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Similar rates for platination of hairpin loops and single-stranded DNA †

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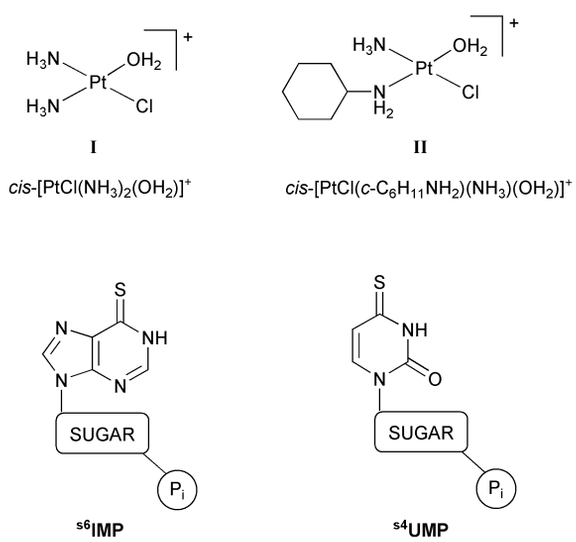
The presence of ribonucleic acid hairpin structures is important for proper processing of the genetic information in living cells. Recent studies indicate that interactions between platinum based anticancer active metal complexes and hairpin motifs result in a change of their three-dimensional structure combined with a decrease of the melting temperature. We here report a study of the reaction between two platinum complexes, *cis*-$[PtCl(NH_3)_2(OH_2)]^+$ **I** and, *cis*-$[PtCl(NH_3)(c\text{-}NH_2C_6H_{11})(OH_2)]^+$ **II**, and a series of short DNA hairpins which all exhibit a documented preference for adduct formation with donor groups located in the loop region; d(CGCGTTXTTCGCG), where X = G-N7, p(S), ^{s6}I or ^{s4}U. The binding kinetics of adduct formation with the hairpins have been compared with that of binding to similar size single-stranded DNA; $d(T_6XT_6)$, where X = G, p(S), ^{s6}I or ^{s4}U. The overall picture reveals rather similar reactivity of the platinum complexes towards these two DNA secondary structures. However, the smaller complex **I** exhibits a tendency for preferential interaction with the hairpins, whereas the more bulky complex **II** shows a higher reactivity towards the single-stranded structures.

Introduction

The anticancer activity of cisplatin (*cis*-$PtCl_2(NH_3)_2$) is most likely a result of its ability to form bifunctional adducts with DNA.[1–7] The preferentially formed crosslink between adjacent guanine bases, the intrastrand GG-adduct, is recognized by the HMG-family of proteins.[8–10] The resulting cellular consequences include interference with the repair process and induction of apoptosis.[8,11–13] Cisplatin is currently used in the clinic worldwide, with a documented high efficacy when used in combination therapy for the treatment of testicular, head and neck and ovarian cancer.[14,15] However, the development of alternative, metal-based drugs still remains an area of intense research. The work is driven by the search for replacement drugs that could be used *e.g.* after development of resistance, or to minimize the toxic side effect.[16–19] Among compounds tested, the family of platinum based metal complexes constitutes the most promising group of coordination complexes with documented anticancer activity.[20] It is likely that many of these compounds exert their activity through interaction with cellular ribonucleic acids. A challenge for future work in this area includes the construction of a metal complex with higher sequence specificity compared with that exhibited by cisplatin.[21]

The distribution of cisplatin adducts on extended DNA-structures is kinetically controlled. Weak, electrostatic interactions play a major role in directing the metal complex towards the DNA surface.[22–26] In addition, factors such as solvent accessibility and DNA deformability around the potential binding site are likely to be of influence on the final distribution of adducts along a given DNA- or RNA-sequence.[27–30] A current goal for work in our group comprises a better understanding for how the metal environment should be modified to optimise the conditions for highly specific interaction with predetermined ribonucleic acid sequences.

