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Unexpected DNA binding properties with correlated downstream biological applications in mono vs. bis-1,8-naphthalimide Ru(II)-polypyridyl conjugates

Gary J. Ryan, Fergus E. Poynton, Robert B. P. Elmes, Marialuisa Erby, D. Clive Williams,* Susan J. Quinn, Thorfinnur Gunnlaugsson**

Introduction

The development of transition metal polypyridyl complexes capable of targeting DNA has been extensively investigated in recent times. This has been largely motivated by the desire to probe DNA-based chemical processes with the view of developing novel therapeutic agents. Ru(II)-polypyridyl complexes show particular promise due to their water solubility, chemical and kinetic inertness and spectroscopic properties such as visible light absorption and photoluminescence. They have been shown to be internalised by cells, with a variety of mechanisms proposed including endocytosis, active transport and passive diffusion, or through the use of vectors or delivery molecules such as polypeptides. Tuning the polypyridyl ligand structure confers diverse functionality leading to intercalating complexes capable of ‘light-switching’ signalling phenomena, and strong DNA binding and photoinduced reactivity with DNA. Furthermore, we have recently shown that such complexes, based on simple bpy and phen ligands, when conjugated via an alkyl thio-linker to the surface of gold nanoparticles can be employed for luminescent imaging within cancer cells. We are interested in combining these features to develop molecules as dual imaging and therapeutic agents. Molecules can bind to DNA through a number of modes. Ru(II)-polypyridyl systems have been shown to bind to DNA by various modes, e.g. through intercalation, groove binding and electrostatic interactions. We have also developed several examples of DNA targeting binders, based on the use of 1,8-naphthalimide derivatives. Such structures have tuneable electronic properties, and have been shown to exhibit good DNA binding affinity through either intercalation or groove binding. In addition to being effective DNA intercalators or groove binders, the 1,8-naphthalimides can also act as sensitising antenna for the Ru(II)-based metal-to-ligand charge transfer (MLCT) emission, allowing for the population of the excited state using two excitation channels. Herein, we present two new DNA targeting Ru(II) complexes, [Ru(bpy)_2(bpy-NAP)]^{2+} (Ru.Nap) and [Ru(bpy)_2(bpy-NAP)_2]^{2+} (Ru.2Nap), Figure 1, based on Pickard he conjugation of one or two 1,8-naphthalimide units, respectively, to the polypyridyl complexes via a flexible alkyl spacer. The rational design of

![Fig. 1](link)
these complexes is based on combining (a) the DNA affinity and (b) the lipophilic nature of the naphthalimide group to facilitate more efficient binding of such Ru(II) complexes to DNA in a cooperative manner,\textsuperscript{38} and potentially enhance cellular uptake.\textsuperscript{36} To fully explore the activity of the complexes a detailed in-vitro photophysical study was completed in the absence and presence of DNA.

Two distinct ligand-dependent DNA binding modes were observed. In the mono-naphthalimide complex intercalation of the naphthalimide brings the Ru(II)+polypyridyl centre in close proximity to the DNA sugar phosphate backbone, which facilitates efficient DNA cleavage upon photoirradiation.

However, then in the case of Ru.2Nap the binding interaction of the second naphthalimide arm to DNA, reduces interaction of the Ru(II)-polypyridyl centre with the DNA backbone. Consequently, this complex is found to be a less effective photocleavage agent. This ‘negative allosteric effect’ leads to different biological activity of the Ru.Nap and Ru.2Nap complexes within HeLa cervical cancer cells and demonstrates how a simple design modification to the polypyridyl ligand can lead to modulated photophysical properties and downstream biological activity of such Ru(II)-polypyridyl-naphthalimide conjugates. This phenomenon, has to the best of our knowledge, not been demonstrated before for such systems and can have significant consequences on the ‘function’ or application (i.e. therapeutic vs. diagnostic) of such complexes in chemical biology.

