Synthesis, Structure, and Biological Activity of New Azine-Bridged Dinuclear Platinum(II) Complexes − a New Class of Anticancer Compounds

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A recently described new class of dinuclear platinum anticancer compounds, represented so far by the isomeric azine-bridged complexes [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-pzn)]Cl_2 (**1**) (pzn = pyrazine), [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-pmn)]Cl_2 (**2**) (pmn = pyrimidine) and [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-pdn)](NO_3)$_2$ (**3**) (pdn = pyridazine), has been added to. Three new dinuclear complexes of this type, [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-2,5pzn)]Cl_2 (**4**) (2,5pzn = 2,5-dimethylpyrazine), [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-qzn)]Cl_2 (**5**) (qzn = quinazoline), and [{*cis*-Pt(NH_3)$_2$Cl}$_2$(μ-pht)](NO_3)$_2$ (**6**) (pht = phthalazine), have been newly synthesized and characterized by ^{1}H and ^{195}Pt NMR spectroscopy. The interaction of the new compounds with 2 equiv. of 9EtG in D_2O at 310 K has been investigated. Complexes **4** and **5** undergo substitution of both chloride ligands by 9EtG similarly to the related complexes **1** and **2**, respectively. The methyl substituents on the pyrazine ring induce steric hindrance in **4** resulting in a slower reaction rate as compared to **1**. Similarly to the case of **3**, interaction of complex **6** with 9EtG results in cleavage of the Pt–N(pht) bond and subsequent formation of the polymeric species. A cytotoxicity assay of **4**–**6** has been performed on seven human tumor cell lines and on L1210 murine leukemia cell lines, sensitive and resistant to cisplatin. Compounds **4** and **5** exhibit lower cytotoxicity than the analogous complexes with unsubstituted azines. Complex **6** is more active: its cytotoxicity in the L1210 cell lines is similar to that of cisplatin. Analysis of nuclear DNA fragmentation in L1210 cells treated with the azine-bridged complexes **1**–**6** has been carried out. The results clearly indicate induction of apoptosis by all the compounds, implying considerable anticancer potential. The structure-activity relationship for this class of dinuclear platinum(II) complexes is discussed.

Introduction

cis-Diamminedichloroplatinum(II), usually referred to as cisplatin, has been used in cancer chemotherapy ever since its approval for the treatment of testicular cancer in 1978.[1] At present, it is one of the most widely utilized antitumor drugs, with especially high efficacy against solid tumors, such as testicular and ovarian cancer.[2] However, several problems have been encountered in clinical use. Firstly, cisplatin treatment is accompanied by severe side effects, e.g. nausea, vomiting, and nephrotoxicity. Secondly, cisplatin anticancer activity is limited to a relatively narrow range of tumors. Besides, some tumors develop resistance after repeated cisplatin administrations.[3,4] These drawbacks have stimulated intensive search for new potential antitumor drugs.

One of the most successful approaches in platinum-based anticancer drug design is the synthesis of polynuclear platinum complexes with bridging linkers.[5] Its success is based on the ability of such complexes to form DNA adducts, which are structurally different from those of cisplatin. The major product of cisplatin interaction with genomic DNA is a 1,2-intrastrand cross-link, which induces a kink on a DNA double helix.[6−9] These conformational changes are believed to play an important role in the development of cisplatin resistance.[10] Thus, the compounds capable of binding to DNA in a significantly different way are expected to overcome the resistance problem. Some of the polynuclear (mostly dinuclear) antitumor active platinum(II) complexes described in the literature contain flexible bridging ligands,[11−15] while others possess rigid linkers together with bridging leaving groups.[16−19] The complexes with flexible linkers (for instance, aliphatic diamines) are designed to form long-range cross-links on DNA,[20,21] and the complexes possessing rigid bridging ligands, such as hydrazine and azoles, are developed to minimize distortion of the DNA double helix in a 1,2-intrastrand cross-link.[22]

Recently, we described a new class of dinuclear platinum(II) complexes with azines as bridging ligands.[23] These complexes present a combination of the two above-mentioned types of compounds: they utilize rigid aromatic rings of azines as linkers between two platinum atoms, featuring

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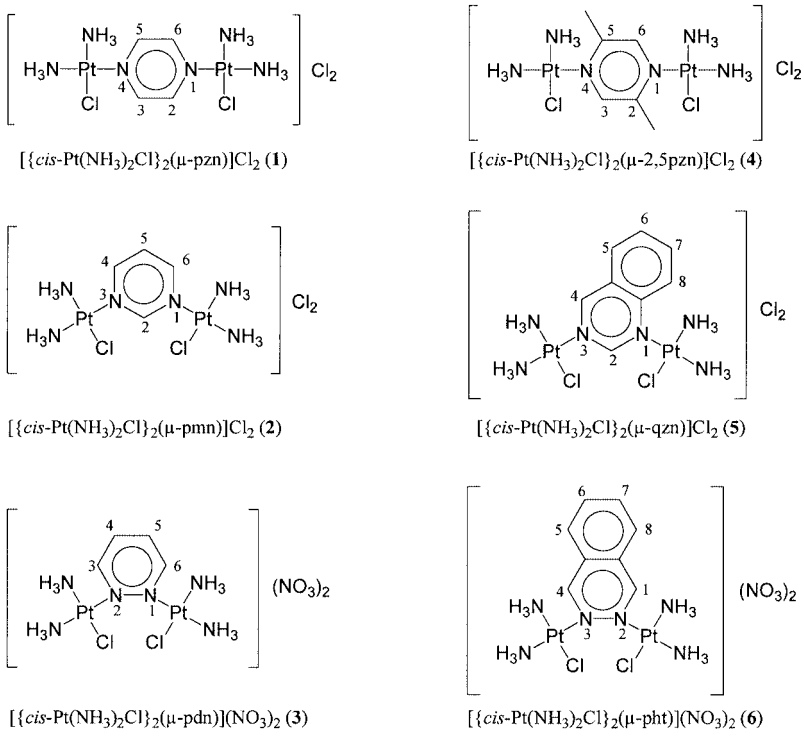


