°C. Anal. ($C_{27}H_{25}F_3N_4O_9S$) C, H, N, S.

Dibromodiphenyl Thioether (60b). The dinitro compound **59** (4.00 g, 0.0063 mol) was hydrogenated in glacial acetic acid (20 mL) over 10% palladium on charcoal (0.40 g) with a Parr apparatus. The mixture was filtered, and the solution was added to a well-stirred solution of sodium nitrite (1.31 g, 0.019 mol) in concentrated sulfuric acid (50 mL) and acetic acid, under N_2, with the temperature being kept at or below −15 °C. After 5 min, the mixture was added to a vigorously stirred mixture of cuprous bromide (14.35 g) and urea (1.5 g) in 47% aqueous hydrobromic acid (70 mL) and chloroform (70 mL). After 2 h, the organic layer was removed and washed successively with water, saturated $NaHCO_3$, 2 N NaOH, water, and then brine. The dried solution was evaporated, and the residue was purified by exhaustive chromatography on silica gel, eluting with EtOAc–petroleum ether, to give the thioether **60b** (0.75 g, 17%): mp 111–113 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (3 H, t, CH_2CH_3), 3.13 and 3.21 (2 H, m, CH_2CH), 3.81 (3 H, s, OMe), 3.82 (2 H, s, CH_2), 3.94 (3 H, s, OMe), 4.30 (2 H, q, CH_2CH_3), 4.80 (1 H, m, CH_2CH), 6.66 (1 H, d, Ar-5′H), 6.77 (1 H, d, Py-5H), 6.96 (1 H, d, Ar-2′H), 7.10 (1 H, d of d, Ar-6′H), 7.19 (1 H, d, NHCO), 7.38 (1 H, d of d, Py-4H), 7.42 (2 H, s, Ar H), and 7.98 (1 H, s, Py-2H). Anal. ($C_{27}H_{25}Br_2F_3N_2O_5S$) C, H, N, Br, S.

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Registry No. 1, 72469-00-2; **2**, 105170-31-8; **3**, 117653-10-8; **4**, 117653-11-9; **5**, 117653-12-0; **6**, 117653-13-1; **7**, 117653-14-2; **8**, 117653-15-3; **9**, 117653-16-4; **10**, 117653-17-5; 11, 117653-18-6; **12**, 117653-19-7; **13**, 117653-20-0; **14**, 105170-26-1; **15**, 105189-53-5; **16**, 117653-21-1; **17**, 117653-22-2; **18**, 117653-23-3; **19**, 105170-16-9; **20**, 117653-24-4; **21**, 105189-37-5; **22**, 105189-56-8; **23**, 105189-72-8; **24**, 105189-60-4; **25**, 105189-67-1; **26**, 105189-91-1; **27**, 105189-93-3; **28**, 105189-96-6; **29**, 105189-99-9; **30**, 117653-25-5; **31**, 117653-26-6; **32**, 105211-23-2; **33**, 105170-24-9; **34**, 35431-26-6; **36**, 105189-48-8; **37**, 117653-28-8; **37** (R′ = CH_2OH), 117653-27-7; **38**, 117653-29-9; **39**, 117653-30-2; **40e**, 117653-31-3; **40e** (acetate), 117653-33-5; **48**, 105189-38-6; **48** (acetate), 105189-39-7; **49**, 105189-40-0; **50**, 105189-42-2; **51b**, 105189-46-6; **52**, 2233-18-3; **53**, 105189-45-5; **54**, 105211-19-6; **55**, 105211-20-9; **56**, 105189-74-0; **56** (α,β-unsaturated), 105189-73-1; **57**, 105189-61-5; **58**, 105189-62-6; **58** (disulfide), 105189-63-7; **59**, 105189-64-8; **60b**, 105189-68-2; 2-$MeOC_6H_4CHO$, 135-02-4; $I(OCOCF_3)_3$, 14353-86-7; CF_3CO-(3,5-Br_2)Tyr-OMe, 105189-44-4; Ac-Gly-OH, 543-24-8; $Pb(SCN)_2$, 592-87-0; CF_3CO-[3,5-$(NO_2)_2$]Tyr-OEt, 105189-50-2; 4-bromo-2-chloroanisole, 50638-47-6; 5-bromo-2-methoxypyridine, 13472-85-0; (5-hydroxy-2-methoxyphenyl)(3-chloro-4-methoxyphenyl)methanol, 117653-32-4; (5-acetoxy-2-methoxyphenyl)(3-chloro-4-methoxyphenyl)methyl acetate, 117653-34-6.

Bifunctional Antitumor Compounds: Interaction of Adriamycin with Metallocene Dichlorides