The present study has been designed with the aim to evaluate the influence of DNA secondary structures on the rate of metallation. The reactivity of a 13-mer DNA hairpin with a binding site incorporated into the middle position was compared with that of a single-stranded DNA of similar size: d(CGCGTTXTTCGCG), and $d(T_6XT_6)$ respectively, where X = G-N7, p(S), ^{s6}I or ^{s4}U, see Scheme 1. Previous studies in our group have shown that selective interaction with the group located in the loop can be obtained due to the kinetic preference for interaction with this site.[24,27,31,32] Two cationically charged platinum complexes, *cis*-$[PtCl(NH_3)_2(OH_2)]^+$, **I** and, *cis*-$[PtCl(NH_3)(c\text{-}NH_2C_6H_{11})(OH_2)]^+$, **II** were used to evaluate the influence from structural changes of the metallation reagent. Compound **I** is the active metabolite of cisplatin, and **II** is



Scheme 1 Representation of studied molecules

† Electronic supplementary information (ESI) available: Tables S1–S3: Observed pseudo-first-order and apparent second-order rate constants. Figs. S1–S3: CD, DSC and UV/vis spectra. Fig. S4: HPLC traces. Fig. S5: Observed pseudo-first-order rate constant plots. See http://www.rsc.org/suppdata/dt/b3/b302477f/

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considered to be the active metabolite of JM216, an orally active Pt(IV) compound currently undergoing Phase III clinical trials.[1–10,33,34] The study reveals similar reactivity towards both types of DNA secondary structures. However, the smaller complex **I** exhibits tendency for preferential interaction with the hairpins, whereas the more bulky complex **II** shows a higher reactivity towards the single-stranded structures.

Experimental

Chemicals

Buffer solutions were prepared from potassium hydrogen phthalate ($KHC_8H_4O_4$, ACROS), and the pH was adjusted by addition of $HClO_4$ (Merck, p.a.).[35] The phthalate buffer was diluted 100 times for the measurements and $NaClO_4$ (Merck, p.a.) was added to adjust the ionic strength (only monovalent cations were used in the buffer typically $[Na^+] = 9.5$ mM and $[K^+] = 0.5$ mM giving [cation] = 10 mM). The pH measurements were performed at 298 K, by use of a Methrom 744 pH meter. Stock solutions were kept at room temperature.

Preparation of platinum compounds

The compound *cis*-$PtCl_2(NH_3)(c\text{-}NH_2C_6H_{11})$ was synthesised according to literature procedures,[36] and *cis*-$PtCl_2(NH_3)_2$ (Sigma) was used as received. The corresponding aqua complexes *cis*-$[PtCl(NH_3)_2(OH_2)]^+$, **I** and *cis*-$[PtCl(NH_3)(c\text{-}C_6H_{11}\text{-}NH_2)(OH_2)]^+$, **II** were prepared as approximately 30 and 50 mM solutions as described previously.[25] Previous studies have shown that the Cl^- ligand *trans* to the coordinated cyclohexylamine-moiety is the most labile one,[37] however, the other Cl^- ligand is also labile giving rise to a mixture of aqua-complexes with similar reactivity in the solution.[32]

Oligonucleotides

The base-modified oligonucleotides $d(T_6{}^{s6}IT_6)$, $d(T_6{}^{s4}UT_6)$, d(CGCGTT^{s6}ITTCGCG), and d(CGCGTT^{s4}UTTCGCG) were synthesised according to procedures described previously.[38–40] The oligonucleotides $d(T_6GT_6)$, d(GCGCTTp-(S)TTGCGC), and d(CGCGTTGTTCGCG) were purchased from Scandinavian Gene Synthesis AB. Stock solutions were kept frozen at −80 °C. The concentrations were determined by use of UV/vis spectroscopy at ambient temperature and pressure, and by using calculated extinction coefficients.[41] Spectra were recorded using a Milton Roy 3000 Diode-array or a Cary 300 Bio Spectrophotometer and thermostated 1.00 cm Quartz Suprasil cells.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter equipped with a Jasco temperature controller peltier. Collected data was evaluated on a computer with a J-700 software. Spectra were recorded from 320 to 220 nm at 25 °C or as a function of temperature between 10 and 90 °C with a heating rate of 1 °C min^{-1}. Five different concentrations within the range of 1.0×10^{-4}–1.0×10^{-5} M were examined. The obtained CD spectra in units of ellipticity were converted to molar ellipticity ($\Delta\varepsilon$). Each reported spectrum is an average of three collected spectra.

