platinum core.

Scheme 1. Synthesis and Numbering of the Series of Dinuclear Platinum Compounds[a]



[a] Compounds **1** and **5** are also known as 1,1/t,t (n = 4) and 1,1/t,t (n = 6), respectively.[8,9]

The synthesis, cytotoxic evaluation, and cellular uptake of dinuclear platinum complexes with the general formula formula [{*trans*-$PtCl_2(NH_3)(L)$}$_2$(μ-$H_2N(CH_2)_n$-NH_2)]$^{2+}$ is described (see Scheme 1), with linker lengths n = 4 and 6 and ligands L = pyridine (**2** and **6**), 2-picoline (**3** and **7**), and 4-picoline (**4** and **8**). Cisplatin, its inactive isomer *trans*-$PtCl_2(NH_3)_2$, and the Farrell type (L = NH_3) compounds 1,1/t,t (n = 4, **1**; n = 6, **5**) are included as controls.

The choice of the pyridine and picoline ligands was inspired by recently reported mononuclear compounds with interesting cytotoxic properties. The cisplatin derivative *cis*-amminedichloro(2-picoline)platinum(II) (ZD0473, AMD473) is currently in phase I clinical trials.[22] The sterically hindering 2-picoline ligand reduces rapid detoxification by thiol-containing molecules.[23] Deactivation by thiols such as glutathione (GSH) is considered to be one of the major resistance mechanisms for platinum compounds, together with

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improved repair of DNA adducts and increased tolerance of DNA damage.[6,7]

4-Picoline was selected because [*cis*-PtCl(4-picoline)$(NH_3)_2$](NO_3) emerged as the most cytotoxic agent in a series of monofunctional platinum compounds.[24] This compound resembles the DNA-binding part of the corresponding dinuclear compound. In structural studies with oligonucleotides [*cis*-PtCl(4-picoline)$(NH_3)_2$](NO_3) induced severe distortions in the helix structure.[25,26]

Two molecules related to the present series have been reported in the literature: [{*trans*-PtCl$(py)_2$}$_2$$H_2N(CH_2)_4NH_2$]$^{2+}$, 1,1/t,t (py,py) ($n = 4$), which has two pyridines coordinated *trans* on the platinum,[27] and [{*trans*-PtCl(NH_3)(quin)}$_2$$H_2N(CH_2)_6NH_2$]$^{2+}$, 1,1/t,t (am,quin), bearing an ammine and a quinoline ligand in *trans* positions.[28] Both compounds showed intermediate cytotoxicity in L1210 mouse leukemia cells.[9] However, no systematic study of a series of compounds has been reported thus far.

The new dinuclear compounds are tested in L1210/0 murine leukemia and the cisplatin-resistant L1201/2 cell line. The L1210/2 line has increased GSH levels and increased DNA repair as its main resistance mechanisms.[29] The substitution of the ammine functionality by pyridine derivatives may influence cellular uptake and DNA binding. Polynuclear compounds are known to bind much faster to DNA in cells than cisplatin.[30] This aspect is investigated by determining the bound drug per DNA base pair ratio after incubation of L1210/0 cells with equimolar drug concentrations.

Results

Synthesis. The dinuclear complexes were obtained using synthetic methods based on literature procedures.[28,33] The diaminobutane ($n = 4$) compounds were obtained as their chloride salts, the diaminohexane compounds as their nitrate salts. Chloride salts were generally easier to purify, while they have less tendency to form hydroxo-bridged polymers.[13] Such polymers are identified by an indicative signal at −2016 ppm in ^{195}Pt NMR.

The diaminobutane nitrate complexes were transformed into their chloride salts by adding an excess of LiCl. As shown in our earlier studies,[19] LiCl is easily removed by extraction with warm ethanol, a solvent in which the diaminobutane complexes hardly dissolve. Unfortunately, the diaminohexane complexes dissolve readily into alcoholic solvents, making LiCl and the platinum chlorides much harder to separate. Therefore, these complexes were isolated as their nitrate salts. Hydroxo-bridged polymers and unreacted starting compounds were removed by dissolving the compound in cold water, followed by filtration and lyophilization. These steps sometimes have to be repeated, to completely remove the polymers. All compounds were obtained in 20−35% yields, except the sterically hindered 2-picoline compounds, which gave substantially lower yields (15%). All these yields are lower than the corresponding L = NH_3 compounds, most likely for steric reasons and differences in solubility.

