Reaction of *cis*-$Ru^{II}(DMSO)_4Cl_2$ with DNA and with some of its Bases in Aqueous Solution*

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Abstract

cis-$Ru^{II}(DMSO)_4Cl_2$ (*cis*-RDT) reacts in aqueous solution with double-stranded DNA forming Ru^{II}–DNA complexes. According to measurements performed with poly(dGdC) and with poly(dAdT) the most likely sites of reaction in the double-stranded polynucleotides are N-7 of both guanine and adenine.

Contrary to what was found with cisplatin (to which *cis*-RDT is comparable in its oncostatic properties) the reaction with *cis*-RDT stabilizes the ordered B structure of DNA. However, the reaction produces a marked cleavage of the chain.

Introduction

Previous investigations have shown that some ruthenium complexes possess good antitumor activity [1–7]. Among these, *cis*-$Ru^{II}(DMSO)_4Cl_2$ (*cis*-RDT) compares well with the properties of *cis*-$Pt^{II}(NH_3)_2Cl_2$ (cisplatin), a well-known and widely used antiblastic substance. The ruthenium complex, however has a remarkably lower toxicity, which makes the drug interesting for possible clinical use [7].

It is now well known that cisplatin reacts with DNA in aqueous solution and forms covalent bonds with some of its bases. It is likely, although not proven, that these reactions are at the root of the biological action of the drug [8].

On the other hand the mutagenic properties of *cis*-RDT [9, 10] suggest that one target of the complex *in vivo* is DNA. The suggestion is also supported by the capability of different Ru^{II} and Ru^{III} complexes to react with nucleosides and nucleotides [11–13]. For these reasons we have undertaken a study of the reaction of *cis*-RDT with natural and synthetic DNAs and with some DNA bases in aqueous solution.

Experimental

cis-RDT was synthesized as already reported [4] and recrystallized from a DMSO–acetone mixture.

Stock solutions of calf thymus DNA (Sigma Chemicals, type I) were prepared by dissolving the polymer in 3 mM NaCl, 1 mM phosphate buffer at pH 7.4 and diluting to a final concentration of about 8 mM (phosphate base).

The ruthenium complexes of nucleotides and polynucleotides were prepared by incubation with freshly prepared solutions of *cis*-RDT at different stoichiometric ratios, r_s. The reaction mixtures were kept in the dark in a water thermostatted bath with occasional manual stirring. The reaction was terminated by raising the chloride concentration to 0.5 M with 4.6 M NaCl and samples were frozen for storage. The separation of the unreacted *cis*-RDT was made by ultrafiltration (stirred ultrafiltration cell Amicon, model 8010) through Diaflo Ultrafilter PM10 membrane using argon as pressurizing gas. The ultrafiltration was carried out first with about 10 ml of 0.5 M $NaClO_4$ solution and then with about 100 ml (10 ml × 10) of 2×10^{-4} M $NaClO_4$. The solutions recovered from ultrafiltration were analyzed by UV absorbance to determine the amount of unbound *cis*-RDT

Absorbance spectra were recorded using a Cary 219 spectrophotometer equipped with a thermostatted cuvette holder and a Haake F3 thermostat provided with a temperature programmer Haake PG 10.

Melting curves were recorded at 257.5 nm by increasing the temperature at a rate of 0.5 °/min.

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CD spectra were obtained using a Jasco J500A dichrograph connected with a Jasco DP-500N data processor and equipped with a thermostatted cell holder.

