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The anticancer Ru(III) complex AziRu was studied in its interaction with model single strand and duplex oligonucleotides in comparison with its analog NAMI-A, currently in advanced clinical trials, using UV-vis, CD and ESI-MS techniques.
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Interaction of anticancer Ru(III) complexes
with single strand and duplex DNA model systems

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Abstract

The interaction of the anticancer Ru(III) complex AziRu - in comparison with its analogue NAMI-A, currently in advanced clinical trials as an antimetastatic agent - with DNA model systems, both single strand and duplex oligonucleotides, was investigated using a combined approach, including absorption UV-vis spectroscopy, circular dichroism (CD) and electrospray mass spectrometry (ESI-MS) techniques. UV-vis absorption spectra of the Ru complexes were registered at different times in a pseudo-physiological solution, to monitor the ligand exchange processes in the absence and in the presence of the examined oligonucleotides. CD experiments provided information on the overall conformational changes of the DNA model systems induced by these metal complexes. UV- and CD-monitored thermal denaturation studies were performed to analyse the effects of AziRu and NAMI-A on the stability of the duplex structures. ESI-MS experiments, carried out on the oligonucleotide/metal complex mixtures under investigation, allowed to detect the formation of stable adducts between the guanine-containing oligomers and the ruthenium complexes. These data unambiguously demonstrate that both AziRu and NAMI-A can interact with the DNA model systems. Although very similar in their structure, the two metal compounds manifest a markedly different reactivity with the examined sequences, with respectively either a naked Ru^{3+} ion or a $Ru(Im)^{3+}$ (Im=imidazole) fragment being incorporated into the oligonucleotide structure *via* stable linkages.

1. Introduction

In the search for novel anticancer agents endowed with high efficacy and reduced side effects, a variety of transition metals derivatives are currently under investigation as promising alternatives to Pt-containing drugs.[1-4] Great interest has been recently focused on Ru complexes, such as KP1019[5] and NAMI-A[6] (Figure 1), which show a remarkable antitumor and antimetastatic activity associated with low toxicity.[7,8] Although the favourable therapeutic properties of these complexes have been demonstrated, their mechanisms of action are still far from being understood. Pharmacokinetic studies carried out within Phase I clinical trials proved that, when these Ru-based compounds are administered intravenously, most of the metal in blood plasma is accumulated in a protein-bound form (>98%).[9,10] Interestingly, in the case of NAMI-A, binding to plasma proteins causes a drastic decrease of its cell uptake and subsequent reduction of its biological activity, thus suggesting that association to these proteins could essentially result into a mechanism of drug inactivation.[11] Conversely, it cannot be excluded that the adducts formed with serum proteins, mainly albumin and transferrin, may represent also an intermediate form protecting the metal from further degradation[12] and allowing its transport to nuclei, with DNA representing the final *in vivo* target, in analogy with the mechanism hypothesized for cisplatin.[13,14] Indeed, even if increasing experimental evidence shows that the main targets for NAMI-A *in vivo* appear to be extracellular matrix components,[15,16] Ru-based drugs are able to bind DNA, producing cell cycle arrest and thus cytotoxicity,[17-20] NAMI-A was reported to interact with DNA and block the template-primer function for DNA polymerase-catalysed DNA synthesis.[21] However, only aged (>20 h) aqueous solutions of the Ru complex rapidly react with DNA; while fresh solutions in water or in acidic buffers (pH 5.7) do not react.[22,23] Interestingly, in cisplatin-resistant MCF-7 tumour cells, treatment with NAMI-A results in increased intracellular ruthenium levels and a higher number of ruthenium-DNA adducts in comparison to cisplatin-sensitive cells.[24] In all these studies, the postulated NAMI-A/nucleic acids adducts resulted to be elusive and were not analysed from a structural point of view. Therefore, although many efforts have been produced to add pieces of information to this intricate puzzle, in-depth investigations on the interactions between Ru-complexes and nucleic acids are still necessary to elucidate their mechanisms of action in detail.

In this context, some of us recently proposed the design, synthesis and characterization of Ru(III)-containing complexes incorporated into nucleolipids as suitable prodrugs for their efficient in vivo delivery.[25-29] In detail, a NAMI-like derivative, with a pyridine replacing the imidazole ring, named AziRu, synthesized by us[25] and others[30] (Figure 1), has been derivatized with an amphiphilic nucleolipid, generating stable liposomes in physiological solutions. *In vitro* biological experiments proved that, if AziRu is not cytotoxic *per se* – similarly to NAMI-A - against MCF-7 human cancer

cells, under the same experimental conditions, the nucleolipidic AziRu-containing complexes exert a high bioactivity, with IC_{50} values in the low micromolar range.[25-29]
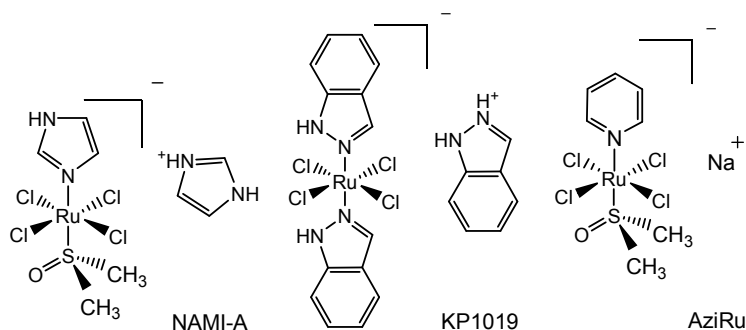

Figure 1. Structures of Ru(III) complexes NAMI-A, KP1019 and AziRu.

Stimulated by these promising results, further refinement of the original design – mainly by varying the Ru(III) ligands and their decorations – is currently in progress to produce new, more efficient Ru(III)-based anticancer agents. This optimization process can greatly benefit from increased knowledge about their reactivity towards all putative biological receptors. In fact the chemical nature and geometry of ligands present in a ruthenium complex can dramatically affect its mechanism of action - as also well exemplified in a recent contribution, in which two similar ruthenium-arene analogues proved to display very different *in vivo* activity, with one targeting the DNA of chromatin, and the other preferentially forming adducts on the histone proteins.[31] Therefore, it is relevant in our opinion to investigate the mechanism of action of AziRu as well as of AziRu derivatives with all the biologically relevant targets, including nucleic acids.

We here present a comparative study in which AziRu has been reacted with model single stranded and duplex oligonucleotides using NAMI-A as a control, and the obtained systems characterised through different biophysical methods including UV-vis and CD spectroscopy, as well as ESI MS.

2. Results and Discussion

2.1 Selection of the model systems

To shed light on the interactions of AziRu and NAMI-A with DNA, oligodeoxyribonucleotides (ODNs) carrying different sequences, in both single strand or duplex form, were incubated with the Ru(III) complexes in a saline phosphate buffered solution, and the resulting mixtures analysed using spectroscopic (UV-vis, CD) and spectrometric (ESI-MS) techniques. Under the assumption that Ru(III) complexes may behave with DNA similarly to Pt-based compounds, which show a marked preference for contiguous guanines within an ODN sequence,[13,14,32] four DNA model systems have been selected for this study: a) a single-stranded 12-mer ODN, containing a single "GG" box in the

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middle of the sequence; b) a single-stranded 12-mer ODN, with two terminal "GG" boxes; c) a 12-mer duplex, overall containing three "GG" boxes; d) a 24-mer single strand without guanines in the sequence.

More in detail, the following ODN sequences have been prepared and characterized by ESI-MS analysis (Figure S1):

- d($^{5'}$CCTCT**GG**TCTCC$^{3'}$) (**1**),

- d($^{5'}$**GG**AGACCAGA**GG**$^{3'}$) (**2**, complementary to ODN **1**), and

- d($^{5'}$TCACACACACACACACACACTT$^{3'}$) (**3**).

Upon hybridization of oligonucleotides **1** and **2** in 1:1 ratio in a saline phosphate buffer (10 mM KH_2PO_4/100 mM NaCl, pH = 7.0), the model duplex **1/2** has been obtained (Figure S2). This system has been shown to react with cisplatin forming a stable adduct, whose structure has been characterized by X-ray crystallography.[33] The resolved structure showed that, in line with the commonly accepted mechanism of action of cisplatin,[13,14] a Pt(II) species coordinates the N7 positions of two contiguous guanines at the level of the central "GG" box within strand **1**.[34] Conversely, sequence **3** has been selected as a control, guanine-free oligomer, having the same number of phosphates as duplex **1/2**.

The ruthenium complex AziRu[25,30] used in this study has been synthesized essentially following reported procedures[35,36] (Scheme S1) and identified by ESI-MS, ^{1}H NMR and UV-vis data (Figures S3-S5).

Each model system – the single strands **1**, **2** and **3**, and the duplex **1/2** – has been incubated with either AziRu or NAMI-A in the saline phosphate buffer at 37 °C and the resulting mixtures analysed over time by UV-vis, CD and ESI-MS techniques.

