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Selective G-quadruplex binding by oligoarginine-Ru(dppz) metallopeptides

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A set of Ru(II) metallopeptides containing the dppz ligand has been synthesized using SPPS methods. Fluorescence titration studies show that those metallopeptides featuring an octaarginine tail display a large binding preference for DNA G-quadruplex structures over those lacking it, and also that the interplay between the octoarginine functionalization and the ancillary ligand in the complex has an essential role in the recognition process. Furthermore, the oligoarginine metallopeptides are also efficiently internalized, causing cell death with signs of apoptosis.

It is known that G-quadruplexes (GQs) play critical roles in a variety of biological processes,[1,2] such as transcriptional regulation, DNA replication, or genome stability. Therefore, the recognition of such non-canonical DNA structures by small molecule binders is currently a hot topic in chemical biology.[3] However, the diversity of GQ structures adds further complexity in the design of selective binding agents,[4] which is why most of the studies focus on the search for agents capable of discriminating between GQs and other DNA secondary structures,[3,4] especially B-DNA, rather than in the selectivity between different structural families of GQs.[5] The typical routes to obtain GQ binders require demanding synthetic procedures that hamper the efficient access to multiple structural variants needed for their functional optimization.[3,4,5] A promising alternative is the decoration of the GQ binders with peptide sequences to modify their physicochemical properties in a more predictable and synthetically-accessible way.[6] However, despite the potential of this approach, G-quadruplex peptide binders have been largely ignored in the literature.[7]

On the other hand, It is known that basic peptides, such as the *Antennapedia* sequence, can be efficiently internalized,[8] and that these oligocationic peptides are useful tools for the transport of a variety of cargoes into the cells. Indeed, the functionalization with octaarginine domains has been successfully applied for the internalization and nuclear delivery of DNA binding agents,[9,10] which also display increased affinity towards the DNA due to the attractive electrostatic interaction between the cationic oligoarginine domains and the anionic phosphate backbone of the DNA.[11] However, to our knowledge, there are no studies about the effect that these oligocationic tags could have on the recognition properties of GQ binders.

We thus focused our attention on dipyridophenazine (dppz) Ru(II) derivatives,[12] which are widely studied models of "light-switch" DNA-binding complexes.[13,14,15] These systems interact with the B-DNA through intercalation of the extended aromatic dppz ligand between consecutive base pairs,[16] and it is also known that these Ru(II)-dppz complexes bind to GQs.[17,18] Therefore, following our interest in the study of DNA binding metallopeptides,[19] we decided to explore the effect of octaarginine modification in the GQ binding properties of these classic Ru(II)-dppz DNA binders. With this aim in mind, we devised the model complex **[Ru(dppz)]-R_8**, (Scheme 1), which in addition to the intercalating dppz ligand, features a dipeptide handle with a chelating bipyridine (βAla-Bpy) that links the complex to a C-terminal (*L*)-octaarginine domain. In short, the precursor peptide ligand was assembled following standard Fmoc/tBu solid phase peptide synthesis protocols by incorporating the protected bipyridine building block (Fmoc-β-Ala-Bpy-OH, **1**) into the pre-assembled octaarginine domain.[11,20] Fmoc-deprotection of the ligand and N-terminal acetylation was followed by the incorporation of the Ru(II) center by reaction with $[Ru(DMSO)_4Cl_2]$,[21] and the installation of the intercalating dppz moiety and the ancillary 1,10-phenanthroline ligand (phen), [22] all transformations being performed while the peptide was still attached to the solid support. Acidic cleavage and deprotection of the complex, followed by reverse-phase HPLC purification, afforded the desired Ru(II) metallopeptide as diastereomeric mixture

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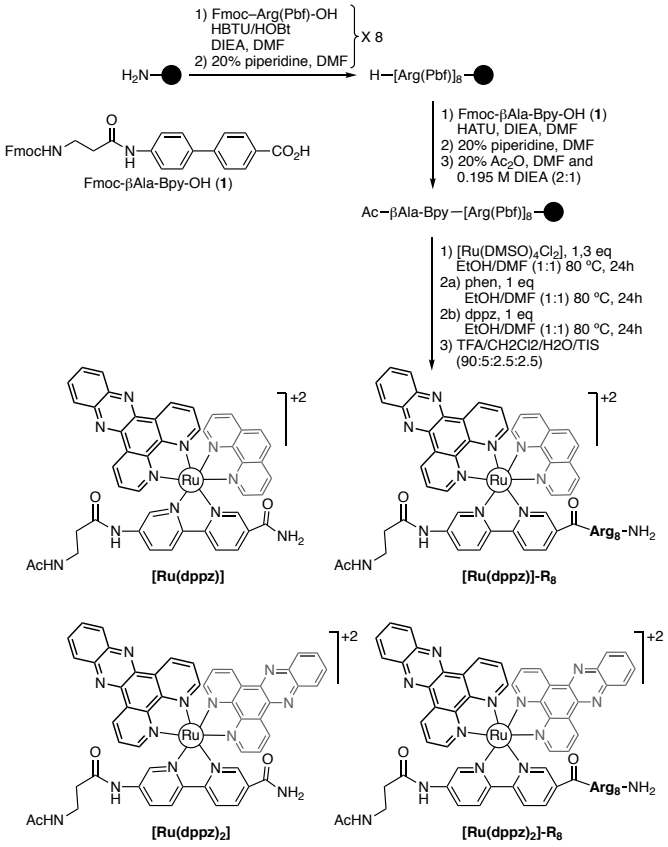
(Scheme 1). In addition to the model **[Ru(dppz)]-R_8** complex, we also synthesized a control complex lacking the octaarginine tail, as well as related analogs featuring two dppz ligands (**[Ru(dppz)]**, **[Ru(dppz)$_2$]-R_8,** and **[Ru(dppz)$_2$]**, Scheme 1).