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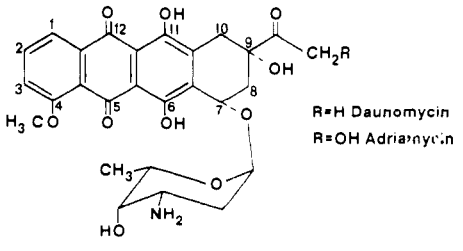
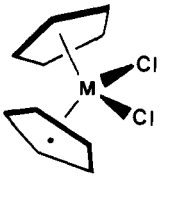
In order to synthesize bifunctional antitumor compounds, the interactions of adriamycin with metallocene dichlorides, Cp_2MCl_2, where M = Zr, Ti, V, have been studied. Using absorption, fluorescence, and circular dichroism measurements, we have shown that adriamycin is able to coordinate to the three metal ions. The interaction of Cp_2ZrCl_2 and Cp_2VCl_2 with adriamycin leads to compounds of 1:2 metal:drug stoichiometry, whereas the interaction of Cp_2TiCl_2 with adriamycin leads to two types of compounds of 1:2 and 1:1 stoichiometry. The Zr–adriamycin complex, which is unable to dissociate, even at a pH lower than 1, does not display antitumor activity against P-388 leukemia. However Ti–adriamycin complexes, which are more susceptible to dissociation in acidic media, exhibit antitumor activity that compares with that of the free drug. These complexes, unlike adriamycin, do not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. In addition, the presence of metal ions promote the binding of the drug to DNA and erythrocyte ghosts.

The anthracycline antibiotic adriamycin (Adr) is an important antitumor agent with marked activity against a wide variety of human neoplasms. Unfortunately, an-



thracyclines exhibit secondary toxic effects, the most serious being cardiotoxicity. A great deal of research effort has been directed toward finding new anthracyclines that might retain the excellent broad-spectrum activity of adriamycin while eliminating its cardiotoxicity.[1-3]

On the other hand, over the past 7 years, Köpf and Köpf-Maier[4] have shown that metallocene dihalides of the constitution Cp_2MX_2, where Cp = η^5-C_5H_5; M = Ti, V, Nb,



Metallocene dichlorides

Mo; X = F, Cl, Br, I, NCS, and N_3, are highly active against Ehrlich ascites tumor cells, lymphoid leukemia L1210, and lymphocytic leukemia P-388. These compounds thus

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constitute a potent new class of organometallic antitumor agents.

On the basis of the observation that the well-known antitumor agent *cis*-dichlorodiammineplatinum(II) and Cp_2MX_2 possess similar *cis*-MX_2 functionalities, it has been postulated that the carcinostatic activity of both type of agents may be similar.[5]

However, a recent study of the fate of the Cp_2TiCl_2, Cp_2VCl_2, and Cp_2ZrCl_2 agents in aqueous solution has shown that, at physiological pH, only Cp_2VCl_2 is appreciably stable to M-C_5H_5 protonolysis;[6] Cp_2VCl_2 is thus the only member of the series for which cyclopentadienyl ligation is likely to remain intact and the predominant species present in plasma is believed to be $Cp_2V(OH)_2$.

Recent reports have shown that the complexation of anthracyclines by metal ions leads to new semisynthetic compounds and that the complexation may thus be considered as a route to new derivatives that may be less cardiotoxic.[7,8] The binding sites available for the complexation of metal ions are formed by the carbonyl oxygen at C_{12} and the phenolate oxygen at C_{11} (site 1), the carbonyl oxygen at C_5 and the phenolate oxygen at C_6 (site 2), and the α-amino sugar (site 3). It has been observed that Fe(III),[9-11] Pd(II),[12] and Yb(III)[13] can be accommodated in the C_{11}–C_{12} binding site only, whereas Cu(II) can be accommodated at C_5–C_6 oxygen atoms and/or C_{11}–C_{12} oxygen atoms.[14-16] In addition, Pd(II) and Pt(II) can be linked to Adr via the α-amino group of the sugar.[12,17]

The complexation of anthracyclines by metal ions prevent their reduction by enzymatic systems and thus the formation of reactive oxygenated species that have been largely postulated as the cardiotoxic agents.[18,19]

All these reports prompted us to study the interaction of adriamycin with Cp_2MCl_2, where M is Zr, Ti, and V. Our aim was twofold: (i) the preparation of bifunctional compounds and (ii) the obtention of less cardiotoxic adriamycin derivatives. This study was thus undertaken with the two following hypothesis. The first hypothesis was that through release of the Cl^- anions the Cp_2M entities would be able to coordinate to the oxygen atoms either at C_5–C_6 or at C_{11}–C_{12}; such a complexation would prevent the reduction of adriamycin by enzymatic systems. The second hypothesis was that, inside the cell, the complex would dissociate, liberating two active antitumor species: Adr and Cp_2M entities.

In this paper we report that Adr is able to coordinate to the three metal ions. The interaction of Cp_2ZrCl_2 and Cp_2VCl_2 with Adr leads to compounds of 1:2 M:Adr stoichiometry, whereas the addition of Cp_2TiCl_2 to Adr leads to two types of compounds of 1:2 and 1:1 stoichiometry. As it was predicted, these compounds cannot be reduced by NADH dehydrogenase. Concerning the bifunctionality of these complexes, it appears that the complex formed between Adr and Cp_2ZrCl_2 is completely inactive, and this can be related to the observation that the dissociation of this complex is not observed even at a pH value as low as 1. However, the species formed between Cp_2TiCl_2 and Adr, which are more susceptible to dissociation in acidic media, exhibit antitumor activity that compares with that of the free drug. These data strongly suggest that our second hypothesis is correct.

