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Metal Anthracycline Complexes as a New Class of Anthracycline Derivatives. Pd(II)-Adriamycin and Pd(II)-Daunorubicin Complexes: Physicochemical Characteristics and Antitumor Activity†

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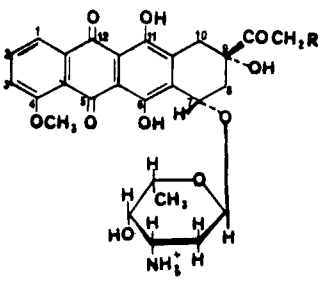
ABSTRACT: Pd(II) complexes of two anthracyclines, adriamycin and daunorubicin, have been studied. Using potentiometric absorption, fluorescence, and circular dichroism measurements, we have shown that adriamycin can form two complexes with Pd(II). The first complex (I) involves two molecules of drug per Pd(II) ion; one of the molecules is chelated to Pd(II) through the carbonyl oxygen on C_{12} and the phenolate oxygen on C_{11}, and the other one is bound to Pd(II) through the nitrogen of the amino sugar. This complexation induces a stacking of the two molecules of drug. In the second complex (II), two Pd(II) ions are bound to two molecules of drug (A_1 and A_2). One Pd(II) is bound to the oxygen on the carbons C_{11} and C_{12} of molecule A_1 and to the amino sugar of molecule A_2 whereas the second Pd(II) ion is bound to the oxygen on C_{11} and C_{12} of molecule A_2 and to the amino sugar of molecule A_1. The same complexes are formed between Pd(II) and daunorubicin. The stability constant for complex II is $\beta = (1.3 \pm 0.5) \times 10^{22}$. Interaction with DNA has been studied, showing that almost no modification of the complex occurred. This complex displays antitumor activity against P-388 leukemia that compares with that of the free drug. Complex II, unlike adriamycin, does not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase.

Adriamycin (Adr) and daunorubicin (Dr) (Chart I) are anthracycline antibiotics widely used in the treatment of various human cancers. Clearly, adriamycin is the antitumor agent with the broadest range of activity clinically. However, a major limitation to its use includes its acute and chronic toxicities. The chronic total dose limiting toxicity is cardiotoxicity, which limits the duration of therapy and consequently the duration of remissions in some situations. Impairment of mitochondrial function in myocardiac cells has been implicated in attempts to explain this cardiotoxicity (Bachmann et al., 1975). Several mechanisms of action have been proposed, including inhibition of electron transfer through the respiratory chain and oxidative phosphorylation (Muhammed et al., 1982), complexation of phospholipid cardiolipin (Goormaghtigh & Ruysschaert, 1983), and initiation of lipid peroxidation (Demant, 1983; Demant & Jensen, 1983). Thus the hope of finding a noncardiotoxic yet active anthracycline antibiotic has prompted the search for new naturally occurring anthracyclines and the development of a large number of semisynthetic analogues. The following structural changes are particularly taken into consideration: (i) derivatization at C_{14} in the side chain, (ii) modification and/or substitution of the amino sugar, and (iii) modification of the substituents in the aglycon moiety.

Chart I



R=H Daunorubicin Dr
R=OH Adriamycin Adr

Thus, varying the number and the position of the hydroxyl groups on the aglycon moiety appears to greatly modify the redox chemistry of these compounds (Ashnagar et al., 1984). This is also true when the anthracycline is modified at the quinone, 5-iminodaunorubicin being thus far the only known one. This modification of the redox properties is an important point since recent investigations of Adr-induced cardiotoxicity have focused on the ability of the drug to be reduced by components of the NADH dehydrogenase system (Doroshow, 1981; Thayer, 1977).

The complexation of anthracycline by metal ions appears to be a route to get new compounds modified simultaneously

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at the quinone, the hydroxyl groups, and even in some cases at the amino sugar moiety. We thus set out to prepare metal anthracycline complexes exhibiting stability constants high enough for the complexes to reach the target without releasing the metal and of course exhibiting cytotoxicity that compares at least with that of the free drug.

The interaction of Adr with Pd(II) ion has been undertaken in this context, and in this paper we report the following experiments: (i) Adr can form two complexes with Pd(II): complex I, which involves 2 mol of Adr per 1 mol of Pd(II) ions and complex II, in which 2 mol of Adr is bound to 2 mol of Pd(II) ions. In both complexes the site of complexation involves the C_{12}-carbonyl oxygen atom and the C_{11}-phenolate group as well as the nitrogen atom of the amino sugar. (ii) The interaction of this complex with DNA has been studied: after 1 week about 20% of the metal was released from the drug. (iii) This complex inhibits P-388 leukemia cell growth in vitro and displays antitumor activity against P-388 leukemia. (iv) This complex unlike free Adr does not catalyze the flow of electrons from reduced nicotinamide adenine dinucleotide (NADH) to O_2 through NADH dehydrogenase.

The same statements are true for daunorubicin, which forms an analogous complex with Pd(II).