Scheme 1. Solid-phase synthesis of the metallopeptide complex **[Ru(dppz)]-R_8**, and structures of the analog complexes studied **[Ru(dppz)]**, **[Ru(dppz)$_2$]-R_8**, and **[Ru(dppz)$_2$]**.

Having at hand the desired Ru(II) metallopeptides, we studied their interaction with synthetic oligonucleotides by taking advantage of the intrinsic environment-sensitive luminescence of the Ru(II)-dppz complexes.[23] As reference GQ structures we selected the *TEL* (telomeric quadruplex DNA) GQ, which is the archetypical model of the mixed parallel/antiparallel GQ topology, as well as oncogenic promoter sequences *c-MYC*,[24] and *c-KIT*,[25] both of them displaying the same parallel quadruplex topology (see ESI for the complete sequences). Although there are several studies about the interaction of enantiopure Ru-dppz complexes with GQs, we have chosen to work with the racemate because it has been previously observed that the chiral selectivity towards parallel and mixed parallel/antiparallel GQ topologies is low.[18b] For comparison purposes, we also studied the binding to a B-DNA oligonucleotide (see ESI, Figures S1 and S2).

Addition of successive aliquots of a stock solution of the *TEL* GQ sequence to a 2 μM solution of **[Ru(dppz)]** in phosphate buffer (pH= 7.5) resulted in a progressive increase in the emission intensity of the Ru(II) complex, which was fitted to a 1:1 binding model consistent with the formation of a specific **[Ru(dppz)]**/*TEL* complex. The affinity of the different complexes for the rest of the oligonucleotides was determined following the same experimental procedure. The analysis of the apparent dissociation constants indicates a clear preference of **[Ru(dppz)]** for *TEL* over both promoter GQ DNAs (Table 1 and Figures 1 and S3-9). On the other hand, **[Ru(dppz)]-R_8** displays a higher affinity for all the DNAs, and once again with a clear preference for the *TEL* GQ over the other two quadruplexes. Therefore, both **[Ru(dppz)]** and **[Ru(dppz)]-R_8** recognize GQ sequences with high affinity, and the presence of the oligoarginine tail induces a significant increase of the binding affinity. Finally, these studies also suggest that both complexes can discriminate between the different quadruplex topologies, and show considerable selectivity for *TEL* over both *c-KIT* and *c-MYC*. It should be noted that, in some cases, **[Ru(dppz)]-R_8** appears to show a slightly higher intrinsic emission than **[Ru(dppz)]** in solution, which might be due to the protection of the dppz ligand from a deactivating solvent protonation process by the large oligoarginine appendage.[26]

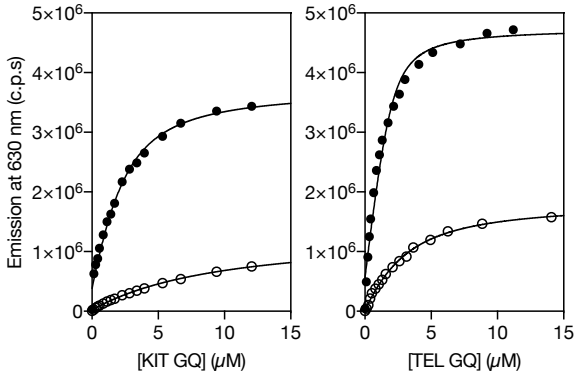


Figure 1. Emission profiles at 630 nm of representative titrations of **[Ru(dppz)]** (○) and **[Ru(dppz)]-R_8** (●) with KIT GQ (left) and TEL GQ (right). The same scale was used in both graphs for comparison. The lines correspond to the best fits to a simple 1:1 binding mode that results in the dissociation constants shown in Table 1 (see ESI for curve fitting procedures using the *DynaFit* program).[27] Similar titrations were performed with **[Ru(dppz)$_2$]** and **[Ru(dppz)$_2$]-R_8**.