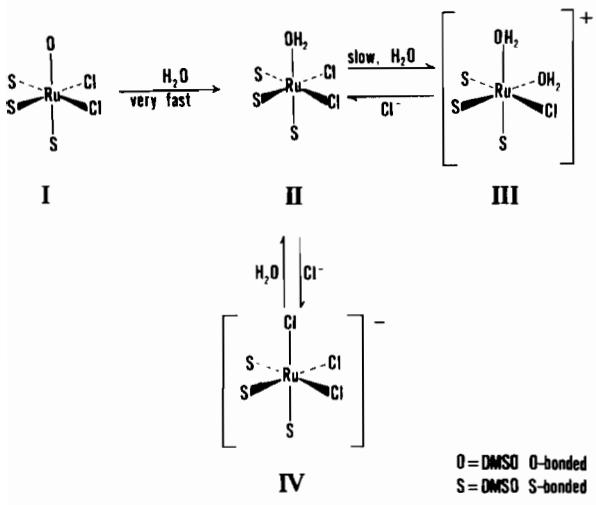
Poly(dGdC), poly(dAdT) and nucleotides were obtained from Pharmacia. pBR322 plasmid DNA (from *Escherichia coli*) was obtained from Boehringer. Restriction enzyme digestion was carried out with BamH1, EcoRI and HindIII (Pharmacia) in 10 mM Tris-HCl, 10 mM $MgCl_2$ and 10 mM NaCl at pH 8 (pH 7.5 in the case of EcoRI). Digestions were quenched by the addition of a 1/4 digest volume of 0.25 M EDTA followed by chilling at −20 °C.

Agarose gel electrophoresis was carried out in submarine horizontal slab gel (BioRad DNA SubCell). 1% gels (Agarose Serva) were prepared and run in 90 mM Tris base, 90 mM borate, pH 8.3. Gels were run at 40 V (2.6 V/cm) at room temperature for about 20 h. The DNA was visualized by staining the gels for 1 h in 0.5 mg/l ethidium bromide and illuminating with ultraviolet light.

Results and Discussion

cis-RDT is a neutral complex with an octahedral structure [15]. Of the four DMSO ligands, one is bonded to the central metal atom through the oxygen, whereas the others are bonded through the sulfur atom with a facial configuration **I**. The oxidation state of ruthenium is +2.

cis-RDT forms bright yellow crystals, very soluble in water. The aqueous solutions, when kept in the dark, are very stable to air oxidation. Once dissolved, the complex immediately dissociates the *O*-bonded DMSO ligand, as demonstrated by NMR studies [14, 16] and gives species **II** of Scheme 1. This compound, according to conductivity measurements [14] and to



Scheme 1.

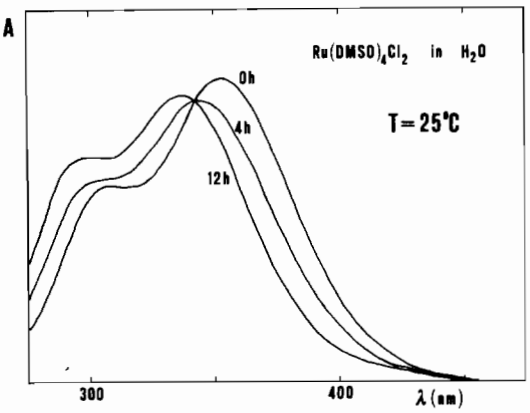


Fig. 1. Time dependency of absorption spectrum of $Ru^{II}(DMSO)_4Cl_2$ in water at 25 °C.

spectral measurements (Fig. 1), undergoes a slow dissociation of a chloride ion to give the cationic species **III**.

The presence of an isosbestic point in Fig. 1 shows that only two species (**II** and **III**) are interchanging in the time range explored. The final equilibrium state is reached within 3 h at 37 °C and within 12 h at 25 °C. The loss of the second chloride ion is not evidenced even after 7 days at 25 °C.

The equilibrium between species **II** and **III** is affected by the Cl^- concentration, as expected. We have measured such an equilibrium at two significant Cl^- concentrations, namely 3 mM and 150 mM, which roughly correspond to intra- and extra-cellular conditions, respectively. At the lowest Cl^- concentration both kinetic and thermodynamic behaviors are almost indistinguishable from those in pure water, whereas in 150 mM Cl^- the dissociation of the first chloride is almost completely suppressed. This behavior might have some biological significance in view of the passive transport of the drug through the plasma membrane.

