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Evidence of the Complexation of the Antitumor Drug Bleomycin with *cis*-[$Pt(NH_3)_2Cl_2$] and with Palladium Derivatives. Physicochemical Characteristics, Interaction with DNA, and Antitumor Activity

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Pt(II) and Pd(II) complexes of bleomycin have been studied. Using potentiometric and spectroscopic measurements, we have shown that *cis*-[$Pd(NH_3)_2Cl_2$], [$enPdCl_2$], and [$PdCl_4$]$^{2-}$ react with bleomycin in a three-step process, forming a 1:1 Pd(II): bleomycin complex. Our data strongly suggest that the pyrimidine nitrogen, the secondary amine nitrogen, the histidine peptide nitrogen, and the adjacent peptide nitrogen are involved in the coordination square. A similar complex is obtained between bleomycin and *cis*-[$Pt(NH_3)_2Cl_2$]. Pt(II)–bleomycin and Pd(II)–bleomycin complexes interact strongly with DNA, but no release of the metal ion from its bleomycin site is observed. The antitumor activity of the Pt(II)–bleomycin complex has been screened. The in vitro inhibition of L 1210 leukemia cell growth by the Pt(II)–bleomycin complex is lower than that induced by *cis*-[$Pt(NH_3)_2Cl_2$]. The in vivo inhibition of Lewis pulmonary carcinomas by the Pt(II)–bleomycin complex is equally lower than that induced by free bleomycin.

Introduction

Bleomycins (BLM) (Chart I) are a family of glycopeptide antibiotics clinically prescribed for the treatment of selected neoplastic diseases.[1] This drug, which both chelates metal ions and binds to deoxyribonucleic acid (DNA), induces a degradation of DNA in a reaction that has been shown to depend, in vitro, on the presence of ferrous ion and molecular oxygen.[2] On the other hand, *cis*-[$PtCl_2(NH_3)_2$] (*cis*-DDPt) has now been proved to be of significant value in treating several human tumors, particularly the seminomas. It has been postulated that the primary chemical lesion is caused to the DNA, inhibiting it as a template for replication.[3,4]

These two antitumor compounds are used in combination chemotherapy to treat malignant tumors.[5-9] The two drugs exhibit synergism. Mascharak et al.[10] have recently demonstrated that *cis*-DDPt alters the sequence specific cleavage of DNA by bleomycin, masking cutting sites near oligo (dG) sequences and stimulating cutting elsewhere.

In the present study we address the question of whether prior covalent binding of *cis*-DDPt to BLM might alter the interaction of these both drugs with DNA and their antitumor activity. Because of the slowness of Pt(II) ligand exchange reactions, parallel studies were conducted on the corresponding Pd(II) complexes, which react 10^5 times faster.[11]

We thus report experiments showing that *cis*-diaminodichloropalladium(II) (*cis*-DDPd) as well as *cis*-ethylenediaminedichloropalladium(II) [$Pd(en)Cl_2$] and potassium tetrachloropalladate(II) [$PdCl_4$]$^{2-}$ react with BLM in a three steps process forming a 1:1 Pd(II):BLM complex. The ligands involved in the coordination square are somewhat different from those suggested for other metal–BLM complexes on grounds of the P3A–Cu(II) model.[12] A similar complex is obtained between BLM and *cis*-DDPt; its antitumor activity has been screened.

Experimental Section

Purified BLM A_2, which contains a (3-aminopropyl)dimethylsulfonium [$-NH(CH_2)_3S^+(CH_3)_2$] group at the terminal amine, depyruvamide bleomycin (depBLM), and *cis*-DDPt were kindly provided by the Laboratoire Roger Bellon. The other Pd(II) complexes were obtained from Johnson Matthey. Calf thymus DNA was purchased from Sigma Chemical Co. All other reagents were of the highest quality available, and deionized distilled water was used throughout the experiments. Samples of Cu(II)–BLM were prepared by the stoichiometric addition of the antibiotics to the Cu(II) salt in 0.05 M Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid] buffer, at pH 7.4. Unless otherwise stated, the Pd(II)–BLM or Pt(II)–BLM complexes were prepared by addition of *cis*-DDPd, *cis*-[$Pd(en)Cl_2$], [$PdCl_4$]$^{2-}$, or *cis*-DDPt to the antibiotic either in aqueous solution or in Hepes buffer.

Absorption spectra were recorded on a Cary 219 spectrophotometer and circular dichroism (CD) spectra on a Jobin Yvon dichrograph, Model Mark V. Results are expressed in terms of ϵ (molar absorption coefficients) and $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficients). Potentiometric measurements were obtained with a Metrohm pH meter, Model E 603, at 25 °C using a Metrohm EA 147 glass electrode.

Uncorrected fluorescence spectra were recorded at 20 °C on a Jobin Yvon Spectrofluo JY3C spectrophotofluorometer equipped with an X-Y recorder and piloted by a microprocessor. All measurements were made in a cuvette with a 1-cm light path.

Tumor Systems. The in vivo inhibition of Lewis pulmonary carcinomas by *cis*-DDPt–BLM carcinomas system and BLM have been compared.

Mice C_{57}/BL are used in experiments and tumors are intramuscularly (IM) implanted consisting of 5×10^6 cells on day 0 (10 mice/group). Compounds are prepared in physiological saline and are intravenously injected (IV) on days 1–4. The pulmonary metastases are counted on day 18.