The different G-quadruplex binding behavior shown by the oligoarginine metallopeptide **[Ru(dppz)]-R_8** is also evident from the UV-vis spectrum, which is characterized by a MLCT band centered at *c.a.* 443 nm that is affected when the complex binds to the GQs or the B-DNA.[5,28,29] More concretely, both the **[Ru(dppz)]** and **[Ru(dppz)]-R_8** complexes experiment an evident hypochromism in their MLCT bands after the addition of *TEL* (Figures S18 and S19), which is indicative of a strong interaction between the metallopeptides and this oligonucleotide.[18] However, the hypochromism phenomena are appreciably more pronounced in **[Ru(dppz)]-R_8** than in **[Ru(dppz)]**. Concerning the interaction of **[Ru(dppz)]-R_8** with *c-MYC* and *c-KIT*, the hypochromism phenomena is slightly less pronounced than in the case of *TEL* (Figures S20 and S21), corroborating the different binding preferences of this metallopeptide for the telomeric DNA over both oncogene promoter sequences.

CD spectroscopy can be used to study the conformation of nucleic acids, providing valuable information particularly in the case of polymorphic GQs.[18,30] Interestingly, the CD spectra of all the studied GQs remained largely unchanged in the presence of either **[Ru(dppz)]-R_8** or **[Ru(dppz)]**, which indicates that the structures of the GQs are not significantly affected by the presence of the Ru(II) metallopeptides (See

ESI†, Figures S22-S27). No CD signals were observed in the visible section of the spectrum.

Having established the beneficial effect of the oligoarginine domain in the GQ recognition properties of the model dppz complexes, we decided to investigate the role of the ancillary ligand in their interaction DNA. To this end, we synthesized two new Ru(II) complexes, **[Ru(dppz)$_2$]** and **[Ru(dppz)$_2$]-R$_8$** (Scheme 1), in which the phenanthroline ligand is replaced by a second dppz unit.[18d] Steady-state luminescence titrations showed that **[Ru(dppz)$_2$]** binds with higher affinity to the different GQ DNAs than the analog complex featuring a single dppz ligand. Furthermore, the binding preference for *TEL* over the promoter quadruplex DNAs (*c-MYC* and *c-KIT*) is maintained, although the selectivity, in this case, was less pronounced than in the case of the phen derivative. In contrast, the replacement of the ancillary phen ligand in the complex **[Ru(dppz)$_2$]-R$_8$** results in a significant increase (over 20-fold) of the binding affinity towards the oncogene promoter sequences *c-MYC* and *c-KIT*, so that the overall selectivity profile is inverted with respect to that of the single dppz analog **[Ru(dppz)]-R$_8$** (Table 1). To our knowledge, this is the first example of this class of GQ selectivity inversion in literature. Thus, the fluorescence data indicates that both the ancillary ligand and the oligoarginine domain are key structural elements that influence the GQ recognition process.

Table 1. Dissociation constants (K_D/μM) of the Ru(II) metallopeptides with selected GQ oligonucleotides.

	TEL	*c-MYC*	*c-KIT*
[Ru(dppz)]	2.72 ± 0.21	11.23 ± 1.03	19.27 ± 1.69
[Ru(dppz)]-R$_8$	0.93 ± 0.06	4.01 ± 0.33	2.05 ± 0.10
[Ru(dppz)$_2$]	0.85 ± 0.05	3.65 ± 0.19	3.06 ± 0.15
[Ru(dppz)$_2$]-R$_8$	1.30 ± 0.07	0.15 ± 0.05	0.14 ± 0.04