Cytotoxicity. The cytoxicities of the compounds were quantified by IC_{50} values in murine leukemia L1210/0 (wild type) and L1210/2 (cisplatin-resistant) cell lines.

Table 1. Cytotoxicities of Cisplatin, *trans*-$PtCl_2(NH_3)_2$, and Dinuclear Compounds **1**−**8** in L1210/0 and Cisplatin-Resistant L1210/2 Cell Lines and Resistance Factors

	IC_{50} (μM)		
compd	L1210/0	L1210/2	RF[a]
1	3.1	1.4	0.5
2	10.7	22.7	2.1
3	7.5	16.5	2.2
4	2.3	7.5	3.3
5	2.4	1.8	0.8
6	19.2	23.8	1.2
7	8.1	9.9	1.2
8	22.2	17.5	0.8
cisplatin	1.5	4.6	3.1
trans-$PtCl_2(NH_3)_2$	15.7	22.0	1.4

[a] RF = IC_{50}(L1210/2)/IC_{50}(L1210/0).

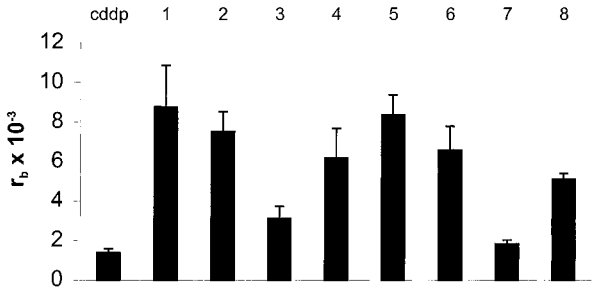

Figure 1. Molecules bound per base pair × 10^{-3} (r_b) to DNA isolated from L1210/0 cells after incubation with 333 μM of various platinum compounds for 1 h at 37 °C.

IC_{50} is defined as the concentration of drug where the growth of the cell line is one-half that of the control experiment. Cytotoxicity results are summarized in Table 1.

The order of the cytotoxicities in L1210/0 is CDDP > **4** ≈ **5** > **1** ≫ **3** ≈ **7** > **2** ≫ **6** ≈ **8**. In the cisplatin-resistant cell line the order is **1** > **5** ≫ CDDP > **4** ≈ **7** > **2** ≈ **3** ≈ **6** ≈ **8**. In general, dinuclear complexes with pyridine or picoline ligands are less active than their ammine counterparts. One exception is **4** in the L1210/0 cell line, which shows a cytotoxicity in the same range as **1**. For the $n = 6$ compounds, **7** is the most active pyridine derivative, although it is still less active than **5**. The resistance profiles for the diaminobutane ($n = 4$) compounds show a change for the pyridine derivatives compared to the ammine compound. The ammine compound is more active in the cisplatin-resistant L1210/2 cell line, whereas the pyridine derivatives show cross-resistance with cisplatin. For the diaminohexane ($n = 6$) compounds, similar cytotoxicity is found for the wild-type and cisplatin-resistant cell lines. Thus, a non-cross-resistant profile is maintained only for the diaminohexane ($n = 6$) series.

Binding to Cellular DNA in L1210/0. After incubation of L1210/0 cells for 1 h at equimolar concentrations of the platinum compounds, DNA was isolated and the r_b value (number of bound drug molecules/base pair) was determined using UV and FAAS. The r_b values are depicted in Figure 1.