In Vitro Inhibition of L 1210 Leukemia Cell Growth. L 1210 leukemia cells can be grown in vitro in RPMI medium supplemented with fetal calf serum (10%) and 10 μM 2-mercaptoethanol. For the growth studies, tubes are seeded with 4.5 mL of cells (approximately 5×10^4 cells/mL); compounds prepared in whole medium are added under a final volume of 0.5 mL (three tubes per concentration). Tubes are incubated at 37 °C for 4 days, and cell numbers are then determined with a Coulter counter.

Results

BLM–[$PdCl_4$]$^{2-}$ System. The addition of [$PdCl_4$]$^{2-}$ to an aqueous solution of BLM gives rise to the rapid formation of a first complex (I), which evolves to a second (II) and then to a third one (III).

(A) Complex I. The formation of complex I is attested by (i) a drop in the pH indicating proton displacement (when [$PdCl_4$]$^{2-}$ is added to a 10^{-3} M BLM solution in a 1:1 molar ratio the pH

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(1) Carter, S. K. In *Bleomycin Current Status and New Developments*; Carter, S. K., Crooke, S. T., Umezawa, H., Eds.; Academic Press: New York, 1978.
(2) Sausville, E. A.; Stein, R. W.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978**, *17*, 2746–2754.
(3) Harder, M. C.; Rosenberg, B. *Int. J. Cancer* **1970**, *6*, 207–210.
(4) Pascoe, J. M.; Roberts, J. J. *Biochem. Pharmacol.* **1974**, *23*, 1345–1350.
(5) Pascoe, J. M.; Roberts, J. J. *Biochem. Pharmacol.* **1974**, *23*, 1359–1362.
(6) Howle, J. A.; Gale, G. R. *Biochem. Pharmacol.* **1970**, *19*, 2757–2762.
(7) Perry, D. J.; Weltz, M. D.; Brown, A. W., Jr.; Henderson, R. L.; Neglia, W. J.; Berenberg, J. L. *Cancer (Philadelphia)* **1982**, *50*, 2257–2260.
(8) Bloch, B.; Nel, C. P.; Kriel, A.; Aatd. J.; Goldberg, G. *Cancer Treat. Rep.* **1984**, *68*, 891–894.
(9) Coonley, C. J.; Bains, M.; Hiloris, B.; Chapman, R.; Kelsen, D. R. *Cancer Res.* **1984**, *54*, 2351–2354.
(10) Marcharak, P. K.; Sugiura, Y.; Kuwahara, J.; Suzuki, T.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6795–6798.
(11) Martin, R. B. In *Platinum, Gold, and Other Metal Chemotherapeutic Agents*; Lippard, S. J., Ed.; ACS Symposium Series 209; American Chemical Society: Washington, DC, 1983; pp 231–244.
(12) Itaka, Y.; Nakamura, H.; Nakatami, T.; Muraoka, Y.; Fujii, A.; Takita, J.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1070–1072.

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Chart I

value decreases from about 5 to 3), (ii) the appearance of a positive band at 365 nm in the CD spectrum, and (iii) the quenching of the pyrimidine fluorescence at 405 nm. The fluorescence spectrum of bleomycin has already been reported and analyzed by Chien et al.[13] With an excitation at 292 nm the emission spectrum exhibits a maximum at 350 nm and a shoulder at 400 nm. These authors assigned these two emissions to the bithiazole rings and the 4-aminopyrimidine group respectively. We have found that an excitation at 307 nm gives rise to a reverse pattern: the emission due to the 4-aminopyrimidine appears as a maximum at 405 nm and that due to the bithiazole rings as a shoulder at 350 nm. We have thus found it more reliable to follow the interaction of BLM with $[PdCl_4]^{2-}$ by monitoring the emission at 405 nm through excitation at 307 nm. Uncorrected fluorescence spectra of bleomycin at various BLM:$[PdCl_4]^{2-}$ molar ratios are shown in Figure 1. Total quenching of the fluorescence at 405 nm occurs for a 1:1 molar ratio. The kinetics of quenching of the fluorescence has been followed at this molar ratio: at 2.8×10^{-5} M BLM 50% of the fluorescence is quenched within 15 s. It should be noticed that, as could be expected, this complexation of BLM to Pd(II) does not modify the fluorescence of the bithiazole rings. A stoichiometry of 1:1 appears to be highly likely for this complex, with 4-aminopyrimidine as one of the ligands.

(B) Complex II. The reaction I → II is characterized by the decrease of a CD band at 290 nm, which becomes negative as time elapses, and the appearance of a positive one at 265 nm; the evolution can conveniently be followed by monitoring these two bands. At 25 °C and at a 10^{-3} M complex concentration, a half-lifetime of 7 min has been determined. The reaction I → II is also characterized by the presence of isodichroic points at 326 and 362 nm. The pH values remain unmodified during the reaction.

(13) Chien, M.; Grollman, A. P.; Horwitz, S. B. *Biochemistry* **1977**, *16*, 3641–3647.

Figure 1. Uncorrected fluorescence spectra of bleomycin in the presence of various amounts of $[PdCl_4]^{2-}$. The spectra have been recorded 2 min after addition of $[PdCl_4]^{2-}$ to BLM at 25 °C; λ_e = 307 nm Experimental conditions: 28 μM BLM in H_2O; $[PdCl_4]^{2-}$:[BLM] = 0 (curve 1), 0.2 (curve 2), 0.4 (curve 3), 0.6 (curve 4), 1 (curve 5). Inset: Relative intensity of fluorescence at 405 nm recorded as a function of time for 28 μM BLM in H_2O and $[PdCl_4]^{2-}$:[BLM] = 1.