Materials and Methods

Purified adriamycin and daunorubicin were kindly provided by Laboratoires Roger Bellon and Rhône Poulenc, respectively. Concentrations were determined by diluting stock solutions to approximately 10 μM and using $\epsilon_{480} = 11\,500\ M^{-1}$ (Chaires et al., 1982). Since anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just prior to use. $K_2[PdCl_4]$, *cis*-$[Pd(NH_3)_2Cl_2]$, and $[Pd(NH_3)_4]Cl_2$ were obtained from Johnson Matthey. Calf thymus DNA, cytochrome *c* (type VI from horse heart), NADH (grade III), cardiac NADH dehydrogenase, L-α-phosphatidylcholine (EPC) from egg yolk type V-E, and cardiolipin (CL) from beef heart were purchased form Sigma Chemical Co., and superoxide dismutase (SOD) was from Miles. All other reagents were of the highest quality available, and deionized bidistilled water was used throughout the experiments. Unless otherwise stated, buffer solutions were 0.05 M Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid].

Absorption spectra were recorded on a Cary 219 spectrophotometer; circular dichroism (CD) spectra on a Jobin Yvon dichrograph Model Mark V. Results are expressed in terms of ϵ (molar absorption coefficient) and $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficients). The values of ϵ and $\Delta\epsilon$ are expressed in terms of [Adr], molar concentrations of adriamycin. Uncorrected fluorescence spectra were recorded at 20 °C on a Jobin Yvon. JY 3C spectrofluorometer equipped with an *xy* recorder and piloted by a microprocessor. All measurements were made in a cell with a 1-cm path length. Potentiometric measurements were obtained with a Metrohm pH meter, Model E 603, at 25 °C, using a Metrohm EA 147 combined glass electrode. Centrifugation was performed with a Beckman Model J2-2I centrifuge.

NADH Dehydrogenase Assay. NADH dehydrogenase activity was determined at 25 °C by modification of a method described previously (Malher, 1955) using cytochrome *c* as the electron acceptor. Adr and complexes I and II were assayed for their NADH–cytochrome *c* reductase activity by following cytochrome *c* reduction at 550 nm. The difference between the extinction coefficients of reduced and oxidized cytochrome *c* was taken to equal 19600. The reaction mixture contained 0.05 M Hepes buffer, pH 7.2, 40 μM cytochrome *c*, 81 μM NADH, 1 unit/L NADH dehydrogenase, and either 0 or *C* μM free or complexed anthracycline (*C* was varied from 0 to 120 μM). The reaction was initiated by addition of the enzyme. Enzymatic activity is expressed in units, such that 1 unit is the amount of enzyme that reduces 1 μM cytochrome *c* per minute at pH 7.2 and 25 °C under the reaction conditions specified above. The production of superoxide anion in the experimental samples was calculated from the rate of cytochrome *c* reduction inhibited by SOD (20 μg/mL).

Binding of Adriamycin and Pd(II)–Adriamycin Complex to Unilamellar Phospholipid Vesicles. Large unilamellar vesicles (LUV) containing egg phosphatidylcholine (EPC) and cardiolipin (CL) in the molar ratio 4:1 at a 12 mM total phospholipid concentration were prepared in pH 7.2 Hepes buffer by the phase-reversion method (Szoka et al., 1980). All the experiments were carried out at 22 °C, and we have checked that the lipids were not peroxidized (Garnier-Suillerot et al., 1984). In these conditions the vesicles were in the liquid-crystalline state. The preparations were diluted in the same buffer to a concentration of 0.25–0.75 mM phospholipid and incubated at 22 °C for 90 min in the presence of about 5 μM adriamycin or Pd(II)–Adr complex. These preparations were then centrifuged at $(4.5 \times 10^4)g$ for 1 h. The concentration of either Adr or Pd(II)–Adr in the supernatant was then determined by spectroscopy absorption.

Tumor Systems. P-388 leukemia provided by the Mason Research Institute (Worcester, MA) is maintained and used for in vivo antitumor testing in accordance with the protocols described by the National Cancer Institute (Geran et al., 1972). Male B6D2FI mice (19–21 g) are used in experiments and are implanted intraperitoneally (ip) with 10^6 P-388 cells on day 0 (5 mice per group). Compounds are prepared in physiological saline and are injected ip on days 1, 2, 3, and 4 (20 mL/kg). The response was measured in median survival time (in days). Results are expressed by $T/C \times 100$ (T = median survival time of treated animals; C = median survival time of control animals). The criterion for significant activity is $T/C \times 100 > 120$.

In Vitro Inhibition of P-388 Leukemia Cell Growth. P-388 cells can be grown in vitro in RPMI 1640 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. Drug effect is expressed by inhibitory dose (ID_{50}), which is obtained by plotting the logarithms of drug concentration against percent inhibition of cell growth and extrapolating the concentration required to inhibit 50% of cell growth.

Results

The addition of $[PdCl_4]^{2-}$ to an aqueous solution of adriamycin gave rise (i) to a shift to higher wavelength of the absorption band in the visible with the appearance of a well-resolved fine structure at 600, 554, and 520 nm, (ii) to modification in the CD spectrum, and (iii) to a drop in the pH, indicating the release of protons. These modifications were rather slow, but after about 5 h no further modification could be detected. In fact, to be sure that the reaction was complete, the spectra and the pH were recorded about 24 h after the addition of $[PdCl_4]^{2-}$ to the solution of adriamycin.