The results show that the cationic dinuclear compounds bind faster to DNA in the L1210/0 cells than cisplatin. Comparing the dinuclear compounds, the sterically hindered 2-picoline complexes bind significantly slower to cellular DNA than the other complexes. The other compounds give similar r_b values. Compounds

1 and **5** seem to bind somewhat better, whereas the 4-picoline compounds are on the lower end of the range. Also, the compounds with the diaminobutane linkers seem to bind slightly better than the corresponding diaminohexane compounds.

Discussion

As a rule of thumb, RF values between 0.5 and 2.0 indicate similar cytotoxicity of a compound in both sensitive and resistant cell lines, whereas compounds with RF > 2 are at least partially cross-resistant with cisplatin. For the diaminobutane ($n = 4$) complexes, only **4** gives an IC_{50} value that is comparable to that of its ammine counterpart. However, it does so only in the L1210/0 cells. In the cisplatin-resistant L1210/2 cell line, all substituted compounds show a moderate cytotoxicity. It seems that the resistance mechanism for cisplatin also affects the diaminobutane compounds. This is in contrast with the ammine compounds that show a non-cross-resistant profile.

For the diaminohexane ($n = 6$) compounds, the non-cross-resistant profile is preserved throughout the series. However, the cytotoxicities are all lower than those for the corresponding ammine compounds. Thus, the introduction of planar ligands appears to have a negative effect on the cytotoxicity properties of dinuclear platinum complexes of the 1,1/t,t type.

The difference in cytotoxicity may be caused by kinetic differences in binding to DNA, which is considered to be the therapeutic target for platinum compounds.[1−4] For example, the highly active trinuclear platinum compound BBR 3464 was found to bind much faster to DNA in L1210 cells than cisplatin.[30] This possibility was investigated by quantification of the amount of platinum compounds bound to cellular DNA in L1210 cells after a 1-h incubation at equimolar concentrations.

From the results presented in Figure 1 it is clear that, compared to cisplatin, the cationic dinuclear compounds have more drug molecules bound to DNA after 1 h of incubation. This is explained by the great affinity of positively charged compounds for DNA,[13] due to strong Coulomb interactions. Surprisingly, the planar ligands do not seem to have a great effect on the ability of the compounds to bind DNA. The only exceptions are the 2-picoline compounds **3** and **7**. The methyl group on the pyridine ring is known to exert steric hindrance on the platinum center, which decreases binding rates to DNA.[34,35]

There appears to be no correlation between the amount of platinum bound to DNA and the cytotoxicity of the compounds. This is seen most clearly by looking at the 2-picoline compounds as these compounds have a much lower r_b than the other compounds. However, **3** does definitely not have the lowest cytotoxicity in the $n = 4$ series, and **7** is the most active compound among the substituted diaminohexane compounds.

The lack of correlation between the quantity of drug bound to DNA and induced cytotoxicity should probably be explained by structural details of the formed adducts. The different DNA adducts formed by polynuclear platinum compounds (compared to cisplatin adducts) are thought to be a key factor in overcoming cisplatin resistance.[16,17] The exact structure of a DNA adduct is likely to be the determining factor for its susceptibility to repair and resistance mechanisms and the potency of the adduct to trigger the mechanisms that lead to cell death.[4]

Conclusion

The substitution of ammine groups by planar ligands did not yield an improvement in the cytotoxic properties of dinuclear platinum complexes of the Farrell 1,1/t,t type. The substituted diaminobutane-linked compounds show a decreased ability to overcome cisplatin resistance in L1210/2 murine leukemia cells compared with their all-ammine counterpart. The diaminohexane compounds retain the non-cross-resistant profile of their parent ammine compounds but showed a significantly lower cytotoxicity than the 1,1/t,t ($n = 6$) compounds.

The ability to bind DNA in L1210/0 cells was not significantly changed by the introduction of planar ligands, except for the sterically hindered 2-picoline compounds. All dinuclear compounds show DNA binding kinetics superior to cisplatin. However, there was no relation found between cytotoxicity and the amount of drug bound to DNA. This result may probably be explained by structural differences in DNA adducts of the investigated compounds. However, the dinuclear compounds may as well have other cellular targets that are of importance. Even though the compounds are structurally very similar, still the small differences are enough to create a large variance in cytotoxicity.