2.2 UV-vis studies

UV-vis analysis can provide precious information about the ligand exchange processes of metal complexes and their general reactivity, by following the evolution over time of the various chromophores. Thus, we first monitored the hydrolysis of NAMI-A and AziRu alone in the saline phosphate buffer, by recording absorption spectra at different times, and successively, under the same experimental conditions, in the presence of each of the four investigated ODN systems.

These experiments allowed to follow the formation of the aqua complexes, obtained through the exchange of the chloride or DMSO ligands with water molecules and postulated to be the species effectively reactive with the *in vivo* targets.[23,37] In the case of NAMI-A, as already documented in previous reports,[23,37-39] the band at 388 nm, attributed to the ligand-to-metal charge transfer transition, disappeared in about 2 h with concomitant increase of the band at 345 nm which in turn then started to decrease after 1 h (Figures 2a and S6a). Successively, a new hydrolysis intermediate

appeared, having a band with a maximum at 266 nm that reached its highest intensity after 6 h (Figure 2a). After longer intervals, increasing scattering phenomena, attributable to the formation of insoluble poly-oxo complexes, were observed (Figure S6b).
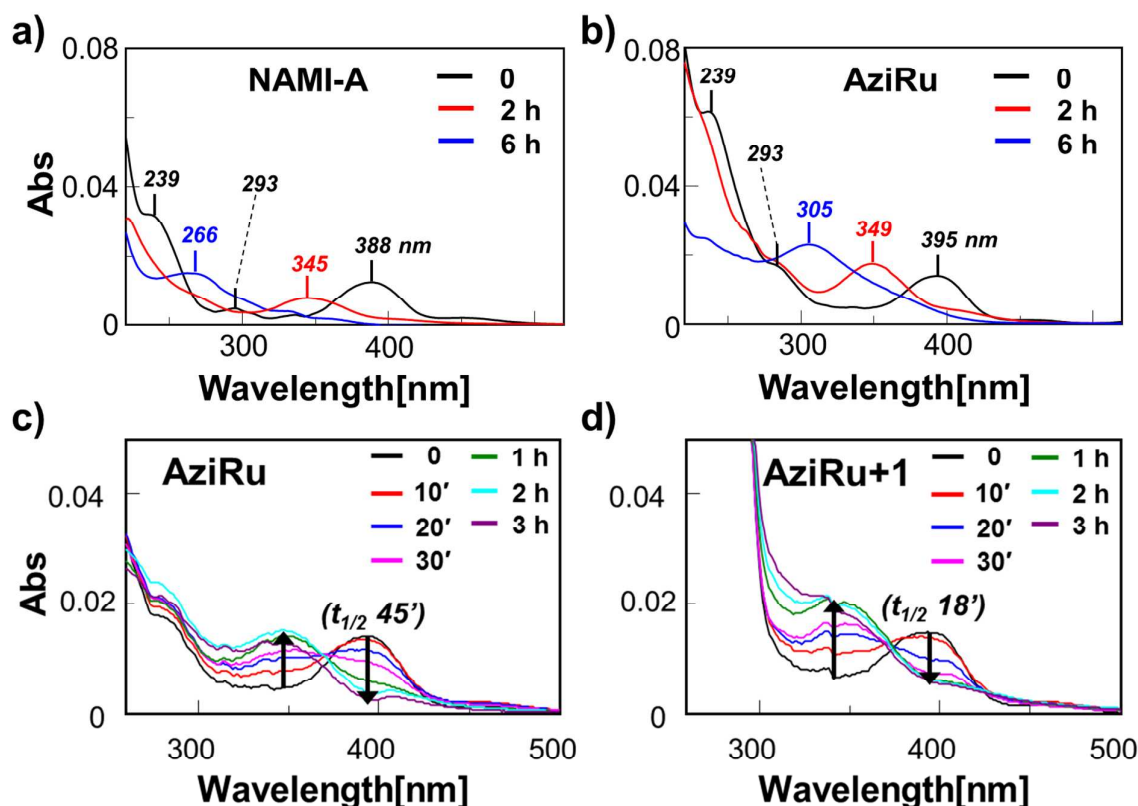

Figure 2. UV-vis absorption spectra of NAMI-A (**a**) and AziRu (**b**), both at 4 μM concentration in the saline phosphate buffer, registered at different times after dissolution; comparison of the UV spectra evolution of AziRu alone (**c**) and in the presence of ODN **1** (**d**) over the first 3 h after dissolution.

To verify whether the studied ODNs could affect the NAMI-A hydrolysis processes, the UV-vis analysis was carried out in the presence of, separately, the single strands **1**, **2** and **3**, and the duplex **1**/**2**, each dissolved at 4 μM concentration. Thus, UV-monitoring showed that the hydrolysis of NAMI-A was generally faster in the presence of ODN systems **1**, **2** and **1**/**2**, as confirmed by the evolution of the band at 388 nm. Remarkably, this band disappeared in ca. 1 h when NAMI-A was mixed with **1**, while for NAMI-A alone this process was complete in ca. 2 h, as showed by the calculated $t_{1/2}$ values, *i.e.* the half-life times of the 388 nm band (Table 1).

In addition, the UV absorbance at 260 nm, where the contribution of the oligonucleotide systems is largely prevalent and the metal complex contributes only to a minor extent, was analysed as a function of the incubation time of the single strands and duplex DNA with NAMI-A. A marked

hypochromic effect with respect to reference curves - obtained by simply adding the absorbance values of each ODN system and of NAMI-A alone, at the same concentration and hydrolysis time - for the duplex and ODN **2** (Figure S7) was observed, thus indicating that a specific interaction at the level of the nucleobases occurred in these ODN systems.

	$t_{1/2}$ [388 nm] (min, ±1)		$t_{1/2}$ [395 nm] (min, ±1)
NAMI-A	60	**AziRu**	45
NAMI-A + 1	33	**AziRu + 1**	18
NAMI-A + 2	50	**AziRu + 2**	17
NAMI-A + 3	58	**AziRu + 3**	45
NAMI-A + 1/2	41	**AziRu + 1/2**	23

Table 1. Comparison of the $t_{1/2}$ values, *i.e.* the half-life time data calculated for the disappearance of the 388 and 395 nm bands of NAMI-A and AziRu, respectively, analysed at 4 μM conc. in the absence or presence of the different ODN systems (1:1 mixtures).

In parallel experiments, AziRu was first examined alone, at 50 (Figure S8a) and 4 μM concentration (Figure 2b). The absorption spectra showed a band centred at 395 nm which, in analogy with NAMI-A, can be attributed to the ligands to Ru charge transfer transition. This band gradually diminished over time, finally disappearing in ca. 2 h, with the concomitant emergence of a new band at 349 nm which reached its highest value after 2.5 h (Figure 2b) and then started to decrease. After 6 h, the formation of a new band centred at 305 nm, attributable to a late hydrolysis product, was observed (Figure 2b). This product resulted to be quite stable in solution for 45 h, then scattering phenomena were revealed, probably due to the formation of insoluble poly-oxo complexes.[22,23] As in the case of NAMI-A, AziRu also proved to be markedly more stable when dissolved in pure water than in buffered solution at pH = 7.0 (Figure S8), as a consequence of the acidic pH produced upon its dissolution in non-buffered solutions (Figure S9). [22,23]

Subsequently, the AziRu hydrolysis process was followed by UV-vis analysis in the presence of, separately, the single strand ODNs **1**, **2** and **3**, and the duplex **1/2**, each dissolved at 4 μM concentration. In particular, in the first 6 h, the disappearance of the band at 395 nm, with the concomitant appearance of a band at 349 nm, was monitored over time, and the analysed systems were compared. As an example, the time evolution of the UV spectra of AziRu alone and in the presence of ODN **1**, over the first 3 h after dissolution, is reported in Figures 2c and 2d, respectively. The disappearance of the 395 nm band proved to be faster for systems **1**–AziRu ($t_{1/2}$ =

18 min), **2**–AziRu ($t_{1/2}$ = 17 min) and (**1**/**2**)–duplex-AziRu ($t_{1/2}$ = 23 min) than for **3**–AziRu, which showed the same $t_{1/2}$ value as AziRu alone ($t_{1/2}$ = 45 min, Table 1).