Adriamycin–$[PdCl_4]^{2-}$ System in Water: Spectroscopic and Potentiometric Studies. In order to determine the stoichiometry of the complex, increasing quantities of $[PdCl_4]^{2-}$ were added to an adriamycin solution at molar ratios of Pd(II)

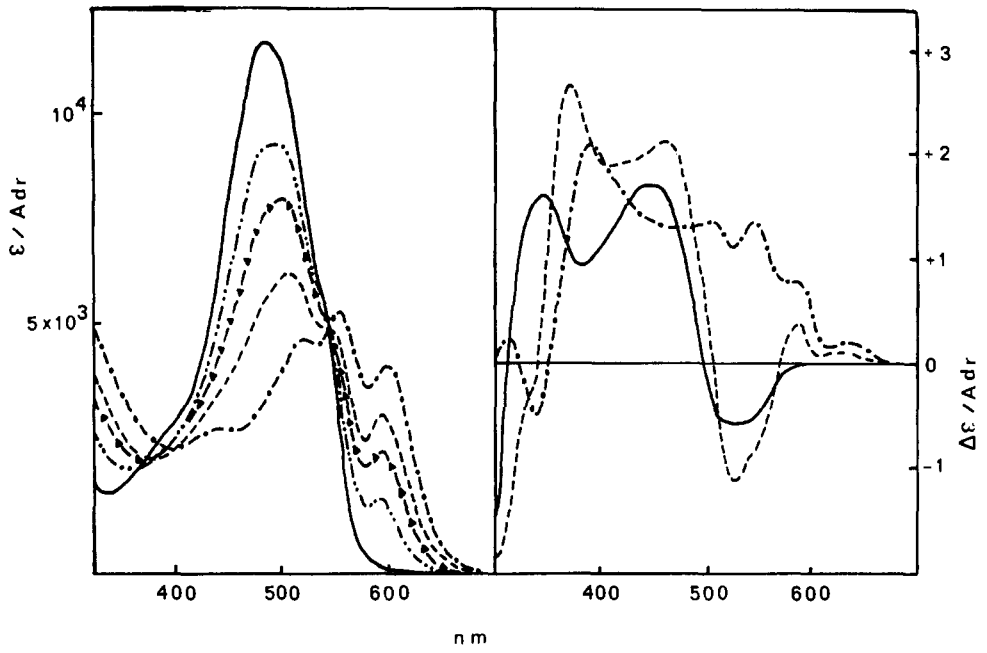

FIGURE 1: Absorption (left) and CD (right) spectra of Adr in the presence of various amounts of $[PdCl_4]^{2-}$. Experimental conditions were as follows: [Adr] = 2×10^{-4} M in aqueous solution; the molar ratio of $[PdCl_4]^{2-}$ to Adr and the pH of the solution were respectively 0, 5.10 (—), 0.2, 4.14 (-···-), 0.4, 3.70 (▲), 0.5, 3.63 (- - -), or 1, 3.46 (-·-). The spectra have been recorded 24 h after the addition of $[PdCl_4]^{2-}$ to Adr.

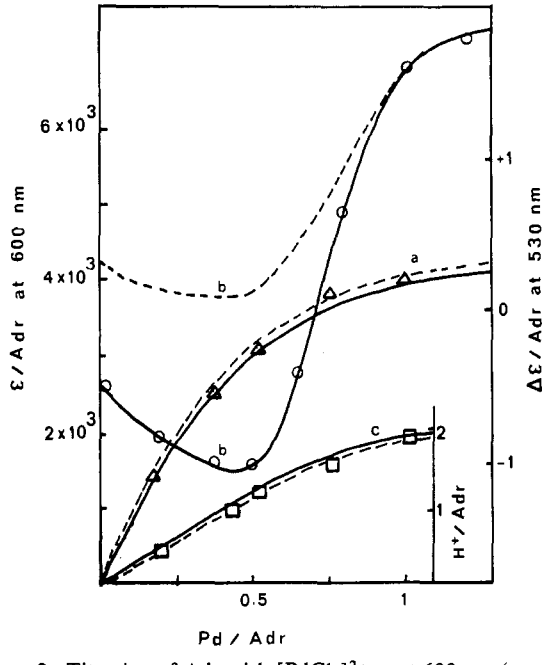

FIGURE 2: Titration of Adr with $[PdCl_4]^{2-}$. ϵ at 600 nm (curves a), $\Delta\epsilon$ at 530 nm (curves b), and the number of protons released per Adr (curves c) have been plotted as a function of the molar ratio of $[PdCl_4]^{2-}$ to Adr. The experimental conditions were the same as in Figure 1. The solvent was H_2O (solid line) or 50% aqueous ethanol (dotted line).