Differential scanning calorimetry

The specific enthalpy of the system was determined as a function of temperature by use of differential scanning calorimetry (DSC). The measurements were carried out on a MicroCal VP-DSC (Microcal Inc.) instrument, equipped with dual 0.5 ml cells for the sample and reference solutions. All solutions were thoroughly degassed prior to measurement. Buffer solutions were introduced into the cells with a Hamilton syringe, and reference thermograms were recorded as a function of time during the set of measurements. The oligonucleotide sample was introduced into the sample cell and measured against buffer under identical conditions with a constant temperature increase of 1 °C min^{-1}. The excess apparent specific heat, c_{ex}, is proportional to the excess heat capacity ΔC_p, and was determined by subtraction of the baseline from the obtained trace. The melting temperature of the oligonucleotide t_m, was determined at the maximum value of c_{ex}, *i.e.* the temperature at which half of the oligomer is denatured. In a two-state process, t_m, should be independent of concentration if no association or dissociation accompanies the transition.

HPLC measurements

The reactions were followed by use of a LaChrom chromatographic system (Merck-Hitachi, working under Microsoft Windows 3.51) with a D-7000 interface and a D-7400 UV/vis detector. Separation was achieved using a reverse phase Protein & Peptide C18 (300 × 4.6 mm I.D., 10 μm particle diameter) column (Vydac) or a reverse phase C18 (250 × 4.6 mm I.D., 5 μm particle diameter) column (Hydrosphere) both equipped with a guard. Solutions of 0.1 M ammonium acetate (NH_4OAc, Merck), **A** adjusted to pH 6.0 with acetic acid (HOAc, Merck), and a 1 : 1 mixture of **A** and acetonitrile (CH_3CN, LAB-SCAN), **B** were used as eluents. Typically, linear gradients were generated, in which the ratio **A** : **B** changed from 84 : 16 or 82 : 18 to 72 : 28 over a period 22 min, by use of a low-pressure gradient system at a constant flow of 1 ml/min, and at a constant temperature of 25.0 ± 0.2 °C. The peak area of eluting reactants and products was determined by use of an on-line HPLC System Manager Software. The time-dependent changes of the peak areas were used for the kinetic evaluation.

Platination reactions followed by HPLC

The oligonucleotides were allowed to react with an approximately 40-fold excess of complex **I** or **II** in dilute phthalate buffer at 25 °C and pH 5.5. Sample aliquots were withdrawn at appropriate time intervals and diluted 10 times in buffer. The samples were stored in liquid nitrogen at −196 °C and injected on the HPLC directly after thawing. Observed pseudo-first-order rate constants were obtained from the fit of a single exponential to the time dependent peak areas corresponding to unreacted oligonucleotide. The stability of the oligonucleotides in the buffer was confirmed by UV/vis spectroscopy.

Results

Structure

The CD spectrum of d(CGCGTTGTTCGCG), is indicative of a B-form structure, with a positive band centred at 280 nm, a negative band at 254 nm, and zero at approximately 270 nm (ESI, † Fig. S1). Thermal melting studies using CD spectroscopy give monophasic transitions between 65.8–67.5 °C. Differential scanning calorimetry (DSC) measurements show a major helix to coil transition at 63.0 ± 1.1 °C (337.1 ± 1.1 K) (ESI, † Fig. S2). Closer inspection of the thermal CD and DSC traces also reveal a small transition at 30 °C. Time dependent CD spectra were recorded for the reaction of **I** and **II** with d(CGCGTT-GTTCGCG) over a period of 60 min, see Fig. 1(A) and (B). The reactions with **I** and **II** result in a decrease of intensities of CD bands related to the presence of B-form DNA, *i.e.* at approximately 280 nm and 254 nm, without formation of any other distinct structure such as A- or Z-DNA.[27,42]

Adducts

Reactions of **I** or **II** with the oligonucleotides result in formation of complexes of the type *cis*-$[PtClL(NH_3)(c\text{-}C_6H_{11}\text{-}NH_2)]$ and *cis*-$[PtClL(NH_3)_2]$, respectively, where L = G-N7,

