Ruthenation of Non-stacked Guanines in DNA G-Quadruplex Structures: Enhancement of *c-MYC* Expression

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Abstract: *Guanine quadruplexes (GQs) are compact four-stranded DNA structures that play a key role in the control of a variety of biological processes, including gene transcription. Bulky ruthenium complexes featuring a bipyridine, a terpyridine, and one exchangeable ligand ([Ru(terpy)(bpy)X]$^{n+}$) are able to metalate exposed guanines present in the GQ of the c-MYC promoter region that are not involved in quadruplex base pairing. qRT-PCR and western-blot experiments indicated that the complexes promote a remarkable increase in the expression of this oncogene. We also show that exchangeable thioether ligands (X = RSR′, Met) allow regulation of the metalating activity of the complex with visible light.*

There is great interest in the development of metal-based DNA binders that show improved selectivity and reduced toxicity relative to *cis*-platinum.[1] Ruthenium complexes are promising alternatives owing to their kinetic stability and rich photochemistry and redox properties.[2] In addition to non-covalent recognition of double-stranded DNA (dsDNA), several ruthenium complexes that form covalent DNA adducts, especially through reaction with the N^7 of guanines, have also been described.[3] While most of these complexes bind dsDNA, the functional relevance of G-quadruplexes (GQs)[4,5] calls for the development of probes capable of targeting these structures.[6] To our knowledge, there is only one precedent for the covalent metalation of a GQ structure with a ruthenium complex, and the reaction presents low selectivity.[7]

Herein, we demonstrate that coordination complexes of the type [Ru(terpy)(bpy)X]$^{n+}$ (X = Cl, RSR′, Met) can selectively metalate unpaired guanines present in parallel GQs, a reaction that is enhanced upon irradiation. Impor-

tantly, we have found that this selective metalation increases the expression of the oncogene *c-MYC*, apparently by disrupting the parallel GQ structure present in its promoter region.

Our work was conceived after learning that while most DNA-metalating ruthenium agents are cytotoxic, the complex [Ru(terpy)(bpy)Cl]$^+$ (**1**) exhibits very low toxicity.[8] We reasoned that the bulky and relatively hydrophobic nature of this sort of complexes could offer excellent opportunities for the selective modification of accessible guanines, thereby promoting specific biological responses with reduced toxicity.

We first studied the ability of chloro complex **1** to metalate guanosine monophosphate (GMP). Mixing complex
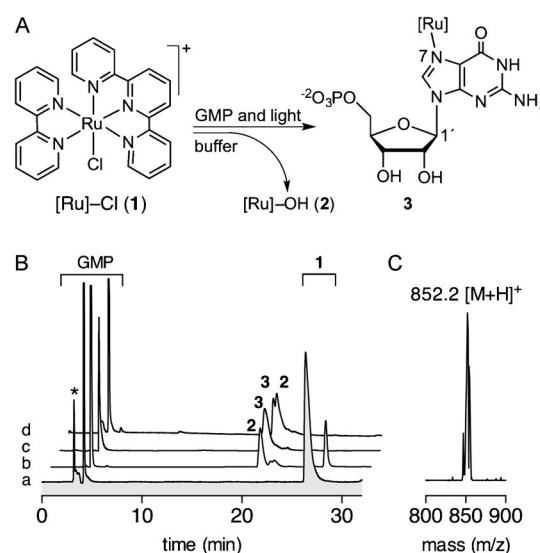

Figure 1. A) GMP metalation reaction with [Ru(terpy)(bpy)Cl]PF$_6$ ([Ru]-Cl, **1**) and formation of the aquo derivative **2**. B) HPLC of the reaction of **1** (250 μм) and GMP (750 μм) in 10 mм phosphate buffer pH 7.5, 100 mм NaCl: in the dark at $t = 0$ (trace a); after 30 min in the dark (trace b); initial mixture after 30 min of irradiation at 455 nm (trace c); and initial mixture after 2 h in the dark (trace d). The injection peak is labeled with an asterisk. C) Signal in the MS spectrum corresponding to the monoadduct **3**.