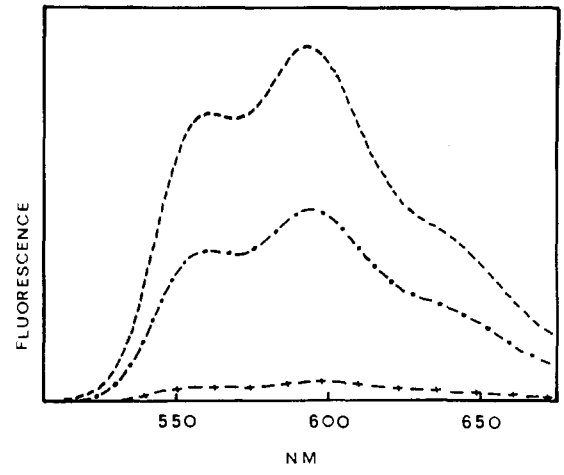

FIGURE 3: Uncorrected fluorescence spectra of Adr in the presence of various amounts of $[PdCl_4]^{2-}$. Experimental conditions were as follows: [Adr] = 5 μM in aqueous solution; the molar ratio of $[PdCl_4]^{2-}$ to Adr was 0 (- - -), 0.25 (-·-), or 0.5 (-+-). The spectra have been recorded 24 h after the addition of $[PdCl_4]^{2-}$ to Adr. Excitation wavelength λ_e = 460 nm.

to Adr varying from 0:1 to 2:1. After 24 h the absorption and CD spectra (Figure 1) and the pH were recorded. Figure 2 shows plots of ϵ at 600 nm, of $\Delta\epsilon$ at 535 nm, and of the number of protons released per Adr as a function of the molar ratio of Pd(II) to Adr.

As can be seen, ϵ at 600 nm and the number of protons released per Adr level off at molar ratios of Pd to Adr higher than 1. This strongly suggests that a 1:1 Pd–Adr complex (II) is formed with the release of two protons per Adr. However, it should be noticed that the plot of $\Delta\epsilon$ at 535 nm does not follow the same trend: this coefficient reaches an extremum at a molar ratio of Pd(II) to Adr equal to 0.5. As is shown in Figure 2, at this molar ratio one proton has been released per Adr, and the value of ϵ at 600 nm is about half that obtained at a 1 to 1 molar ratio. So, most probably a 1:2 Pd–Adr complex (hereafter labeled I) is also formed. The CD spectrum of this complex resembles that of Adr at high concentration, i.e., when Adr is in a dimeric form with a CD signal of the couplet type (Martin, 1980; Henry et al., 1985).

The fluorescence spectra of Adr in the presence of $[PdCl_4]^{2-}$ were recorded under similar conditions. The fluorescence spectrum of Adr is well-known: with an excitation at 460 nm, the emission spectrum exhibits a maximum at 595 nm and shoulders at 640 and 560 nm. Uncorrected fluorescence spectra of Adr at various molar ratios of Adr to $[PdCl_4]^{2-}$ are shown in Figure 3. As can be seen, quenching of the fluorescence at 595 nm is reduced to about 5% of the initial value at a 0.5 to 1 molar ratio of $[PdCl_4]^{2-}$ to Adr. No

fluorescence is observed with an excitation line at 600 nm.

Adriamycin-$[PdCl_4]^{2-}$ System in 50% Aqueous Ethanol Solution. For the free drug the association depends on the dielectric constant of the solvent. Martin (1980) has shown that 300 μM daunorubicin, which is in the dimeric form in aqueous solution, converted to the monomeric form in 50% aqueous methanol solution. In order to determine whether the structure of the two complexes depends on the dielectric constant, we performed experiments similar to those reported above in 50% aqueous ethanol solution. ϵ at 600 nm, $\Delta\epsilon$ at 530 nm, and the number of protons released as a function of the molar ratio of $[PdCl_4]^{2-}$ to Adr are shown in Figure 2: ϵ at 600 nm and the number of protons released follow the same trend as in pure water while $\Delta\epsilon$ at 530 nm does not exhibit its two-step behavior. Thus the CD pattern of complex I depends on the dielectric constant of the solvent while that of complex II does not.

Adriamycin-$[PdCl_4]^{2-}$ in Water as a Function of Time. The evolution of the absorption and CD spectra and of the pH of a solution containing $[PdCl_4]^{2-}$ and Adr in a 1:1 molar ratio was followed as a function of time. This evolution occurs in two steps: the first step is characterized by (i) the appearance of the absorption at 600 nm and the presence of isosbestic points at 544 and 372 nm, (ii) the increase of the amplitude of the negative band at 532 nm in the CD spectrum, and (iii) the release of one proton per adriamycin. With a 2×10^{-4} M solution this first step is reached after about 30 min. In fact, the spectral pattern thus obtained and the pH value are similar to those obtained in a 1:2 molar ratio of $[PdCl_4]^{2-}$ to Adr (after 5 h). The second step is characterized by (i) a further increase of the absorption band at 600 nm with the presence of isosbestic points at 528 and 412 nm, (ii) the decrease of the amplitude of the CD band at 530 nm, and (iii) the release of a second proton per Adr. It thus appears that the formation of complex II occurs via complex I.

The following equilibria may thus already be suggested:

$$\tfrac{1}{2}Pd^{2+} + AdH_3^+ \rightleftharpoons \underset{I}{\tfrac{1}{2}[Pd(AdH_2)_2]^{2+}} + H^+$$

$$\underset{I}{\tfrac{1}{2}[Pd(AdH_2)_2]^{2+}} + \tfrac{1}{2}Pd^{2+} \rightleftharpoons \underset{II}{[Pd\text{-}AdH]^+} + H^+$$

where AdH_3^+, AdH_2, and AdH^- stand for adriamycin fully protonated, once deprotonated, and twice deprotonated, respectively.