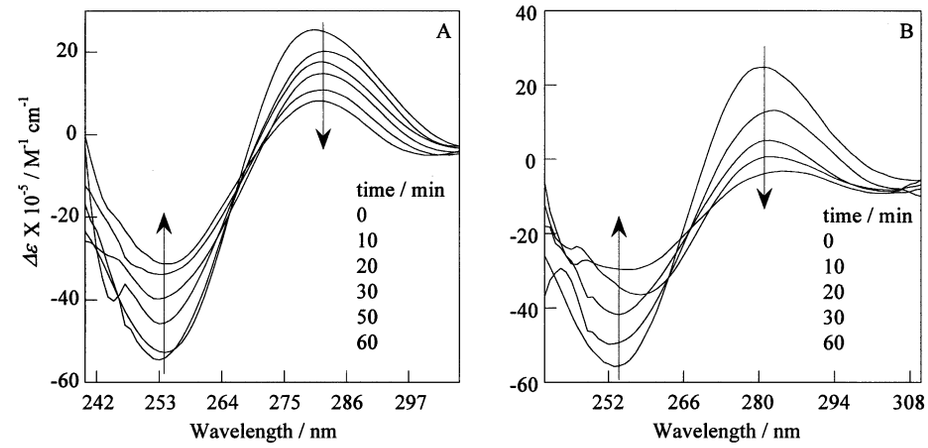

Fig. 1 CD spectra at different times for the reaction of d(CGCGTTGTTCGCG) with (A) **II** and (B) **I** at 25 °C and pH 5.5, [Pt(II)] = 5.5×10^{-4} M, [oligo] = 4.5×10^{-5} M and [cation] = 10 mM.

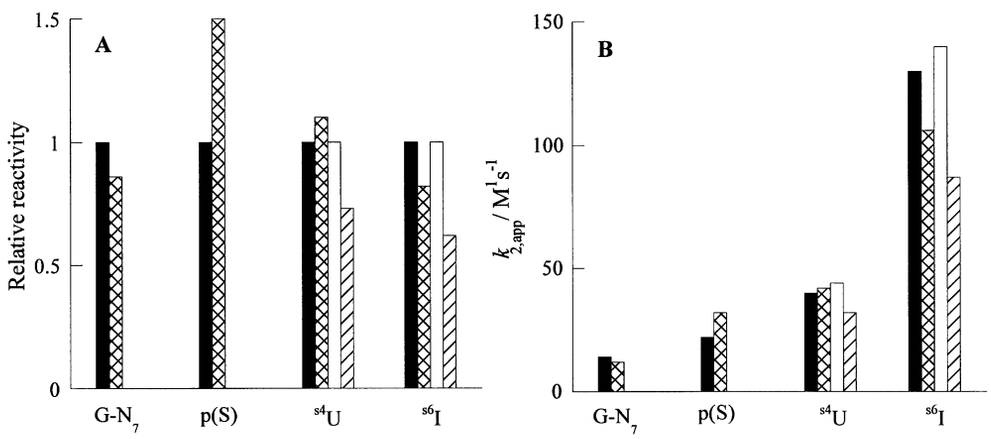

Fig. 2 (A) Relative reactivity, and (B) apparent second-order rate constants for adduct formation of **I** with hairpins (white) and single-stranded (hatched) and **II** with hairpins (black) and single-stranded (crossed), as a function of donor groups, [cation] = 10 mM.

p(S), ^{s4}U or ^{s6}I is incorporated into the central position of the studied oligomers. Coordination to ^{s4}U and ^{s6}I was confirmed by UV/vis spectroscopy and by ^{1}H NMR spectroscopy.[32] Reactions with the thiones were studied by observation of the decrease in absorbance at 322 or 332 nm, and the concomitant increase at 350 or 370 nm, for ^{s6}I or ^{s4}U respectively (Typical example given in ESI,†Fig. S3 of the reaction between **I** and d(CGCGTT^{s6}ITTCGCG)). Selective platination of the centrally located both guanine base and p(S)-linkage in d(T_6XT_6) and d(CGCGTTGTTCGCG) has been confirmed previously by us.[23,27]

