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Interaction of an anticancer ruthenium complex HInd[RuInd$_2$Cl$_4$] with cytochrome *c*✪

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Cytochrome *c* is an important electron transfer protein in the respiratory chain, shuttling electrons from cytochrome *c* reductase to cytochrome *c* oxidase. Extensive chemical modification studies indicate significant electrostatic interactions between these proteins and show that all structural and conformational changes of cytochrome *c* can influence the electron transport. In the present work we examine the effect of an anticancer ruthenium complex, *trans*-Indazolium (bisindazole) tetrachlororuthenate(III) (HInd[RuInd$_2$Cl$_4$]), on the conformation of cytochrome *c*, the state of the heme moiety, formation of the protein dimer and on the folding state of apocytochrome *c*. For this purpose, gel-filtration chromatography, absorption second derivative spectroscopy, circular dichroism (CD) and inductively coupled plasma atomic emission spectroscopy (ICP(AES)) were used. The present data have revealed that binding of the potential anticancer drug HInd[RuInd$_2$Cl$_4$] complex to cytochrome *c* induces a conformation of the protein with less organized secondary and tertiary structure.

Ruthenium(III) complexes are presently an object of great attention in the field of medicinal chemistry, as antitumor agents with selective antimetastatic properties and low systemic toxicity. Ruthenium compounds appear to penetrate reasonably well the tumor cells and bind effectively to DNA. While the initial DNA binding site of many ruthenium com-

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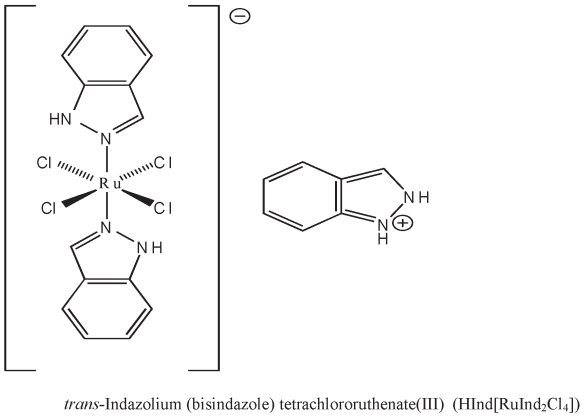
Abbreviations: HInd[RuInd$_2$Cl$_4$], trans-Indazolium (bisindazale) tetrachlororuthenate(III); ICP(AES), inductively coupled plasma atomic emission spectroscopy.

plexes is the same as that of cisplatin, i.e. the N7 site of Gua, their antitumor mechanism is most likely distinctly different (Clarke, 2002). *trans*-Indazolium (bisindazole) tetrachlororuthenate(III) (HInd[$RuInd_2Cl_4$]) complex (Fig. 1) for a long time investigated by the group of Bernard Keppler, showing encouraging pharmacological properties and low toxicity (Keppler *et al.*, 1990). This complex has now entered phase I clinical trials. In preclinical investigations, the activity of HInd[$RuInd_2Cl_4$] complex was observed against non-small cell lung, breast and renal cancers (Depenbrock *et al.*, 1997). This has inspired considerable interest in the study on the biochemical behavior of the HInd[$RuInd_2Cl_4$] complex including its interactions with proteins. The binding to proteins might result in drastic modifications or even loss of the biological activity of the starting biomolecules (Kratz *et al.*, 1994; Smith *et al.*, 1996). The major fraction of ruthenium(III) species (80–90%) is bound to albumin and a much smaller amount to transferrin (Kratz *et al.*, 1994). It has been proposed that cell surface transferrin receptors bind ruthenium-loaded transferrin with high affinity; the transferrin-receptor complexes are subsequently endocytosed and transferred to acidic non-lysosomal compartments where ruthenium is released (Klausner *et al.*, 1983). Binding of ruthenium(III) species has a strong impact on albumin structure and influences considerably its binding of other molecules including drugs (Trynda-Lemiesz *et al.*, 2000).