Results and Discussion

Synthesis of Ru.Nap and Ru.2Nap

The mono-substituted naphthalimide complex Ru.Nap and the bis-substituted complex Ru.2Nap were synthesised in high yield, as demonstrated in Scheme 1. Firstly, the 1,8-naphthalimide 1, was formed using the mono-protected Boc diamine, N-(tert-butoxycarbonyl)-1,5-diaminopentane, giving 2 after BOC-deprotection using TFA. The naphthalimide 2 was then reacted with the appropriate 2,2’-bipyridine acid chloride under anhydrous conditions to give 3a and 3b in 83 and 95 % yield, respectively, after initial aqueous acid workup, followed by further purification using silica column chromatography. This was followed by complexation of 3a and 3b with Ru(bpy)$_2$Cl$_2$. For both, the reaction mixture was heated at reflux under an argon atmosphere for 20 hrs, followed by purification involving, treatment with concentrated aqueous solution of NH$_4$PF$_6$, which resulted in the formation of the PF$_6^-$ salts. These were purified using silica flash column chromatography by eluting the sample using the solvent mixture CH$_3$CN:H$_2$O:NaNO$_2$(sat) (40:4:1).

The Cl$^-$ salts of these complexes were then regenerated by stirring a solution of either Ru.Nap and Ru.2Nap in MeOH with Amberlite ion exchange resin. The Cl$^-$ salts were then further purified by column chromatography on Sephadex LH-20, eluting with MeOH, giving the products as red/brown solids in 86 and 76 % yields, respectively. Both Ru.Nap and Ru.2Nap were fully characterised (See Experimental). The distinct units of the complexes were readily discerned by NMR (CD$_3$CN and CD$_3$OD, 400 MHz) (See ESI† Figure S1-S7). CHN analysis showed the formation of the desired products in high purity.

UV-Vis Absorption Spectra

Having successfully synthesised both Ru.Nap and Ru.2Nap their various photophysical properties were evaluated. The UV-Vis absorption spectra were recorded in buffered pH 7.0 aqueous solution, and show distinctive spectroscopic signals for both the Ru(II) MLCT-based absorption and that of the naphthalimide, see Figure 2a and 2b. In the case of Ru.Nap an intense band was observed at 286 nm, which was mainly attributed to π-π* intraligand (IL) transitions. The band at 338 nm was assigned to the π-π* absorption and that of the naphthalimide, see Figure 2a and 2b. In the case of Ru.Nap an intense band was observed at 286 nm, which was mainly attributed to π-π* intraligand (IL) transitions. The band at 338 nm was assigned to the π-π*
The presence of the amide substituted bipyridine ligand causes 1,8-naphthalimides to interact through π+-stacking and in Ru.2Nap this can occur intramolecularly between the two naphthalimides, or between a naphthalimide and a bipyridine ligand. For this reason, the existence of electronic interactions in the ground state were considered by constructing an additive spectrum using a suitable Ru(II) complex, Ru and a water soluble 1,8-naphthalimide derivative, Nap, see Figure 3. Figure 3 compares the summed spectra with that of Ru.2Nap reveals a number of differences. Firstly, the MLCT region is found to be less absorbing (by 22 %) in Ru.2Nap. This strongly suggests electronic interaction between the Ru(II) centre and the 1,8-naphthalimide in the ground state. It is also worth noting that the IL transitions at 286 nm are also affected. Secondly, the band hypochromism was found to be more pronounced in the region of the 1,8-naphthalimide absorptions, at 230 and 345 nm, respectively. In particular, the absorbance at 345 nm is reduced by ca. 47 %, in comparison to that observed for the additive spectrum. These observations suggest that significant intramolecular stacking interactions of the 1,8-naphthalimides occur in solution.

**Emission Spectra**

Excitation of Ru.Nap and Ru.2Nap at 450 nm results in single band emission at 645 and 670 nm, respectively, Figure 2. The amide substituted bipyridine ligands results in a red-shifted emission compared to that observed for Ru(bpy)$_2$ which typically emits at ca. 605 nm. For both complexes significant contribution to MLCT-based emission is observed upon naphthalimide excitation, which suggests the presence of efficient singlet – singlet energy transfer, from the naphthalimide to the MLCT excited state. Evidence for this is clearly seen in the excitation spectra of these derivatives, see Figure 2a and 2b, and by comparing these excitation spectra to that of the control Ru complex (See ESI† Figure S8). Indeed, the absorption and excitation spectra are close to being super-imposable. The contribution to the Ru(II) emission from the 1,8-naphthalimides was found to be much greater for Ru.2Nap than Ru.Nap, which indicates that the second 1,8-naphthalimide enhances this effect. The observation of very weak naphthalimide emission for both complexes is a further indication of energy transfer to the Ru(II)-polypyridyl centre. This process is represented schematically in Figure S9 ESI†.