(C) Complex III. The reaction II → III is very slow and is characterized by the appearance of a negative band at 320 nm and the presence of an isodichroic point at 310 nm. The half-lifetime of the reaction is about 20 h. The pH remains unchanged. In fact, the kinetics of this reaction is pH-dependent: up to pH 5.5 no modification is observed, but when the pH is raised above

Table I. Absorption and CD Data for Complexes I, II, III, I′, II′, III′, and d

complex	absorption		CD	
	λ, nm	ε	λ, nm	Δε
I	325 (sh)	2000	367	+0.5
	292	14000	290	+1.4
II	325 (sh)	2000	355	+0.6
			317 (sh)	−0.4
	292	16000	296	−1.1
			265	+1.1
III			345	+1.1
	325	4000	320	−1
	297	14000	296 (sh)	+1
	260	18500	265	+2.6
I′	335 (sh)	1700	360	+0.5
	292	14000	290	+1.2
II′	335 (sh)	3000	350	+0.5
	292	16000	295	−1.1
			265	+1.1
III′	335	4200	345	+1
			318	−1
	295	14000	295 (sh)	+1.3
	265	13000	270	+3
d	325	5000	315	−2.4
	290	14000	276	+2.1

6.5 the reaction becomes fast. The pK of the reaction II → III has been determined spectroscopically and is equal to 6.1. This reaction is not reversible.

It should be noticed that if $[PdCl_4]^{2-}$ is added to an Hepes buffer BLM solution (pH 7.4), the formation of complex III occurs with a half-lifetime equal to 10 min. This value has been determined by using the quenching of 4-aminopyrimidine fluorescence. The CD spectra of complexes I–III are shown in Figure 2. Data are collected in Table I.

depBLM–$[PdCl_4]^{2-}$ System. The addition of $[PdCl_4]^{2-}$ to 10^{-3} M depBLM in aqueous solution in a 1:1 molar ratio gives rise, as in the previous case, to a decrease of the pH from 5 to 3. A negative CD band appears at 515 nm. The formation of this complex is fast and takes place within the 2 min following the mixing. No evolution either of the CD spectrum or of the pH is observed as time elapses. Moreover an increase of the pH up to 7 does not modify the CD spectrum. It thus appears that in that case, with no dependence on pH or time, only one complex, hereafter labeled "d", is formed. The spectral data are shown in Table I.

BLM–$[Pd(en)Cl_2]$ and BLM–*cis*-$[Pd(NH_3)_2Cl_2]$ Systems. As in the previous case the reaction of BLM with one or the other of these two complexes has been followed as a function of time and molar ratio of BLM to Pd(II). Here again a three-step process and a 1:1 stoichiometry were observed for the complex ultimately obtained. The first complex (I′) is characterized by the quenching of pyrimidine fluorescence ($t_{1/2}$ = 7 min). The reaction I′ → II′ takes place within the following hours and the II′ → III′ reaction within about 3 days. As can be seen in Table I, the spectral data for complexes II′ and III′ are identical with those of complexes II and III, respectively. This strongly suggests that the ligands involved in the square of coordination are the same in complexes II and II′ on one hand and in complexes III and III′ on the other. This means that the complexes formed are independent of the starting complexes used, i.e. $[PdCl_4]^{2-}$, *cis*-$[Pd(NH_3)_2Cl_2]$, or $[Pd(en)Cl_2]$. When the starting complex used is either *cis*-$[Pd(NH_3)_2Cl_2]$ or $[Pd(en)Cl_2]$, the complexation of Pd(II) to BLM occurs with almost no variation of the pH value, which remains near 5. This can easily be explained by taking into account the fact that (i) when *cis*-$[Pd(NH_3)_2Cl_2]$ is dissolved in water, the most predominant species present at pH 5 are *cis*-$[Pd(NH_3)_2Cl_2]$ and *cis*-$[Pd(NH_3)_2ClH_2O]^+$ [11] and (ii) the pK of deprotonation of NH_4^+ is about 10.[14] When complex II′ is formed the four original ligands are removed from *cis*-$[Pd(NH_3)_2Cl_2]$ and cis-$[Pd(NH_3)_2ClH_2O]^+$, and NH_3 immediately forms NH_4^+. Thus the two protons that are released by BLM are immediately bound to the leaving groups. This explains why a decrease of pH is not observed.

(14) Sillen, L. G.; Martell, A. E. in *Stability Constants of Metal Ion Complexes*. The Chemical Society: London, 1964.