Figure 1. Schematic representation of the azine-bridged dinuclear platinum complexes [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-pzn)]Cl_2 (**1**), [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-pmn)]Cl_2 (**2**), [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-pdn)]$(NO_3)_2$ (**3**), [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-2,5pzn)]Cl_2 (**4**), [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-qzn)]Cl_2 (**5**), and [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-pht)]$(NO_3)_2$ (**6**)

at the same time non-bridging chloride ions as leaving groups. These compounds exhibit considerable activity in vitro in several human tumor cell lines. The complex [{*cis*-$Pt(NH_3)_2Cl\}_2$(μ-pzn)]Cl_2 (pzn = pyrazine) overcomes cisplatin resistance in the L1210/DDP murine leukemia cell line (Figure 1). We have now extended this new class of dinuclear platinum complexes applying other azines, such as quinazoline (qzn), phthalazine (pht), and 2,5-dimethylpyrazine (2,5pzn), as bridging ligands. These azines have different streric and electronic properties caused by substituents on the aromatic ring. In this paper, we describe the synthesis and characterization of three new azine-bridged dinuclear platinum(II) complexes, their interaction with 9-ethylguanine (9EtG), the results of cytotoxicity tests for these compounds, and the analysis of nuclear DNA fragmentation induced by the azine-bridged complexes. Structure-activity correlations for azine-bridged dinuclear platinum(II) complexes are presented.

Results

Synthesis and Characterization of the Complexes

The synthetic procedure used for the preparation of the new azine-bridged dinuclear platinum(II) complexes is similar to the previously described method for the synthesis of **1**−**3**.[23] In all cases the formation of the desired compounds was accompanied by a series of side reactions, and repeated recrystallizations were necessary to purify the complexes.

The complexes were characterized by ^1H and ^{195}Pt NMR spectroscopy (Table 1) and elemental analysis. ^1H NMR spectra show a downfield shift of the signals of the aromatic protons of the ligands as compared to the respective signals for the free azines. ^{195}Pt NMR shifts are in the range typical for an $[N_3Cl]$ coordination environment around the platinum atom.[24] The smaller upfield shift of **6** (δ = −2232 ppm) compared to the shifts of the two other platinum complexes (δ ≈ −2330 ppm) might result from the close proximity of the platinum atoms in **6**. Thus, NMR spectroscopy presents clear evidence of the formation of the desired dinuclear platinum(II) complexes.

Interaction with 9EtG

^1H NMR spectra, run over the course of the reaction, of the complexes **1** and **4**−**6** with 2 equiv. of 9EtG in 0.1 M $NaClO_4$ solution in D_2O at 310 K are shown in Figure 2, a−d, respectively. The ^1H and ^{195}Pt NMR spectroscopic data for the final products are given in Table 2.

Complex 1

As the reaction proceeds, the intensity of the 9EtG signal (δ = 7.82 ppm) decreases, and a signal at δ = 8.27 ppm from the H8 protons of the guanines in the disubstituted product **I** increases in intensity (Figure 2, a). The structure of **I** was confirmed by ^{195}Pt NMR spectroscopy: the ^{195}Pt chemical shift of the product is typical of the $[PtN_4]$ unit.[19,25] In the first hours of interaction of **1** with 9EtG a signal at δ = 8.34 ppm corresponding to the intermediate

Table 1. ^{1}H and ^{195}Pt NMR spectroscopic data for the dinuclear azine-bridged complexes **4**−**6** and the respective free ligands (D_2O)

Free azines, δ(^{1}H) [ppm]	Complex	Coordinated azine ligands, δ(^{1}H) [ppm]	δ(^{195}Pt) [ppm]
8.27, s (H3, H5); 2.47, s (CH_3)	**4**	9.11, s (H3, H5); 3.09, s (CH_3)	−2346
9.08, s (H2); 8.84, s (H4); 7.85, t (H7); 7.76, d (H8); 7.62, d (H5); 7.59, t (H6)	**5**	10.21, s (H2); 10.09, s (H4); 9.56, d (H8); 8.53, t (H7); 8.41, d (H5); 8.12, t (H6)	−2317, −2339
9.44.08, s (H3, H8); 8.05, m (H4, H7, H5, H6)	**6**	10.08, s (H3, H8); 8.41, m (H4, H7); 8.36, m (H5, H6)	−2232