Determination of the pK of Formation of the Complexes. Due to the rather slow kinetics of formation of these complexes it was impossible to perform the usual potentiometric titrations. So, we operated in the following way. Solutions of adriamycin at pHs ranging from 2 to 7 were prepared, and $[PdCl_4]^{2-}$ was then added at a 1:1 molar ratio of Pd(II) to Adr. The solutions were allowed to stand for 1 day, and then absorption and CD spectra and pH were recorded. Figure 4 shows the plot of ϵ at 600 nm as a function of pH. A pK of formation equal to 2.3 is thus determined for complex II.

The same statements are true for daunorubicin, which forms analogous complexes with Pd(II).

Interaction of Adriamycin with cis-$[Pd(NH_3)_2Cl_2]$ and with $[Pd(NH_3)_4]^{2+}$. Experiments similar to those described above were performed with either *cis*-$[Pd(NH_3)Cl_2]$ or $[Pd(NH_3)_4]^{2+}$ as starting material. Similar results are obtained, i.e., formation of two complexes, one involving one Pd(II) ion per two molecules of Adr and the other involving one Pd(II) ion per molecule of Adr. There are some slight differences between these systems in their kinetics of formation. On the other hand,

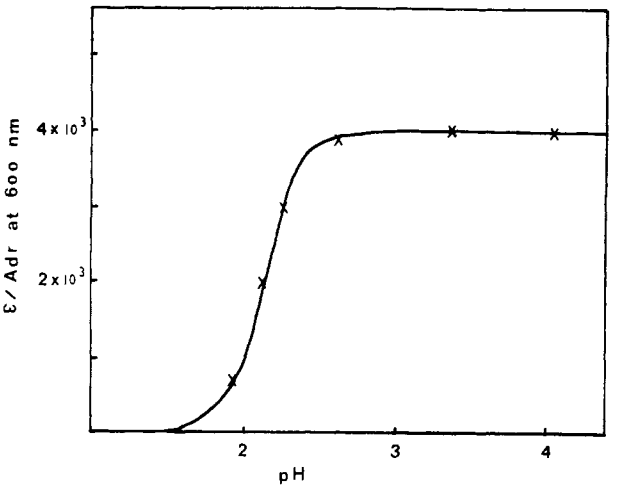

FIGURE 4: Spectroscopic titrations of the 1:1 $[PdCl_4]^{2-}$–Adr system. ϵ at 600 nm has been plotted as a function of pH. Experimental conditions were as follows: [Adr] = 2×10^{-4} M; [KCl] = 0.02 M. Solutions of adriamycin at pHs ranging from 2 to 7 were prepared, $[PdCl_4]^{2-}$ was then added, and the solutions were left for 1 day. Absorption spectra and pH were then recorded.

in these two systems we did not observe the release of protons. 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 formed is *cis*-$[Pd(NH_3)_2Cl(OH)]^+$ (Martin, 1983) and (ii) the pK of deprotonation of NH_4^+ is about 10 (Sillen & Martell, 1964). When complex II is formed, three original ligands at least are removed from *cis*-$[Pd(NH_3)_2Cl(OH)]^+$, the most likely being Cl^-, OH^-, and NH_3. In fact, OH^- cannot be released as OH^- but as H_2O (Martin, 1983), and NH_3 immediately froms NH_4^+. Thus the two protons that are released by Adr are immediately bound to the leaving groups. This explains why a decrease in pH is not observed.

Pd(II)–Adr (1:1)–DNA Interactions. Since DNA has been postulated as one of the sites of action of anthracyclines in vivo, we have undertaken to examine the interaction of complex II with DNA. The experiments were carried out under conditions for total binding of the free drug to DNA, i.e., at a nucleotide to drug molar ratio higher than 7 (Fritzsche et al., 1982). The interaction has been monitored by using the ϵ at 600 nm, which is highly characteristic of the presence of the complex. The solution contained 2×10^{-5} M complex II and DNA at a molar ratio of nucleotides to Adr equal to 10. A very slow decrease in the absorption is observed: after 5 days a decrease of 25% is observed. This can be taken as an indication that some release of Pd(II) from Adr has occurred.

Effects of the Two Complexes on Superoxide Production by NADH. It is well documented that a component of mitochondrial NADH dehydrogeanse actively reduces Adr to semiquinone, initiating a free-radical cascade (Davies et al., 1983; Doroshow, 1983). We have recently shown that, once complexed to Fe(III) forming $Fe(HAd)_3$ complex, Adr lost its ability to be reduced by NADH dehydrogenase, and no superoxide production is detected (Beraldo et al., 1985). It was thus interesting to check whether the same phenomenon occurred when Adr was complexed to Pd(II). We compared the effect of the Adr–palladium complexes on superoxide formation by mitochondrial NADH dehydrogenase with that of the free drug.