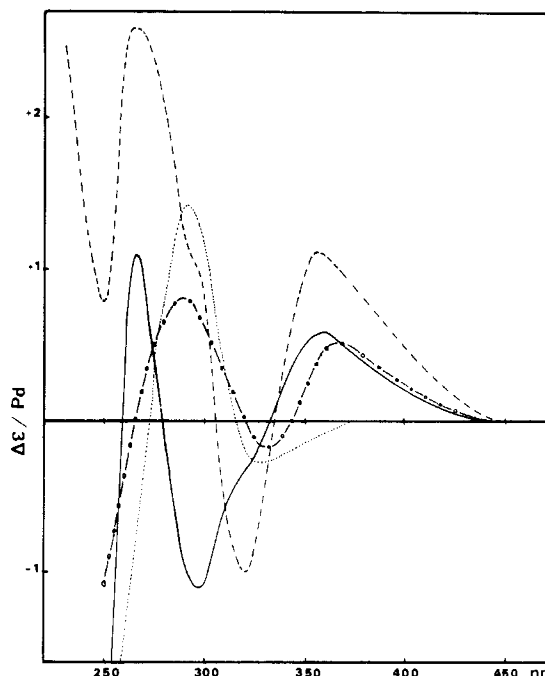


Figure 2. Circular dichroism spectra of free bleomycin (···), complex I (-·-), complex II (–), and complex III (---). Experimental conditions: 10^{-3} M $[PdCl_4]^{2-}$ was added to 10^{-3} M BLM in H_2O. The time elapsed after mixing is less than 10 s for complex I, 10 min for complex II, and 3 days for complex III.

Here again complex III formation occurs at once when the pH is raised above 7. The reaction II′ → III′ has been monitored spectroscopically, and a pK equal to 6.2 has thus been obtained.

Titration of the BLM–$[PdCl_4]^{2-}$ System. The potentiometric titration of complex II was performed in 0.1 M $NaClO_4$ in the pH range 2–10. The pH of the BLM solution was first adjusted to 2 by addition of HCl.

$[PdCl_4]^{2-}$ at a 1:1 BLM:$[PdCl_4]^{2-}$ molar ratio was subsequently added, and the solution was left for 1 h to ensure complete formation of complex II (complex II formation was checked by CD spectroscopy). The solution was then slowly titrated up to pH 10 by addition of 0.1 M NaOH. In Figure 3, the number of protons $\bar{n}$ released per bleomycin has been plotted as a function of pH ($\bar{n}$ has been calculated classicaly as $([H^+] + [Na^+]_{added} - [Cl^-]_{initial} - [OH^-]/[BLM-Pd])$).[15] As can been seen, at pH 2, two protons have been released with regard to free BLM. From pH 3 to 5 another is released with a pK value of about 4. In the case of free BLM one proton, which has been assigned to the deprotonation of imidazole of histidine,[16] is titrated in this pH range with a pK of 4.7. Since the spectroscopic titration of complex II in this pH range does not reveal any modification of the complex, we can suggest that the third titrated proton has been released by imidazole, which is not involved in the coordination to Pd(II). A further increase of the pH from 5 to 9 gives rise to the release of a fourth proton, which occurs concomitantly with the reaction II → III. In the absence of metal ion, in this pH range, deprotonation of the α-amino nitrogen of the β-aminoalanine portion occurs with a pK of 7.5 (curve a, Figure 3).

All these data and the observation that the reaction of depBLM with $[PdCl_4]^{2-}$ yields one complex, which does not evolve either with time or with pH (in the range pH 2–9), led us to already suggest the following:

(15) Bjerrum, J. In *Metal Amine Formation in Aqueous Solution*; Haasen: Copenhagen, 1941.
(16) Chen, D. M.; Hawkins, B. J.; Glickson, J. D. *Biochemistry* **1976**, *16*, 2731–2736.

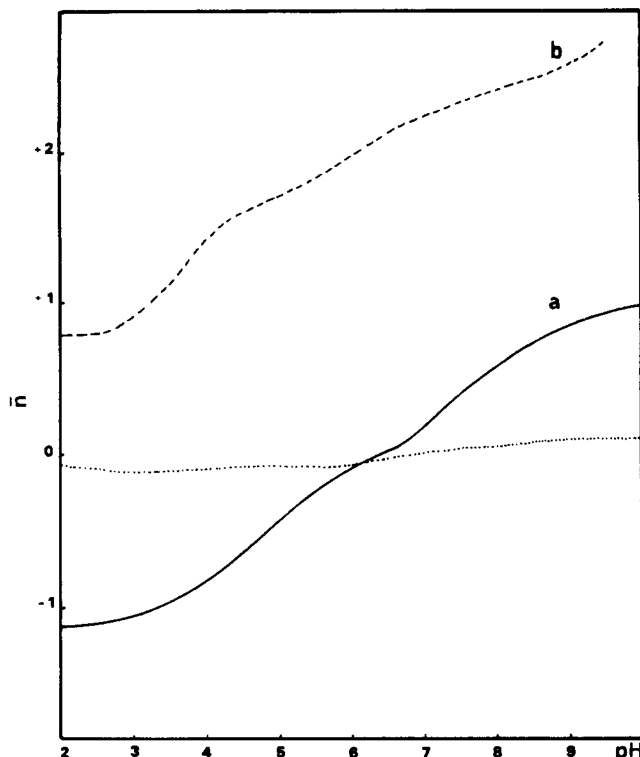
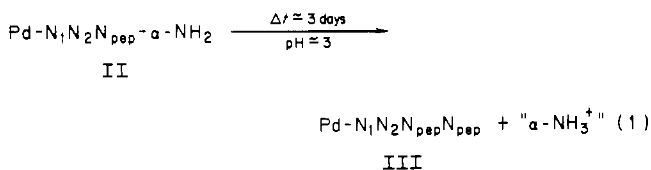
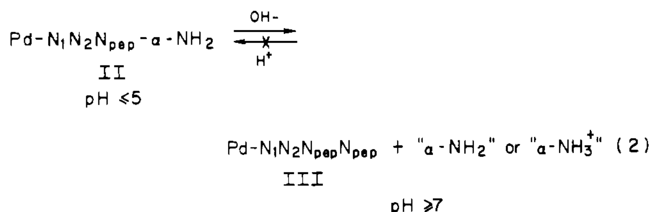