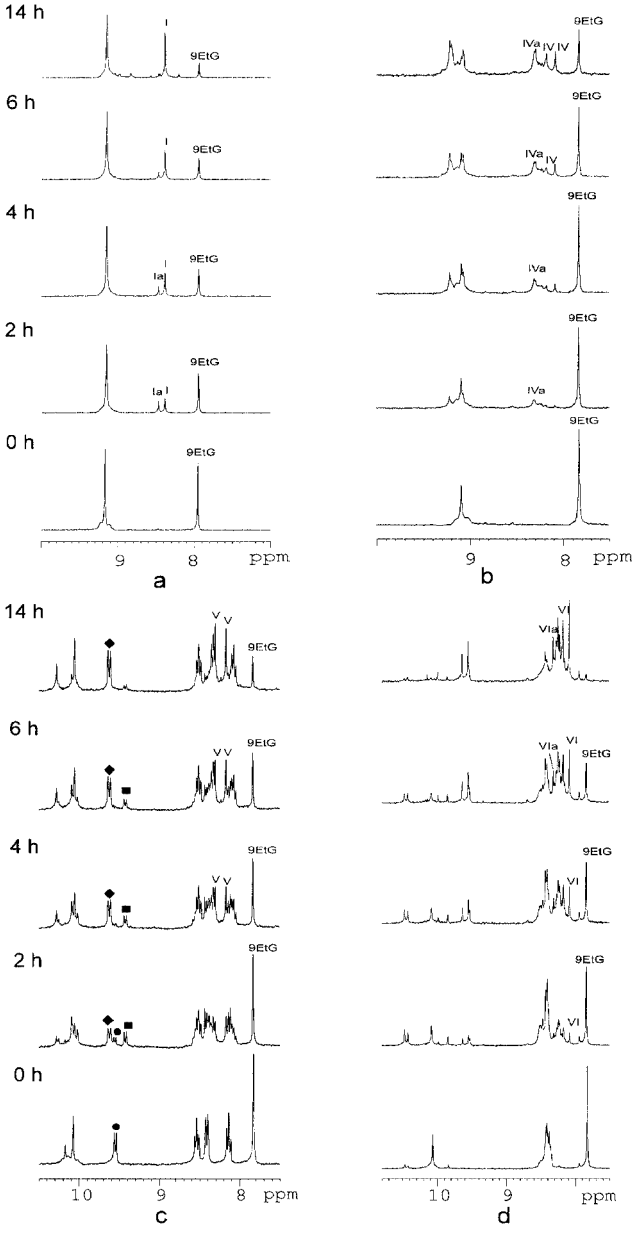


Figure 2. ^{1}H NMR spectra taken over the course of the reaction of **1** (a), **4** (b), **5** (c), and **6** (d) with 2 equiv. of 9EtG in 0.1 M $NaClO_4$ solution in D_2O at 310 K; the H8 signals of the reaction products **I**, **Ia**, **IV**, **IVa**, **V**, **VI**, and **VIa** are shown with the respective roman numbers; the symbols refer to $H8_{(qzn)}$ protons in **5** (circle), **Va** (square), and **V** (diamond)

product **Ia** (see Scheme 1) was also observed, while during the reaction of this complex with GMP no intermediate species were detected.

Table 2. ^{1}H and ^{195}Pt NMR spectroscopic data for the final products of the reaction with 9EtG, **I**, **IV**, **V**, **VI** and **VIa**, in 0.1 M $NaClO_4$ solution in D_2O at 298K

Complex	H8 of 9EtG, δ(^{1}H) [ppm]	δ(^{195}Pt) [ppm]
I	8.27	−2438
IV	8.08; 8.18	−2458
V	8.19; 8.31	−2456
VI	8.10	n.o.[a]
VIa	8.33	−2422

[a] Not observed.

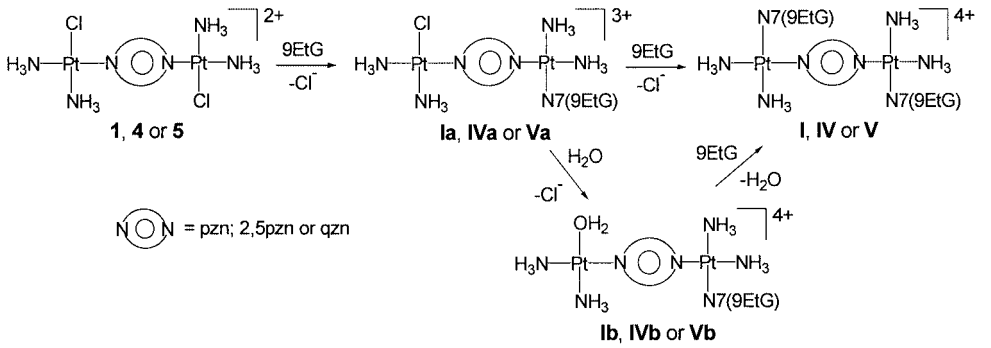
The half-life of 9EtG was determined graphically based on relative integration values of the H8 proton, and is presented in Table 3. As compared to the reaction with GMP [$t_{1/2}$(GMP) = 100 min] previously described,[23] binding of 9EtG to **1** is slower than GMP binding to this complex. A certain amount of the monosubstituted complex was observed in the case of 9EtG, indicating a slower substitution of the second chloride ion as compared to the reaction with GMP.

Table 3. The times of disappearance of 50% 9EtG ($t_{1/2}$) during the reactions of **1** and **4** with 2 equiv. of 9EtG in 0.1 M $NaClO_4$ solution in D_2O at 310 K

Complex	$t_{1/2}$ [min]
1	160
4	210

Complex 4

The substitution of the first chloride ion by a 9EtG molecule yields an intermediate complex **IVa** (see Scheme 1). As the reaction proceeds further, two new signals at δ = 8.08 and 8.18 ppm appear and gradually increase in intensity. One of them was initially tentatively assigned to the hydrolyzed intermediate **IVb**. However, no peaks corresponding to the platinum nucleus in the [N_3O] coordination environment[19,23,25] were found in the ^{195}Pt NMR spectrum. Thus, **IVb** is not present in the reaction mixture, and the signals at δ = 8.08 and 8.18 ppm can only be assigned to the non-equivalent H8 protons of the two guanine bases in the disubstituted product **IV**. In contrast to **I** where free rotation around Pt−N bonds is present, complex **IV** is sterically hindered, and it might exist in a certain thermo-



Scheme 1. The reaction scheme for the interaction of complexes **1**, **4**, and **5** with 2 equiv. of 9EtG in aqueous solution

dynamically favored conformation, in which the two guanine molecules have a different configuration with respect to the CH_3 groups.

The half-life of 9EtG was determined graphically based on relative integration values of the H8 proton, and is given in Table 3. As is clear from Table 3, complex **4** reacts much slower with 9EtG than the analogous complex **1**, without methyl substituents on the azine ring. It has been shown previously that the rate-limiting step of guanine binding to the azine-bridged complexes is a direct substitution of the chloride ion by N7 of a guanine base, and therefore the reaction rate is mainly determined by steric factors.[23] Apparently, methyl groups of the 2,5-dimethylpyrazine in **4** hamper chloride substitution by 9EtG, resulting in a much slower interaction as compared to **1**.