Methods and Materials

Abbreviations: py, pyridine; 2pic, 2-methylpyridine, 2-picoline; 4pic, 4-methylpyridine, 4-picoline; CDDP, *cis*-diamminedichloroplatinum(II), cisplatin; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetate; FAAS, flameless atomic absorption spectroscopy; GSH, glutathione; IC_{50}, drug concentration that reduces cell growth to 50% of control; PBS, phosphate buffer in saline; r_b, bound drug/DNA base pair; RF, resistance factor, IC_{50}(resistance line)/IC_{50}(parent line); SDS, sodium dodecyl sulfate; TEN, Tris−EDTA−NaCl buffer.

General. NMR: NMR spectra were taken on a Bruker DPX 300 spectrometer with a 5-mm multinucleus probe. Temperature was kept constant at 25 °C using a variable temperature unit. ^{195}Pt spectra were calibrated with respect to external K_2PtCl_4 at $\delta = -1614$ ppm. ^{1}H NMR was measured in D_2O using TMS as an external reference at $\delta = 0$ ppm.

FAAS: Platinum concentrations were determined using graphite oven flameless atomic absorption spectroscopy.[31] Measurements were carried out on a Perkin-Elmer 3100 AAS apparatus, equipped with a platinum hollow cathode lamp and a AS-60 graphite oven autosampler. For each determination, 40 μL of sample was injected. The furnace program was: drying 120 °C/90 s, ashing 1300 °C/60 s, 20 °C/15 s, atomization and measurement 2650 °C/5 s, purging 2600 °C/5 s. The furnace was purged with argon gas.

Syntheses. Dinuclear platinum compounds were prepared from the appropriate mononuclear compounds and diamines. 1,4-Diaminobutane and 1,6-diaminobutane were obtained from Aldrich. Mononuclear starting compounds of the general formula [*trans*-$PtCl_2(NH_3)$(L)] (L = pyridine, 2-picoline, or 4-picoline) were prepared according to literature procedures.[32] The dinuclear compounds 1,1/t,t ($n = 4$, 6) were prepared according to the general method as described by Qu and Farrell.[33] Syntheses of the (am,L) dinuclear compounds were based on a previously published method.[28] Diaminobutane compounds were prepared as chloride salts, diaminohexane compounds as nitrate salts.

Diaminobutane ($n = 4$) Compounds. [{*trans*-PtCl(NH_3)(pyridine)}$_2$(μ-$H_2N(CH_2)_4NH_2$)]Cl_2 (2). To a solution of 0.4 g (1.11 mmol) of *trans*-[$PtCl_2(NH_3)$(pyridine)] in 10 mL of DMF was added 0.18 g (1.05 mmol) of $AgNO_3$. The solution

was stirred in the dark overnight at room temperature. After cooling at 5 °C the precipitated AgCl was filtered off. A yellow filtrate remained. To the filtrate was added 44 mg (0.51 mmol) of diaminobutane in 2 mL of DMF dropwise while stirring. After 6 h of stirring in the dark, the reaction was quenched by the addition of 1.50 g of LiCl. Stirring was continued for 1 h. Then DMF was evaporated in vacuo. To the remaining white solid was added about 50 mL of ethanol and the suspension was boiled for 1 h. A white compound was filtered off and washed with ethanol and diethyl ether: yield 35%; ^{195}Pt NMR (D_2O) δ −2343; ^{1}H NMR (D_2O) δ 8.72 (d, J = 5.4 Hz, 4H, 3), 7.98 (t, J = 7.6 Hz, 2H, 5), 7.53 (t, J = 6.75 Hz, 4H, 4), 2.43 (s, 4H, 1), 1.60 (s, 4H, 2). Calcd for $C_{14}H_{28}N_6Pt_2Cl_4$: C, 20.70; H, 3.47; N, 10.34. Found: C, 20.60; H, 3.62; N, 10.07.

The other two diaminobutane compounds were prepared using the same procedure, starting from the appropriate *trans*-$PtCl_2(NH_3)(L)$ precursors.