The quantum yields for both systems, recorded in aerated and de-gassed solution, compared well with that obtained for the control Ru $\Phi_{Ru,MLCT} = 0.014$ (all excitation at 450 nm), see Table 1. These results confirm the absence of any quenching of the MLCT based emission by the naphthalimide, which is expected as the naphthalimide centre is not sufficiently oxidising to accept an electron from the excited states of the Ru(II) complex (as deduced from a simplified Rehm-Weller equation, where the driving force for electron transfer was calculated to be approximately 0.12 eV for Ru.Nap and 0.26 eV for Ru.2Nap, see ESI Equation S1†).

**Table 1** Emission properties of Ru(II) complexes in aerated and de-gassed solution at 298 K. All at 6.5 µM concentration. λ$_{ex}$ is 450 nm.

<table>
<thead>
<tr>
<th>Complex</th>
<th>λ$_{em}$ (nm)</th>
<th>$\Phi_0$ (±10%) aerated buffer</th>
<th>$\Phi_0$ (±10%) aerated acetonitrile</th>
<th>$\Phi_0$ (±10%) de-gassed acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ru</td>
<td>670</td>
<td>0.014</td>
<td>0.021</td>
<td>0.061</td>
</tr>
<tr>
<td>Ru.Nap</td>
<td>645</td>
<td>0.018</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ru.2Nap</td>
<td>670</td>
<td>0.014</td>
<td>0.021</td>
<td>0.064</td>
</tr>
</tbody>
</table>

**Fig. 2** UV/Visible, excitation and emission spectra of (a) Ru.Nap (6.5 µM) and (b) Ru.2Nap (6.5 µM) in 10 mM phosphate buffer, at pH 7.

**Fig. 3** UV/Visible absorption spectra of Ru.2Nap (6.5 µM) (---), Ru (6.5 µM) (---), Nap (13 µM) (---) and Ru + Nap (---). All samples recorded in 10 mM phosphate buffer at pH 7.

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The positive free energy changes calculated for both systems render electron transfer from the excited Ru(II) complex to the 1,8-naphthalimide thermodynamically unfavourable in both cases. Quantum yield determination for Ru.2Nap in dry acetonitrile under aerated and degassed conditions revealed the complex emission to be quenched by dissolved oxygen (Table 1). Furthermore, the close agreement in the quantum yield values determined for Ru.2Nap and Ru suggests the absence of an equilibrium between the triplet excited state of the 1,8-naphthalimide and that of the Ru(II) complex. The presence of such equilibrium would be expected to greatly enhance the lifetime of Ru.2Nap over that of the reference complex. Having established the steady-state spectroscopic properties, the complexes were next studied in the presence of DNA to establish the influence of binding interactions.

**DNA Binding: Absorption Titrations**

The DNA binding properties of the complexes was probed by monitoring absorption bands of the naphthalimide and the Ru(II) centre. In the case of the mono-naphthalimide Ru.Nap, the addition of increasing concentrations of salmon testes’ DNA (st-DNA) resulted in a 37 % hypochromism of the 1,8-naphthalimide band at 338 nm (Figure 4a). Such a large change in the absorbance is characteristic of intercalation. A significant change was also observed in the absorbance of the MLCT band, which decreased by 16 %. In comparison the control Ru complex undergoes a 9 % hypochromic shift at the MLCT band, which is attributed to electrostatic interactions with the phosphate backbone of DNA. The intrinsic binding constant of 9.0 × 10^6 M^-1 was derived from a binding isotherm using the model of Bard et. al. and a binding site size of 2.18 base pairs was calculated, see Table 2. This is an order of magnitude higher than the binding observed for either the naphthalimide or ruthenium metal centre, Ru(bpy)_2^2+. Ru separately and confirms the presence of cooperative binding.