Materials and Methods

Purified adriamycin was kindly provided by Laboratoire Roger Bellon. Concentrations were determined by diluting stock solutions to approximately 10 μM and using ϵ_{480} = 11500 M^{-1}.[20] Since anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just prior to use. Cp_2TiCl_2 was obtained from K&K Laboratories and used as received. Cp_2VCl_2 was obtained from Strem Laboratories and recrystallized twice from chloroform according to Wilkinson and Birmingham.[21] Synthesis of Cp_2ZrCl_2 was carried out by published procedure.[22] Calf thymus DNA, cytochrome *c* (type VI from horse heart), NADH (grade III), cardiac NADH dehydrogenase, and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. All other reagents were of the highest quality available, and deionized double-distilled water was used throughout these 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 and circular dichroism (CD) spectra on a Jobin Yvon dichrograph Model Mark V. Results are expressed in terms of ϵ (molar absorption coefficient) and $\Delta\epsilon = \epsilon_L - \epsilon_L$ (molar CD coefficient). 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 JY3C spectrofluorimeter. Potentiometric measurements were obtained with a Metrohm pH meter, Model E603, at 25 °C, using a Metrohm EA 147 combined glass electrode. Centrifugations were performed with a Beckman Model J2-21 centrifuge.

Erythrocyte Ghosts. Erythrocytes (RBC), drawn from human healthy donors of the Seine Saint Denis blood bank and collected on adenosine citrate dextrose, were washed three times via 1000*g* centrifugation in phosphate buffer saline, and the buffy coat was discarded after each centrifugation. Packed erythrocytes were hemolyzed in a 9-fold volume of 1 mM Tris–0.1 mM HCl buffer (pH 7.4). Membranes were sedimented by centrifugation at 20000*g* for 20 min and then washed four times by centrifugation at 20000*g* for 20 min in a 9-fold volume to Tris buffer. White membranes were obtained as described by Hamaguchi and Cleve.[23] Protein concentration was determined according to the Lowry procedure;[24] 10^{10} cells yielded a mean of 4.6 ± 0.6 mg of protein (10 experiments).

NADH Dehydrogenase Assay. NADH dehydrogenase activity was determined at 25 °C by modification of a method

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described previously[25] using cytochrome c as the electron acceptor. Adr and its complexes 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, 25 units/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).

DNA Samples. DNA samples were prepared by dissolving DNA in 0.15 M HEPES with 4–5 h of stirring at room temperature. The concentrations of the solutions were determined spectrophotometrically at 260 nm (ϵ = 6600 cm^{-1} per nucleotide). Aliquots of 1 mM DNA solution were added to 200 μM drug solution (complexed or free). After each addition a precipitate appeared at once that was removed by centrifugation at 900 rpm for 10 min. The amount of drug or metal–drug complex remaining in the supernatant was determined spectroscopically.

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/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

Interaction of Adriamycin with Cp_2ZrCl_2, Cp_2TiCl_2, and Cp_2VCl_2. (A) Cp_2ZrCl_2. Cp_2ZrCl_2 solutions were prepared by dissolving a weighed amount of the compound in water. A decrease of the pH was observed which can account for the release of about one proton/molecule of Cp_2ZrCl_2. According to Toney and Marks[6] ring protonolysis is fast in unbuffered solutions. In order to prevent formation of hydroxide, the mother solution was prepared at pH near 4. Adr was dissolved in HEPES buffer solution, pH 7.2. The two solutions were mixed in such a way that the molar ratio of Zr(IV) to Adr was varied from 0 to 2. The slow formation of a complex was monitored by absorption and CD spectroscopy. In a typical experiment, 300 μM Adr solution in the presence of zirconocene dichloride was left at room temperature. After about 5 days, the formation of the complex was complete as indicated by the absence of further modification of the spectrum.

The formation of the complex was attested by a shift of the absorption band of Adr to a higher wavelength and the appearance of a CD spectrum very different from that of free Adr (Figure 1). In particular, a strong CD band at 550 nm as well as an absorption band at 586 nm can be used to monitor the complex formation. The plot of the amplitude as a function of the molar ratio show that the stoichiometry of the Zr:Adr complex is 1:2 (Figure 2); this complex will hereafter be labeled $Zr(Adr)_2$. This stoichiometry was confirmed by fluorescence study: the fluorescence emission of Adr through excitation at 480 nm was completely quenched in the 1:2 complex (Figure 2).

When the metal ions solution was added to Adr dissolved in unbuffered aqueous solution, the formation of the complex was even slower when the pH was lower. The formation of the complex did not give rise to the release of proton.

This complex was particularly stable and a decrease of the pH of the solution down to 1.2 did not give rise to the release of the metal ion from its binding site, even after 3 days.

(B) Cp_2TiCl_2. This interaction was less simple than the preceding one. This was due in part to the pH dependency of the kinetics of ring protonolysis. Depending on the pH value of the titanocene dichloride solution, two types of complexes could be obtained through interaction with Adr. In order to determine the role of cyclopentadienyl ring protonolysis in the complex formation, we performed a preliminary study of this protonolysis in the absence of Adr and under the conditions used hereafter. The absorption spectrum of titanocene dichloride aqueous solution exhibited a strong band at 240 nm (ϵ = 32000) which is due to a large extent to a charge-transfer transition