As for NAMI-A, the trend of the 260 nm band, where the main contribution to the absorbance is given by the oligonucleotide systems while that of the metal complex is only marginal, was monitored for a long time interval (0-72 h, solid lines of Figure 3). Reference curves, obtained by simply adding the absorbance values of each ODN system and of AziRu alone, at the same concentration and hydrolysis time, have been derived (dashed lines of Figure 3). From these data it emerged that the experimental and reference curves were almost superimposable only for the mixture **3**–AziRu, while significant differences, especially after 6 h incubation, were observed for the **1**–AziRu, **2**–AziRu and (**1**/**2**)–AziRu systems, suggesting that an interaction between the metal complex and these oligonucleotides occurred (Figure 3).
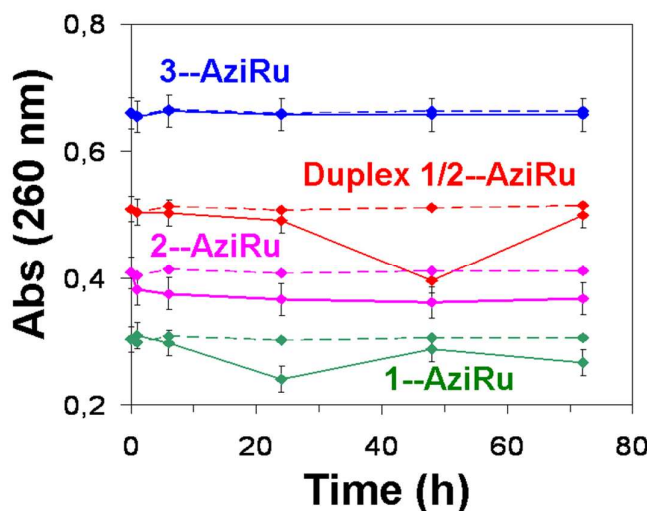

Figure 3. Incubation time dependence of the absorbance at 260 nm relative to the solutions containing ODNs **1**, **2** and **3**, and the duplex **1**/**2**, each separately mixed 1:1 with AziRu (solid lines); dashed lines are reference curves obtained by adding the absorbance of each oligonucleotidic system with that of AziRu alone under the same concentration and incubation time conditions (error bars = ±0.025).

Taken together, the UV-vis data revealed a similar behaviour for AziRu and NAMI-A alone, when dissolved in buffered solutions. Furthermore, both ruthenium complexes showed clear evidence of interaction with the single strands **1** and **2** as well as with the duplex **1**/**2**, with AziRu proved to be approximately 2-fold more reactive than NAMI-A, as determined on the basis of their $t_{1/2}$ values. In all cases, no reactivity was apparent with the guanine-free oligonucleotide **3**.

2.3 CD studies

Interactions of AziRu/NAMI-A with single strand ODNs

CD experiments were used to evaluate how and to what extent the ruthenium complexes could affect the conformation of the studied ODN model systems. To this purpose, CD spectra of single strands **1**, **2** and **3** (20 μM) were compared with those obtained upon incubation with 1 eq of the Ru complex.

In the case of **1**, containing one central "GG" box, the CD spectra of the ODN with the Ru complexes showed a gradual reduction of the 273 nm-band over time (Figure S10 for AziRu) with a concomitant red-shift of the maximum (2 nm for AziRu and 1 nm for NAMI-A, Figure 4a) and signal stabilization after 48 h (ΔCD_{max}=1.05 mdeg for AziRu, and 0.93 for NAMI-A). This behaviour is indicative of an interaction of **1** with both metal complexes.
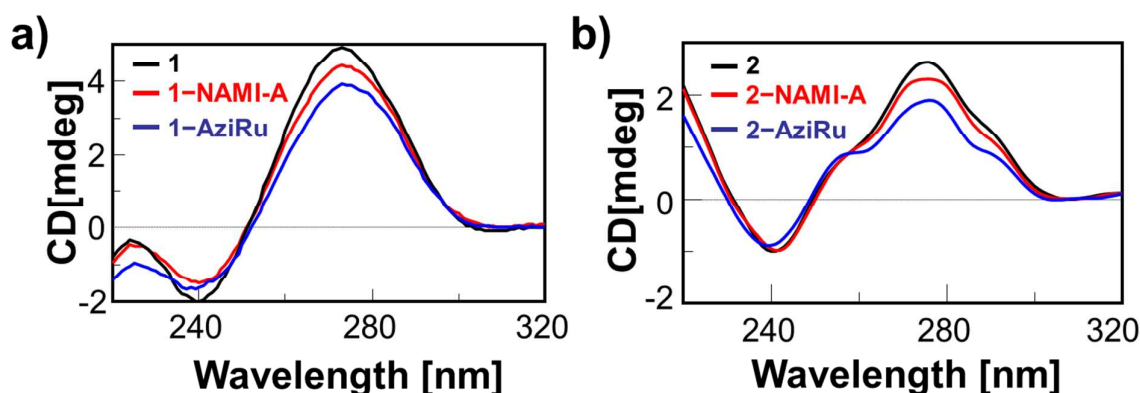

Figure 4. Comparison of the CD spectra of ODNs **1** (**a**) and **2** (**b**), at 20 μM concentration, in the absence (black curves) and presence of 1 eq of NAMI-A (red) and AziRu (blue) after 48 h incubation.

Also for ODN **2**, containing two "GG" boxes at the ends of the sequence, the spectra of the oligonucleotide in the presence of NAMI-A or AziRu showed a reduced intensity of the CD positive band (ΔCD_{276}= 0.72 mdeg for AziRu, and 0.39 mdeg for NAMI-A), accompanied by a 1 nm bathochromic shift of its maximum, compared with the oligonucleotide alone (Figure 4b). These results suggest that an interaction with both the metal complexes occurs also in this case.

On the contrary for oligonucleotide **3**, which is guanine-free, almost no difference in the CD spectra was observed even after 72 h incubation with 1 eq of either AziRu or NAMI-A in the saline phosphate-buffered solution. No effect was detected even in the presence of an excess (2 eq) of the metal complexes (Figure S11). These findings, associated with previously described UV-vis analysis results, suggest that these ruthenium salts display selective recognition towards guanine-containing ODNs, with AziRu being more reactive than NAMI-A.

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To further characterize the binding between the ruthenium metal complexes and ODNs **1** and **2**, CD titration experiments were performed by recording spectra of the ODNs treated with increasing amounts (from 0 to 10 eq) of AziRu and NAMI-A after 72 h incubation (Figures S12 and S13). In general, a reduction of the CD bands was observed, with a more pronounced effect for the incubation with AziRu compared to NAMI-A. In particular, for **1**, the 273 nm-band showed an overall reduction in intensity – larger for AziRu than for NAMI-A – with a concomitant bathochromic shift of 2 nm for AziRu and 1 nm for NAMI-A upon addition from 0 to 10 eq of the metal complex. The CD spectra did not show significant changes after addition of 9-10 eq of both AziRu and NAMI-A (Figure S12). In the case of ODN **2**, a reduced intensity of the CD band at 276 nm, more pronounced for AziRu than for NAMI-A, as well as a 2 nm blue-shift of the maximum of the positive band, were observed (Figure S13).

Subsequently, the ability of the ruthenated ODNs, **1**–AziRu and **2**–AziRu, to form duplex structures with the corresponding complementary sequence (free **2** and **1**, respectively), was evaluated through CD experiments carried out in tandem cuvettes. This kind of cuvettes allowed to register the *sum* and *mix* spectra of the two components (*i.e.*, the spectrum obtained by maintaining the two oligomers in separate compartments of the cell, and the one obtained after mixing the two solutions) for each system (**1**–AziRu/**2** and **2**–AziRu/**1**, Figure S14). In both cases, the comparison between the *sum* and *mix* spectra proved that duplex structures were formed, each with a peculiar conformation, overall slightly different from natural **1**/**2** duplex (Figure 5a). Similar results were obtained also in the case of NAMI-A (Figures S15 and 5b).
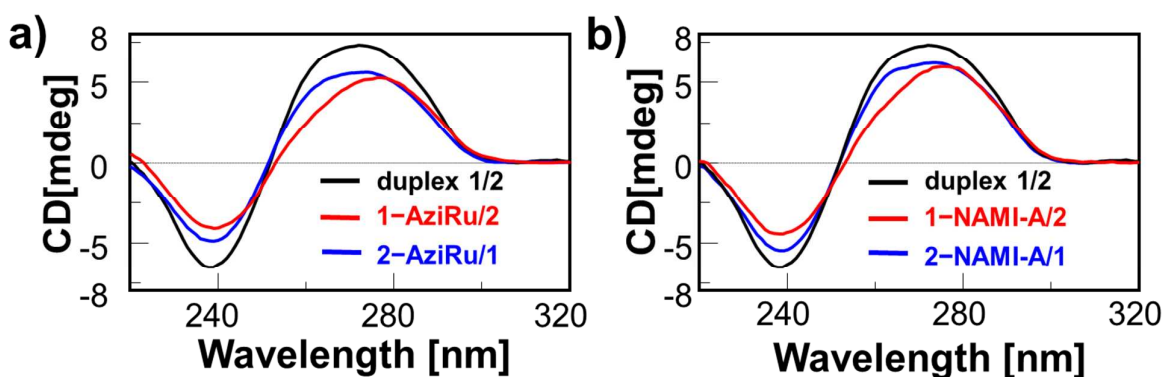

Figure 5. Comparison of CD spectra of the natural duplex **1**/**2** (black lines) and of the duplex structures derived from hybridization of the ruthenated single strands **1** or **2** with free ODNs **2** or **1**, respectively, registered at 2.5 μM concentration in the saline phosphate-buffered solution.