[{*trans*-PtCl(NH$_3$)(2-picoline)}$_2$(μ-H$_2$N(CH$_2$)$_4$NH$_2$)]Cl$_2$ (3): yield 16%; ^{195}Pt NMR (D_2O) δ −2342; ^{1}H NMR (D_2O) δ 8.77 (d, J = 5.6 Hz, 4H, 7), 7.83 (t, J = 7.7 Hz, 2H, 6), 7.48 (d, J = 7.80 Hz, 2H, 4), 7.33 (t, J = 6.7 Hz, 2H, 5), 3.06 (s, 6 H, 3), 2.43 (s, 4 H, 2), 1.54 (s, 4H, 1). Calcd for $C_{16}H_{32}N_6Pt_2Cl_4$: C, 22.87; H, 3.84; N, 10.00. Found: C, 21.69; H, 4.35; N, 9.84.

[{*trans*-PtCl(NH$_3$)(4-picoline)}$_2$(μ-H$_2$N(CH$_2$)$_4$NH$_2$)]Cl$_2$ (4): yield 23%; ^{195}Pt NMR (D_2O) δ −2342; ^{1}H NMR (D_2O) δ 8.52 (d, J = 8.0 Hz, 4H, 3), 7.36 (d, J = 6.0 Hz, 4H, 4), 2.43 (s, 10H, 1+5), 1.60 (m, 4H, 2). Calcd for $C_{16}H_{32}N_6Pt_2Cl_4$: C, 22.87; H, 3.84; N, 10.00. Found: C, 22.68; H, 3.44; N, 9.95.

[{*trans*-PtCl(NH$_3$)(pyridine)}$_2$(μ-H$_2$N(CH$_2$)$_6$NH$_2$)](NO$_3$)$_2$ (6). To a solution of 0.3 g (0.83 mmol) of *trans*-[$PtCl_2$-(NH_3)(pyridine)] in 15 mL of DMF was added 0.13 g (0.79 mmol) of $AgNO_3$. The solution was stirred in the dark for 24 h. After cooling at 5 °C for 1 h the precipitated AgCl was filtered off. To the yellow filtrate was added dropwise 44 mg (0.39 mmol) of diaminohexane in 5 mL of DMF while stirring. The solution was stirred in the dark for 24 h. Solvent was removed using a rotatory evaporator. The remaining yellowish oil was dissolved in ice-cold water. Any undissolved particles were filtered off over a 0.1-μm filter. The colorless filtrate was lyophilized, obtaining a off-white compound. In some cases, the dissolving−filtration−lyophilization cycle had to be repeated in order to obtain a good purity: yield 36%; ^{195}Pt NMR (DMF:D_2O = 10:1) δ −2342; ^{1}H NMR (D_2O) δ 8.79 (d, J = 5.2 Hz, 4H), 8.04 (t, J = 7.8 Hz, 2H), 7.59 (t, J = 6.8 Hz, 4H), 2.52 (m, 4H), 1.60 (m, 4H), 1.16 (m, 4H). Calcd for $C_{16}H_{32}N_8Pt_2$-Cl_2O_6: C, 21.51; H, 3.61; N, 12.54. Found: C, 21.39; H, 3.67; N, 12.55.

The other two diaminohexane compounds were prepared using the same procedure, starting from the appropriate *trans*-$PtCl_2(NH_3)(L)$ precursors.

[{*trans*-PtCl(NH$_3$)(2-picoline)}$_2$(μ-H$_2$N(CH$_2$)$_6$NH$_2$)](NO$_3$)$_2$ (7): yield 14%; ^{195}Pt NMR (DMF:D_2O = 10:1) δ −2328; ^{1}H NMR (D_2O) δ 8.83 (d, J = 5.5 Hz, 2H), 7.87 (t, J = 8.9 Hz, 2H), 7.53 (d, J = 7.8 Hz, 2H), 7.38 (t, J = 6.5 Hz, 2H), 3.12 (s, 6H) 2.47 (m, 4H), 1.60 (m, 4H), 1.20 (m, 4H). Calcd for $C_{18}H_{36}N_8Pt_2Cl_2O_6$: C, 23.46; H, 3.94; N, 12.16. Found: C, 22.84, H, 3.94; N, 11.86.