When the titration was repeated for Ru.2Nap even greater changes in the absorption spectrum were observed, see Figure 4b. A large, 56 %, hypochromic shift was observed in the naphthalimide band at 345 nm. However, more strikingly, a 41 % decrease was observed for the MLCT band. The magnitude of the hypochromic shift is more than twice that observed for the mono-naphthalimide complex. These observations show that the environment of both the naphthalimide and the metal centre of Ru.2Nap is significantly altered in the presence of DNA, which also is the case for Ru.Nap. While, partial intercalation of the Ru(II) complex may also be possible, it is unlikely due to the minimal extended planar nature of the bipyridine ligands. The binding constant K for these interactions was determined as 1.5 × 10^7 M^-1, with a slightly larger calculated binding site size of 2.48 base pairs. The binding curve profiles obtained from the UV-visible titrations indicate that both complexes bind very strongly to DNA. Notably, in the case of Ru.2Nap changes in the UV-vis absorption spectra are complicated by the existence of intramolecular π-stacking interactions between the naphthalimides and/or with the bpy ligand to which they are coupled. Furthermore, the lack of a clear isosbestic point in the case of Ru.2Nap possibly reflects the fact that the flexible linker allows multiple modes of binding to occur.

**DNA Binding: Emission Titrations**

Fluorometric DNA titrations were followed by exciting both chromophores independently at 338 nm and 450 nm. Having observed that the emission from the Ru(II) centre is sensitised by energy transfer from the 1,8-naphthalimides, it was expected that any intercalation of the naphthalimide group with DNA would result in changes in the photophysical properties of the complexes. Direct excitation of Ru.Nap metal centre at 450 nm resulted in a two phase increase in intensity coinciding with a more gradual increase after the addition of ca. one base pair equivalent. The emission was found to plateau at ca. 10 base pair equivalents with an increase in MLCT emission intensity of 20 % observed (Figure S10 ESI†). It is possible that the modest increase in intensity observed for Ru.Nap, is due to protection of the metal centre from quenching by oxygen and solvent molecules upon DNA binding.

**Table 2** DNA binding parameters from fits to absorbance data

<table>
<thead>
<tr>
<th>Complex</th>
<th>%ΔAbs</th>
<th>%ΔMLCT</th>
<th>Binding constant K (M^-1)</th>
<th>Binding site size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ru.Nap</td>
<td>37 %</td>
<td>16 %</td>
<td>9.0 × 10^6 (± 1.0) M^-1</td>
<td>2.18 (± 0.02)</td>
</tr>
<tr>
<td>Ru.2Nap</td>
<td>56 %</td>
<td>41 %</td>
<td>1.5 × 10^7 (± 0.5) M^-1</td>
<td>2.48 (± 0.02)</td>
</tr>
</tbody>
</table>
between the metal complex centre and naphthalimide. The proposed to result in more efficient energy transfer to the emission behaviour. Direct excitation of the MLCT b and at metal.

quenching of the 1,8+naphthalimide singlet excited state by absence of electron transfer from a suitable nucleotide the changes in emission intensity, see Figure 6. An initial increase in intensity represented a decrease of 46% from that of the 1,8+naphthalimide band at 345 nm yields more pronounced decrease of 73% in intensity (14%) was observed at base equivalents (up to 0.45 base pairs), followed by a subsequent decrease of 73% compared to the initial intensity. The behaviour at low Bp/D equivalents is attributed to the disruption of intramolecular stacking of the 1,8-naphthalimides, which occurs in the presence of a small quantity of DNA. This unstacking is proposed to result in more efficient energy transfer to the metal.

The overall decrease in intensity may be attributed to quenching of the 1,8-naphthalimide singlet excited state by electron transfer from the nucleotides. However, in the absence of electron transfer from a suitable nucleotide the decrease in intensity may also arise due to disruption of energy transfer from the naphthalimide to the metal centre due to the presence of the DNA environment as observed in the excitation spectrum (Figure S13 ESI†).

Thermal Denaturation Studies
Thermal denaturation studies were carried out to further elucidate

MLCT emission observed upon excitation of the Ru.Nap naphthalimide group at 338 nm, in the presence of DNA, was found to decrease (ca. −43%) in intensity (Figure S11 ESI†).