For all the duplex structures indicated in Figure 5, CD melting experiments were carried out (Figures 6a and 6b). In general, the metallated duplexes were slightly more stable than the natural

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duplex, and this effect was more pronounced for both the **2**–AziRu/**1** and **2**–NAMI-A/**1** duplexes (Table 2), *i.e.* the structures derived from hybridization of free ODN **1** with the metallated single strand containing the highest guanine content (**2**). Valuably, the variation of the CD signal at 276 nm between 15 and 75 °C (ΔCD) for all the ruthenated duplexes resulted to be lower with respect to the natural one. This behaviour could be probably ascribed to overall stabilizing effects of a metal-guanine interaction, in turn producing partial loss of some stacking and/or Watson-Crick interactions.
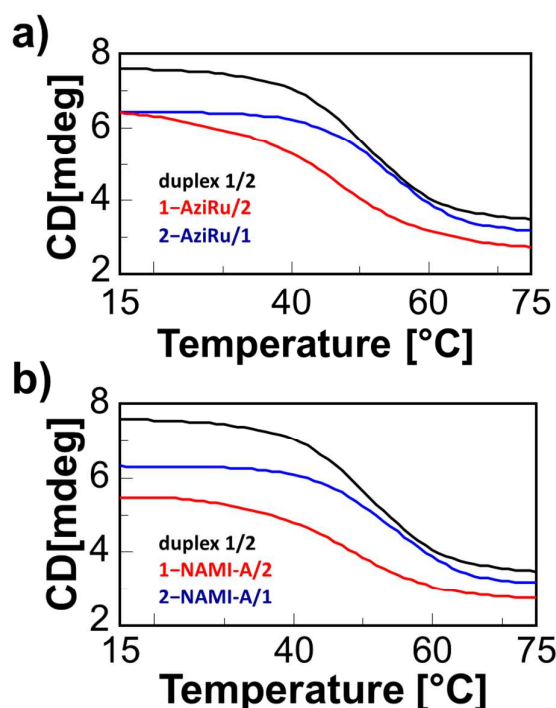

Figure 6. **a**) and **b**) Overlapped CD melting curves of the ruthenium-containing duplexes, **1**–Ru/**2** (red lines), and **2**–Ru/**1** (blue lines), and of the natural duplex **1/2** (black lines), at 2.5 μM concentration in 10 mM phosphate buffer/100 mM NaCl.

Interactions of AziRu/NAMI-A with the duplex DNA model system

Subsequently, CD measurements were carried out also to analyse the interaction of the metal complexes with the preformed duplex **1/2**, which was thus incubated with 1 eq of AziRu and, in a parallel experiment, with 1 eq of NAMI-A. The CD spectra of the metallation reaction on duplex **1/2**, monitored over time, showed a slight reduction of the positive band at 272 nm with respect to the natural duplex, with stabilization of the CD signal after 48 h. Interestingly, no shift of the positive and negative bands was observed in both cases, essentially proving that the overall conformation of the duplexes resulted almost unaffected upon incubation with the Ru(III) complexes (Figure 7).
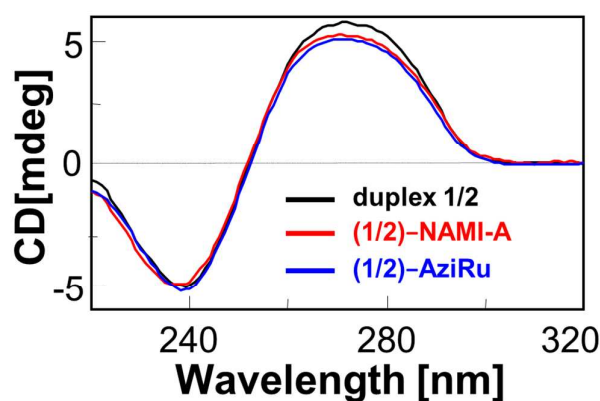
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Figure 7. Overlapped CD spectra of natural **1**/**2** duplex (20 μM), and of metallated duplexes (**1**/**2**)–AziRu and (**1**/**2**)–NAMI-A after 48 h incubation.

CD melting curves of the (**1**/**2**)–AziRu and (**1**/**2**)–NAMI-A duplexes showed slightly higher T_m, but lower ΔCD_{272} (15-75 °C), than the natural duplex (Figure 8). Thus, the interaction of the metal complex with the duplex structure, in 1:1 ratio, did not perturb the general conformation of the natural double helix, but probably determined localized, partial loss of some stacking and/or Watson-Crick interactions.
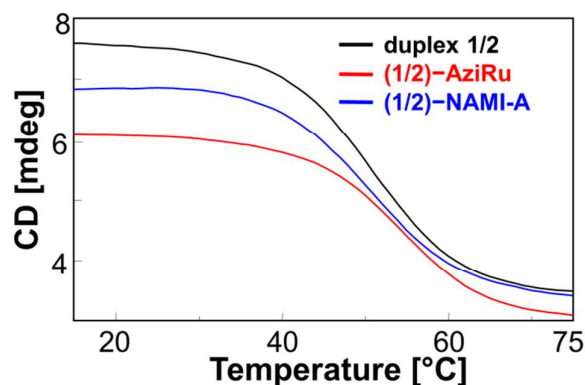
Figure 8. Overlapped CD melting curves of the metallated duplexes, (**1**/**2**)–AziRu (red) and (**1**/**2**)–NAMI-A (blue), and of the natural duplex **1**/**2** (black) at 2.5 μM concentration in 10 mM phosphate buffer/100 mM NaCl.

To get further information on the binding of the Ru(III) complexes with duplex **1**/**2**, CD titration experiments were performed (Figure S16). CD spectra of the duplex treated with increasing amounts, from 0 to 10 eq, of AziRu and NAMI-A were recorded after 72 h incubation time. A reduction in the intensity of the 272 nm band of the duplex was observed, with a red shift of its maximum of 3 nm in the case of AziRu and 5 nm for NAMI-A. Stabilization of the CD signal

occurred upon addition of 9-10 eq of both the metal complexes with an overall ΔCD of 1.4 mdeg in the case of AziRu and 1.3 mdeg for NAMI-A (Figure S16).

CD data overview

From an overview of the CD data, it emerged that, upon ruthenium complex binding, the % CD band variation, indicative of the conformational change, for the studied ODNs was more marked for ODN **2** (with the highest guanine content) than for ODN **1**, while ODN **3**, not containing guanines, remained almost unaffected in all cases (Figure 9 and S17). Smaller conformational changes were observed for the duplex structure **1/2** with respect to single strands **1** and **2**.

As far as the metal complexes are concerned, AziRu proved to be always more reactive than NAMI-A, inducing higher conformational changes in all the investigated ODN systems (Figure 9).
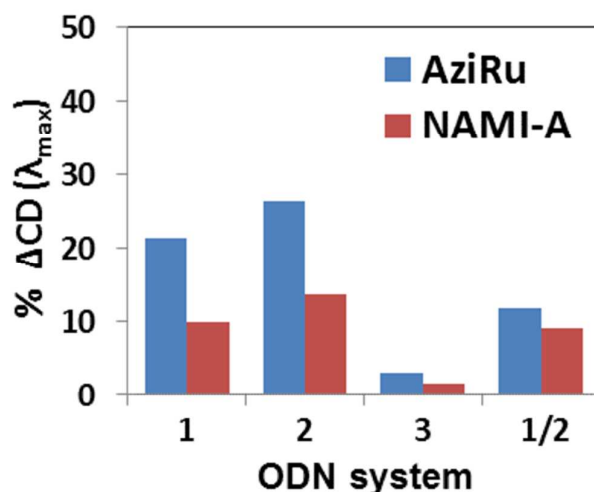

Figure 9. Comparison of the % CD band variation observed in the different ODN systems investigated, upon binding with the ruthenium complexes AziRu and NAMI-A. The errors associated with these data are within 3%.

The ruthenated single strands **1** and **2** in all cases maintained their ability to hybridize the complementary ODN, even though the resulting duplexes were sensibly distorted with respect to the natural one, particularly when the treatment with the Ru complexes was carried out on ODN **1**.

In general, all the ruthenated duplex structures - either formed by metallation of a preformed duplex or of a single strand subsequently hybridized with its complementary sequence - contained a lower degree of Watson-Crick and/or stacking interactions (< ΔCD), but were somehow stabilized by the interaction with the ruthenium complex, showing higher melting temperatures with respect to the natural duplex (Table 2).

	ΔCD(15-75) (mdeg)	T$_m$ (°C, ± 0.5)
duplex 1/2	4.10	51.1
1–AziRu/2	3.67	52.5
1–NAMI-A/2	2.70	52.0
2–AziRu/1	3.25	55.2
2–NAMI-A/1	3.12	54.6
(1/2)–AziRu	2.99	54.3
(1/2)–NAMI-A	3.42	51.2

Table 2. CD melting data of duplex **1/2** and of the metallated duplex structures here studied.

2.4 ESI-MS studies

The interaction of AziRu/NAMI-A with single strands **1**, **2** and **3** has been investigated also by ESI-MS. For all the experiments, each ODN – analysed in a 50 μM aq. solution - was incubated with 1 eq of either NAMI-A or AziRu at 37 °C and the resulting multi-charged spectra recorded after 48 h.