When DNA is incubated with *cis*-RDT a reaction takes place. This can be demonstrated by the absorption spectrum of the incubated and then ultrafiltered DNA solution, which shows an excess of absorbance in the wavelength range beyond 300 nm. The time course of the reaction between *cis*-RDT and DNA can be followed by recording the changes produced in the absorption spectra of the DNA adduct, as, for example, the ratio between the absorbance at 230 nm and that at 258 nm, as a function of time (Fig. 2). The data show that, at 25 °C, the reaction proceeds rapidly within the initial 10 h and then tends to level off. After 2 days the amount of ruthenium complex bound to DNA corresponds to a value of about 0.25 mol of metal per mol of phosphate group.

The CD spectra of both native and heat-denatured DNA before and after incubation with *cis*-RDT are shown in Fig. 3. The data suggest that the B confor-

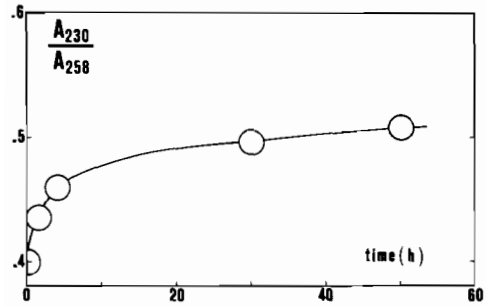


Fig. 2. Time dependency of A_{230}/A_{258} for Ru^{II}–DNA complexes.

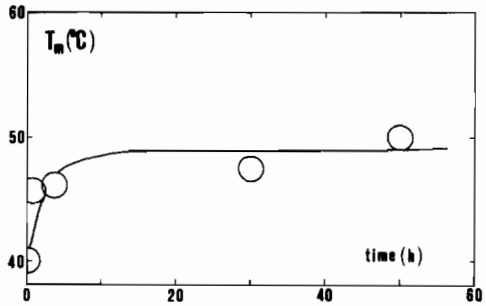


Fig. 4. Melting temperatures of Ru^{II}–DNA complexes in 2×10^{-4} M $NaClO_4$, as a function of incubation time.

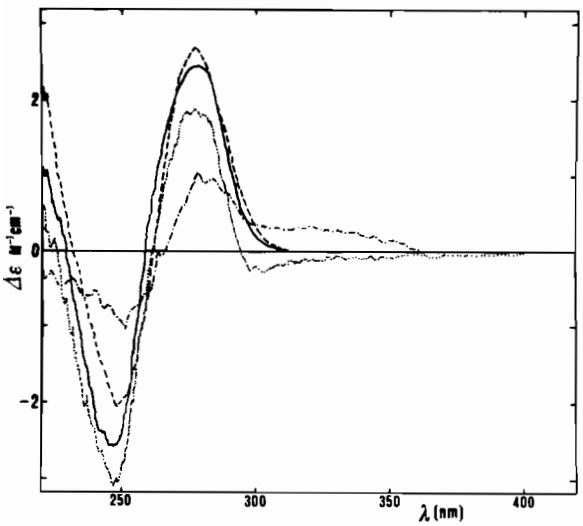


Fig. 3. CD spectra of native (·····) and heat-denatured (– · –) calf thymus DNA complexed with *cis*-RDT. For comparison the spectra of untreated native (——) and heat-denatured (- - - -) DNA are reported. All measurements were performed at 25 °C.

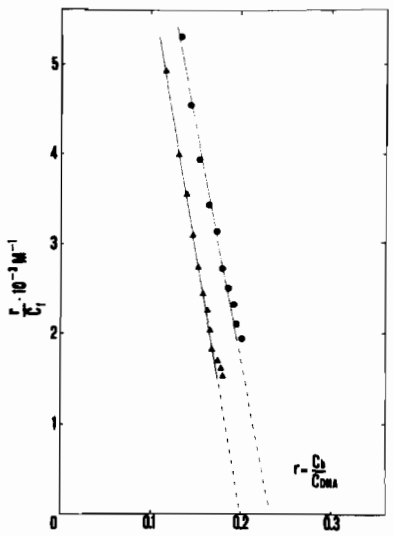


Fig. 5. Scatchard plots for ethidium bromide binding to DNA untreated (●) and treated (▲) with *cis*-RDT. Solvent: 0.5 M NaCl, T = 25 °C. The amount of ethidium bound was determined by absorption measurements.