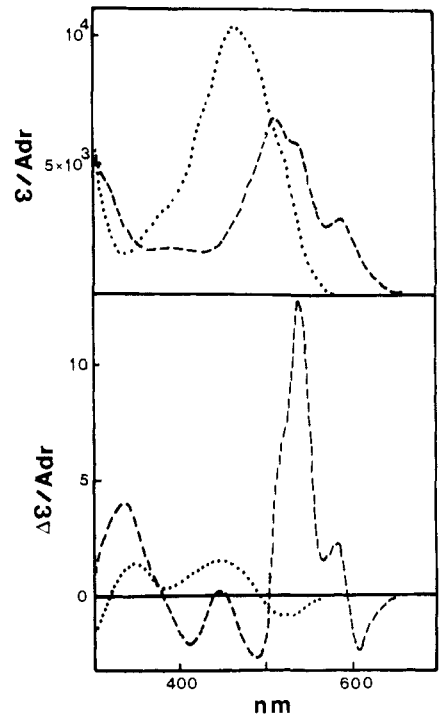

Figure 1. Absorption (upper) and CD (lower) spectra of Adr–Cp_2ZrCl_2 system. Experimental conditions were as follows: [Adr] = 3 × 10^{-4} M in Hepes buffer solution (0.05 M, pH 7.2). The molar ratio of Cp_2ZrCl_2 to Adr was respectively 0 (···) and 0.5 (- - -). The spectra have been recorded 5 days after the addition of Cp_2ZrCl_2 to Adr.

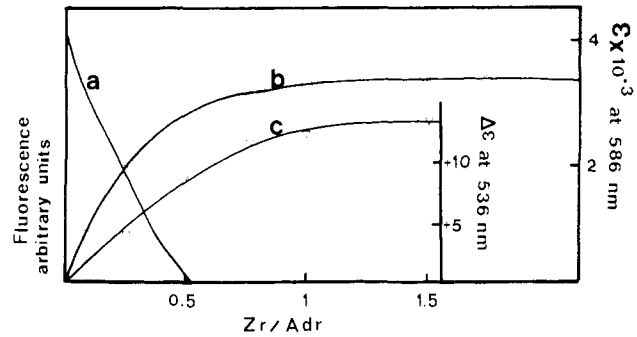

Figure 2. Titration of Adr with Cp_2ZrCl_2. Relative fluorescence (curve a) (excitation wavelength λ_e = 460 nm), ϵ/Adr at 586 nm (curve b), and $\Delta\epsilon$/Adr at 536 nm (curve c) have been plotted as a function of the molar ratio of Cp_2ZrCl_2 to Adr. Experimental conditions: [Adr] = 300 μM, 0.05M HEPES, pH 7.2. The spectra were recorded 5 days after the addition of Cp_2ZrCl_2 to Adr.

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between Ti(IV) and the cyclopentadienyl ring.[26] The amplitude of this band can be easily used to monitor the release of cyclopentadiene. For a 3×10^{-4} M titanocene dichloride aqueous solution at pH 4, the decrease of ϵ at 240 nm is slow, i.e. about 12% in 20 h. This is shown in Figure 3 where ϵ at 240 nm has been plotted as a function of time. This kinetics did not depend on Cl^- concentration. When Cp_2TiCl_2 was dissolved in pH 7.4 HEPES buffer, the decrease of ϵ at 240 nm occurred in two steps: a fast decrease of 50% of the initial value took place within the first 30 min and then a very slow decrease of ϵ occurred with a rate that compared with that observed at pH 4 (Figure 3). These data corroborate those of Toney and Marks[6] showing that, in Cp_2TiCl_2, the Ti–C_5H_5 bond is expected to be stable over a period of days in unbuffered, low-pH solutions and that, at physiological pH, protonolysis of the first cyclopentadienyl ring is very rapid, while loss of the second one proceeds at a rate comparable to that of the initial ring loss in unbuffered solutions.

The titanocene solution used to prepare the complexes was either 3×10^{-4} M in pH 4 unbuffered aqueous solution, which was used 15 min after the dissolution of the compound, or 3×10^{-4} in pH 7.4 HEPES buffered solution, which was used 30 min after the dissolution of the compound. With use of the first solution the Cp_2Ti framework was intact and with the second solution one cyclopentadienyl ring was lost.

In a first set of experiments, titanocene dissolved in pH 7.4 HEPES buffer was added to an HEPES–Adr solution at a molar ratio of Ti(IV) to Adr ranging from 0 to 2. The formation of a complex was complete within 30 min as was attested by the variation of the absorption and CD spectra. Analysis of CD and absorption data show that this complex has a 1:2 stoichiometry. This complex will hereafter be labeled $Ti(Adr)_2$. We observed that after 30 min, i.e. once $Ti(Adr)_2$ complex was formed, the value of ϵ at 240 nm decreased from the initial ϵ_i value to ϵ_i minus 14000. This decrease can be attributed to the release of the cyclopentadienyl ring, which was still bound to the metal ion at the beginning of the reaction and was displaced by Adr from its coordination site to Ti(IV) as the reaction proceeded.

In a second experiment titanocene dichloride dissolved in unbuffered pH 4 aqueous solution was added to an HEPES–Adr solution. The final concentration of Adr was 300 μM. Here again the analysis of the absorption and CD data as a function of time and the molar ratio of Ti to Adr ranging from 0 to 2 showed that a Ti:Adr complex (hereafter labeled Ti(Adr)) of 1:1 stoichiometry was formed within 20 min. The decrease of ϵ at 240 nm, once the complex is formed, can account for the release of one cyclopentadienyl ring by the Cp_2Ti^{2+} entity. So, in this experiment, only one Cp was released through complex formation. Absorption and CD spectra of complex Ti(Adr) and $Ti(Adr)_2$ are shown in Figure 4. We found that these conditions were the best to prepare solutions containing more than 90% of either complex $Ti(Adr)_2$ (experiment 1) or complex Ti(Adr) (experiment 2). Other conditions yielded a mixture of both complexes.