Interactions of NAMI-A with single strands

The interaction of NAMI-A with ODN **1** produced four peaks (Figure 10). The peaks at 728.09 and 741.69 m/z correspond to adducts characterized by the coordination to the ODN of, respectively, one naked Ru^{3+} and one [C$_3$H$_4$N$_2$Ru]$^{3+}$ fragment (in which the imidazole ring is still bound to the metal centre) to the ODN molecule. The peaks at 745.50 and 749.10 m/z correspond to ODN/Ru-complex 1:1 adducts in which the metal centre of the [C$_3$H$_4$N$_2$Ru]$^{3+}$ fragment is coordinated by one or two water molecules, respectively.
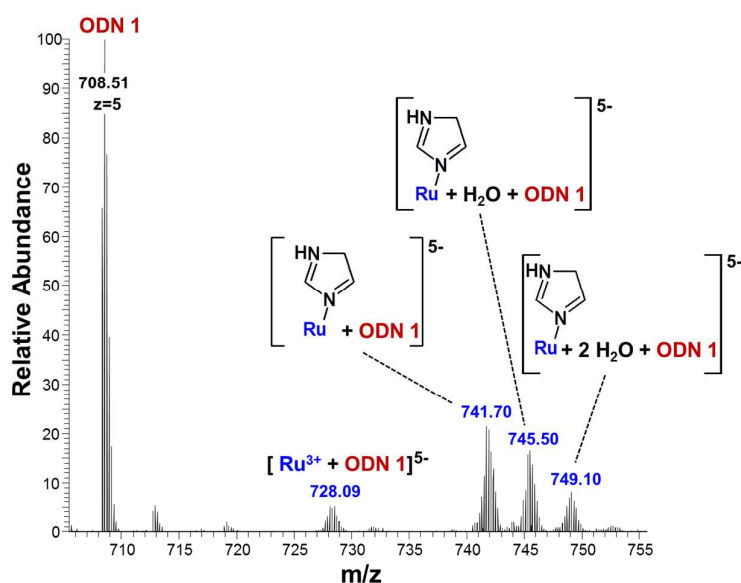
Figure 10. ESI-MS spectrum of ODN **1** (50 µM), incubated with 1 eq of NAMI-A in water at 37 °C for 48 h. CH₃OH was added to the reaction mixture before the analysis, carried out at a final concentration of 25 µM in CH₃OH/H₂O, 1:1 (v/v). Theoretical and deconvoluted peaks for the found m/z values are reported in Table S1.

From inspection of the spectra obtained after incubating NAMI-A with ODN **2**, three significant peaks, additional with respect to the spectrum of **2** alone, were detected: these can be assigned, as in the case of the experiment with ODN **1**, to the oligonucleotide bound to $[C_3H_4N_2Ru]^{3+}$, $[C_3H_4N_2Ru(H_2O)]^{3+}$ and $[C_3H_4N_2Ru(H_2O)_2]^{3+}$ fragments (Figure 11).

Remarkably, ODN **3**, incubated with NAMI-A under the same conditions used for **1** and **2**, did not show any adduct.
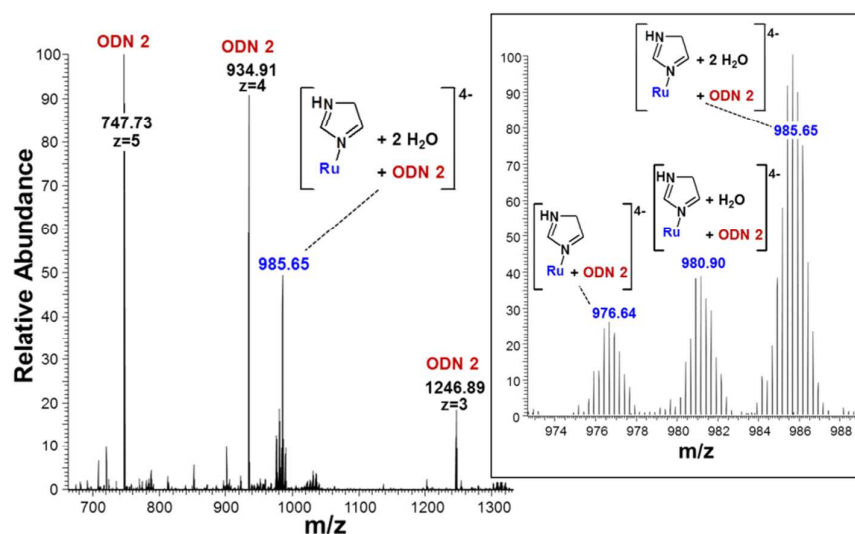

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Figure 11. ESI-MS spectrum of ODN **2** (50 µM), incubated with 1 eq of NAMI-A in water at 37 °C for 48 h. The analysis was carried out at final concentration of 25 µM in CH_3OH/H_2O, 1:1 (v/v). Theoretical and deconvoluted peaks for the found m/z values are reported in Table S2.

Interactions of AziRu with single strands

The same conditions described to investigate NAMI-A were used to assess the reactivity of AziRu with oligonucleotides. Coordination of naked Ru^{3+} was clearly detected with both ODNs **1** and **2** (Figure 12). Thus, in the case of AziRu, coordination occurs mainly through a kind of activation that involves full detachment of ligands.
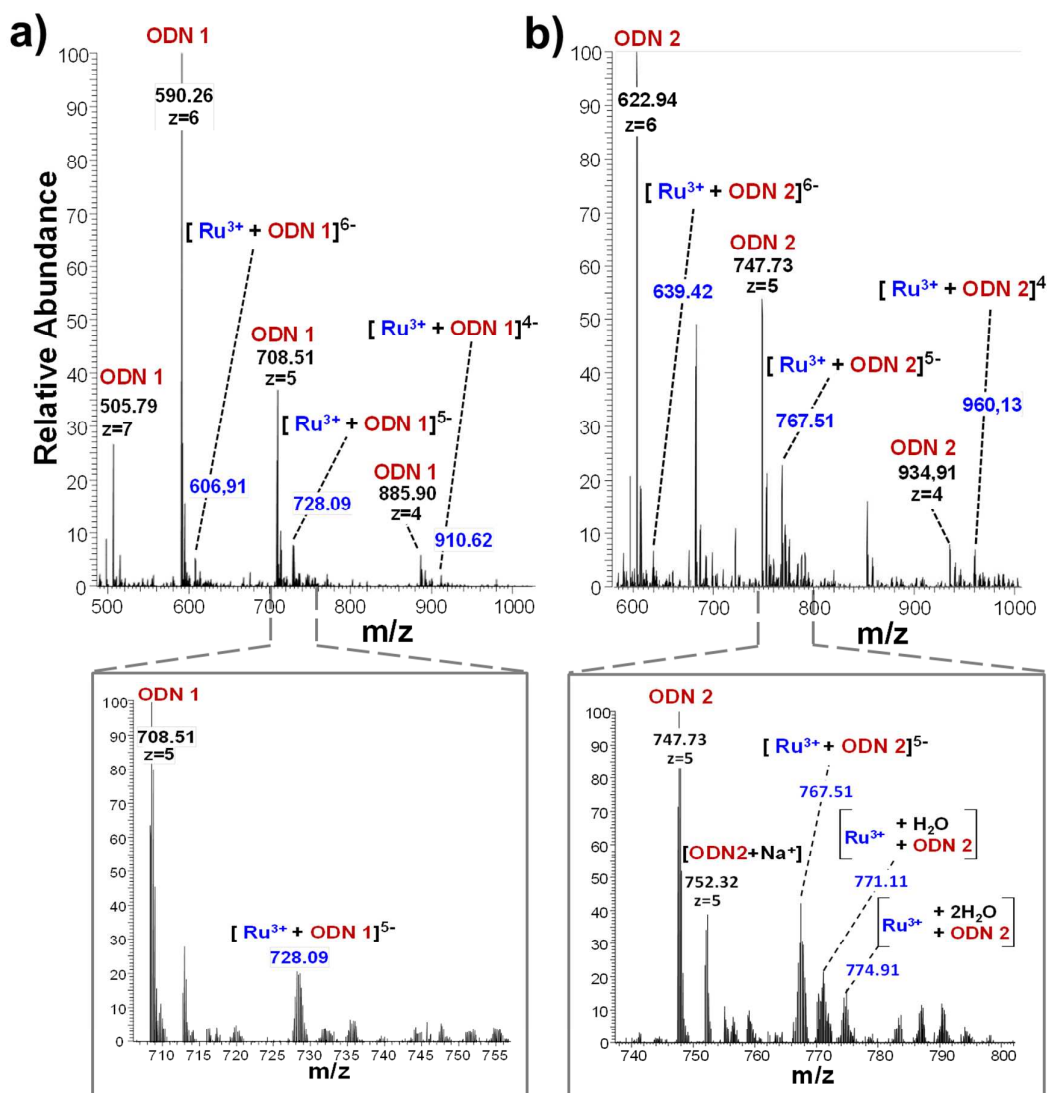
Figure 12. ESI-MS spectra of **1** (**a**) and **2** (**b**), both at 50 µM concentration, incubated with 1 eq of AziRu in water at 37 °C for 48 h, with magnification of the 700-750 or 735-800 m/z intervals, respectively. The analyses were carried out at a final concentration of 25 µM in CH_3OH/H_2O, 1:1 (v/v). Theoretical and deconvoluted peaks for the found m/z values are reported in Table S3.