A large decrease in intensity was observed up to the addition of ca. 3 base pair equivalents, followed by a more gradual decrease up to ca. 15 base pairs. No concomitant changes were seen in the 1,8-naphthalimide emission. We can consider that the binding of the naphthalimide, in the Ru.Nap complex, to DNA results in (1) positioning of the naphthalimide in a new chemical environment and (2) the disruption of coupling between the metal complex centre and naphthalimide. The effect on the intramolecular coupling is most clearly seen by comparing the excitation spectra of the complexes recorded in the presence, and absence of DNA (see Figure 5). It can be seen that in the presence of higher concentrations of DNA the excitation spectrum for Ru.Nap closely resembles that of Ru, which has no appended 1,8-naphthalimides.

Ru.2Nap in the presence of DNA also exhibited complex emission behaviour. Direct excitation of the MLCT band at 450 nm in the presence of low concentrations of st-DNA (0–0.45 base pair equivalents) resulted in a modest (25%) increase in the MLCT emission intensity (Figure S12 ESI†). However, in contrast to Ru.Nap, further additions of st-DNA resulted in a decrease in the emission intensity. The final intensity represented a decrease of 46% from that of the initial intensity. In Ru.2Nap excitation of the 1,8-naphthalimide band at 345 nm yields more pronounced changes in emission intensity, see Figure 6. An initial increase in intensity (14%) was observed at base equivalents (up to 0.45 base pairs), followed by a subsequent decrease of 73% compared to the initial intensity. The behaviour at low Bp/D equivalents is attributed to the disruption of intramolecular stacking of the 1,8-naphthalimides, which occurs in the presence of the DNA environment as observed in the excitation spectrum (Figure S13 ESI†).

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Circular Dichroism (CD) and Linear Dichroism (LD) Studies

Circular and linear dichroism\textsuperscript{53} DNA titrations were also carried out for both complexes to determine the nature of any induced chirality in the presence of DNA and to attempt to elucidate their specific mode of DNA interaction. Keeping the concentration of DNA constant, increased amounts of the complexes were added to give a range of Bp/D ratios. Both conjugates were found to display similar CD behaviour. In the case of Ru.Nap a small induced CD signal was observed at long wavelength with the maximum appearing at approximately 470 nm for the MLCT absorption band, see Figure 8a. This observation confirms that the Ru(II) component of Ru.Nap is associated with the DNA structure. A small induced CD signal was observed in the region of absorption of the 1,8-naphthalimide at 350 nm, however larger signal changes were observed at lower wavelengths where absorption arises due to both the naphthalimide and bpy \(\pi\rightarrow\pi^*\) intraligand transitions. In contrast, larger changes in the CD signal were seen in the region of absorption of DNA, which is possibly due to a combination of conformational changes of the DNA induced by the bound complex, or alternatively due to ICD transitions of the bound complexes themselves. These changes can be clearly seen in the difference spectra shown in Figure 8b. The CD spectra obtained for Ru.2Nap revealed significant changes below 300 nm in the region where DNA absorbs.

Interestingly, weaker signals were observed in the naphthalimide and the MLCT region, which suggests that the two complexes bind differently to DNA (see Figure S14 ESI†).

In an attempt to understand in greater detail the different binding modes involved, linear dichroism (LD) measurements were also performed on Ru.Nap and Ru.2Nap in the presence of DNA. Using this technique intercalative binding gives rise to absorption minima, while groove binding interactions tend to result in absorption maxima. The LD spectra of Ru.Nap in the presence of DNA are shown in Figure 8c. The DNA bands exhibit the characteristic absorption minima below 300 nm. In addition, an absorbance minimum is found in the region of the 1,8-naphthalimide at 350 nm. This suggests a perpendicular orientation of the naphthalimide chromophore relative to the bases \textit{(i.e.} intercalated between the bases). Significantly, the MLCT absorption region possesses structured bands with an absorbance minimum at 420 nm and a maximum at 475 nm. The presence of this structure indicates that the metal complex is closely associated with the DNA with interactions in the groove and possible partial intercalation. These results support our finding above that both components (naphthalimide and Ru(II) polypyridyl) are contributing to the overall binding interaction with DNA, and that the Ru(II) complex is tightly bound.