mation of the DNA chain remains basically unaltered following the reaction, the small changes in the amplitude of both positive and negative bands and the appearance of a negative band at 300 nm being noticed only after 2 days of incubation. On the other hand, the differences in CD spectra are more marked in the denatured state as if the reaction proceeded further when the conformational order was disrupted. Both these findings confirm results recently obtained by Clarke *et al.* with the system $[(NH_3)_5Ru^{III}]_n$–DNA [17].

That the binding of *cis*-RDT to the B conformation does not alter significantly the ordered B state is confirmed also by the results of the melting experiments carried out on the Ru–DNA complexes at different incubation times. The data (Fig. 4) show that the increased binding of *cis*-RDT to DNA progressively increases the stability of the chain. This result, when compared to those obtained with DNA after reaction with cisplatin [18] or with other ruthenium complexes [17], is rather surprising. The weakening of the B conformation, in the case of cisplatin, has been attributed to the formation of intrastrand bonds between two DNA bases and the metal complex. In the case of *cis*-RDT the increased stability can be due either to a partial screening of the negative phosphate charges from the positively charged base–Ru^{II} complexes and/or to an energetically very favorable location of the complexes in the DNA major groove. A possible interstrand crosslink by the *cis*-RDT as a cause of helix stabilization can be ruled out as the melting of the chain is completely irreversible.

Despite the absence of relevant modifications in the B structure the complexed DNA shows a lower number of sites available for intercalation, as shown in Fig. 5, where the Scatchard plots for the interaction of ethidium bromide with complexed and un-

complexed DNA are shown. The data have been obtained in high ionic strength (0.5 M NaCl) in order to provide the presence of only intercalating binding.

In the case of cisplatin it has been shown that circular DNA, after reaction with the metal complex, appears modified in its topological properties as if it had reacted with a typical intercalator [19, 20]. *cis*-RDT, however, does not produce any relaxation of the pBR322 supercoiled form even after prolonged time of reaction. Again this experiment shows that reaction of *cis*-RDT with DNA does not lead to substantial changes in the geometry of the B double helix.

The same conclusion can be drawn by a study of the possible protection of specific base sequences on DNA by the reacted *cis*-RDT. pBR322 was incubated with the ruthenium complex and then assayed with different restriction enzymes (EcoRI, HindIII and BamH1). None of the restriction sites appeared particularly protected by the presence of the ruthenium complexes, even for prolonged incubation time.

In both experiments with circular DNA, however, a marked increase in the nicked form was noticed after reaction with *cis*-RDT. This result, already noticed with a different Ru^{II} complex [17], might have biological significance.

Having assessed that *cis*-RDT reacts with DNA, the next step was the identification of the reacting groups in the chain. It is well known that Ru^{II} complexes react with many nitrogen bases [21]. In the case of DNA bases, Ru^{II} complexes react preferentially with the N-7 of guanine [11], N-1 of adenine [22] and N-4 of cytosine [22], although other positions can be used. By far the most reactive position is the N-7 of guanine [23].

We have incubated both 5′-GMP and 5′-AMP with *cis*-RDT under the same experimental conditions used for the reaction with DNA. In both cases the reaction is fast but that with 5′-GMP appears faster judging by the rate of growth of the CD signal in the wavelength range 300–450 nm. After 2–3 h both reaction mixtures were eluted through a Biogel P2 column. Several peaks were collected in each case, some of them showing ellipticity at wavelengths beyond 300 nm (where the single nucleotides do not absorb). The absorption and CD spectra of the most representative peaks (one in the case of AMP and two in the case of GMP) are shown in Figs. 6 and 7 (together with the corresponding spectra of the nucleotides alone).

These complexes in aqueous solution show fairly good stability to air oxidation, although lower than that exhibited by the Ru^{II}–DNA complexes.