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3. Conclusions

In a scenario of growing interest for Ru-based drugs, with promising data emerging from preliminary Phase II clinical trials on NAMI-A,[40,41] we have here investigated the interactions of the Ru(III) complex AziRu and of its anticancer analogue NAMI-A with DNA model systems, either single strand or duplex oligonucleotides, by means of UV, CD and ESI-MS analysis.

In line with previous reports on NAMI-A,[22,23] the hydrolysis of both metal complexes turned out to be faster in a saline phosphate buffered solution than in pure water. Interestingly, the early aquation steps, assumed to provide the effectively reactive species able to interact with *in vivo* targets, were always faster in the presence of guanine-containing oligonucleotides and showed AziRu to be ca. 2-fold more reactive than NAMI-A.

In the interactions of AziRu/NAMI-A with DNA model systems, the base composition played a key role: when incubated with ODNs **1** and **2**, AziRu and NAMI-A showed a higher reactivity in the ligand exchange processes and produced a general hypochromic effect in the 260 nm band of the oligonucleotides. On the contrary, **3** did not affect the hydrolysis rate of the two Ru(III) complexes nor experienced any UV change in the 260 nm band. These results are consistent with the CD data, which confirmed guanines as a preferential target in the reactions of AziRu/NAMI-A with single strands. For ODNs **1** and **2**, in fact, the single strand conformation was always altered upon interaction with these complexes, as revealed by the reduced intensity of the CD positive band of both ODNs, with concomitant bathochromic shift (with AziRu) and hypsochromic shift (with NAMI-A) of their maximum. In the case of **3**, whose sequence does not contain guanines, no difference was observed in the CD spectra recorded before and after incubation with both Ru complexes, in the used experimental conditions.

Subsequently, through CD experiments, we evaluated the ability of the ruthenated ODNs **1** and **2** to form duplex structures with the corresponding complementary sequence (free **2** and **1**, respectively). In both cases, duplex structures were obtained, but somehow distorted with respect to the untreated duplex and having higher T_m values and lower variation of the CD signal between 15 and 75 °C. This behaviour could be attributed to overall stabilizing effects of the ruthenium-guanine coordination, which however locally determined, presumably by steric hindrance, a partial loss of duplex structure.

Both Ru complexes also proved to interact with the preformed duplex **1**/**2**, as confirmed by UV and CD analysis. The CD spectra of the duplex **1/2** treated with either NAMI-A or AziRu showed a small reduction of the band at 272 nm, but a substantially unchanged conformation with respect to the natural duplex. CD melting curves also in these cases showed slightly higher T_m values, but

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lower ΔCD_{272} for the metallated duplexes in comparison with the untreated duplex. In conclusion, the interaction of these metal complexes with a preformed duplex did not detectably perturb the overall duplex conformation, which resulted to be more stable than the untreated double helix, even if local, partial loss of some Watson-Crick H-bonding and/or stacking interactions occurred.

ESI-MS spectra confirmed the coordination of mainly naked Ru^{3+} to ODNs **1** and **2**, in no case showing the formation of adducts with **3**. Comparing the spectra recorded for the two Ru-based compounds, it can be concluded that NAMI-A and AziRu present different activation profiles; notably, the latter reacts with oligonucleotides essentially through full detachment of ligands, thus showing pyridine to be a more labile ligand compared to imidazole.

Taken together, these data clearly demonstrate that both AziRu and NAMI-A are able to selectively interact with GG-containing DNA model systems, with AziRu sensibly more reactive than NAMI-A. Thus, the simple replacement of an imidazole ring with a pyridine moiety in axial position of the Ru(III) complex is responsible for a marked increase in the reactivity of the metal centre, able to form stable adducts with guanine-containing oligonucleotides. Interestingly, some of us have recently shown that AziRu and NAMI-A also differently react with model proteins.[42,43] These results can be useful to provide a rational basis to design novel, optimized Ru-based anticancer agents, taking into account that also tiny, point structural modifications may result into markedly different reactivity, eventually determining very different *in vivo* mechanisms of action.

4. Experimental Section

Abbreviations

Circular Dichroism (CD); DMSO (DiMethylSulfOxide); ElectroSpray Ionization Mass Spectrometry (ESI-MS); Imidazole (Im); ODN (OligoDeoxyriboNucleotide).

(**1**/**2**)–AziRu and (**1**/**2**)–NAMI-A stand for: preformed duplex coordinated with the ruthenium complexes.

1–AziRu/**2**, **1**–NAMI-A/**2**, **2**–AziRu/**1** and **2**–NAMI-A/**1** are used for: the duplex formed between the ruthenium complex-coordinated ODN (ruthenated ODNs, **1** or **2**) and the corresponding free complementary strand.

4.1 Materials, Apparatus, and General Methods

GlenPack cartridges for ODN purification were purchased from Primm (Milan, Italy). Dialysis was performed in 1 mL-Float-A-Lyzer G2 column with a molecular weight cut-off of 0.5 kDa (Spectrum laboratories Inc, Germany). All the other reagents and solvents were from Sigma-Aldrich (DMSO, NaCl, KH_2PO_4, K_2HPO_4). NAMI-A was a generous gift from prof. E. Alessio.

UV measurements were registered on a JASCO V-550 UV/vis spectrophotometer equipped with a Peltier block (PTC-348WI) by using 1 cm quartz cells of 1 ml internal volume (Hellma). UV melting curves were recorded by following the absorbance changes at a determined wavelength on increasing the temperature (heating rate 0.5 °C/min, monitoring wavelength 260, bandwidth 2 nm, response 16 s). T_m values were calculated as the maxima of the plots of the first derivative of the absorbance *vs.* temperature (error ±0.5 °C).

All the CD spectra and CD melting curves were collected on a Jasco J-715 spectropolarimeter equipped with a Peltier element PTC-348WI for temperature control, using a Teflon stoppered 0.1 cm quartz cuvette of 0.35 ml internal volume (Hellma), and a tandem cell (2 × 0.4375 cm, Suprasil quartz, Hellma). CD parameters were the following: spectral window 200–320 nm, data pitch 1 nm, band width 2 nm, response 4 s, scanning speed 50 nm/min, 5 accumulations.

ESI-MS spectra of the ODNs and of the ODN-ruthenium complex mixtures were recorded by direct injection at 30 μl min^{-1} flow rate in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. The working conditions were as follows: spray voltage 3.1 kV, capillary voltage 45 V and capillary temperature 220 °C. The sheath and the auxiliary gases were set at 17 and 1 (arbitrary units), respectively. ESI-MS spectra of the ruthenium complexes alone were recorded in negative ion mode by direct injection at a 5 μl min^{-1} flow rate in a LCQ Advantage ion trap (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. The working conditions were as follows: spray voltage 4.5 kV, capillary voltage -10 V and capillary temperature 270 °C. The sheath gas was set at 10 (arbitrary units) whereas auxiliary gas was kept at 5 (arbitrary units). All ESI spectra were elaborated using Xcalibur software (Thermo).

4.2 Preparation of the ODN systems and ruthenium complexes

The following ODN sequences have been synthesized in the solid phase following a standard phosphoramidite protocol (1 μmol scale) using an ABI Expedite 8909 oligosynthesizer:
d($^{5'}$CCTCT**GG**TCTCC$^{3'}$) (**1**),

d($^{5'}$GGAGA**CC**AGAGG$^{3'}$) (**2**),

d($^{5'}$TCACACACACACACACACACACTT$^{3'}$) (**3**).

The ODNs were purified by GlenPack cartridges (C18), desalted by dialysis versus H_2O and lyophilized. Quantification of the ODNs was performed by UV measurements (λ = 260 nm, 85 °C) of a stock solution obtained by dissolving the oligomers in a known volume of bidistilled H_2O.

Data on the identity and purity of the ODNs were obtained by ESI-MS analysis (Figure S1).

The model duplex structure was obtained by mixing ODNs **1** and **2** in 1:1 ratio in a 10 mM KH$_2$PO$_4$/100 mM NaCl, pH = 7.0 buffer and annealed by keeping the resulting solution at 95 °C for 5 min followed by slow cooling to room temperature.

The ruthenium complex AziRu was prepared in our laboratories using reported procedures[30,25] (Scheme S1) starting from ruthenium trichloride.

Stock solutions of AziRu and NAMI-A for UV and CD experiments were prepared by freshly dissolving 1.0 mg of each metal complex in anhydrous DMSO at 2 and 4 mM concentrations depending on the analysis to be performed.