Complex 5

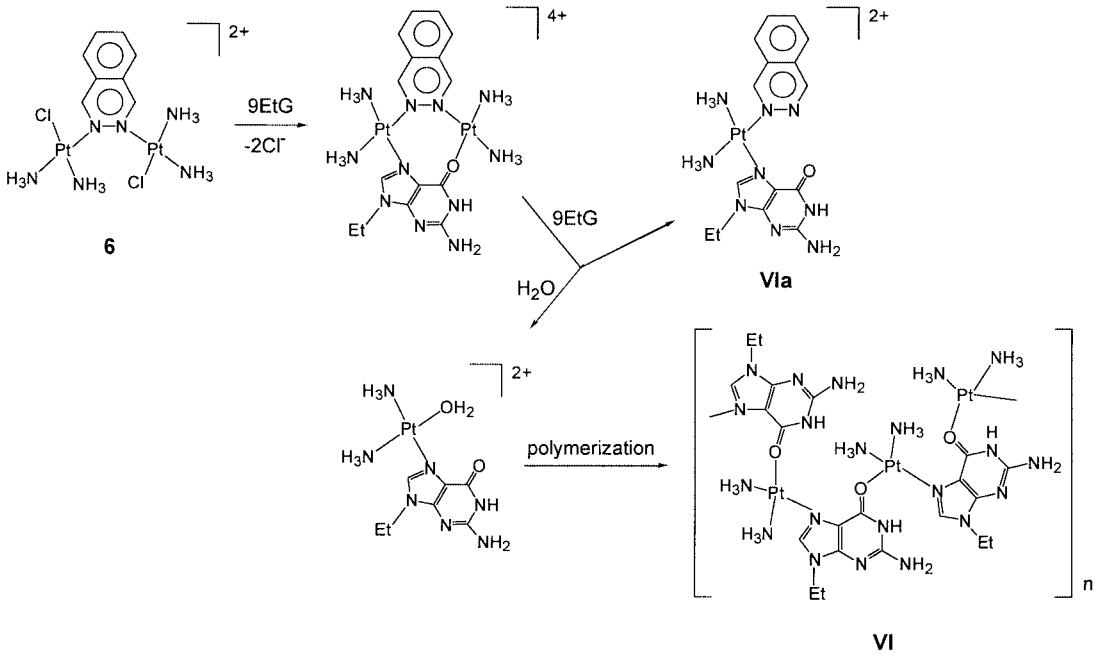
The reaction of **5** with 9EtG provides the disubstituted complex **V** as confirmed by ^1H and ^{195}Pt NMR spectroscopy (Table 2). An interesting feature of **V** is that the H8 protons of the two guanine molecules in this complex are not equivalent. This is not unexpected, as one of them is shielded by an aromatic ring of quinazoline and its signal is, therefore, shifted upfield. Complex **5** reacts with guanine via the intermediate species **Va**. This is clearly seen from the signals of the $H8_{(qzn)}$ proton of the quinazoline. As the reaction proceeds, the intensity of the peak corresponding to the starting complex **5** ($\delta = 9.56$ ppm) decreases, and at first the signal of the $H8_{(qzn)}$ proton of the intermediate **Va**, at $\delta = 9.42$ ppm, increases in intensity. Later in the course of the reaction the intensity of this signal diminishes, while the peak ($\delta = 9.63$ ppm) corresponding to the $H8_{(qzn)}$ proton of the final product **V** builds up in intensity. It should be noted that only one intermediate was observed, whereas two different intermediates would be expected due to the asymmetry of the starting complex. This indicates that the least hindered chloride ion is substituted first. The substitution of the second chloride ion is more difficult for steric reasons and is, therefore, slower. It may be concluded that the reaction of 9EtG with complex **5** proceeds slower than the same reaction with the related compound **2**. However, no quantitative confirmation was possible due to an overlap between the signals of the aromatic protons of quinazoline and H8 signals of the guanine bases of the products.

Complex 6

Complex **6** interacts with 9EtG in a similar manner to that previously found for the reaction of the related compound **3** with GMP,[23] i.e. with the cleavage of the Pt−N(pht) bond and subsequent polymerization. First, the N7,O6-platinated 1:1 complex $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}9EtG\text{-}N7,O6)(\mu\text{-}pht)]^{4+}$ is formed. Binding of another 9EtG molecule to this complex breaks the Pt−N(pht) bond, yielding two mononuclear fragments, $[cis\text{-}Pt(NH_3)_2(pht)(9EtG\text{-}N7)]^{2+}$ (**VIa**) and $[cis\text{-}Pt(NH_3)_2(9EtG)(H_2O)]^{2+}$. The latter species polymerizes to form product **VI**, $[\{cis\text{-}Pt(NH_3)_2\}(\mu\text{-}9EtG\text{-}N7,O6)\{cis\text{-}Pt(NH_3)_2\}(\mu\text{-}9EtG\text{-}N7,O6)]_n$ (see Scheme 2).

In contrast to the previously described reaction of **3** with GMP, in the case of interaction between **6** and 9EtG the second guanine molecule predominantly attacks the platinum atom bound to O6 of the bridging guanine. The Pt−N7(9EtG) bond of the initially formed 1:1 complex $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}9EtG\text{-}N7,O6)(\mu\text{-}pht)]^{4+}$ remains intact, and therefore, the mononuclear complex $[cis\text{-}Pt(NH_3)_2(pht)(9EtG\text{-}O6)]^{2+}$ was not observed. This phenomenon appears to be related to the guanine base rather than to the bridging azine, as during the reaction of **3** with 9EtG no species $[cis\text{-}Pt(NH_3)_2(pdn)(9EtG\text{-}O6)]^{2+}$ was detected (spectra not shown).