Kinetics

The difference in retention time between unreacted oligonucleotides and the corresponding platinated ones allows for determination of rate constants for the metallation reactions by use of HPCL methodology. Pseudo-first-order rate constants, k_{obs}, were obtained by a fit of a single exponential to the normalised HPLC peak areas (ESI, † Fig. S4). Apparent second-order rate constants were calculated according to $k_{2,app} = k_{obs}/[Pt(II)]_{tot}$ (ESI, † Table S1 and Fig. S5), *c.f.* discussion below concerning rate law. The data allows for construction of a reactivity series in order of decreasing platination rates; $k_{2,app}$: ^{s6}I > ^{s4}U > p(S) > G-N7 see Fig. 2. For example, the second-order rate constants for reactions of **II** with d(CGCGTT^{s6}ITTCGCG), d(CGCGTT^{s4}UTTCGCG), d(GCGCTTp(S)TTGCGC) and d(CGCGTTGTTCGCG) are 130 ± 9, 40 ± 2, 22 ± 1, and 14 ± 1 M^{-1} s^{-1}, respectively. The rate constants for the corresponding single-stranded oligomers exhibit the same overall trend with similar absolute rate constants; 106 ± 5, 42 ± 3, 32 ± 1, and 12 ± 2 M^{-1} s^{-1}, for reactions with d(T_6^{s6}IT_6), d(T_6^{s4}UT_6), d(T_6p(S)T_6), d(T_6GT_6), respectively (complete data in ESI,† Tables S2 and S3).

Discussion

Structure of unreacted DNA

The structure of the oligonucleotides d(T_6XT_6), where X = G-N7 p(S), ^{s6}I or ^{s4}U, is likely to be rather flexible in solution under the salt conditions employed, [cation] = 10 mM.[43] Conformational energy minima may however exist for both helical structures[43] and bent ones.[44,45] In contrast, the CD measurements of the oligonucleotide d(CGCGTTGTTCGCG) indicates that this DNA exhibits a well defined B-DNA structure (ESI, † Fig. S1), due to formation of the base-paired stem region in the hairpin.[27,46,47] The thermal melting studies confirm this picture with loss of both the positive and negative maximum, at λ = 281 and 253 nm respectively, when monitored by CD. Further, the DSC-studies allows for the melting process to be fitted to a two-state model with only the initial and final states significantly populated (t_m = 63.0 ± 1.1 °C). It should be noted though, that the DSC-data also allows for a fit to a non two-state model, *i.e.* unfolding in a step-wise manner. It might thus be possible that this method allows for resolution of the individual contributions to the melting process from at least two of the sub-regions of the hairpin; the stem, stem-loop junction and/or loop region, all in agreement with observations made by others.[43,44,46]

Structural changes accompanying platination

The circular dichroism (CD) spectra show that d(CGCGTT-GTTCGCG) changes its structure after reaction with **I** and **II**. The band intensities decrease with half-lives ($t_{1/2}$) of approximately 20 min, which is in good agreement with the half-life that can be estimated from the obtained rate constants, *vide supra*, giving $t_{1/2} \approx 15$ min under our experimental conditions with $C_{Pt} = 5.3 \times 10^{-5}$ M. These observations clearly show that an initial change of the oligonucleotide structure occurs as a result of formation of the covalent monoadduct. Further, in the presence of monovalent cations, the platination step occurs in the absence of formation of any other distinct structural DNA-type.[42] Previous studies by us have indicated that platination of DNA-hairpin structures may result in formation of Z-DNA.[27] The latter type of structure was observed as the thermodynamically stable one only in the presence of Mg^{2+}, however. The here obtained results thus confirm the presence of Mg^{2+} as crucial for the induction of Z-DNA during the time-span covering both the initial platination step and the subsequent rearrangement of the DNA-structure that may follow platination.

Kinetics

Rate law. The studied reactions are all relatively fast, with half-lives in the range of 1.5–20 min. The reaction time is thus short enough to take place before any significant hydrolysis of **I** or **II** has taken place.[48] Further, the pH chosen for the study, pH 5.5, is well below the pK_a-values for **I** and **II**, which allows the aqua complexes to be the dominating species in solution.[37,49] The reaction studied can thus be simplified to the direct replacement of the aqua-ligand on the cationically charged platinum complex (Pt–OH_2^+) by the approaching nucleophile (L^{-n}; n = 0, 11 or 12) in a bimolecular, one-step process as schematically outlined in eqn. (1). The corresponding rate of the reaction is $v = k$[Pt–OH_2^+][L^{-n}]. Support for this reaction model is given by the experimentally observed linear correlation between the observed pseudo-first-order rate constant (k_{obs}) and the excess concentration of the metal reagent, and allows for determination of the apparent second-order rate constant according to $k_{2,app} = k_{obs}$/[Pt–OH_2^+].

$$\text{Pt–OH}_2 + \text{L} \longrightarrow \text{Pt–L} + \text{H}_2\text{O} \qquad (1)$$