As is shown on Figure 5, Adr increased superoxide formation by NADH dehydrogenase in a dose-dependent fashion that follows saturation kinetics. Complex I also increased the superoxide formation to about 50% of the quantity induced

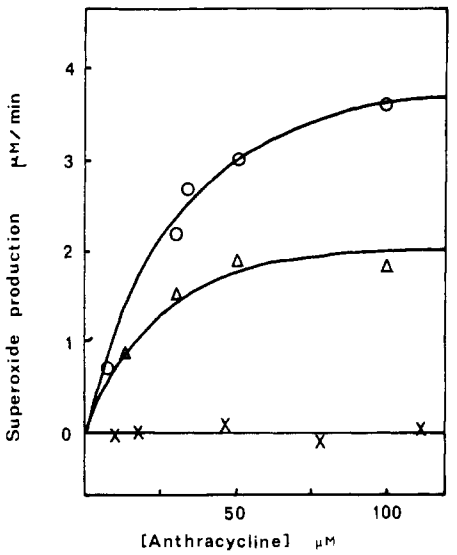

FIGURE 5: Effect of drug concentration on superoxide formation by NADH dehydrogenase. Superoxide formation was determined spectrophotometrically by the rate of SOD-inhibitable cytochrome *c* reduction as described under Materials and Methods. The reaction mixture contained Hepes buffer (0.05 M), pH 7.2, cytochrome *c* (40 μM), NADH dehydrogenase (1.0 unit/L), SOD (0 or 20 μg/mL), NADH (81 μM), and the indicated amount of adriamycin (○), $Pd(H_2Ad\text{-}NH_2)(HAd\text{-}NH_3^+)$, complex I (△), or $(Pd\text{–}HAd\text{-}NH_2)_2$, complex II (×).

by the free drug. To the contrary, complex II does not increase superoxide formation over control levels.

Binding of Complex II to Cardiolipin-Containing LUV. The binding of complex II to CL-containing LUV was compared with that of free Adr by determining the concentration of free or complexed drug that remained in the supernatant after centrifugation, as described under Materials and Methods. The pK_a value of the two phosphate groups of cardiolipin is 4. Thus, at pH 7.4 cardiolipin carries two negative charges. The experiments were performed at three values of the molar ratios of CL to Adr: 8, 16, and 24. These results obtained were independent of this molar ratio. Under the conditions used, we measured that the presence of Pd(II) helped the binding of the drug to the LUV: 75% of the free drug remained in the supernatant whereas only 10% of complex II could be detected. After the centrifugation the concentration of phospholipids that may remained in the supernatant was determined by the method of Steward (1980). In the conditions used, about 30% of phospholipids remained in the supernatant when the experiment was performed in the absence of any drug. The percentage remained about the same when the same experiment is performed in the presence of free drug and decreased to 20% in the presence of complex II. This strongly suggests that neither Adr nor the complex remove cardiolipin from the liposomal membranes.

Antitumor Activity. The in vitro inhibition of P-388 leukemia cell growth by complex II was compared with that induced by the free drug. ID_{50} equal to 0.018 μg/mL was found for both compounds. Furthermore, they both display antitumor activity with $T/C \times 100 \simeq 160$ for a dose of 5 mg/kg. No significant differences, in terms of therapeutic efficacy and general toxicity, were observed between the complex and the free drug.

Discussion

Although the mechanism of adriamycin and daunorubicin cardiac toxicity remains incompletely understood, recent studies have suggested that the cytotoxic effects of these agents may be related to the formation of semiquinone free-radical intermediates in vivo (Sato et al., 1977) and/or to the association of the drug with cardiolipin [for a review, see Goormaghtigh and Ruysshaert (1984)]. There has thus been a continuing interest in the improvement of the chemotherapeutic index of adriamycin and daunorubicin via analogue synthesis.

It has recently appeared that the complexation of anthracyclines by metal ions could be a route to get new compounds modified simultaneously at the quinone, the hydroxyl groups, and even, in some cases, at the amino sugar moiety.

The first reported observation of metal binding to anthracyclines was made by Calendi et al. (1965), who found that large spectral changes occurred in the visible spectrum of daunorubicin upon the addition of an excess of Al(III), Fe(II), Fe(III), and Mg(II) in aqueous solution. Two systematic studies of the interaction of Adr and Dr with Cu(II) were made by Greenaway and Dabrowiak (1982) and Beraldo et al. (1983), respectively. Both teams have reported the formation of two types of complexes: one involving two molecules of drug per Cu(II) ion and the other, one molecule of drug per Cu(II) ion. These complexes exhibit very high stability constants; however, Cu(II) ions are immediately picked up by albumin once the complexes are injected into the plasma (Beraldo, 1984).