The structures of the products were confirmed by ^1H and ^{195}Pt NMR spectroscopy, mass spectrometry and pH titration. The ^1H NMR spectrum of **VIa** shows an asymmetric profile of the pht ligand. The ^{195}Pt shift of this complex is typical of $[N_4]$ coordination surrounding a platinum atom.[18,23,25] A signal assigned to $[cis\text{-}Pt(NH_3)_2(pht)(9EtG)]^+$ was observed in the mass spectrum of **VIa**. ^1H NMR spectroscopic data for **VI** confirm the absence of phthalazine. The H8 signal of this complex is shifted by 0.26 ppm downfield compared with free 9EtG, indicating an inductive effect of platinum coordinated to guanine. As in the case of the similar complex $[\{cis\text{-}Pt(NH_3)_2\}(\mu\text{-}GMP\text{-}N7,O6)\{cis\text{-}Pt(NH_3)_2\}(\mu\text{-}GMP\text{-}N7,O6)]_n$,[23] no signal was found in the ^{195}Pt NMR spectrum of **VI**. This phenomenon



Scheme 2. The proposed reaction scheme for the interaction of **6** with 2 equiv. of 9EtG in aqueous solution

is a result of ^{195}Pt relaxation, and is often observed in the case of polymerized compounds.[26] The mass spectrum of complex **VI** gives a signal, which corresponds to $[Pt(\mu\text{-}9EtG)Pt(NH_3)]^+$.

Thus, the close proximity of the platinum atoms does not allow coordination of two 9EtG ligands to the Pt−(μ-pht)−Pt moiety resulting in cleavage of the Pt−N(pht) bond upon binding of the second 9EtG, followed by polymerization. It should be noted that this is the first example of the formation of polymeric species in the reactions involving platinum complexes with 9EtG.

pH Titrations

The chemical shift of the signal of the H8 proton of 9EtG is strongly dependent on (de)protonation of the heterocyclic nitrogen atoms. Therefore, a pH titration is a very convenient method of determining the platinum binding position of the guanine.[27,28] The pH titration was performed on the final products of the reaction of 9EtG with compounds **1** and **4**−**6**. The plots of the chemical shifts of the signal of the H8 protons as a function of pD are shown in Figure 3. For all the products analyzed, the N7 (de)protonation in the acidic area, as it is known for free 9EtG, is absent. This clearly indicates that in all of these compounds the platinum atoms are bound to the guanines at the N7 position. The N1 (de)protonation in basic solution was observed for **I**, **IV**, and **VIa**, confirming the proposed structures. Complex **V** was found to be unstable at pD > 8.5, therefore, the (de)protonation process at N1 in the basic region could not be detected.

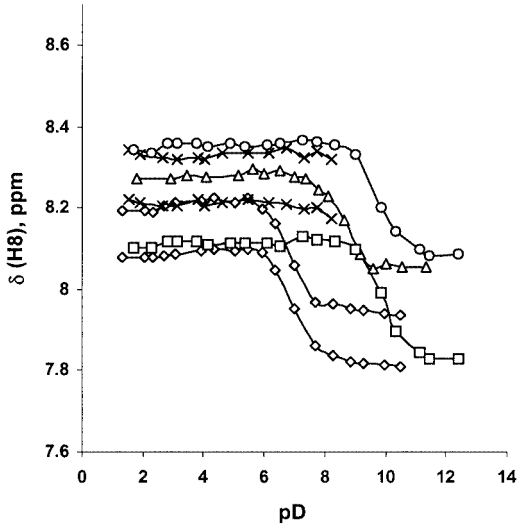


Figure 3. Plots of the chemical shift of H8 protons vs. pD for **I** (triangle), **IV** (diamond), **V** (cross), **VI** (square), and **VIa** (circle); pH titrations were performed in 0.1 M $NaClO_4$ solutions in D_2O at 298 K; pD was adjusted with 1 M and 0.1 M DCl, 1 M and 0.1 M NaOD

The results from the pH titration of **VI** present evidence of the binding positions of the platinum atoms in this complex. For **VI**, the N7 (de)protonation process was not observed indicating that platinum binds to guanine at the N7 position. Another platinum atom can possibly be bound to either N1 or the O6 position. N1 (de)protonation was detected upon pH titration of **VI**, strongly suggesting that the binding position of another platinum atom is the O6 atom of a guanine ligand.

In vitro Cytotoxicity Assay in Human Tumor Cell Lines

The cytotoxicity of **4**−**6**, and cisplatin as a reference, in several human tumor cell lines, MCF and EVSA-T (breast cancer), WIDR (colon cancer), IGROV (ovarian cancer), M19 (melanoma), A498 (renal cancer), and H226 (non-small cell lung cancer), is summarized in Table 4. All three azine-bridged complexes were found to be in the active range in all the cell lines, except for **4** in A498. Among the three dinuclear compounds tested, **4** was the least cytotoxic in all of the cell lines, and **6** exhibited a slightly higher activity than **5** in five cell lines. The cytotoxicity of the complexes is lower than that of cisplatin. However, similarly to the related complexes **1**−**3**,[23] compounds **4**−**6** show a high specificity for the IGROV cell line. They are approximately half as cytotoxic as cisplatin in this cell line, whereas their activity compared to cisplatin in the other cell lines is 4−5 times lower.

Table 4. In vitro cytotoxicity assay of complexes **4**−**6** and cisplatin on human tumor cell lines

Test compound	IC_{50} [μM]						
	A498	EVSA-T	H226	IGROV	M19	MCF-7	WIDR
4	11.9	6.3	19.6	1.1	8.4	9.0	5.4
5	21.8	5.7	17.8	1.0	5.8	7.3	5.8
6	> 88.3	16.7	53.7	3.2	23.5	23.4	7.5
Cisplatin	5.7	1.4	4.5	0.4	1.8	1.4	1.8

In vitro Cytotoxicity Assay in L1210 Cell Lines Sensitive and Resistant to Cisplatin

The cytotoxicity tests of complexes **4**−**6** were performed on L1210 murine leukemia cells, sensitive (L1210/0) and resistant (L1210/DDP) to cisplatin. Cisplatin and the azine-bridged complexes **1**−**3** were included as reference compounds. The results are presented in Table 5. The IC_{50} values for the reference complexes **1**−**3** correlate with the previously reported data.[23] Complex **4** exhibits significantly lower activity compared with the related compound **1** in both L1210/0 and L1210/DDP cell lines. Compound **5** is half as active as the analogous complex **2**. Both **4** and **5** are approximately 5 times less cytotoxic than cisplatin. Nevertheless, these complexes are still in the active range. Compound **6** was found to be twice as active as the related complex **3**. Its cytotoxicity is similar to that of cisplatin. As expected, in the resistant cell line cisplatin is significantly less active, with the resistance factor (*RF*) equal to 4.7. The resistant factors of the azine-bridged complexes are lower (around 3) indicating that they partially circumvent cisplatin resistance.