The preferred binding sites for Ru(III) complexes are histidine residues of the proteins (Smith *et al.*, 1996; Yocom *et al.*, 1982). It is known that at pH 7 a stable complex between Ru(III) and histidine-33 in ferricytochrome *c* is formed (Yocom *et al.*, 1982). In previous investigations (Tian *et al.*, 2000) ruthenium-cytochrome *c* derivatives were used to define the interaction domain for cytochrome *c* on the cytochrome bc_1 complex. Cytochrome *c* is a mitochondrial peripheral membrane protein functioning in the respiratory chain in



trans-Indazolium (bisindazole) tetrachlororuthenate(III) (HInd[$RuInd_2Cl_4$])

Figure 1. *trans*-Indazolium (bisindazole) tetrachlororuthenate(III) (HInd[$RuInd_2Cl_4$]).

the inner mitochondrial membrane, shuttling electrons from cytochrome *c* reductase to cytochrome *c* oxidase. However, in 1996 it was found that cytochrome *c*, when released from mitochondria to the cytosol, activates a programmed cell death cascade (apoptosis) (Cai *et al.*, 1998). The binding of the ruthenium complex to cytochrome *c* may change considerably the structure of the protein and affect its biological function. In the present work I examine the effect of HInd[$RuInd_2Cl_4$] on the conformation of cytochrome *c*, its heme state, the interactions with apocytochrome *c* and cytochrome *c* dimer formation. For this purpose, gel-filtration chromatography, absorption second derivative spectroscopy, circular dichroism (CD) and inductively coupled plasma atomic emission spectroscopy (ICP (AES)) methods have been used.

MATERIALS AND METHODS

Cytochrome *c* from horse heart was purchased from Fluka Chem. Co. (Neu-Ulm, Switzerland). The concentration of native ferricytochrome *c* was determined using $\varepsilon_{410} = 1.06 \times 10^5\ M^{-1}cm^{-1}$ (Babul & Stellwagen, 1972). Heme was obtained from Serva (Heidelberg, Germany), its concentration was evaluated spectrophotometrically in 0.01 M

NaOH, using ε_{385} = 5.84 × 10^4 $M^{-1}cm^{-1}$ (Beaven *et al.*, 1974). Apocytochrome *c* was prepared by chemical removal of the heme group from cytochrome *c* (silver sulfate method) as described by Fisher (Fisher *et al.*, 1973). The protein was purified by a modified version of the procedure described previously (Rankin *et al.*, 1998). Apocytochrome *c* was stored in aliquots at –20°C and at 0°C on the day of the measurements to prevent protein aggregation. The concentration of purified apocytochrome *c* was determined spectrophotometrically using ε_{277} = 1.05 × 10^4 $M^{-1}cm^{-1}$ (Stellwagen *et al.*, 1972). The purity of apocytochrome *c* was calculated by comparison of A_{410} (cytochrome *c*) and A_{277} (apocytochrome *c*).

HInd[$RuInd_2Cl_4$] was synthesized by B. K. Keppler *et al.* as described earlier (Keppler *et al.* 1989). Stock solutions were prepared in 0.9% NaCl and were used in all experiments from a freshly prepared 5 × 10^{-4} M solution. In all the experiments a phosphate buffer was used so that the final concentrations were 0.004 M NaH_2PO_4, 0.1 M NaCl and 0.025 M $NaHCO_3$, pH 7.4. All chemicals used were reagent grade.

Absorption and difference spectra were recorded on Beckman DU-650 spectrophotometers. Second derivative spectra were obtained using the software package provided by the manufacturer. Determination of tyrosine exposure in the protein by second-derivative spectroscopy was carried out as described by Ragone (Ragone *et al.*, 1984). The state of Tyr residues can by evaluated by second derivative absorption spectroscopy. One estimates the ratio of heights of second-derivative positive and negative peaks (the arithmetic sums) at 283 *vs.* 287 nm (a) and 290.5 *vs.* 294–5 nm (b). The ratio a/b is then a factor reporting the exposure of the tyrosine residues into the water environment. Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectropolarimeter in the range of 190–250 and 300–600 nm, using 0.1 and 1.0 cm cuvettes, respectively. The amounts of ruthenium bound per mole of cytochrome *c* were evaluated with a Spectrometer 3410 ICP (AES) (Fisions, Vienna, Austria).

The complex of cytochrome *c* with HInd[$RuInd_2Cl_4$] was prepared by incubation of a two-fold excess of the ruthenium compound with the protein for 24 h at 37°C. The sample was then chromatographed on a Sephadex G-25 column (2 × 50 cm) equilibrated and eluated with 4 mM phosphate buffer at pH 7.4. Absorbance of cytochrome *c* and HInd[$RuInd_2Cl_4$]-cytochrome *c* were measured at 410 nm. The ruthenium content in selected fractions was determined by the ICP(AES) method.