Recently, several studies have been devoted to the complexation of anthracyclines with Fe(III). Gosalvez et al. (1978) were the first to propose that the addition of Fe(III) to Adr could yield a less cardiotoxic compound; thus, they prepared a compound named quelamycin by addition of three Fe(III) to one molecule of drug. This compound has been used clinically. However, it has recently been shown that, at physiological pH, one Adr molecule could not accommodate three Fe(III) ions and that in fact the complex formed involved three molecules of drug per Fe(III) ion (Myers et al., 1982; Beraldo et al., 1985). This complex exhibits a very high stability constant, and Fe(III) is not removed from the complex by plasma components; it displays antitumor activity against P-388 leukemia, and the complexation of Adr to Fe(III) prevents the reduction of Adr by a component of mitochondrial NADH dehydrogenase (Beraldo et al., 1985). In this complex Fe(III) is chelated to three molecules of Adr through one carbonyl oxygen and one phenolate oxygen (Beraldo et al., 1985). Moreover, Muindi et al. (1984) have shown that the removal of the C_{11}-hydroxy group of adriamycin is associated with complete loss of iron binding to the drug chromophore. It has thus been inferred that the complexation of Fe(III) to Adr occurs through the carbonyl oxygen on C_{12} and the phenolate oxygen on C_{11}. Analogous data have been obtained with the carminomycin–Fe(III) system (Fiallo & Garnier-Suillerot, 1985). It thus appears that the complexation of Fe(III) at the quinone moiety gives rise to a modification of the redox properties. The complexed drug cannot be further reduced by NADH dehydrogenase. This compares with 5-iminodaunorubicin, which is modified at the quinone moiety and cannot be reduced by NADH dehydrogenase (Davies et al., 1983).

The complexation of anthracycline by metal ions can thus be a promising route to get therapeutically interesting new derivatives provided that the complexes exhibit high stability constants. In fact, the problem is that the complex has to reach the target without releasing the metal ion.

The data reported in that paper appear to establish that Pd(II) forms two complexes, I and II, with Adr. Complex I is formed through complexation of two molecules of Adr to

one Pd(II) ion and the concomitant release of one proton per Adr. The formation of complex II occurs at a molar ratio of Pd(II) to Adr equal to 1 and with the release of two protons per Adr. In both complexes these protons may have been released by (i) the hydroxyl group on C_6, (ii) the hydroxyl group on C_{11}, or (iii) the ammonium group of the sugar moiety. In order to determine whether both hydroxyl groups have the ability to bind Pd(II) ion, we performed some experiments with an anthracycline devoid of a C_{11}-hydroxyl group: aclacinomycin (Oki, 1980). It would have been, in fact, more valuable to examine the behavior of 11-deoxyadriamycin with Pd(II); unfortunately, this compound was not available to us. We thus estimated that aclacinomycin most probably exhibits the same behavior toward complexation with metal ions especially after we had checked that, like 11-deoxyadriamycin, aclacinomycin is unable to complex Fe(III) (Fiallo and Garnier-Suillerot, unpublished data). Similarly, the addition of $[PdCl_4]^{2-}$ to aclacinomycin did not give rise to the formation of complex. This strongly suggests that the complexation of Pd(II) to Adr occurs at the C_{12}-carbonyl oxygen and the C_{11}-phenolate oxygen and/or at the amino group of sugar.

Concerning the structure of complex I, there are three possibilities, all of them giving rise to the release of one proton per Adr: (i) Pd(II) ion is bound to the amino group of the sugar of each molecule, (ii) Pd(II) ion is bound at the C_{12}-carbonyl oxygen and C_{11}-phenolate oxygen of each molecule, or (iii) Pd(II) ion is bound at the C_{12}-carbonyl oxygen and C_{11}-phenolate oxygen of one molecule and to the amino sugar of the other molecule. The first possibility can be ruled out by the observation that complexation gives rise to a shift of the absorption band to higher wavelengths which is assigned to deprotonation of the hydroxyl group. The second possibility can also be ruled out by the observation that the addition of a second Pd(II) ion, yielding complex II, gives rise to an increase in the absorption at 600 nm, suggesting that complex I still has some possibility of deprotonation of one hydroxyl group through complexation. Moreover, the observation that complex I is still able to catalyze the transfer of electrons from NADH to molecular oxygen through NADH dehydrogenase gives evidence that one of the two anthraquinones does not have its redox properties modified by complexation. We thus infer that, in complex I, Pd(II) ion is bound to one molecule of Adr through the C_{12}-carbonyl oxygen and the C_{11}-phenolate oxygen, thus forming a six-membered chelate ring, and to the other molecule of drug through the amino nitrogen of the sugar.

It is obvious that such a molecule may adopt different conformations depending on the solvent properties; the two molecules of drug can be either stacked or extended. For the free drug the association process depends on the dielectric constant of the solvent. Martin (1980) has shown that 300 μM daunorubicin, which is in the dimeric form in aqueous solution, converted to the monomeric form in 50% aqueous alcohol solution. This can be monitored by CD spectroscopy: when Adr (or Dr) is in the dimeric form, the CD signal is of the couplet type with a positive band at 445 nm and a negative one at 530 nm; when the drug is in the monomeric form, only one positive band at 480 nm is present in the CD spectrum.

The CD spectrum of complex I exhibits in water a negative band at 530 nm, indicating a stacking of the two molecules of drug. This band disappears by addition of 50% ethanol to the aqueous solution, indicating a change in the conformation of the molecule.

Concerning now the structure of complex II, here again there are three possibilities: (i) Pd(II) ion is bound to the

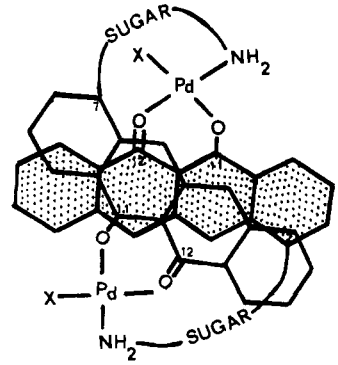

FIGURE 6: Schematic drawing of the $(Pd–HAd-NH_2)_2$ complex.