Table 5. In vitro cytotoxicity assay of complexes **1**−**6** and cisplatin on L1210 murine leukemia cell lines, sensitive (L1210/0) and resistant (L1210/DDP) to cisplatin

Test compound	IC_{50} [μM] L1210/0	L1210/DDP	*RF*[a]
1	1.0±0.1	3.5±0.1	3.5
2	5.2±0.2	18.4±1.0	3.5
3	5.7±0.6	21.3±3.0	3.7
4	9.3±1.6	> 38	> 4
5	12.2±0.4	34.6±1.0	2.8
6	2.8±0.1	8.4±0.3	3.0
Cisplatin	2.0±0.2	9.4±0.8	4.7

[a] *RF* − resistance factor, $RF = IC_{50}(L1210/DDP)/IC_{50}(L1210/0)$.

Induction of Apoptosis by the Platinum Complexes in L1210/0 Cells

One potentially important manner by which platinum−DNA adducts may kill the cells is by induction of programmed cell death or apoptosis. Activation of apoptosis results in nuclear disintegration and eventual fragmentation of the dying cell. Irreversible cleavage of nuclear DNA into nucleosomal oligomers is a distinct feature of apoptotic cell death.

The L1210/0 cells were exposed to the azine-bridged complexes **1**−**6** and cisplatin for 48 h at the concentrations equal to their IC_{50}. The low-molecular weight DNA extracted from the cells was taken for agarose electrophoresis. As shown in Figure 4, DNA fragmentation with a characteristic laddering pattern was observed for all the compounds. DNA laddering is a frequently used indicator of apoptosis.[29,30] Therefore, it can be concluded that all the azine-bridged complexes as well as cisplatin trigger apoptotic pathways in L1210/0 cells.

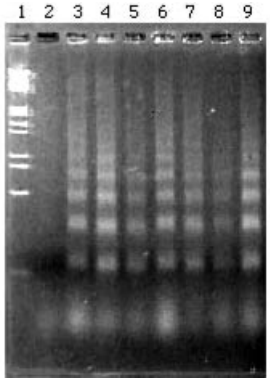


Figure 4. Analysis of nuclear DNA fragmentation induced by the azine-bridged dinuclear platinum complexes **1**−**6** and cisplatin; gel analysis was carried out as described in the Exp. Sect.; cells were incubated for 48 h with cisplatin (lane 3) and compounds **1**−**6** (lane 4−9, respectively) at the concentrations equal to their IC_{50}; lane 1: DNA marker; lane 2: control

Discussion

Compared to the previously described azine-bridged dinuclear complexes **1**−**3**, the newly synthesized complexes **4**−**6** possess additional substituents (e.g. additional aromatic ring or methyl groups) on the azine ligand. Investigation of their interaction with 9EtG shows that the steric hindrance induced by additional groups hampers the substitution of chloro ligands by guanine molecules. Binding

of these complexes to DNA is supposed to be sterically even more restricted, and they might prefer to react with relatively flexible protein molecules than with rather rigid dsDNA. Therefore, a very small amount of Pt−DNA adducts is expected to be formed resulting in a lower cytotoxicity as compared to the parent compounds. This was observed for complexes **4** and **5**, which were found to be less active than their analogues **1** and **2**, respectively. The methyl groups of 2,5-methylpyrazine in **4** induce steric hindrance on both platinum atoms, while in the case of **5** only the second substitution step is supposed to be affected by the presence of an additional aromatic ring. This difference reflects on the activity of the complexes: **4** is much less cytotoxic than **5**.

In the case of **6**, the close proximity of two platinum atoms rather than the presence of an additional aromatic ring determines the outcome of the interaction with 9EtG. In contrast to the reaction with 9EtG, it is unlikely that complex **6** would form an N7,O6-platinated complex [{*cis*-$Pt(NH_3)_2$}$_2${μ-G(dsDNA)-N7,O6}(μ-pht)] on double-stranded DNA, because the O6 atom of the guanine is involved in Watson−Crick base paring. As suggested earlier for the related complex **3**,[23] the initial substitution of the first chloride ion will probably be followed by the Pt−N(pht) bond cleavage upon nucleophilic attack of the neighbouring guanine molecule. This will result in the formation of the two mononuclear fragments, [*cis*-$Pt(NH_3)_2$-(pht){G(dsDNA)-N7}] and [*cis*-$Pt(NH_3)_2${G(dsDNA)-N7}(H_2O)]. The latter one is expected to coordinate to another nucleobase producing cross-links on DNA similarly to cisplatin. The former will apparently stay as a monofunctional adduct as in the case of the analogous complex **3**. Since in this monofunctional adduct the phthalazine ligand occupies the *cis* position with respect to the guanine base, it might act as an intercalator on DNA improving the cytotoxicity of **6** compared with complex **3**, which cannot provide any additional interaction with nuclear DNA.

The azine-bridged dinuclear platinum(II) complexes present a new class of platinum anticancer compounds. In human tumor cell lines they exhibit cytotoxic profiles, which differ from that of cisplatin, and some of them largely or partly circumvent the cross-resistance with cisplatin in murine leukemia cells, due to the formation of DNA adducts different from those of cisplatin. Our results clearly show that the azine-bridged complexes induce apoptosis in L1210/0 cells. Apoptosis in cancer cells is more difficult to trigger because of their ability to either block pro-apoptotic pathways or stimulate anti-apoptotic ones. Thus, induction of apoptosis in tumor cells by the azine-bridged complexes presents clear evidence of considerable anticancer activity of these compounds.