To gain more insight into the GQ recognition process, we performed computational studies with **[Ru(dppz)$_2$]** and **[Ru(dppz)$_2$]-R$_4$** (the R$_8$ domain was reduced to four residues for computational feasibility). These complexes were docked to the crystalline structure of *TEL*.[31] The four different stereoisomers (two Λ and two Δ) of **[Ru(dppz)$_2$]** and **[Ru(dppz)$_2$]-R$_4$** were computed. In agreement with the experimental results, the binding profiles of both metallopeptides seem to be entirely different. In the case of **[Ru(dppz)$_2$]**, our model indicates that one dppz ligand fits snugly in the pocket created by the sequence GTTAG near the end of the quadruplex for all stereoisomers (Figure S29). The value of the intermolecular binding energy provide an estimate of the strength of this binding, being of 11-12 kcal/mol for **[Ru(dppz)$_2$]**. The free binding energy is also estimated by AutoDock yielding similar values: 9-10 kcal/mol. Due to its large flexibility, the docking poses in **[Ru(dppz)$_2$]-R$_4$** are only indicative, as it is not possible to assert how far or near we are of the global docking minima. The binding mode of **[Ru(dppz)$_2$]-R$_4$** seems to be different to that of **[Ru(dppz)$_2$]**, as in none of the lowest energy poses there is a dppz ancillary ligand in a CTTAG, or similar, pockets. In fact, the binding mode of **[Ru(dppz)$_2$]-R$_4$** appears to be clearly dominated by the interactions between the positively charged guanidinium groups from the arginine side chains and the anionic phosphate groups from the GQ backbone. The value of the intermolecular binding energy of **[Ru(dppz)$_2$]-R$_4$** is 19-20 kcal/mol, and the free binding energy is 10-11 kcal/mol, both indicating an enhanced interaction with the TEL GQ sequence compared to **[Ru(dppz)$_2$]**. Thus, the computational results support our initial assumption that the oligocationic domain would establish constructive electrostatic interactions with the DNA backbone, resulting in enhanced binding affinity. In an attempt to decipher the origin of the inversion in selectivity resulting from the introduction of the ancillary dppz ligand, we also performed the docking of the **Λ-Ru(dppz)$_2$]-R$_4$** stereoisomer to c-*KIT*.[32] The docking of **Λ-Ru(dppz)$_2$]-R$_4$** to *TEL* and *c-KIT* yields virtually the same binding energy regardless of the different binding geometries: *on-top* for *TEL* and *partially-sandwiched* for *c-KIT* (Figure 2). In line with the experimental results, this suggests a similar binding mechanism despite the substrate differences.[33]

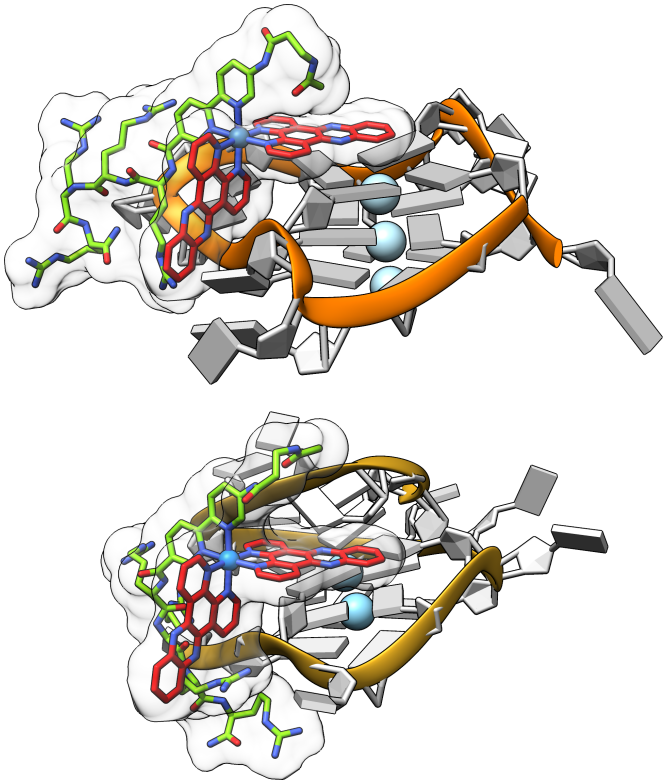


Figure 2. Top: best docking pose (lowest energy) of **[Λ-Ru(dppz)$_2$]-R$_4$** to the TEL GQ (PDB 1KF1). The dppz ligands are shown in red with one of them sitting on top of the GQ. Bottom: best docking pose of **[Λ-Ru(dppz)$_2$]-R$_4$** to the c-KIT GQ (PDB 4WO3). In this case, one of the dppz ligands is partly intercalating in the GQ structure. Light blue spheres represent the potassium ions at the center of the G-quartets, the bases are shown as grey slabs, and the DNA backbone as an orange or light orange ribbons, respectively. The Arg$_4$ domain makes in both cases some electrostatic contacts with the backbone that stabilize the complexes.

Conclusions

We report herein the first study about the effect of the presence of an oligoarginine functionalization in the recognition properties of a GQ binder. We have found that the appending of a R$_8$ tail to a Ru-dppz complex increase its affinity for GQ sequences and that the interplay between the ancillary ligand and the R$_8$ tail is key to control the selectivity between different GQ structures. Moreover, the R$_8$ functionalization endowed the GQ binders with cell-internalization properties and, interestingly, with appreciable cytotoxic capabilities. We believe that the metallopeptide approach report herein will open new perspectives for designing selective GQ binders.

Acknowledgements

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31 DNA sequence: GGGTTAGGGTTAGGGTTAGGG; PDB ID: 1kf1
32 DNA sequence: AGGGAGGGCGCTGGGAGGAGGG; PDB ID: 4wo3
33 Due to the exponential relationships between K_D and ΔG, the 20-fold difference in K_D can arise from relatively small differences in free energy of binding.