1 with 3 equiv of GMP in phosphate buffer (pH 7.5), led to the partial formation of the aquo complex [Ru(terpy)-(bpy)H$_2$O]$^{+2}$ (**2**; over 50% after 30 min at RT), while the guanosine remained essentially unreacted (Figure 1B, trace b). Further incubation for 2 h afforded the metalated product **3** and the aquo derivative **2** (Figure 1A), with total consumption of the starting chloride (Figure 1B, trace d).

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Interestingly, irradiation of the initial mixture for 30 min ($\lambda = 455$ nm) led exclusively to the formation of the monoadduct **3** (approximate conversion of 80% based on the disappearance of GMP; Figure 1 B, trace c and Figure S5 in the Supporting Information). Importantly, the reaction is fully orthogonal, so competitive control experiments in the presence of excess adenosine monophosphate (AMP), cytidine monophosphate (CMP), and thymidine monophosphate (TMP; Figure S6), as well as lysine or even cysteine (Figure S7), led exclusively to the formation of the GMP derivative. Moreover, experiments with double-stranded oligonucleotides (dsDNAs) presenting different arrangements of guanines revealed modest reactivity, but only with those featuring terminal guanines (Table S1, and Figures S10–S13 in the Supporting Information). This selectivity most likely stems from the bulkiness of complex **1**, which cannot reach the sterically hindered nucleophilic N^7 site in paired, internal guanines.[9]

Remarkably, irradiation of a mixture of the parallel *c-MYC* quadruplex d[TTGAG$_3$TG$_3$TAG$_3$TG$_3$TA$_3$][10] (10 μM) with 5 equiv of **1** in 10 mM phosphate buffer (pH 7.5) and 100 mM KCl led to the clean formation of a product with a mass corresponding to a monoadduct derivative (Figure 2 A, trace C, peak at 22 min, *MYC*-[Ru], 81% conver-
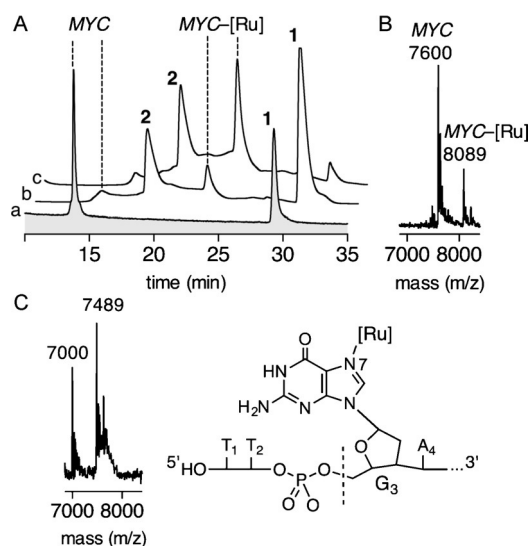

Figure 2. A) HPLC traces of a mixture of the GQ of *c-MYC* (*MYC*, 10 μM) and **1** (5 equiv) in 10 mM phosphate buffer pH 7.5, 100 mM KCl, at RT: in the dark at $t = 0$ (trace a); after 30 min in the dark (trace b), initial mixture after irradiation for 30 min at 455 nm (trace c). B) MS of the metalated product (*MYC*-[Ru]) showing the peaks corresponding to the complex ($m/z = 8089$) and the demetalated fragment ($m/z = 7600$). C) MS of the products after BSP digestion of *MYC*-[Ru] showing the peaks corresponding to the digested complex ($m/z = 7489$) and non-metalated digested product ($m/z = 7000$) resulting from the cleavage at G$_3$ as shown by the dashed line in the structure.

sion). We also observed reaction in the absence of light (approx. 41% conversion after 30 min at RT, Figure 2 A, trace b). Importantly, MS analysis of the bovine spleen

phosphodiesterase (BSP) digestion of the ruthenated oligo-nucleotides lead to identification of the first guanine of the sequence (5′-TT**G**A..., Figure 2 C) as the metalation site (Figure S24).[11] No reaction was observed under the same conditions with a mutated *c-MYC* GQ containing a C instead of a G in position 3 (*MYCm*, d[TT**C**AG$_3$TG$_3$TAG$_3$TG$_3$TA$_3$]; Figure S18).