RESULTS AND DISCUSSION

Interaction of HInd[$RuInd_2Cl_4$] with cytochrome *c*

Cytochrome *c* reacts with HInd[$RuInd_2Cl_4$] producing a blue-green product with d-d transition around 585–590 nm, indicating protein binding. The spectra of cytochrome *c* with HInd[$RuInd_2Cl_4$] complex in phosphate buffer pH 7.4 (not shown) were found to be essentially similar to those previously reported for the serum protein complexes where coordination *via* imidazole nitrogen atoms was taking place (Kratz *et al.*, 1994; Trynda-Lemiesz *et al.*, 2000). The amounts of the ruthenium compound per mole of protein determined using the ICP(AES) method after being separated by gel-filtration chromatography, ranged between 0.89 and 0.96. This result supports the binding of one HInd[$RuInd_2Cl_4$] molecule by one molecule of the protein at low molar excess of ruthenium.

It is known that cytochrome *c* can be cross-linked selectively with rhodium and platinum complexes (Chen & Kostic, 1988; Chen *et al.*, 1997). The data obtained for the cytochrome *c*-HInd[$RuInd_2Cl_4$] system indicated that a di-protein complex is not formed *via* the ruthenium species.

Structural properties of cytochrome *c* modified with HInd[RuInd$_2$Cl$_4$] complex

In order to obtain information about the structural perturbation of cytochrome *c* upon HInd[RuInd$_2$Cl$_4$] binding, CD and second derivative of absorption spectra measurements were performed. Figure 2 shows

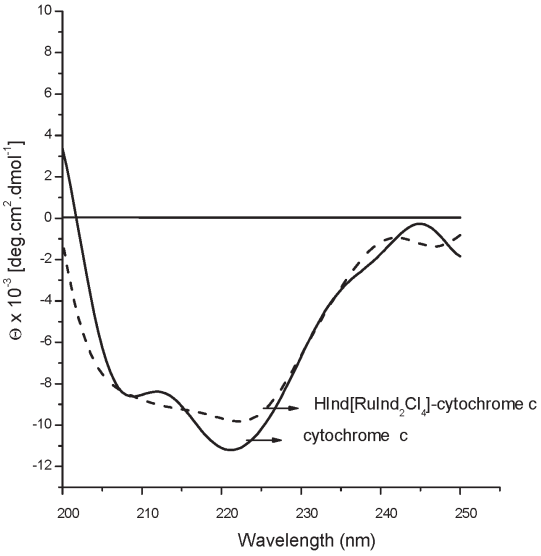


Figure 2. UV-CD spectra of cytochrome *c* (—) and HInd[RuInd$_2$Cl$_4$]-cytochrome *c* (– – –) at a molar ratio 2:1, after 24 h incubation at 37°C.

Concentration of cytochrome *c* = 80 mM

changes in α-helical content as measured at 222 nm. Cytochrome *c* has the α-helical content of about 35% and a decrease of 15% in ellipticity is observed when the ruthenium complex is added. This indicates distinct changes in the protein secondary structure.

Second-derivative spectroscopy was used to examine the state of Tyr residues in the protein. The method is based on the greater sensitivity of Tyr to the solvent environment than that of Trp. It is well known that the absorption of tyrosyl residues is largely masked by the stronger absorption of Trp. A second-derivative spectrum of proteins containing both tyrosyl and tryptophanyl residues shows two minima centered around 283 and 290.5 nm and two maxima around 287 and 295 nm. Since the position of the peaks does not change much after exposure to the perturbing agent, I analyzed the second-derivative spectra of the protein in terms of the ratio between the peak to peak distance *a* and *b* (see Fig. 3). The ratio determined for native cytochrome *c* (about 0.46) is lower than observed for the ruthenium-modified protein