C_{12}-carbonyl oxygen, the C_{11}-phenolate oxygen, and the amino group of the same molecule, forming a $Pd–HAd-NH_2$ complex, where $HAd-NH_2$ stands for adriamycin deprotonated at the C_{11}-hydroxyl group and at the amino sugar, or (ii and iii) Pd(II) ion can be bound to the oxygen atoms at C_{11} and C_{12} of one molecule and to the amino group of another one molecule, forming either (ii) a dimer $(Pd–HAd-NH_2)_2$ or (iii) a polymer $(Pd–HAd-NH_2)_n$. The first hypothesis can be ruled out by the observation that due to steric hindrance it is impossible to bind Pd(II) to these ligands when they are on the same molecule. Of the two other possibilities, it is easy to see that the dimeric structure is rigid, i.e., the two Pd(II) ions maintain the two molecules of drug in fixed position with regard to each other. One may expect that such a structure will not be solvent-dependent. At the opposite, the polymeric complex may, depending on solvent, adopt several conformations: (i) a pleated sheet conformation with the molecules of drug stacked or (ii) an extended conformation. The observation that the spectral patterns of complex II are the same if the complex is dissolved either in water or in 50% aqueous ethanol solution strongly suggests that the dimeric structure is the most likely (Figure 6).

Having identified the ligands and the structure of complex II, it is now possible to calculate its stability constant. The formation constants β and β^* are defined by the equilibria:

$$Pd^{2+} + HAd-NH_2 \rightleftarrows {}^1/_2(Pd–HAd-NH_2)_2$$

$$\beta = \frac{[(Pd–HAd-NH_2)_2]^{1/2}}{[Pd^{2+}][HAd-NH_2]}$$

and

$$[PdCl_4]^{2-} + H_2Ad-NH_3^+ \rightleftarrows {}^1/_2(Pd–HAd-NH_2)_2 + 4Cl^- + 2H^+$$

$$\beta^* = \frac{[(Pd–HAd-NH_2)_2]^{1/2}[Cl^-]^4[H^+]^2}{[PdCl_4^{2-}][H_2Ad-NH_3^+]}$$

Taking into account the first phenolic deprotonation and the amino sugar deprotonation (Kiraly & Martin, 1982)

$$H_2Ad-NH_3^+ \rightleftarrows HAd-NH_2^- + 2H^+$$

$$\frac{[H^+]^2[HAd-NH_2^-]}{[H_2Ad-NH_3^+]} = k_1k_2 = 2.4 \times 10^{-19}$$

and the fact that palladium is added as $[PdCl_4]^{2-}$ from a starting solution containing 2.5×10^{-2} M $[PdCl_4]^{2-}$ and 0.2 M KCl (Sillen & Martell, 1964)

$$Pd^{2+} + 4Cl^- \rightleftarrows [PdCl_4]^{2-}$$

$$K = \frac{[PdCl_4^{2-}]}{[Pd^{2+}][Cl^-]^4} = 1.65 \times 10^{13}$$

it follows that

$$\beta = \beta^*(K/k_1k_2)$$

On the other hand, owing to the low value of the deprotonation constants k_1 and k_2 of adriamycin one can state that, below pH 7, $[Adr]_t = [H_2Ad\text{-}NH_3^+] + 2[(Pd\text{–}HAd\text{-}NH_2)_2]$. The stability constant thus obtained is

$$\beta = (1.3 \pm 0.5) \times 10^{22}$$

Thus the four ligands involved in the coordination square of Pd(II) are two oxygens and one amine nitrogen from Adr, the fourth ligand being either Cl^- or H_2O if the starting material is $[PdCl_4]^{2-}$.

One of the important points that emerges from this study is that complex II exhibits a very high stability constant. Owing to this high stability constant and despite (i) the affinity of Adr for DNA and (ii) the ability of Pd(II) to complex to nitrogen of DNA (Martin, 1985), incubation of DNA with complex II gives rise to only very weak dissociation of the complex. Nevertheless, the complex displays antitumor activity in vivo against P-388 leukemia that compares with that of the free drug.

Concerning the points that may be connected with cardiac toxicity, our data show that complex II unlike free Adr does not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. This result could appear as an improvement of the therapeutic index of Adr through complexation with Pd(II). On the other hand, the association of the drug with CL appears to be enhanced by the presence of Pd(II). This result can appear as unfavorable. Nevertheless, the question of whether there is a direct correlation between the affinity of anthracycline drugs for CL and cardiotoxicity is still a matter of debate. Recent experiments (Kolodziejezyk and Garnier-Suillerot, unpublished data) have shown that a compound such as mitoxantrone, which seems to be less cardiotoxic than Adr, exhibits higher affinity for CL-containing LUV than Adr.

In conclusion, we can say that the complexation of anthracyclines by metal ions appears to be a route to get new compounds modified simultaneously at the quinone, at the hydroxyl groups, and even in the case of Pd(II) at the amino sugar moiety. This yields modifications of the redox properties and of the biological properties of these potent antitumor antibiotics.

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