The variety of compounds which A and G can form with *cis*-RDT makes identification of the complexes formed with DNA difficult. It can be done, however, by enzymatic hydrolysis of the Ru^{II}–DNA chain, followed by separation and identification of the Ru^{II}–base compounds. This work is under way in our laboratory. Some useful information in this respect, however, has been obtained by studying ruthenium complexes formed by reaction with synthetic DNAs of definite base sequence.

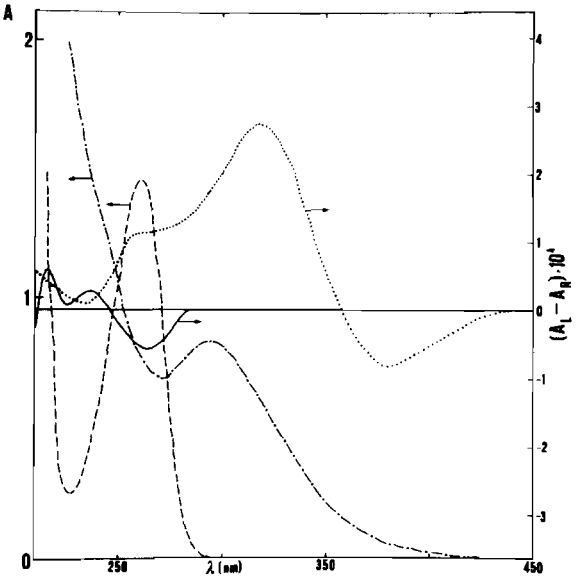


Fig. 6. Absorption (- - -) and CD spectra (——) of 5′-AMP. Absorption (– · –) and CD (·····) spectra of a reaction product between *cis*-RDT and 5′-AMP.

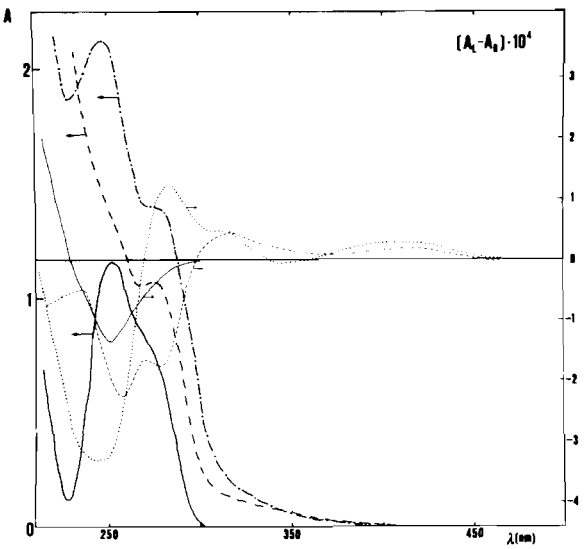


Fig. 7. Absorption (——) and CD (——) spectra of 5′-GMP. Absorption (– – –) (– · –) and CD (·····) (- - - -) spectra of two reaction products between *cis*-RDT and 5′-GMP.

Figure 8 shows the change with time of the CD spectrum of poly(dGdC) dissolved in 2×10^{-4} M $NaClO_4$ in the presence of *cis*-RDT at $r_s = 1$. The polymer undergoes a complete B-to-Z transition within 10 h at 37 °C. The transition is not due to the presence of trace amounts of di or trivalent cations as it is not reversed by addition of EDTA [24]. This