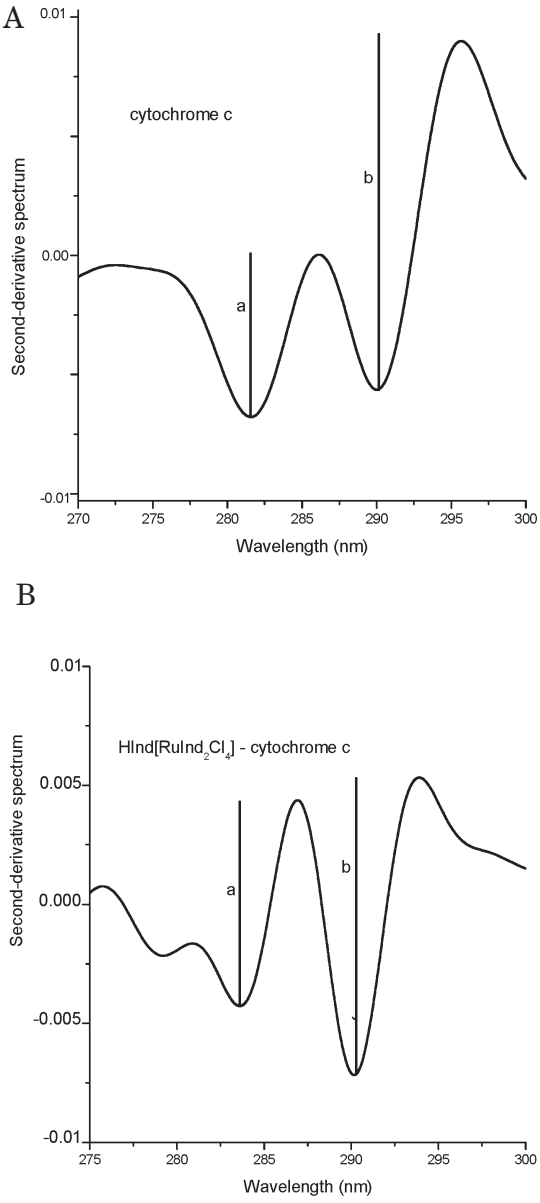


Figure 3. Second-derivative of absorption spectra of native cytochrome *c* (A) and HInd[RuInd$_2$Cl$_4$]-cytochrome *c* (B).

The second-derivative spectra are identified by the two lines **a** and **b,** which indicate the peak distances between the maximum at 287 nm and the minimum at 283 nm, and the maximum at 295 nm and the minimum at 290.5 nm, respectively. The values of the ratio **a**/**b** determined for the native protein = 0.46, for the HInd[RuInd$_2$Cl$_4$]-modified cytochrome *c* = 0.7.

(about 0.7). These data suggest that the increase of the *a/b* ratio between the native form of cytochrome *c* and that perturbed by the ruthenium complex is related to changes occurring in the tyrosyl microenvironment, and indicate a more open conformation compared to that of the native protein. The ratios determined for native proteins by Ragone (Ragone *et al.*, 1984) are in all cases lower than those observed for unfolded proteins. The ratio a/b determined for cytochrome *c* in 6 M guanidine hydrochloride was 2.2.

Influence of HInd[RuInd$_2$Cl$_4$] complex on the heme state

Absorption and CD spectra of the heme group in cytochrome *c* can be used to monitor changes in the state of this prosthetic group and gain information about the tertiary structure around the heme moiety (Dysan & Beattie, 1982). Circular dichroism of the heme group in cytochrome *c* was measured in order to monitor structural changes in cytochrome *c* after HInd[RuInd$_2$Cl$_4$] modification. A Cotton effect in the Soret region, with a positive band at 400 nm and a negative band at 420 nm is exhibited in the CD-visible spectra. The spectrum for HInd[RuInd$_2$Cl$_4$]-bound cytochrome *c* displayed similar features as native cytochrome *c*, but with decreased peak intensities. More specifically, the intensity of the wide negative band around 420 nm decreased distinctly, while the positive band shifted to 405 nm (Fig. 4). The exact mechanism resulting in the optical activity in the Soret region around 400 nm is uncertain, and the cytochrome *c* spectrum cannot be assigned with absolute precision to a specific transition. Nevertheless, CD spectra in this region are strongly dependent on the immediate conformational environment of the heme group and they can serve as an indicator of even small conformational variations in the protein structure (Barker & Ferguson, 1999). Thus, coordination of the ruthenium complex changes the heme environment, suggesting

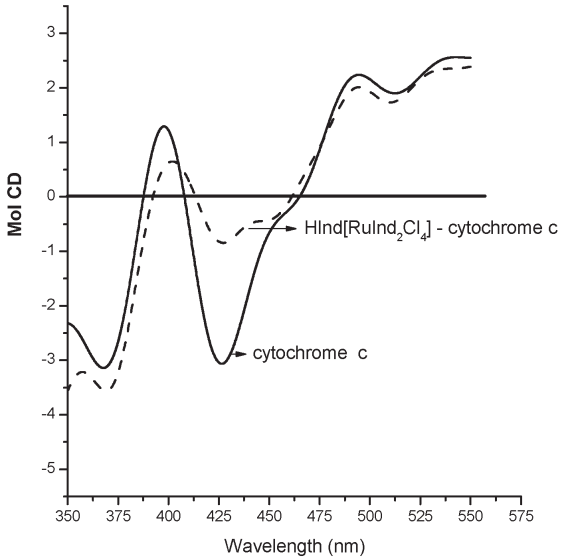


Figure 4. Effect of HInd[RuInd$_2$Cl$_4$] on the Soret region CD spectrum of cytochrome *c*.