Figure 3. Potentiometric titration of bleomycin (curve a) and complex II (curve b) ([BLM] = 10^{-3} M; $[[PdCl_4]^{2-}]$ = 0 (curve a) and 10^{-3} M (curve b); $[NaClO_4]$ = 0.1 M). $\bar{n}$ is the number of protons released per bleomycin, as a function of pH. Three independent measurements have been performed.

(i) The ligands involved in the coordination square of complex II most probably are the pyrimidine nitrogen (since we observed the quenching of the pyrimidine fluorescence) and the secondary amine nitrogen; the coordination of these two ligands occurs without release of proton. The other two ligands bind to metal through release of protons and are suggested to be the peptide nitrogen of the histidine residue and the α-amino nitrogen of the β-aminoalanine portion. In that scheme of binding, the metal site has a square-plane structure with three chelate rings of 5–5–5-members. The coordination of a peptide nitrogen at this pH value is not to be precluded since it has been estimated that favorable Pd^{2+}-promoted peptide deprotonations take place with $pK_a \simeq 2$.[17]

(ii) The reaction II → III, which occurs either as time elapses at pH 3 or at once when the pH is raised up to 7, is suggested to be due to a rearrangement of the four ligands around Pd(II), i.e. the substitution of the α-amino nitrogen of β-aminoalanine portion by a second peptide nitrogen, most probably that adjacent to the one already coordinated. This can be summarized by the following equilibria:





where "α-NH_2" stands for the α-amino nitrogen of the β-aminoalanine, N_1 and N_2 for the secondary amine nitrogen and pyrimidine nitrogen, and N_{pep} for the peptide nitrogen. This proposition is supported by the following observation: the complex formed between $[PdCl_4]^{2-}$ and depBLM does not evolve when the pH is raised from 2 to 10. Moreover the special role played by the α-amino nitrogen of the β-aminoalanine portion of BLM is emphasized by the following experiment.

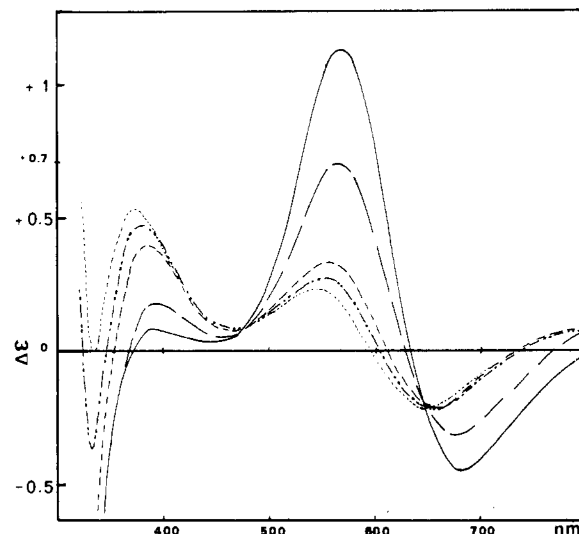


Figure 4. Circular dichroism spectra of the BLM–Cu–α-NH_2–$[PdCl_4]^{2-}$ system as a function of time ([BLM–Cu–α-NH_2] = 10^{-3} M; $[[PdCl_4]^{2-}]$ = 10^{-3} M; Hepes buffer pH 7.4). Δt = 0 (–), 1 h (– –), 19 h (- - -), 25 h (–··–), and 24 h (···).

Interaction of BLM–Cu(II)–α-NH_2 with $[PdCl_4]$. We have recently demonstrated that the reaction of BLM with Cu(II) occurs as a function of pH, in a three-step process;[18] a complex, hereafter labeled BLM–Cu(II), is formed at pH 2.5, which most probably involves in its coordination square the pyrimidine nitrogen, the secondary amine nitrogen, the peptide nitrogen of the histidine residue, and the histidine imidazole nitrogen. The increase of the pH gives rise to the fixation of the α-amino nitrogen of β-aminoalanine in the apical position. The formation of this complex, hereafter labeled BLM–Cu(II)–α-NH_2, occurs with a pK of 2.7. The reaction of BLM–Cu(II)–α-NH_2 with $[PdCl_4]^{2-}$ at a 1:1 molar ratio in pH 7.4 Hepes buffer has been monitored by using CD spectroscopy. Since the ligand field (d–d) transitions of Pd(II) complexes occur from about 290 to 350 nm[17] and those of Cu(II) complexes at wavelengths higher than 500 nm, there is no overlapping between them. The visible CD spectroscopy can thus conveniently be used to monitor the ligand field modification around Cu(II) ion. As can be seen in Figure 4 the reaction of BLM–Cu(II)–α-NH_2 with $[PdCl_4]^{2-}$ gives rise to a slow modification of the CD spectra, which evolves from that characteristic of BLM–Cu(II)–α-NH_2 to that of BLM–Cu(II). At a 5×10^{-4} M concentration the BLM–Cu(II) complex is fully formed after 24 h ($t_{1/2} \simeq 5$ h). We can thus infer that through interaction with Pd(II) the α-amino nitrogen of β-aminoalanine has been removed from its coordination site to Cu(II). Given that the experiments are performed at pH 7.4 and that, at this pH value, $[PdCl_4]^{2-}$ complexes have a tendency to form hydroxo-bridged complexes, the problem becomes very difficult to solve and we will not try to determine what are the other ligands of the Pd(II) ion.