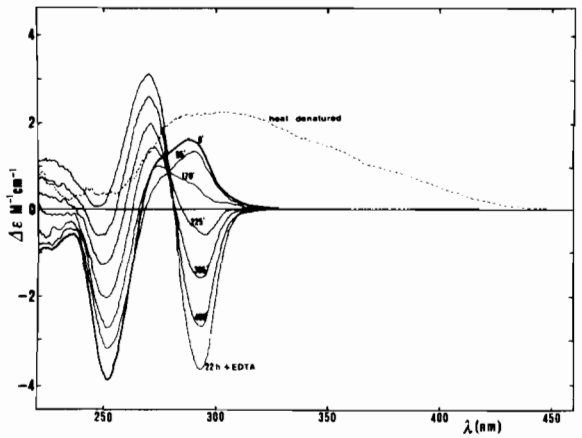


Fig. 8. CD spectra, registered at different times, of poly(dGdC) ($c = 1.18 \times 10^{-4}$ M) dissolved in 2×10^{-4} M $NaClO_4$ and in the presence of equimolar amount of *cis*-RDT. At t = 22 h EDTA was added to a final concentration of 2×10^{-4} M and then the solution was heated to 90 °C to produce the denatured form whose CD spectrum was recorded at room temperature.

finding is strongly in favor of a reaction of *cis*-RDT with the N-7 of guanine. It is, in fact, known that substitution reactions at the N-7 of guanine by bulky groups stabilize the *syn*-conformation of the *N*-glycosidic bond which, in turn, favors the left-handed form of the alternating polynucleotide [25–29]. The absorption spectrum of the ultrafiltrated polymer shows a small absorbance beyond 300 nm, which means that the conformational change is obtained with small amounts of bound *cis*-RDT. If the complexed poly(dGdC) is heated to 90 °C a denatured state is obtained (whose CD spectrum is also shown in Fig. 8) which does not reverse to the ordered state on cooling, contrary to what was observed with the uncomplexed polymer. This result suggests that the reaction of *cis*-RDT with the single-stranded polynucleotide produces molecular complexes which prevent the reformation of Crick and Watson hydrogen bonds.

Similar results have also been obtained with poly(dAdT) in 0.1 M $NaClO_4$. In this case no change in the conformation of the polymer was noticed. The excess absorbance measured beyond 300 nm after filtration and the stabilization of the B structure towards heat suggest that *cis*-RDT is bound to poly(dAdT), very likely at the N-7 of adenine, as binding to the preferred N-1 position would produce a destabilization of the double helix. Again a different binding of the complex must occur following denaturation by heating, as reversibility of the process is canceled.

Conclusions

The preliminary results shown in this work allow the following conclusions:

(1) *cis*-RDT reacts with DNA. This is demonstrated by the retention of the long wavelength absorption band after ultrafiltration of the Ru^{II}–DNA complex with 0.5 M $NaClO_4$, which should remove all the positively charged ruthenium complex electrostatically bound to negatively charged DNA.

(2) At $r_s = 1$ the amount of bound *cis*-RDT is about 0.2 mol/mol of phosphate after about 10 h of incubation at 25 °C. The reactive forms of the complex should be species **II** and **III** of Scheme 1. In both cases the complex formed on DNA should bear a positive charge.

(3) *cis*-RDT probably reacts with the N-7 of guanine and, at a lower rate, with the N-7 of adenine with ordered forms of DNA. G7 has already been demonstrated as the binding site for other ruthenium complexes [11, 17, 30]. A7 is suggested by the experiments carried out on poly(dAdT). Taking for granted that *cis*-RDT cannot react with thymine [17], a reaction with A1 and/or A6 would destabilize the B conformation of the chain, which is contrary to what is observed. On the same grounds, cytosine should be discarded as a possible reaction target in the structured polymer.

(4) The increase in denaturation temperature, the absence of a relaxation in the cccDNA, the absence in protection against cleavage by restriction enzymes, all suggest a good fitting of the bulky Ru^{II} complex on the major groove of DNA. The positive charge of the complex and the possibility to form hydrogen bonds with neighboring groups can explain the slightly increased stability of the structure.

(5) The marked increase in the amount of the nicked form noticed with plasmid DNA is indicative of a weakening of the phosphate ester bond following reaction with *cis*-RDT. An analogous result has been attributed to oxidation of Ru^{II}–DNA by molecular oxygen, via formation of HO· and subsequent sugar fragmentation [17]. Although this type of mechanism cannot be excluded, we have no evidence for an appreciable oxidation in our case. Whatever the cause, the cleavage of the DNA chain by the Ru^{II} complexes remains an interesting observation with possible biological implications.

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