[{*trans*-PtCl(NH$_3$)(4-picoline)}$_2$(μ-H$_2$N(CH$_2$)$_6$NH$_2$)](NO$_3$)$_2$ (8): yield 30%; ^{195}Pt NMR (DMF:D_2O = 10:1) δ −2342; ^{1}H NMR (D_2O) δ 8.59 (d, J = 6.5 Hz, 4H), 7.43 (d, J = 6.0 Hz, 4H), 2.49 (s, 10H), 1.64 (s, 4H), 1.20 (s, 4H). Calcd for $C_{18}H_{36}N_8$-$Pt_2Cl_2O_6$: C, 23.46; H, 3.94; N, 12.16. Found: C, 23.55; H, 3.49; N, 11.86.

Growth Inhibition Assays in L1210 and L1210/CDDP. Cell lines L1210/0 (wild-type) and L1210/2 (cisplatin-resistant) were a gift from The Netherlands Cancer Institute (NKI, Amsterdam). The cell lines were cultured in McCoy's 5a medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland), penicillin (100 units/mL; Duchefa, The Netherlands) and streptomycin (100μg/mL; Duchefa, The Netherlands). During growth, the cells grew partly in suspension and partly weakly adherent to the flasks.

Cytotoxicities of the platinum compounds were determined by measuring the inhibition of cell growth. Cells (5×10^4/mL) were pre-cultured in 24 multiwell plates for 24 h at 37 °C in an incubator with 5% CO_2 and subsequently treated with at least three different concentrations of platinum compound. Fresh stock solutions of platinum compounds in sterile Millipore-filtered water were diluted with medium prior to incubation. Cell numbers were determined after 72 h using a counting chamber. Each platinum concentration was tested in at least two independent experiments.

Binding to Cellular DNA in L1210. Cells were grown to a concentration of 20×10^5 cells/mL in Falcon flasks and then collected by centrifugation (10 min, 1000*g*) in 50-mL flasks. Medium without serum was added until the concentration of the cells was 2×10^7 cells/mL. From this suspension aliquots of 1 mL were taken for treatment with platinum complexes.

Stock solutions of platinum complexes in medium without serum were added to the platinum complexes to obtain a drug concentration of 333 μM and a cell concentration of 2×10^7 cells/mL. This high drug concentration was used to make sure that the final platinum content of the isolated DNA yielded signals well above the detection limits of the FAAS technique. The cells were incubated for 1 h at 37 °C. Each experiment was done in duplicate.

After 1 h cells were isolated by centrifugation (1000*g*, 5 min). Medium was discarded and the cells were suspended in 1 mL of PBS (0.1 M phosphate buffer in 0.15 M NaCl) and centrifuged again. This washing step was repeated twice. After centrifugation cells were taken up in 500 μL of TEN buffer, pH = 8 (10 mM Tris, pH = 8, 10 mM EDTA, and 150 M NaCl), and subsequently 5 μL of proteinase K and 50 μL of 10% SDS. The lysed cells were kept at 55 °C for 1 h. Proteins were then removed by chloroform/phenol (1:1) extraction followed by an extraction with chloroform alone. DNA was precipitated from the aqueous layer by adding an equal volume of isopropyl alcohol. Finally, precipitated DNA was removed from the solution, washed with 70% ethanol and dissolved in 1 mL of water.

The DNA concentration was determined by measuring the UV absorption at 260 nm. From this absorption, the concentration of base pairs was calculated using mean molar extinction coefficient per base pair ϵ_{260} = 16800 M^{-1} cm^{-1}. The platinum concentration was measured by FAAS. From the platinum concentration, the actual drug concentration was derived. Combining FAAS and UV results, the drug molecules per base pair ratio (r_b) was calculated.

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