It should be noted that the addition of Adr to Ti(Adr) did not yield $Ti(Adr)_2$, showing that the formation of $Ti(Adr)_2$ did not occur via Ti(Adr). The spectral pattern of both complexes was independent of pH values in the range 9–3. However, a decrease of the pH to very acidic values gave rise to modification; i.e., as the pH was decreased, the spectrum characteristic of the free drug was recovered, indicating a dissociation of the complex. The reaction being rather slow, solutions of Ti(Adr) and $Ti(Adr)_2$ at pH ranging from 1 to 7 were thus prepared and allowed to stand for 5 h, and then the absorption spectra and pH were recorded. Fifty percent of the free drug was recovered at pH 2 and 2.5 for Ti(Adr) and $Ti(Adr)_2$, respectively.

(C) Cp_2VCl_2. Vanadocene dichloride was dissolved in pH 4 unbuffered aqueous solution. It has been shown that, at acidic and up to physiological pH value, the Cp_2V framework is preserved. No complex formation was observed when this solution was added to a pH 7.4 HEPES buffer solution of adriamycin. In order to determine if the formation of the complex could occur at other pH values, unbuffered solutions of 300 μM Adr of pH ranging from 4 to 7 were prepared and added to the V(IV) solution. This was done at various molar ratios of vanadium to Adr. The solutions were left at room temperature. After 3 days a 1:2 complex (hereafter labeled $V(Adr)_2$) was formed and the pH values of all the solutions decreased to about 4. The CD and absorption spectra of this complex are shown in Figure 5. Once formed, the complex was stable from pH 4 to 6; i.e., no spectral modifications were observed. However, at pH higher than 6, dramatic spectral modifications were observed, and the spectra of free Adr was recovered, indicating the release of V(IV) from its binding site.

The modification of the absorption spectrum of Adr through complexation, i.e. the shift of the whole visible absorption band to higher wavelengths, and more precisely the appearance of a band at about 600 nm, is characteristic of the deprotonation of one of the two hydroxyl groups at either C_6 or C_{11}. Previous reports have shown that site I is involved in the complexation of anthracycline with Fe(III), Pd(II), and Y(III) and that only Cu(II) is able to bind in either site I and/or site II. In the present case we observed that aclacynomycin, which lacks an hydroxyl group at C_{11}, is not able to bind metallocene dichloride. This suggests that site I of Adr is involved in the complexation to Zr(IV), Ti(IV), and V(IV). On the basis of the similarities of their CD spectra, we propose for the three complexes, $Zr(Adr)_2$, $Ti(Adr)_2$, and $V(Adr)_2$, a common structure in which the metal ion is octahedrally surrounded by two molecules of Adr, the metal ion being bound to two molecules of Adr through the C_{12}-carbonyl oxygen and the C_{11}-phenolate oxygen, thus forming a six-membered chelate ring, and to the amino nitrogen of the sugar. A space-filling model shows that such a structure is possible. On the other hand, this proposition is supported by the following arguments. During the preparation of $V(Adr)_2$, achieved by mixing an aqueous vanadocene dichloride solution to an adriamycin one at pH 6.5, we observed that the formation of the complex occurred concomitantly with a slow decrease of the pH value down to 4. In the case of a 3×10^{-4} M Adr solution, this accounts for the release of about 0.3 proton/Adr molecule, if we take into account the facts that (i) when Cp_2VCl_2 is dissolved in water at pH 6.5, the most predominant species formed is $Cp_2V(OH)_2$[6] and (ii) when $V(Adr)_2$ is formed, four original ligands are removed, i.e. 2 OH^- and 2 Cp^- groups and, in water at pH about 6, they will pick up 4 H^+. On the other hand, through complexation, each Adr molecule releases 2 H^+, if the amino group is involved in the complexation, and this can explain the very slight decrease of pH. In contrast, if the amino group is not involved in the coordination, only one proton is released per Adr molecule and an increase of the pH value would thus be expected. On these grounds, the above-

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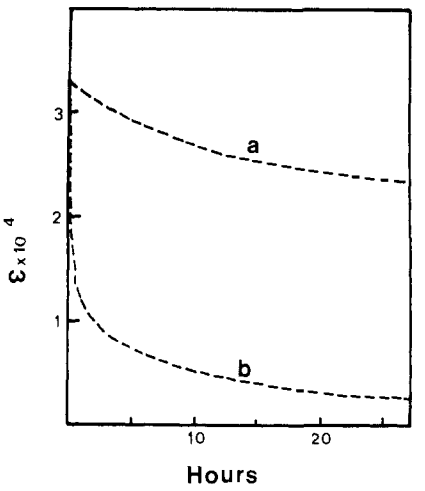

Figure 3. Variation of the absorption spectra of Cp_2TiCl_2 in aqueous solution as a function of time and pH ϵ at 240 nm was plotted as a function of time for different pH values. Experimental conditions were as follows: Cp_2TiCl_2 (2.7×10^{-4} M) was dissolved in 0.1 M KCl, pH 4 aqueous solution (curve a) or in 0.1 M KCl, HEPES buffer, pH 7.2 (curve b).