Conclusion

In this paper, we have described the synthesis of three new azine-bridged dinuclear platinum complexes, their interaction with 9EtG, and biological activity of these compounds. The structure-activity relationship within this class of dinuclear platinum anticancer compounds has been established. The complexes possessing additional groups in the azine ring, which induce steric hindrance and hamper binding of the complex to nuclear DNA, are less cytotoxic than the complexes with unsubstituted azines. The substituents on the ligand, that can provide additional interaction with DNA such as intercalation, significantly improve antitumor properties of the complex. In this study, we have also shown that all the azine-bridged dinuclear platinum complexes are able to induce apoptosis in tumor cells indicating a considerable anticancer potential of this class of compounds.

Experimental Section

General: K_2PtCl_4 was used as obtained from Johnson&Matthey (Reading, U.K). Silver nitrate was purchased from Acros, 2,5-dimethylpyrazine, quinazoline, and phthalazine were received from Aldrich, 9EtG was purchased from Sigma, and further purification was not carried out. *cis*-$[Pt(NH_3)_2Cl_2]$ was synthesized according to a literature method.[31] The dinuclear platinum complexes [{*cis*-$Pt(NH_3)_2Cl$}$_2$(μ-pzn)]Cl_2 (**1**), [{*cis*-$Pt(NH_3)_2Cl$}$_2$(μ-pmn)]Cl_2 (**2**), [{*cis*-$Pt(NH_3)_2Cl$}$_2$(μ-pdn)]$(NO_3)_2$ (**3**) were prepared as described previously.[23]

Instrumentation: All NMR spectra were recorded with a 300-MHz Bruker DPX300 spectrometer with a 5-mm multi-nucleus probe. The temperature was kept constant by a variable-temperature unit. ^1H and ^{195}Pt chemical shifts were referenced to TSP and $Na_2[PtCl_6]$ (δ = 0 ppm), respectively. C, H, and N analyses were performed with a Perkin−Elmer 240B microanalyzer. Mass spectrometric measurements were performed with a ThermoFinnigan AQA spectrometer using an Electrospray Interface ionization technique.

***cis*-$[Pt(NH_3)_2Cl(dmf)](NO_3)$:** A solution of $AgNO_3$ (0.08 g, 0.47 mmol) in 3 mL of dimethylformamide (DMF) was added dropwise over 3 h to a stirred solution of *cis*-$[Pt(NH_3)_2Cl_2]$ (0.15 g, 0.5 mmol) in DMF (7 mL) at room temperature in the dark. The mixture was stirred overnight in the dark, and the AgCl precipitate was then filtered off. The resulting pale-yellow DMF solution of *cis*-$[Pt(NH_3)_2Cl(dmf)](NO_3)$ was used as a starting material for the preparation of each azine-bridged dinuclear platinum(II) complex described below.

[{*cis*-$Pt(NH_3)_2Cl$}$_2$(μ-2,5pzn)]Cl_2 (4): 2,5pzn (23.0 mg, 0.21 mmol) in DMF (1 mL) was added dropwise to the stirred DMF solution of *cis*-$[Pt(NH_3)_2Cl(dmf)](NO_3)$, obtained as above. The resulting solution was stirred at room temperature for 6 h in the dark, then concentrated in a rotary evaporator, and the residue was washed with diethyl ether. A crude product was recrystallized twice from a minimal amount of 0.5 M aqueous solution of LiCl yielding a pale-yellow powder of **4**, which was washed with MeOH and diethyl ether, dried in vacuo. Yield: 0.03 g (17%). $C_6H_{20}Cl_4N_6Pt_2$ (708.2): calcd. C 10.18, H 2.85, N 11.87; found C 10.13, H 2.82, N 11.94. ^1H NMR (D_2O): δ (2,5pzn resonance) = 3.09 (s, 6 H), 9.11 (s, 2 H) ppm. ^{195}Pt NMR (D_2O): δ = −2346 ppm.

[{*cis*-$Pt(NH_3)_2Cl$}$_2$(μ-qzn)]Cl_2 (5): qzn (28.3 mg, 0.22 mmol) in DMF (1 mL) was added dropwise to the stirred DMF solution of *cis*-$[Pt(NH_3)_2Cl(dmf)](NO_3)$, obtained as above. The resulting solution was stirred at room temperature for 6 h in the dark. This solution was then concentrated in a rotary evaporator, and the resi-

due was washed with diethyl ether. The crude product was dissolved in a minimal amount of 1 M aqueous solution of LiCl, then excess of MeOH was added. The volume of the resulting solution was reduced to yield a yellow precipitate, which was purified in the same way once more to obtain a yellow precipitate of **5**. This precipitate was collected, washed with MeOH and diethyl ether, and dried in a dessicator over KOH. Yield: 0.05 g (25%). $C_8H_{18}Cl_4N_6Pt_2$ (730.2): calcd. C 13.16, H 2.48, N 11.51; found C 13.02, H 2.27, N 11.41. ^{1}H NMR (D_2O): δ (qzn resonance) = 8.13 (t, 1 H), 8.41 (d, 1 H), 8.53 (t, 1 H), 9.56 (d, 1 H), 10.09 (s, 1 H), 10.21 (s, 1 H) ppm. ^{195}Pt NMR (D_2O): δ = −2317, −2339 ppm.