The chemoselectivity of the reaction can be rationalized by considering the secondary structure of the *c-MYC* GQ (Figure 3), which shows that G3 is exposed to the solvent and
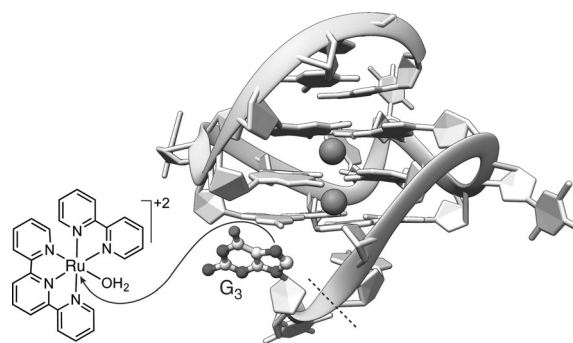

Figure 3. Structure of the GQ of c-*MYC* (PDB ID: 1XAV), highlighting the exposed G$_3$ and the metalation selectivity. The large spheres in the quadruplex structure represent potassium ions.

does not participate in the formation of the G-quartets. As a consequence, it has a highly accessible N^7 nucleophile that can react with the bulky ruthenium complex. Importantly, circular dichroism experiments revealed that the GQ secondary structure is disrupted in the ruthenium adduct *MYC*-[Ru] (Figure S3, right). Control experiments with other related parallel quadruplex such as *c-KIT1*, which also presents non-stacked guanines, revealed a similar reactivity pattern. Mass spectrometry shows that the promoter is modified by the ruthenium complex at the expected positions (Figures S21 and S22), although curiously, in this case, CD analysis revealed that the quadruplex remained mostly intact (Figure S4).[12]

With the above molecular information, we explored whether Ru complex **1** could affect the expression of the oncogene *c-MYC*, since this oncogene is involved in many important cellular processes.[13] This was analyzed by real-time quantitative reverse transcription PCR (qRT-PCR) in HeLa and Vero cells (Figure 4 and Figure S26, respectively), using porphyrin TMPyP4, a GQ binder that is known to repress the expression of c-*MYC*,[14] as a control. As shown in Figure 4, HeLa cells treated with **1** (100 μM) in Dulbecco's modified Eagle's medium (DMEM) presented a modest but significant increase in the transcription of *c-MYC* compared to untreated cells (80% at 16 h and 200% at 48 h).[15] As expected, treatment with TMPyP4 led to a 60% decrease in the cellular levels of c-*MYC* mRNA after 48 h.

We also analyzed the expression of c-MYC protein by western blot. In agreement with the qRT-PCR results, treatment of cells with 100 μM of **1**, led to a noticeable increase in the levels of c-MYC protein (Figure 4, averaged 40%
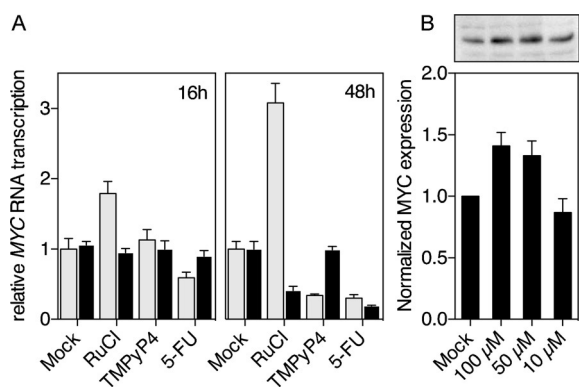
Figure 4. A) qRT-PCR analysis of *c-MYC* (light bars) and *ALAS1* (black bars) transcription. Hela cells were incubated for 16 h or 48 h with either 100 μM RuCl, TMPyP4, or 50 μM 5-fluorouracil (5-FU, a disruptor of RNA synthesis). Amounts are relative to the expression levels of the housekeeping gene *GAPDH*. Values are the average of three experiments, and error bars indicate the standard error. B) Analysis of the expression of c-MYC by western-blot. HeLa cells were incubated for 16 h with 100 μM compound **1** and then lysed, and c-MYC was detected by SDS-PAGE, followed by western blot with an anti-MYC Antibody (upper panel). The relative amount of protein in two independent experiments was quantified by densitometry (lower panel). Data are represented as the fold change with respect to untreated controls. Error bars indicate the standard deviation of the fold change with respect to untreated controls.