Cytochrome *c* in the absence of ruthenium complex (—) and with HInd[RuInd$_2$Cl$_4$] at a molar ratio ruthenium complex/protein, 2:1, after 24 h incubation at 37°C (- - -). Concentration of cytochrome *c* = 0.4 mM. Mol CD units are: dm^{3}mol^{-1}cm^{-1}.

that conformation of cytochrome *c* coordinated with ruthenium is different from that of native cytochrome *c*.

Interaction of HInd[RuInd$_2$Cl$_4$] complex with apocytochrome *c*

Apocytochrome *c* is the haem-free precursor of cytochrome *c*. In contrast to the holoprotein, apocytochrome *c* is largely unstructured in aqueous conditions, with a residual amount of α-helix likely to be associated with the C-terminus (Fisher *et al.*, 1973). Interactions between the polypeptide chain and the heme moiety are needed to achieve a folded state. The non-covalent binding of heme to apocytochrome *c* promotes a structural organization of the polypeptide chain, particularly an increase in the α-helical content (Dumount *et al.*, 1994). Although non-covalent binding of heme to apocytochrome *c* has been shown to induce folding of the protein, the resulting conformation certainly differs from that of native cytochrome *c*. The occurrence of secondary structures in purified

apocytochrome *c*, heme-apocytochrome *c* and HInd[$RuInd_2Cl_4$]-apocytochrome *c* was investigated by CD measurements. As seen in Fig. 5 the apoprotein, in contrast to native

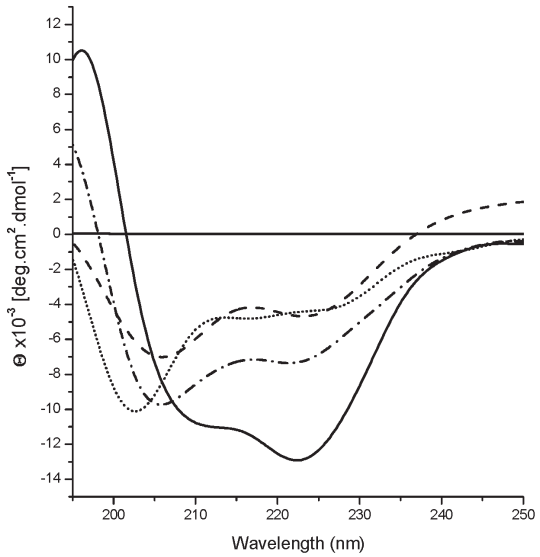


Figure 5. Reconstitution of cytochrome *c* by the heme and HInd[$RuInd_2Cl_4$] binding to apocytochrome *c*.

UV-CD spectra of native cytochrome *c* (—), apocytochrome *c* (• • • • •), apocytochrome *c*-heme (– · –) and apocytochrome *c*-HInd[$RuInd_2Cl_4$] (- - -). Spectra were recorded after 1 h of incubation at 37°C. Molar ratio apocytochrome *c*/heme and apocytochrome *c*/HInd[$RuInd_2Cl_4$] = 1:2. Concentration of cytochrome *c* = 50 μM, apocytochrome *c* = 48 μM.

cytochrome *c*, exhibits negative ellipticities around 200 nm. A minimum at this wavelength is characteristic of a random coil conformation. The far UV-CD spectra of the heme-apocytochrome *c* and HInd[$RuInd_2Cl_4$]-apocytochrome *c* complexes showed an increase in the amplitude of the ellipticity in the 220 and 208 nm regions. Changes in the CD spectrum of apocytochrome *c* are consistent with an increase in the α-helical content of the protein on binding of heme or the ruthenium complex. The results indicate that both the heme- and ruthenium-apocytochrome *c* complexes, although quite different from native holocytochrome *c*, do have some organized secondary structure. Comparison of the spectra of heme- and HInd[$RuInd_2Cl_4$]-apocytochrome *c* indicates that formation of the ruthenium complex increases the secondary structure content but distinctly less efficiently than the noncovalent heme binding. The UV-visible difference spectra (not shown) of heme binding to apocytochrome *c* and ruthenium-modified apocytochrome *c* show that the effect of the ruthenium complex on the heme binding is very weak, suggesting that the binding sites of heme and the ruthenium complex are different.

The present data reveal that binding of ruthenium complexes (a potential new metallopharmaceutical) to cytochrome *c* can induce a conformational change of the protein with a loss of organized tertiary structure, change of the heme group state, and increase in the α-helical content of apocytochrome *c*. It seems plausible that the coordination of the HInd[$RuInd_2Cl_4$] complex and the consequent conformational changes can influence the biological function of cytochrome *c*.

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