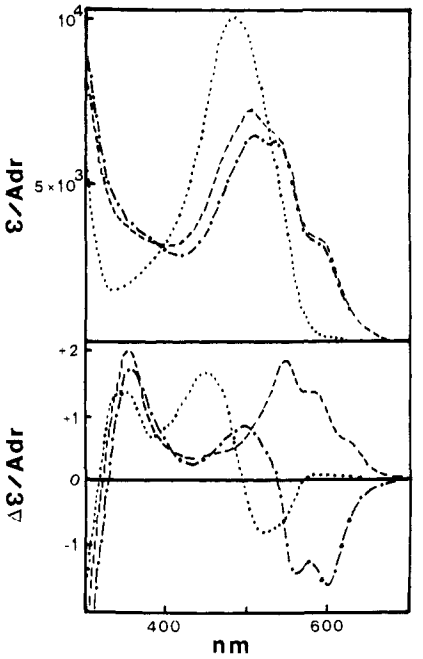

Figure 4. Absorption (upper) and CD (lower) spectra of Adr in the presence of various amounts of Cp_2TiCl_2. Experimental conditions were as follows: [Adr] = 3.2×10^{-4} M in HEPES buffer solution. The molar ratio of Cp_2TiCl_2 to Adr was respectively 0 (···), 0.5 (---), or 1 (-·-). The spectra were recorded 3 days after the addition of Cp_2TiCl_2 to Adr.

proposed structure appears reasonable. However, we should emphasize that the evidence for the participation of the amino group in the complexation is a bit weak. In the case of $Zr(Adr)_2$ and $Ti(Adr)_2$, it is not possible to use the same considerations on the pH because the reactive species are not as well defined as in the case of the vanadocene dichloride–Adr system.

Concerning now the Ti(Adr) complex, Figure 4 shows that its CD spectrum is of the couplet type, suggesting a stacking of the molecules of drug. It is well known that for the free drug the association depends on the dielectric constant of the solvent, and it has been shown that 300 μM adriamycin, which is in the dimeric form in aqueous solution, converted to the monomeric form in 50% aqueous

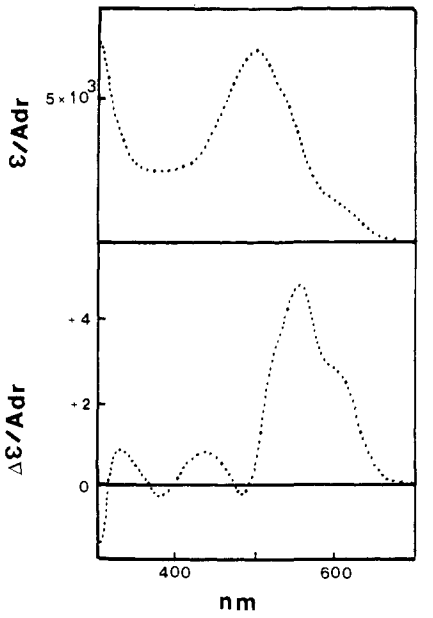

Figure 5. Absorption (upper) and CD (lower) spectra of the Adr–Cp_2VCl_2 system. Experimental conditions were as follows: [Adr] = 3×10^{-4} M in aqueous solution, pH 4.1. The molar ratio of Cp_2VCl_2 to Adr was equal to 0.5. The spectra were recorded 7 days after the addition of Cp_2VCl_2 to Adr.

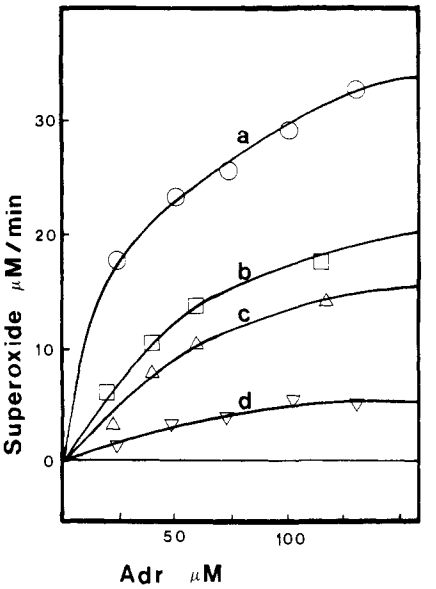

Figure 6. Effect of drug concentration on superoxide formation by NADH dehydrogenase. Superoxide formation was determined spectrophotometrically by the rate of SOD-inhibitable cytochrome *c* reduction as described in the Materials and Methods section. The reaction mixture contained 0.05 M HEPES buffer, pH 7.2, cytochrome *c* (40 μM), NADH dehydrogenase (250 unit/L), SOD (0 or 10 μg/mL), NADH (81 μM), and the indicated amount of adriamycin (a), $Ti(Adr)_2$ (b), Ti(Adr) (c), and $Zr(Adr)_2$ (d).

methanol solution.[27] The addition of 50% ethanol to an aqueous solution of Ti(Adr) did not modify its CD spectrum, indicating that the CD pattern did not depend on the dielectric constant of the solvent. This suggests a dimeric structure in which two Ti(IV) ions maintain two molecules of drug in fixed positions with regard to each other. Such a structure could be achieved if one Ti(IV) ion is bound to the oxygen atoms at C_{11} and C_{12} of one

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molecule of drug and to the amino group of the second molecule of drug. The ligands involved in the coordination sphere of Ti(IV) are two oxygens and one amino from Adr and one cyclopentadienyl.