Interaction of BLM–Pd(II) with Iron. Since the antitumor activity of BLM has been related, at least in vitro, to a free radical formed by the iron complex,[19] it was interesting to study the behavior of BLM–Pd(II) complex in the presence of iron as well as that of BLM–Fe(III) in the presence of Pd(II). The reaction of BLM–Pd(II) with Fe(II) at a 1:1 molar ratio was monitored by using CD spectroscopy. No modification of the CD spectrum of the BLM–Pd(II) complex was observed even after several days. For the opposite reaction, the addition of $[PdCl_4]^{2-}$ to BLM–Fe(III) complex gave rise to the slow appearance of the CD

(17) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *32*, 385–426.

(18) Albertini, J. P.; Garnier-Suillerot, A. *J. Inorg. Biochem.* **1985**, *1*, 15–24.

(19) Onishi, T.; Iwata, H.; Takagi, Y. J. *J. Biochem.* **1975**, *77*, 745–749.

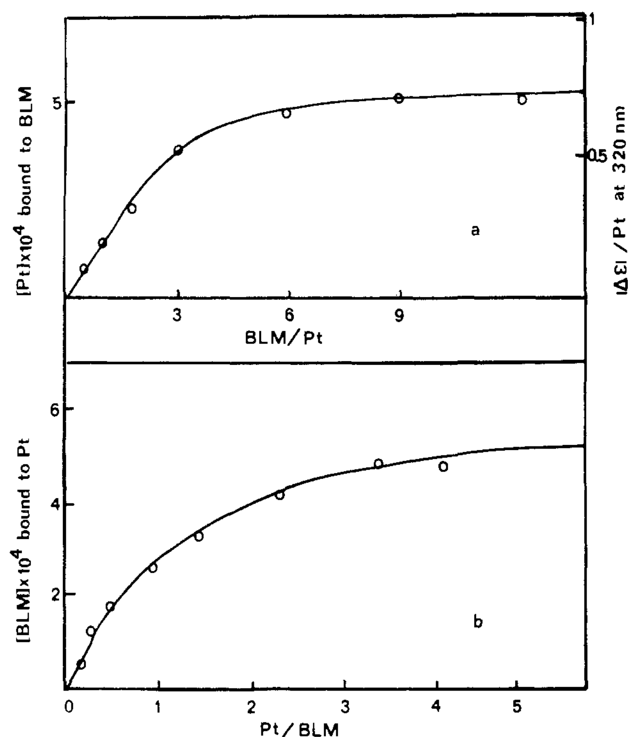


Figure 5. Spectroscopic titration of the bleomycin-*cis*-[$Pt(NH_3)_2Cl_2$] system. Curve a: $\Delta\epsilon$ at 320 nm as a function of the molar ratio of BLM to *cis*-DDPt. ([*cis*-DDPt] = 5×10^{-4} M; Hepes buffer pH 7.4; Δt = 7 days at 37 °C). Curve b: [BLM] bound to Pt as a function of the molar ratio of *cis*-DDPt to BLM ([BLM] = 5×10^{-4} M; Hepes buffer pH 7.4; Δt = 7 days at 37 °C).

spectrum characteristic of the BLM–Pd(II) complex ($t_{1/2} \simeq 3$ h at [BLM] = 5×10^{-4} M), indicating that Pd(II) displaced Fe(III) from its coordination site. This is not meant to imply that the BLM–Pd(II) complex will be inactive in vivo. It is worthwhile to compare these data with those of the BLM–copper–iron system: BLM–Cu(II) does not degrade DNA in vitro, but Cu(II) is a potent inhibitor of the DNA breakage reaction presumably because it displaces the Fe(II) required for activity.[20] In contrast, in vivo studies in tissue culture and animals reveal that BLM–Cu(II) is as effective as metal-free BLM for the breakage of DNA.[21] In order to explain the discrepancy between the in vitro and in vivo activity of the Cu(II) complex, Umezawa and co-workers[22] hypothesized that, in vivo, BLM–Cu(II) is activated in a series of reactions giving rise to the release of Cu(II) by BLM. Thus in the case of the BLM–Pd(II) complex, we can also hypothesize a series of reactions giving rise to the release of Pd(II) by BLM.

BLM and *cis*-[$Pt(NH_3)_2Cl_2$]. The reaction of *cis*-DDPt with BLM is very slow and, as we shall see below, remains uncomplete even after a long period of incubation. This reaction has been followed at different molar ratios of BLM to *cis*-DDPt in pH 7.2 Hepes buffer. The mixtures were left for 1 week at 37 °C in the dark, under permanent stirring. These conditions were chosen in order to be as close as possible to the physiological one. The aim of this section was to determine in these conditions (i) the stoichiometry of the complex formed and (ii) the amount of complex formed in any mixture of BLM and *cis*-DDPt.