Figure 5. A) Thioether complexes **4** and **5**. B) *c-MYC* transcription as measured by qRT-PCR in the dark (black bars) or after irradiation (60 min, light bars). Hela cells were incubated for 16 h with 100 μM RuMet (**5**) or aquo complex **2**. RNA levels are given relative to the expression levels of the housekeeping gene *GAPDH*. C) Expression of c-MYC as measured by western blot in the presence or absence of compound **5**. The relative amount of protein with respect to that of β-actin was quantified by densitometry (lower panel). The experimental procedures are as described in Figure 4.

increase). These data confirm that, in contrast to most quadruplex targeting agents, complex **1** promotes an increase instead of a decrease in the level of gene expression, thereby acting as a transcriptional activator.[16] ICP-MS measurements of isolated nuclei and chromatin obtained after treatment with complex **1** confirmed the presence of relatively significant amounts of ruthenium, which is consistent with efficient cellular uptake and nuclear delivery of the complex (Tables S2 and S3). In agreement with the early studies,[8] cell viability assays confirmed that **1** is essentially non-cytotoxic (Figure S27).

While the above data indicate that **1** is capable of altering the expression of *c-MYC*, the development of derivatives that could be activated by using external irradiation would be highly attractive.[17] Towards this aim, we prepared complexes **4** and **5**, which feature a thioether ligand (Figure 5) and are kinetically stable but undergo rapid ligand exchange upon irradiation with visible light.[18] In contrast to the chloride complex **1**, in vitro experiments with the thioether derivative **4** showed that it does not react with the *c-MYC* quadruplex in the dark after 30 min, but yields the desired GQ monoadduct upon irradiation (71% conversion, Figure S17). The acetyl-methionine derivative **5** presents even higher kinetic stability than complex **4**, and no traces of the metalation adducts, or even of the aquo derivative **2**, were observed after several hours in the dark. However, irradiation for 30 min triggers efficient covalent metalation of the GQ (Figure S20). We also analyzed the effect of complex **5** on transcription of the *c-MYC* gene by qRT-PCR in Hela cells. As shown in the Figure 5 (left), *c-MYC* mRNA levels increased after irradiation, becoming similar to those observed after treatment with the aquo complex **2**. Western-blot analysis confirmed
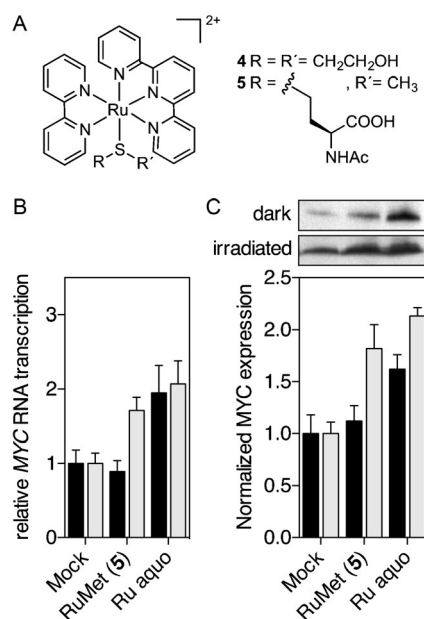
that enhancement of the gene expression only took place in the presence of light, while the levels of the protein c-MYC did not change when the cells were kept in the dark (Figure 5 and Figure S29). As expected, both qRT-PCR and western-blot experiments showed that the aquo compound **2** is active both in the dark and under irradiation. Moreover, control experiments confirmed that the irradiation does not have any measurable effect on cell viability (Figure S28), and that the complex does not generate significant amounts of singlet oxygen upon irradiation (Figure S25).

In conclusion, we have demonstrated the selective modification of accessible guanines flanking the GQ of c-*MYC* with designed bulky ruthenium complexes. Importantly, the bioorthogonal metalation enhances the expression of the oncogene c-*MYC*. Given that an increase in c-*MYC* transcription has been shown to be important in several cancers, in particular for the renewal ability of cancer stem cells,[19] our discovery might lead to interesting biological applications.

Acknowledgements

for her help with the biological experiments and Verónica Piñeiro of the Unidad de Análisis Elemental (RIAIDT-USC) for the ICP analysis.

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