Biochemical Properties of the Metallocene Dichlorides–Adriamycin Systems. $V(Adr)_2$ is not stable at pH values higher than 6 and this is probably due to the formation of vanadium hydroxide. For this reason, in the following, we will not examine its biochemical and biological properties.

(A) Effect of the Complexes on Superoxide Production by NADH Dehydrogenase. We have investigated the effect of the complexes on superoxide anions formation by mitochondrial NADH dehydrogenase. This effect was compared with that of the free drug. Adr increased superoxide formation by NADH dehydrogenase in a dose-dependent fashion that appeared to follow saturation kinetics (Figure 6). On the contrary, $Zr(Adr)_2$ and, to a less extent, TiAdr and $Ti(Adr)_2$ complexes did not increase superoxide formation over control levels.

(B) Interaction of the Metallocene Dichloride–Adriamycin Complexes with DNA. Since DNA has been postulated as one of the sites of action of anthracyclines in vivo, we have undertaken the examination of the interactions of these complexes with DNA.

A first set of experiments was 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.[28] The interaction has been monitored by using the CD spectrum of the complexes, which can be used as a fingerprint. The solution contained 2×10^{-5} M complex and DNA at a molar ratio of nucleotides to Adr equal to 10. In the case of $Zr(Adr)_2$ complex, no modification of the CD spectrum was observed, even after several days, suggesting that the presence of the metal ion precluded the intercalation of Adr between the base pairs of DNA. Under the same experimental conditions, small modifications of the Ti(Adr) and $Ti(Adr)_2$ spectra were observed, indicating that, in these cases, some metal ions were released from their Adr binding sites.

In a second set of experiments, we compared the tendency of these complexes to aggregate and precipitate nucleic acids to that of free Adr. In order to characterize the precipitates formed, we performed the titration of a 200 μM concentration of the free drug and of its metallocene dichlorides complexes with a range of DNA concentrations. In every case, a precipitate immediately appeared, which was removed by centrifugation at 900 rpm for 10 min. In the case of the complexes, the pellets had a color similar to that of the complex in water, indicating that the drug and the metal ion were bound to DNA in the form of the metal–drug complex. The binding of the nucleic acid to the drug, or metal–drug complex, was detected by checking the amount of free drug or free drug–metal complex in the supernatant after centrifugation. This allowed the determination of the amount of drug that precipitated with DNA. We observed that in the case of the free drug the formation of the pellet occurred through the binding of one molecule of drug per one nucleotide (i.e., in the precipitate the molar ratio of drug to nucleotide was equal to 1). However, in the case of the complexes the formation of the pellets occurred at a stoichiometry of Adr per nucleotide equal to 1.65, 1.25, and 1.5 for Ti(Adr), $Ti(Adr)_2$, and $Zr(Adr)_2$, respectively.

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(C) Binding of Adr and Complexes to Erythrocyte Ghosts. The binding of complexes $Zr(Adr)_2$, $Ti(Adr)_2$, and Ti(Adr) to erythrocyte ghosts 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 in the Materials and Methods section. Under the conditions used, we found that the presence of metal ions helped the binding of the drug to the membrane and that an amount of 1.9×10^8, 3.8×10^8, 4.1×10^8, and 6.0×10^8 molecules of Adr are bound per erythrocyte ghost in the case of free Adr, $Ti(Adr)_2$, Ti(Adr), and $Zr(Adr)_2$, respectively.

(D) Antitumor Activity. The in vitro inhibition of P-388 leukemia cell growth by $Zr(Adr)_2$, Ti(Adr), and $Ti(Adr)_2$ was compared with that induced by the free drug. An ID_{50} equal to 0.02 μg/mL was found for Ti(Adr), $Ti(Adr)_2$, and the free drug. However, an ID_{50} higher than 0.2 μg/mL was found for $Zr(Adr)_2$.

Discussion

The aim of this work was to synthesize bifunctional compounds by association of a DNA intercalative agent, adriamycin, with a metallocene dichloride, compounds of the "alkylating" type. The idea was that such an association could have several roles: (i) it would prevent each of the drugs from reacting with nontarget agents and eliminate the ability of Adr to produce superoxide and (ii) at the level of the target, the complex would dissociate, liberating two active antitumor species.

Concerning the first point, our data show that $Zr(Adr)_2$, Ti(Adr), and $Ti(Adr)_2$ cannot be reduced by NADH dehydrogenase. These data compared with those previously obtained, showing that once complexed, either to Fe(III)[9] or to Pd(II),[12] Adr lost its ability to be reduced by NADH dehydrogenase, and no superoxide production was detected. As the production of superoxide, in the respiratory chain of the mitochondria, is one of the mechanisms the most largely accepted to explain the cardiotoxic effect of anthracycline, the inability of the complexes to be reduced by NADH dehydrogenase can appear as an improvement of the therapeutic index of these drugs.

Now concerning the second point, $Zr(Adr)_2$ appears to be devoid of antitumor activity, and this is most probably related to the observation that no dissociation of the complex occurred even when it is left for a long period at pH 1. In contrast, Ti(Adr) and $Ti(Adr)_2$, which are more sensitive to low pH media (50% dissociation is observed around pH 2.5), exhibit antitumor activity that compares with that of the free drug. However, the observation that $Zr(Adr)_2$, which does not dissociate, is not cytotoxic strongly suggests that the cytotoxicity is related to the dissociation of the complex. This is an argument in favor of the generation of two active species at the level of the target.