4.3 UV-vis absorption experiments

UV-vis absorption analysis of AziRu and NAMI-A alone was performed by dissolving each metal complex (2 μl withdrawn from a 2 mM stock solution), directly in the quartz cell, in 1 ml of the saline phosphate buffer (10 mM KH$_2$PO$_4$/100 mM NaCl solution, pH = 7.0), obtaining a 4 μM final ruthenium concentration, and immediately registering the spectra from t = 0 to 1 h every 10 min, then from 1 to 6 h every 60 min, and finally at t = 24, 30, 48, 54, 72 and 78 h (selected spectra were reported in Figures 2 and S6 for AziRu and NAMI-A, respectively). The UV-vis experiments in the presence of the ODNs were carried out in the same manner as described above, except that in the quartz cell the ODNs (**1**, **2**, **3** or the duplex **1/2**) at the proper concentration (4 μM) were already present in the 1 ml-incubation solution.

AziRu alone was also analysed at 50 μM concentration, obtained by withdrawing 12.5 μl from the 4 mM AziRu stock solution, and dissolving them in both 10 mM KH$_2$PO$_4$/100 mM NaCl solution (pH = 7.0) and pure H$_2$O (Figure S8).

All the UV-vis experiments were performed in triplicate.

4.4 Circular dichroism experiments

CD analysis was performed as follows: solutions of the ODNs at 20 μM concentration in 10 mM KH$_2$PO$_4$/100 mM NaCl solution (pH = 7.0) were prepared withdrawing 17.5 μl from a 400 μM stock solution of each ODN system (**1**, **2** and **3**) and dissolving them in an eppendorf tube in order to have a final volume of 700 μl. The 20 μM solution of duplex **1/2** was obtained by mixing 17.5 μl of ODNs **1** and **2**, withdrawn from each of the 400 μM stock solution, to a 700 μl final volume of 10 mM KH$_2$PO$_4$/100 mM NaCl solution (pH = 7.0), followed by annealing procedure (see above). For each prepared ODN solution, half (350 μl) was used as reference during the analysis, and to the remaining part, 1.75 μl of AziRu (withdrawn from the 4 mM stock solution) were added, then leaving the resulting solution in the dark at 37 °C. CD spectra of the ruthenium-treated solutions were then registered at 24, 48 and 72 h in the 0.1 cm quartz cuvette, and compared with the

corresponding ODNs reference solutions at the same incubation times. The same experiments were carried out also with NAMI-A (addition of 1.75 μl of the complex withdrawn from a 4 mM stock solution). Each experiment was performed in triplicate.

The CD experiments assessing the formation of the duplex structure between the ruthenated ODNs (**1**–Ru or **2**–Ru) with the corresponding complementary strands (free **2** or **1**, respectively) were performed in the tandem cell by placing, in one chamber, the metallated ODN (diluted from the 20 μM solution), and in the other chamber the free ODN (diluted from the 400 μM stock solution), each at 5 μM concentration in 10 mM KH_2PO_4/100 mM NaCl solution (pH = 7.0). Two CD spectra were registered for each experiment: the first one resulting as the sum of the separate components, here named *sum* spectrum, and the second one recorded after mixing the two solutions until stabilization of the signal, here named *mix*. After mixing the two solutions, the ODNs concentration resulted to be reduced by a factor of 2, going from 5 to 2.5 μM, and the solution-light path increased from 0.437 to 0.875 cm (Figures 5, S14 and S15).

All the CD melting experiments, relative to the reference duplex **1/2** and all the ruthenated duplexes were performed in triplicate in the tandem cell at 2.5 μM concentration in 10 mM KH_2PO_4/100 mM NaCl solution (pH = 7.0) (Figures 6 and 8).

4.5 ESI-MS experiments

Metal complex/ODN adducts were prepared by mixing equivalent amounts of the ODN (50 μM) and Ru complexes in water incubated for 48 h at 37 °C. Before the analysis, CH_3OH was added to the reaction mixture, with 2-fold dilution so to obtain a final concentration of 25 μM in CH_3OH/H_2O, 1:1 (v/v). The ODNs alone, as references, were dissolved in water at 50 μM concentration and injected after dilution 1:2 with CH_3OH directly to the ESI source (30 μl min^{-1} flow rate). For acquisition, Xcalibur 2.0 software (Thermo) was used and monoisotopic and average deconvoluted masses were obtained by using the integrated Xtract tool. For spectrum acquisition a nominal resolution (at m/z 400) of 100000 was used.

Ruthenium complexes alone were analysed by ESI-MS at a final concentration of 50 μM in CH_3OH/H_2O, 1:1 (v/v).

References

1.   C. Nardon, G. Boscutti, D. Fregona, Beyond platinums: gold complexes as anticancer agents, *Anticancer Res.*, **2014**, *34*, 487-492.

2.   S. Komeda, A. Casini, Next-generation anticancer metallodrugs, *Curr. Top. Med. Chem.* **2012**, *12*, 219-235.

3.   A. Bergamo, C. Gaiddon, J. H. Schellens, J. H. Beijnen, G. Sava, Approaching tumour therapy beyond platinum drugs: status of the art and perspectives of ruthenium drug candidates, *J. Inorg. Biochem.*, **2012**, *106*, 90-99.

4.   L. Ruiz-Azuara, M. E. Bravo-Gómez, Copper compounds in cancer chemotherapy, *Curr. Med. Chem.*, **2010**, *17*, 3606-3615.

5.   C. G. Hartinger, M. A. Jakupec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P. J. Dyson, B. K. Keppler, KP1019, a new redox-active anticancer agent-- preclinical development and results of a clinical phase I study in tumor patients, *Chem. Biodivers.,* **2008**, *5*, 2140-2155.

6.   E. Alessio, G. Mestroni, A. Bergamo, G. Sava, Ruthenium antimetastatic agents, *Curr. Top. Med. Chem.*, **2004**, *4*, 1525-1535.

7.   W. H. Ang, A. Casini, G. Sava, P. J. Dyson, Organometallic ruthenium-based antitumor compounds with novel modes of action, *J. Organomet. Chem.,* **2011**, *696*, 989-998.

8.   A. Levina, A. Mitra, P. A. Lay, Recent developments in ruthenium anticancer drugs, *Metallomics*, **2009**, *1*, 458-470.

9.   F. Lentz, A. Drescher, A. Lindauer, M. Henke, R.A. Hilger, C. G. Hartinger, M. E. Scheulen, C. Dittrich, B. K. Keppler, U. Jaehde, Pharmacokinetics of a novel anticancer ruthenium complex (KP1019, FFC14A) in a phase I dose-escalation study, *Anticancer Drugs*, **2009**, *20*, 97-103.

10.   J. M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J. H. Beijnen, J. H. Schellens, A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent, *Clin. Cancer Res.*, **2004**, *10*, 3717-3727.

11.   A. Bergamo, L. Messori, F. Piccioli, M. Cocchietto, G. Sava, Biological role of adduct formation of the ruthenium(III) complex NAMI-A with serum albumin and serum transferrin, *Invest. New Drugs*. **2003**, *21,* 401-411.

12.   M. I. Webb, C. J. Walsby, Control of ligand-exchange processes and the oxidation state of the antimetastatic Ru(III) complex NAMI-A by interactions with human serum albumin, *Dalton Trans.*, **2011**, *40*, 1322-1331.

13.   V. Cepeda, M. A. Fuertes, J. Castilla, C. Alonso, C. Quevedo, J. M. Pérez, Biochemical mechanisms of cisplatin cytotoxicity, *Anticancer Agents Med. Chem.*, **2007**, *7*, 3-18.

14.   L. Kelland, The resurgence of platinum-based cancer chemotherapy, *Nature Rev. Cancer*. **2007**, *7*, 573–584.

15.   J. B. Aitken, S. Antony, C. M. Weekley, B. Lai, L. Spiccia, H. H. Harris, Distinct cellular fates for KP1019 and NAMI-A determined by X-ray fluorescence imaging of single cells, *Metallomics*, **2012**, *4*, 1051-1056.

16.   M. I. Webb, C. J. Walsby, EPR as a probe of the intracellular speciation of ruthenium(III) anticancer compounds, *Metallomics*, **2013**, *5*, 1624-1633.

17.   A. Bergamo, R. Gagliardi, V. Scarcia, A. Furlani, E. Alessio, G. Mestroni, G. Sava, In vitro cell cycle arrest, in vivo action on solid metastasizing tumors, and host toxicity of the antimetastatic drug NAMI-A and cisplatin, *J. Pharmacol. Exp. Ther.*, **1999**, *289*, 559–564.

18.   J. Malina, O. Novakov, B. K. Keppler, E. Alessio, V. Brabec, Biophysical analysis of natural, double-helical DNA modified by anticancer heterocyclic complexes of ruthenium(III) in cell-free media, *J. Biol. Inorg. Chem.,* **2001**, *6*, 435-445.