In contrast to these results, the LD spectra obtained for Ru.2Nap in the presence of DNA contained significantly less

Fig. 8 Circular dichroism curves of (a) st-DNA (150 \(\mu\)M) in 10 mM phosphate buffer, at pH 7 in the absence and presence of Ru.Nap at varying ratios and (b) the difference spectra obtained. Linear dichroism curves of st-DNA (150 \(\mu\)M) in 10 mM phosphate buffer, at pH 7.4 in the absence and presence of (c) Ru.Nap at varying ratios and (d) Ru.2Nap at varying ratios.
structure, see Figure 8d. This is somewhat surprising giving that the UV-Vis and emission titrations demonstrated that Ru.2Nap has higher binding affinity for DNA. The LD showed a weakly absorbing minimum in the region of 1,8-naphthalimide absorption at 365 nm. This may be explained by the overlap of the absorbance of the two naphthalimide with different binding modes. If multiple binding interactions occur with DNA, for example where only one naphthalimide is bound to the DNA by intercalation and the other binds via a different mode e.g. groove binding, the overlap of the negative LD signal of the intercalating and positive signal of the groove binding naphthalimides would be expected to reduce the observed LD signal. The presence of a negative LD signal does, however, suggest that at least one naphthalimide moiety is binding via an intercalative mode of binding.

Significantly, no LD signal was observed in the MLCT region of the spectrum for Ru.2Nap. Indeed, both CD and LD reveal increased optical activity in the MLCT region of the spectrum for Ru.Nap compared to the Ru.2Nap. We speculate that there are two possible origins for this observation. Firstly, that the binding of the second naphthalimide causes the position and orientation of the metal complex change significantly. Secondly, as noted for the naphthalimide signal, the presence of multiple binding orientations may result in overlapping signals from groove binding and intercalating contacts. This second observation would be consistent with strong changes observed for the complex in the MLCT region in the UV-visible absorption titration (Figure 4b). In summary, such behaviour supports the observations from both CD and Tm measurements above, where such differing changes were attributed to the fact that Ru.Nap is most likely bound by a classical intercalative mode, whereas Ru.2Nap likely binds in a more complicated and possibly multimodal manner. The ground and excited state DNA binding results and the differences seen in the LD titrations for Ru.Nap and Ru.2Nap clearly demonstrate that while strong binding can be achieved using two naphthalimides, the second naphthalimide moiety can also influence the binding mode of the Ru(II) centre itself with DNA. Such a phenomenon has not, to the best of our knowledge, been demonstrated before using Ru(II) polypyridyl complexes, flanked with two identical binding ligands such as the naphthalimide units in Ru.2Nap. This is a feature that can have the potential to modulate or tune the ability of such Ru(II)-polypyridyl complexes to interact with DNA, such as their ability to cleave or damage DNA either in the dark or upon light activation. With this in mind we set out to probe these potential effects further using both Ru.Nap and Ru.2Nap.

**Photocleavage of DNA by Ru.Nap and Ru.2Nap**

The ability of Ru.Nap and Ru.2Nap to cleave pBR322 plasmid DNA was investigated and compared to that of Ru(bpy)$_3^{2+}$, see Figure 9. The amounts of Form I vs Form II present are detailed in Table S1 ESI†, as determined from densitometry measurements of the relative fluorescence intensity of the resulting bands, that were compared to the control photocleaver Ru(bpy)$_3^{2+}$, which was used to evaluate the relative efficiency of cleavage of the conjugate systems. The presence of Ru(bpy)$_3^{2+}$ resulted in an increase in Form II after 5 minutes irradiation. In the presence of Ru.Nap (Lane 3) a small amount of damage was also apparent after only 1 min of irradiation, the percentage of Form II having increased to 35%. After 3 minutes irradiation, this had increased to 49% Form II, and at the longest irradiation time of 5 minutes had increased to 61% Form II. From these results, it can be concluded that Ru.Nap displayed a reasonably efficient photocleavage of DNA, leading to more pronounced changes than the reference Ru(bpy)$_3^{2+}$.