Concerning the active metal species release from $Ti(Adr)_2$ or Ti(Adr) complexes, it is obvious that in the first case Ti(IV) ion is released, which will have a tendency to bind to the target; in the case of Ti(Adr) it is possible that a cyclopentadienyl group is still bound to Ti(IV). However, in any case it is highly probable that the metal species released from Ti(Adr) or $Ti(Adr)_2$ complexes compares with that which is formed when Cp_2TiCl_2 is injected into the plasma at pH near 7. As it has been shown that such Ti(IV) species were active, we believe that in the present case this is also true.

Our data suggest that the association of the two drugs Adr and Cp_2TiCl_2 can lead to an improvement of the therapeutic index of both drugs. Moreover, the presence of metal ions has another advantage, which is to promote

the binding of the drug to susceptible targets such as DNA and membrane.

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Cardiotonic Agents. 9. Synthesis and Biological Evaluation of a Series of (*E*)-4,5-Dihydro-6-[2-[4-(1*H*-imidazol-1-yl)phenyl]ethenyl]-3(2*H*)-pyridazinones: A Novel Class of Compounds with Positive Inotropic, Antithrombotic, and Vasodilatory Activities for the Treatment of Congestive Heart Failure

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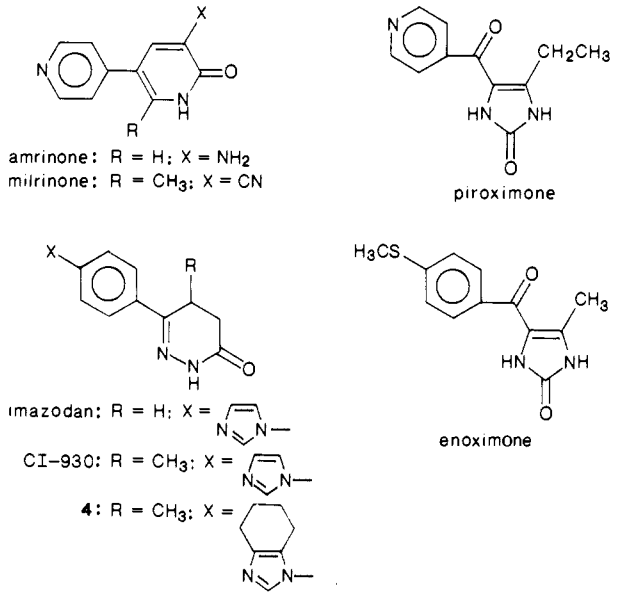
A novel series of analogues of (*E*)-4,5-dihydro-6-[2-[4-(1*H*-imidazol-1-yl)phenyl]ethenyl]-3(2*H*)-pyridazinone was synthesized as a variation on the imazodan series. The compounds were evaluated for (i) hemodynamic activity, (ii) cyclic AMP-phosphodiesterase inhibitory activity (human platelets and guinea pig heart tissue), and (iii) platelet aggregation inhibitory activity. The insertion of the ethenyl moiety between the phenyl and dihydropyridazinone rings produced novel compounds that retained the potent inotropic/vasodilator activity of the parent imazodan series and enhanced the platelet aggregation inhibitory potency. Compound **3d**, the most potent in this series, demonstrated in vivo antithrombotic activity. The synthesis and the biological activity of these new pyridazinone analogues are reported.

The extensive search to find a nonglycoside, noncatecholamine digitalis replacement led to the discovery of several new cardiotonic drugs,[1-6] including amrinone,[7,8] milrinone,[9,10] enoximone,[11,12] piroximone,[13,14] and imazodan (Chart I). The positive inotropic and vascular relaxant actions of these agents are apparently due to the selective inhibitory effects on the low k_m cyclic AMP-specific, cyclic GMP-inhibited form of phosphodiesterase (PDE IIIC) present in cardiac and vascular muscle.[15] Although clinical studies have shown that these agents provide both acute and sustained hemodynamic improvement,[16] their influence on the natural history of heart failure is unclear, and the precise role of these agents in the long-term management of heart failure remains controversial.[17] We have previously reported on imazodan and CI-930, two selective PDE IIIC inhibitors for the treatment of congestive heart failure.[18,19] The favorable clinical results seen with imazodan[20] and CI-930[21] encouraged the development of a second generation cardiotonic that would possess a greater balance of inotropic and vasodilator activity, as well as additional actions that might influence the underlying pathology and progression of congestive heart failure. From a therapeutic perspective, it may be advantageous to administer a cardiotonic agent with platelet aggregation inhibitory activity to those patients with a history of myocardial infarction and an increased risk of coronary or pulmonary thrombosis.[22-27]

Recently, the synthesis and the biological activity of lixazinone (RS-82856), a potent and selective inhibitor of the type IIIC phosphodiesterase, has been reported.[28,29] This agent, which contains major structural elements of two structurally dissimilar compounds, cilostamide and anagrelide (Chart II), exhibits potent inotropic and antithrombotic properties. In this compound the *N*-cyclohexyl-*N*-methyl-4-oxybutyramide side chain was of significant value as a steric and/or lipophilic pharmacophore

Chart I



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