Inherent reactivity of I and II. The similar coordination environment of **I** and **II**, with one H_2O molecule, one chloride ligand, and two nitrogen donors coordinated to the metal, gives rise to similar reactivity of these two complexes. A comparison of their reactivity towards the sterically less demanding single-stranded poly d(T) fragments shows however a systematic higher reactivity of **II**, see Fig. 2, that is likely an effect of the more pronounced *trans*-effect exerted by the cyclohexylamine ligand compared with that of ammine.[37,50]

Inherent reactivity of DNA target. The presently investigated systems reveal that the adduct formation rate is dominated by type of target d(^{s6}I), d(^{s4}U), p(S) and G-N7 rather than detailed structure of neither DNA nor the metal complexes, see Fig. 2(B). The relative reactivity of the nucleophiles agrees well with previous studies of related systems, *i.e.* showing a higher reactivity for the sulfur-donors compared to reaction with G-N7. Of the three sulfur-donors studied, ^{s6}I exhibits the highest reactivity, exceeding that of G-N7 with almost one order of magnitude for reactions in both single-stranded and hairpin structures, see Fig. 2 (complete data in ESI,† Tables S2 and S3). For the least reactive sulfur-donor, the p(S) linkage, the kinetic preference is only a factor of 2–3, but still allowing for platination of these groups prior to substantial interaction with closely located G-N7 has occurred.[51]

DNA structural influence. The distribution of platinum adducts on DNA is likely to be controlled by kinetic factors. The apparent reactivity of a binding site is a combination of factors such as (*i*) electrostatic conditions for approach, (*ii*) solvent accessibility in the close vicinity of the reactive site, (*iii*) inherent reactivity of the donor-group and, (*iv*) the ability of the molecule to accommodate for the sterics imposed on the adduct formation reaction. In the present study, the molecular environment in the direct vicinity of the platination site is identical for all oligomers studied, with two thymine bases flanking the binding site; the TTXTT-sequence, *cf*. Scheme 1. Further, the sequence belongs to a single-stranded part in both the hairpin and single-stranded oligomers. The overall picture of reactivity dominated by the type of DNA target indicates that the steric requirements imposed by the loop formation do not dramatically change the condition for the approaching metal complex. It is obvious however, that the relative effect of hairpin formation on the kinetics is a delicate interplay between (*i*) the nature of the approaching metal complex and (*ii*) exact location of the adduct formation site, see Fig. 2(B). For the more bulky complex **II**, a tendency for facilitated adduct formation is observed with the single-stranded structures during adduct formation with in particular p(S), but possibly also ^{s4}U. In contrast, adduct formation with ^{s6}I is slightly enhanced by hairpin formation, and a similar tendency is observed for adduct formation with G-N7. For the less sterically demanding complex **I**, a common trend of facilitated adduct formation in the hairpin environment is observed for reaction with both ^{s4}U and ^{s6}I. One explanation for obtained similar rates for platination of hairpin loops and single-stranded DNA might be that the single-stranded oligomers bend to hairpin-like ones in solution,[45] thus making approach to and adduct formation with these two types of DNA-structures relatively similar.

Conclusions

Adduct formation reactions with donor-atoms located on DNA are often referred to as being kinetically controlled. We here present a study of the kinetics for adduct formation between two kinetically similar anticancer active complexes, **I** and **II**, and a series of reactive groups incorporated into the middle of short 13-mer oligonucleotides. The reactivity of single-stranded poly d(T)-fragments is compared with that of hairpin structures. The present study shows that adduct formation is mainly dominated by the inherent reactivity of the reactive group on the DNA. The most rapid reaction is observed for adduct formation with ^{s6}I, exceeding that of adduct formation with G-N7 with about one order of magnitude. The effect of hairpin formation on the kinetics seems to be the result of a delicate interplay between the exact location of donor-atom in the loop and the sterics imposed on the reaction by the reacting metal complex. More specifically, facilitated adduct formation with the hairpin is observed for the smaller complex **I**, whereas the bulkier complex **II** reacts more rapidly with the open single-stranded structure. The results have implications for the understanding of biological effects of small molecule interactions with readily accessible ribonucleic acids in the living cell. For example, it seem likely that both single-stranded stretches of transcriptionally active DNA as well as the transient structures involved in processing of RNA could be potential targets. Work in our laboratory is currently underway to explore this hypothesis.

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