[{*cis*-Pt(NH$_3$)$_2$Cl}$_2$(μ-pht)](NO$_3$)$_2$ (6): pht (28.3 mg, 0.22 mmol) in DMF (1 mL) was added dropwise to the stirred DMF solution of *cis*-[Pt(NH$_3$)$_2$Cl(dmf)](NO$_3$), obtained as above. The resulting solution was stirred overnight in the dark at room temperature. Then it was concentrated in a rotary evaporator and the residue extracted with MeOH (15 mL). The extract was filtered, concentrated in a rotary evaporator, and extracted again with MeOH (15 mL). The resulting extract was filtered, concentrated in a rotary evaporator, and the residue washed with a small amount of EtOH, and thoroughly washed with diethyl ether to obtain a yellow powder of **6** which was dried in a dessicator over KOH. Yield: 0.06 g (30%). $C_8H_{18}Cl_2N_8O_6Pt_2$ (783.3): calcd. C 12.27, H 2.32, N 14.30; found C 12.22, H 2.40, N 14.40. ^{1}H NMR (D_2O): δ (pht resonance) = 8.36 (m, 2 H), 8.41 (m, 2 H), 10.08 (s, 2 H) ppm. ^{195}Pt NMR (D_2O): δ = −2232 ppm.

Reactions with 9EtG: The reactions of the complexes **1**, **4**, **5**, and **6** (4 mM) with 2 equiv. of 9EtG (8 mM) were monitored over 14 h at 310 K by ^{1}H NMR spectroscopy in a 0.1 M $NaClO_4$ solution in D_2O (pD ≈ 7). The reactions of **1** and **4** with 9EtG were quantified using relative integration values of the signals of the H8 protons.

pH Titrations: The pH titrations were carried out in 0.1 M $NaClO_4$ solutions in D_2O by adjustment of the pD with 0.1 M and 1 M DCl, 0.1 M and 1 M NaOD. The pD values were measured at 298 K using a PHM 80 pH meter (Radiometer) before and after each ^{1}H NMR measurement. The pH values were not corrected for H/D isotope effects.

In vitro Cytotoxicity Assay in Human Tumor Cell Lines: In vitro cytotoxicity assays in human tumor cell lines were performed at the Dr. Daniel den Hoed Kliniek (Rotterdam Cancer Institute), Department of Medical Oncology (Rotterdam, the Netherlands). The seven well-characterized cell lines used were MCF7 and EVSA-T (breast cancer), WIDR (colon cancer), IGROV (ovarian cancer), M19 (melanoma), A498 (renal cancer), and H226 (non-small cell lung cancer). All cell lines were maintained in a continuous logarithmic culture in RPMI 1640 medium with Hepes and phenol red (Paisley, Scotland). The medium was supplemented with 10% FCS (Paisley, Scotland), penicillin 100 IU/mL and streptomycin 100 μg/mL (Sigma). Cisplatin and the dinuclear platinum complexes **4** and **5** were dissolved in water (1 mg/200 μL), complex **6** was dissolved in DMSO (1 mg/200 μL) and finally diluted in full medium. After 48 h of preincubation of the tumor cells in 96-well flatbottom microtiter plates, the solutions of the test compounds were added. The plates were incubated at 37 °C, 8.5% CO_2 for 120 h. IC_{50} values were determined using the microculture sulforhodamine-B test (SRB).[32]

In vitro Cytotoxicity Assay in L1210 Cell Lines Sensitive and Resistant to Cisplatin: The L1210/0 murine leukemia cell line (JCRB 9026) was obtained from the Health Science Research Resources Bank (Osaka, Japan). The cisplatin-resistant L1210/DDP cell line was obtained by exposure of L1210/0 cells to cisplatin at the concentration of 10 μM over a period of 3 months and subsequent cloning. L1210/0 and L1210/DDP were cultured in a suspension in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum and no antibiotics. The cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. Cell growth inhibition was determined by an MTT assay [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide].[33] In brief, the cells were divided into 96 well plates at the concentrations of 10^5 cells/mL in 100 μL of growth medium and were allowed to attach for 16 h at 37 °C in a 5% CO_2 humidified atmosphere. Then the cells were incubated with the platinum complexes at various concentrations for 48 h. Following exposure to the drugs, 10 μL of a 5 mg/mL MTT solution was added to each well, and the cells were incubated for 4 h. Then the culture media were removed, and the plates were washed with PBS. 200-μL aliquots of 2-propanol containing 0.04 M HCl were pipetted into each well. The absorbance was measured at 570 nm using a Biorad 550 microplate reader. The IC_{50} values were defined as the drug concentrations that reduced the absorbance by 50% compared to the drug-free control.

Induction of Apoptosis by the Platinum Complexes in L1210/0 Cells: The cells were exposed to the concentrations equal to IC_{50} of each azine-bridged complex. For the analysis of DNA fragmentation, DNA of a low molecular weight extracted from the cells was taken for agarose electrophoresis. The cells were cultured at the concentrations of 10^5 cells/mL in 24 mL for 16 h, followed by incubation with complexes **1**−**6** for 48 h. The cells (10^7 cells) were then harvested by centrifugation and resuspended in 0.1 mL of lysis buffer [0.5% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl buffer (pH = 7.4)]. Afterwards the suspension was incubated at 4 °C for 10 min. In order to remove DNA of a high molecular weight, the suspension was centrifuged at 16300 rpm at 4 °C for 5 min. Then 10 μL of RNase A (10 mg/mL) in Tris-EDTA buffer [1 mM EDTA, 10 mM Tris-HCl buffer (pH = 8.0)] was added to the supernatant and incubated at 37 °C for 1 h. 10 μL of Proteinase K solution (10 mg / mL) was then added, and further incubation was performed at 50 °C for 30 min. Afterwards 20 μL of 5 M NaCl and 120 μL of 2-propanol were added, and the resulting mixture was left overnight at −20 °C. After centrifuging at 16300 rpm at 4 °C for 15 min, the DNA pellet was obtained and subsequently resuspended in 100 μL of 10 mM loading buffer [2 mM EDTA, 90 mM Tris-boric acid buffer (pH = 8.0)]. The DNA was loaded onto a 2% agarose gel, and electrophoresis was carried out in a loading buffer at 100 V for 1 h. The DNA was observed by ethidium bromide solution staining (1 μg/mL, 20 min). The photographs were obtained under UV illumination by a Kodak EDAS-120 system.

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