The binding of Pt(II) to BLM is attested by the appearance of a negative band at 320 nm. The amount of Pt(II) bound to BLM can thus be determined by using $\Delta\epsilon$ at 320 nm. Curve a of Figure 5 shows the plot of $\Delta\epsilon$ at 320 nm as a function of the molar ratio of BLM to *cis*-DDPt. As can bee seen with the experimental conditions used and with 5×10^{-4} M *cis*-DDPt, a molar ratio of BLM to *cis*-DDPt higher than 3 is needed to ensure the binding of about 90% of Pt(II) to BLM. This is not meant to imply however either that the stoichiometry of the complex is 1:3 or that the stability constant is low. In fact, the complex formed between Pt(II) and BLM is very strong. This is attested by the following experiment: when an excess of Cu(II) is added to a mixture of BLM and *cis*-DDPt, Cu(II) reacts very rapidly with free BLM in solution; this does not give rise to any noticeable shift of the equilibrium between Pt(II) and BLM since the amplitude of $\Delta\epsilon$ at 320 nm is not modified. This strongly suggests that the stability constant between Pt(II) and BLM is at least as high as that between Cu(II) and BLM. Free BLM was thus titrated as the copper–BLM complex by using the amplitude of the CD spectrum at 650 nm.[18] Curve b of Figure 5 shows the plot of the concentration of BLM bound to Pt(II) as a function of the molar ratio of *cis*-DDPt to BLM. In any mixtures of BLM and *cis*-DDPt it is then possible to determine independently the concentration of BLM bound to Pt(II) and that of Pd(II) bound to BLM. This allowed us to check that the stoichiometry of the complex formed was 1:1. This was expected since Pt(II) and Pd(II) ion are well-known to form similar complexes. The observation that, despite the strong stability constant of the BLM–Pt complex, in a mixture of BLM and *cis*-DDPt at a 1:1 molar ratio only about 50% of the complex is formed can be justified by the fact that Pt(II) has a high tendency to form very stable polymeric hydroxy compounds.

BLM–Pd(II)–DNA System. The interaction of complex III with DNA has been followed with fluorescence and CD spectroscopy. As previously reported, excitation at 292 nm gives rise to an emission spectrum exhibiting a maximum at 350 nm due to the fluorescence of the bithiazole rings. The effect of varying concentrations of DNA on the fluorescence of complex III was measured. Addition of DNA to this complex quenches the fluorescence at 350 nm; a maximal quenching, which resulted in a residual fluorescence of 25%, was obtained at a molar ratio of nucleotide to complex III equal to 5. In free BLM–DNA interaction, the quenching of the fluorescence at 350 nm has been interpreted as an intercalation of the bithiazole rings between DNA base pairs.[23] The same interpretation held for complex III–DNA interaction, suggesting that the complexation of Pd(II) to BLM does not prevent the intercalation of the bithiazole rings. Measurements of the fluorescence quenching of complex III by DNA at 350 nm allowed the concentration of free and bound complex to be determined. The binding data were analyzed by Scatchard plots.[24] Complex III binding to DNA appears to be a noncooperative phenomenon with an apparent equilibrium constant of 9.6×10^5. One molecule of complex III binds for every 1.5 base pairs. This is somewhat at variance with the results obtained previously in the case of other metal–BLM complexes: Roy et al.[25] found that one Cu(II)–BLM molecule binds for every 2.8 base pairs, and we found almost the same number of nucleotides/site in the case of Co(III)–BLM and Fe(III)–BLM complexes.[26,27] The interaction of complex III with DNA has also been followed with CD spectroscopy. No modification either of the CD spectrum of DNA, in the 230–300-nm range, or of that of complex III, in the 300–400-nm range, can be detected. These results are obtained whatever the value of the molar ratio of nucleotide to complex and whatever the time elapsed after the mixing. The CD spectra of the DNA complex III mixture can be roughly fit by the addition of the DNA and complex III spectra.

These data suggest that the interaction of complex III with DNA occurs without modification of the DNA conformation as well as the ligand field symmetry around the Pd(II) ion. We can

(20) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978**, *17*, 2740–2746.
(21) Rao, E. A.; Saryan, L. A.; Antholine, W. E.; Petering, D. *J. Med. Chem.* **1980**, *23*, 1310–1318.
(22) Takahasi, K.; Yoshio, A. O.; Matsuda, A.; Umezawa, H. *J. Antibiot.* **1977**, *30*, 861–869.
(23) Povirk, L. F.; Hogan, M.; Dattagupta, N. *Biochemistry* **1979**, *18*, 96–101.
(24) Scatchard, G. *Ann. N.Y. Acad. Sci.* **1949**, *51*, 660–672.
(25) Roy, S. N.; Orr, G. A.; Breuver, F.; Horwitz, S. B. *Cancer Res.* **1981**, *41*, 4471–4477.
(26) Albertini, J. P.; Garnier-Suillerot, A. *Biochemistry* **1982**, *21*, 6777–6782.
(27) Albertini, J. P.; Garnier-Suillerot, A. *Biochemistry* **1984**, *23*, 47–53.