19.   G. I. Pascu, A. C. G. Hotze, C. Sanchez-Cano, B. M. Kariuki, M. J. Hannon, Dinuclear ruthenium(II) triple-stranded helicates: luminescent supramolecular cylinders that bind and coil DNA and exhibit activity against cancer cell lines, *Angew. Chem. Int. Ed.*, **2007**, *46*, 4374-4378.

20.   H. Lai, Z. Zhao, L. Li, W. Zheng, T. Chen, Antiangiogenic ruthenium(II) benzimidazole complexes, structure-based activation of distinct signaling pathways, *Metallomics*, **2015**, *7*, 439-447.

21.   D. Pluim, R. C. van Waardenburg, J. H. Beijnen, J.H. Schellens, Cytotoxicity of the organic ruthenium anticancer drug NAMI-A is correlated with DNA binding in four different human tumor cell lines, *Cancer Chemother. Pharmacol.*, **2004**, *54*, 71-78.

22.   M. Bacac, A. C. Hotze, K. van der Schilden, J. G. Haasnoot, S. Pacor, E. Alessio, G. Sava, J. Reedijk, The hydrolysis of the anti-cancer ruthenium complex NAMI-A affects its DNA binding and antimetastatic activity: an NMR evaluation, *J. Inorg. Biochem.*, **2004**, *98*, 402-412.

23.   H. Velders, A. Bergamo, E. Alessio, E. Zangrando, J. G. Haasnoot, C. Casarsa, M. Cocchietto, S. Zorzet, G. Sava, Synthesis and chemical−pharmacological characterization of

the antimetastatic NAMI-A-type Ru(III) complexes (Hdmtp)[*trans*-RuCl$_4$(dmso-S)(dmtp)], (Na)[*trans*-RuCl$_4$(dmso-S)(dmtp)], and [*mer*-RuCl$_3$(H$_2$O)(dmso-S)(dmtp)] (dmtp = 5,7-Dimethyl[1,2,4]triazolo[1,5-*a*]pyrimidine), *J. Med. Chem.*, **2004**, *47*, 1110-1121.

24.   M. Groessl, O. Zava, P. J. Dyson, Cellular uptake and subcellular distribution of ruthenium-based metallodrugs under clinical investigation versus cisplatin, *Metallomics*, **2011**, *3*, 591-599.

25.   L. Simeone, G. Mangiapia, G. Vitiello, C. Irace, A. Colonna, O. Ortona, D. Montesarchio, L. Paduano, Cholesterol-based nucleolipid-ruthenium complex stabilized by lipid aggregates for antineoplastic therapy, *Bioconjug. Chem.*, **2012**, *23*, 758-770.

26.   G. Mangiapia, G. D'Errico, L. Simeone, C. Irace, A. Radulescu, A. Colonna, D. Montesarchio, L. Paduano, Ruthenium-based complex nanocarriers for cancer therapy, *Biomaterials,* **2012**, *33*, 3770-378.

27.   G. Mangiapia, G. Vitiello, C. Irace, R. Santamaria, A. Colonna, R. Angelico, G. D'Errico, D. Montesarchio, L. Paduano, Anticancer cationic ruthenium nanovectors: from rational molecular design to cellular uptake and bioactivity, *Biomacromolecules*, **2013**, *14,* 2549-2560.

28.   D. Montesarchio, G. Mangiapia, G. Vitiello, D. Musumeci, C. Irace, R. Santamaria, G. D'Errico, L. Paduano, A new design for nucleolipid-based Ru(III) complexes as anticancer agents, *Dalton Trans.,* **2013**, *42*, 16697-16708.

29.   G. Vitiello, A. Luchini, G. D'Errico, R. Santamaria, A. Capuozzo, C. Irace, D. Montesarchio, L. Paduano, Cationic liposomes as efficient nanocarriers for the drug delivery of an anticancer cholesterol-based ruthenium complex, *J. Mater. Chem. B*, **2015**, *3*, 3011-3023.

30.   M. I. Webb, R. A. Chard, Y. M. Al-Jobory, M. R. Jones, E. W. Y. Wong, C. J. Walsby, Pyridine analogues of the antimetastatic Ru(III) complex NAMI-A targeting non-covalent interactions with albumin, *Inorg. Chem.,* **2012**, *51*, 954-966.

31.   Z. Adhireksan, G. E. Davey, P. Campomanes, M. Groessl,.C. M. Clavel, H. Yu, A. A. Nazarov, C. Hui Fang Yeo, W. Han Ang, P. Dröge, U. Rothlisberger, P. J. Dyson, C. A. Davey, Ligand substitutions between ruthenium–cymene compounds can control protein versus DNA targeting and anticancer activity, *Nat. Commun.*, **2014**, *5*, 3462-3474.

32.   M. Groessl, Y. O. Tsybin, C. G. Hartinger, B. K. Keppler, P. J. Dyson, Ruthenium versus platinum: interactions of anticancer metallodrugs with duplex oligonucleotides characterised by electrospray ionisation mass spectrometry, *J. Biol. Inorg. Chem.*, **2010**, *15*, 677-688.

33.   R. C. Todd, S. J. Lippard, Structure of duplex DNA containing the cisplatin 1,2-
        $\{Pt(NH_3)_2\}^{2+}$-d(GpG) cross-link at 1.77 Å resolution, *J. Inorg. Biochem.*, **2010**, *104*, 902-
        908.

34.   Unfortunately all the attempts we have carried out to obtain crystals of the same duplex
        metallated with the ruthenium complexes under investigation, suitable for X-rays diffraction
        analysis, have been so far unsuccessful.

35.   E. Alessio, G. Balducci, M. Calligaris, G. Costa, W. M. Attia, G. Mestroni, Synthesis,
        molecular structure, and chemical behavior of hydrogen *trans*-bis(dimethyl
        sulfoxide)tetrachlororuthenate(III) and *mer*-trichlorotris(dimethyl sulfoxide)ruthenium(III):
        the first fully characterized chloride-dimethyl sulfoxide-ruthenium(III) complexes, *Inorg.
        Chem.,* **1991**, *30*, 609-618.

36.   E. Alessio, G. Balducci, A. Lutman, G. Mestroni, M. Calligaris, W. M. Attia, Synthesis and
        characterization of 2 new classes of ruthenium(III)-sulfoxide complexes with nitrogen donor
        ligands (L)sNa[trans-RuCl$_4$(R$_2$SO)(L)] and mer,cis-RuCl$_3$(R$_2$SO)- (R$_2$SO)(L) the crystal-
        structure of Na[trans-RuCl$_4$(DMSO)- (NH$_3$)],2DMSO, Na[trans-
        RuCl$_4$(DMSO)(Im)],H$_2$O,Me$_2$CO (Im) imidazole) and mer,cis-
        RuCl$_3$(DMSO)(DMSO)(NH$_3$), *Inorg. Chim. Acta*, **1993**, *203*, 205-217.

37.   M. Bouma, B. Nuijen, M. T. Jansen, G. Sava, A. Bult, J. H. Beijnen, Photostability profiles
        of the experimental antimetastatic ruthenium complex NAMI-A, *J. Pharm. Biomed. Anal.*,
        2002, *30*, 1287-1296.

38.   V. Vargiu, A. Robertazzi, A. Magistrato, P. Ruggerone, P. Carloni. The hydrolysis
        mechanism of the anticancer ruthenium drugs NAMI-A and ICR investigated by DFT-PCM
        calculations, *J. Phys. Chem. B.*, **2008**, *112*, 4401-4409.38.

39.   R. Ajibola Adigun, B. Martincigh, V. O. Nyamori, B. Omondi, C. Masimirembwac, R. H.
        Simoy. Kinetics and mechanistic investigation into the possible activation of imidazolium
        trans-[tetrachloridodimethylsulfoxideimidazoleruthenate(III)], NAMI-A, by 2-
        mercaptoethane sulfonate, *Dalton Trans*., **2014**, *43*, 12943-12951.

40.   S. Leijen, S. A. Burgers, P. Baas, D. Pluim, M. Tibben, E. van Werkhoven, E. Alessio, G.
        Sava, J. H. Beijnen. J. H. M. Schellens, Preliminary Phase II studies in combination with
        gemcitabine in non-small cell lung cancer patients, *Invest. New Drugs*, **2015**, *33*, 201–214.

41.   A. Bergamo, G. Sava, Linking the future of anticancer metal-complexes to the therapy of
        tumour metastases, *Chem. Soc. Rev*., **2015**, doi number: 10.1039/C5CS00134J.

42.   A. Vergara, G. D'Errico, D. Montesarchio, G. Mangiapia, L. Paduano, A. Merlino,
        Interaction of anticancer ruthenium compounds with proteins: high-resolution X-ray

structures and raman microscopy studies of the adduct between hen egg white lysozyme and AziRu, *Inorg. Chem.*, **2013**, *52*, 4157-4159.

43.   L. Messori, A. Merlino, Ruthenium metalation of proteins: the X-ray structure of the complex formed between NAMI-A and hen egg white lysozyme, *Dalton Trans.*, **2014**, *43*, 6128-6131.