Table II. Antitumor Activity of the BLM–Pt(II) Complex

	cis-DDPT	mixture		
		cis-DDPt	BLM–Pt(II)	BLM
concn in Pt(II), μg/L	0.65	0.13	0.52	
cytotoxicity,[a] %	82		45	
	BLM	mixture		
		cis-DDPt	BLM–Pt(II)	BLM
dose of BLM per injection, mg/kg	2.5		0.7	1.7
inhibition,[b] %	100		88	

[a] In vitro inhibition of L 1210 leukemia cell growth by *cis*-DDPt and BLM–Pt(II) complex. [b] Antitumor activity against the pulmonary carcinoma of Lewis. The experimental conditions are described in the Experimental Section.

already suggest that Pd(II) ion has not been released from its BLM binding site, and the following experiment supports this assumption. When Cu(II) ions are added to a DNA–BLM mixture, one observes the immediate complexation of Cu(II) to BLM; for the opposite case, when Cu(II) ions are added to a DNA–BLM–Pd(II) mixture, one does not observe the formation of the copper–BLM complex. All these data allows us to conclude that through interaction with DNA, Pd(II) ion has not been released from its BLM binding site.

BLM–Pt(II)–DNA System. Similar experiments have been performed with the BLM–Pt(II) complex; in that case, in order to ensure complete binding of Pt(II) ion to BLM, a 3 molar excess of BLM was used. CD spectra do not reveal any modification either of DNA conformation or of the ligand field symmetry around the Pt(II) ion. Here again from our data, we can infer that Pt(II) ion has not been released from its BLM binding site.

Antitumor Activity. The in vitro inhibition of L 1210 leukemia cell growth by the BLM–Pt(II) complex was compared with that induced by *cis*-DDPt. The in vivo inhibition of Lewis pulmonary carcinomas by the BLM–Pt(II) complex was compared with that induced by free BLM. As we have stated previously, the addition of *cis*-DDPt to BLM yields a mixture of BLM–Pt(II) complex, BLM, and *cis*-DDPt. For these tests a mixture containing BLM and *cis*-DDPt at initial concentrations equal to 1.5×10^{-3} M and 0.5×10^{-3} M, respectively, was used. The solution was left for 1 week in the dark, at 37 °C, under permanent stirring. The concentration of BLM–Pt(II) thus formed was checked by CD spectroscopy and the data of Figure 5. We have then stated that the solution contained 0.4×10^{-3} M BLM–Pt(II) complex, 0.1×10^{-3} M *cis*-DDPt and 1.1×10^{-3} M BLM (i.e. 80% of the initial *cis*-DDPt and 27% of the initial BLM are engaged in the complex). The data are collected in Table II. As it can be seen the mixture is less cytotoxic than *cis*-DDPt toward L 1210 leukemia cell growth. Since in the mixture about 80% of the initial *cis*-DDPt present is engaged in the complex, we can infer that through complexation with BLM the cytotoxicity of *cis*-DDPt has been decreased by a factor of 2. The same conclusion can be drawn concerning the antitumor activity of BLM: the mixture is about 12% less efficient that free BLM; since in the mixture about 27% of the starting BLM is complexed to Pt(II), the conclusion can be drawn that the complexation of BLM to Pt(II) gives rise to a decrease of its antitumor activity.

Discussion

The present study was undertaken in order to determine whether prior covalent binding of *cis*-DDPt to BLM might alter the interaction of these both drugs with DNA and their antitumor activity. Since on one hand Pd(II) ions react 10^5 faster than Pt(II) and on the other it is well-known that these two ions form the same kind of complexes with various ligands, most of the physicochemical studies have been conducted with Pd(II) ions. The first result drawn from our experiments is that, as a function of time, the complexation of $[PdCl_4]^{2-}$ to BLM occurs in a three-step process. The kinetics of the last step is greatly enhanced by an increased of the pH up to 7. The same behavior holds for the interaction of BLM either with *cis*-DDPd or *cis*-$[PdenCl_2]$. On the other hand, the complexation of $[PdCl_4]^{2-}$ to depbleomycin seems to take place in only one step.

The CD spectra of the three complexes III, III′, and "d" are closely related, strongly suggesting that the same four ligands are involved in the square of coordination: i.e. the pyrimidine nitrogen, the secondary amine nitrogen, the histidine peptide nitrogen, and the adjacent peptide nitrogen forming 5–5–5– chelate rings. The same coordination site can be suggested for Pt(II). The conclusion can be drawn that the coordinaton site of complex III does not depend on the nature of the initial Pd(II) complex added to BLM since all the initial ligands are substituted by those of BLM.

In that scheme the CD bands lying at about 350, 320, and 290 nm can be assigned to d–d transitions. Their positions are consistent with a ligand field induced by four nitrogen atoms.[17] It must be pointed out that in complex III the α-amino nitrogen of the β-aminoalanine moiety is not a ligand of Pd(II). This is a variance with the observations that this group acts as a ligand in most of the complexes of BLM with other metal ions.

The last points relevant to this study are that (i) BLM–Pd(II) as well as the BLM–Pt(II) complex exhibits a strong affinity for DNA, this interaction occurring without release of the metal ion from its binding site to BLM, and (ii) the complexation of BLM and *cis*-DDPt gives rise to a decrease of the cytotoxicity of both drugs. The conclusion therefore follows that prior covalent binding of the two drugs alters their cytotoxicity but in a way that appears to decrease the therapeutic index, unless this prior covalent binding can help to reduce the secondary toxic effects.

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Registry No. *cis*-DDPd, 15684-18-1; *cis*-DDPt, 15663-27-1; Pd(en)-Cl_2, 15020-99-2; $K_2[PdCl_4]$, 10025-99-7.