In contrast, complex Ru.2Nap resulted in only a minor cleavage of the nucleic acid, with Form I being the predominant DNA species present, even after 5 minutes irradiation. The different photocleaving ability exhibited by the two complexes is attributed to their different DNA binding modes which position the Ru(II)-polypyridyl centre in different environments. A reduction in activity is likely to be due to the proximity of the metal complex to the DNA. This could arise if the complex was poorly positioned or held away from the DNA bases. On the other hand, if the complex is very tightly associated with the DNA groove structure, then access to molecular oxygen and the photoreactivity would be reduced. This may be the case for Ru.2Nap. This is a somewhat unexpected result as it demonstrates that the ‘cooperativity’ that results in increasing binding affinity can have major and ‘negative’ secondary effects. Which, as stated above, we believe to be a direct consequence of the positioning of Ru(II)-polypyridyl centre along the DNA backbone when the second naphthalimide unit is bound.

**Cellular Uptake, Localisation and Viability Studies**

With the aim of using Ru.Nap and Ru.2Nap as dual functioning imaging and therapeutic agents, their ability to be internalised in target cells is of vital importance. In order to evaluate the ability of Ru.Nap and Ru.2Nap to localise within cells, fluorescence confocal microscopy and cell viability analyses were undertaken. Confocal microscopy was carried out in order to provide visual evidence of the
localisation of Ru.Nap and Ru.2Nap in HeLa cervical cancer cells. Populations of live cells (0.5 × 10^5) were incubated with both complexes (30 µM) at 37 °C for 4 and 24 hrs before being treated with the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope with a 60× oil immersion lens. The results obtained are exemplified in Figure 10, which shows the fluorescence confocal laser scanning microscopy images of live HeLa cells after incubation with Ru.Nap for 4 and 24 hrs (the corresponding images for Ru.2Nap are shown in Figure S15 ESI†). The observed images show the presence of red luminescence emanating from within the cells’ interior, associated around the nucleus. Although we do not demonstrate co-localisation with any specific organelles for this study, our previous work in this area has demonstrated that related Ru(II) complexes localise predominantly within the mitochondria, leading to perinuclear clustering, with some localisation observed in the lysosomes and the endoplasmic reticulum. Similar localisation results has been seen by several other researchers. Additionally, cellular viability was measured using an AlamarBlue assay. Results showed a significant reduction in IC_{50} values following light activation with compound Ru.Nap, compared to cells maintained in the dark (7.8 µM versus 30 µM). In contrast, for compound Ru.2Nap, there was no difference between light and dark IC_{50} values, with the cells showing only a small degree of toxicity at the highest concentration tested, 30 µM, see Table 3. The production of reactive oxygen species (ROS) may be a potential mechanism through which these novel compounds induce apoptosis. Taken together, these results are of considerable significance in providing evidence for our assertion that the use of such bifunctional complexes, comprising separate hydrophilic and hydrophobic centres is a viable means of effecting cellular accumulation of Ru(II) based probes and reactive agents within cells for potential diagnostic and therapeutic use.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>IC_{50} value (µM)</th>
<th>Dark</th>
<th>Light activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ru.Nap</td>
<td>29.8</td>
<td>7.81</td>
<td></td>
</tr>
<tr>
<td>Ru.2Nap</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td></td>
</tr>
</tbody>
</table>

**Conclusions**

In conclusion, we have reported the preparation of two new DNA binding ruthenium complexes appended with one (Ru.Nap), or two (Ru.2Nap) naphthalimides through flexible alkyl chain linkers, and investigated their photophysical and biological properties. The luminescence of the complexes is found to be solely MLCT based with excitation of the naphthalimide centres resulting in MLCT emission, as evidenced from their excitation spectra. This presents the opportunity to obtain MLCT-based emission from both complexes through excitation into either the MLCT or the naphthalimide absorption bands. These complexes were found...
to bind strongly to DNA; demonstrating that our design supports cooperativity, where the naphthalimide moiety greatly assists in inducing strong association with DNA by displaying large changes in the steady state absorption and emission of both complexes. Both complexes were also shown to exhibit CD activity in the presence of DNA further confirming their ability to bind strongly to DNA. The binding mode of these complexes with DNA was further investigated by observing the changes in the LD spectra. From the relatively large negative band in the LD spectrum when bound to DNA, Ru.Nap appeared to bind to DNA via intercalation of the naphthalimide moiety; this being further supported by a large $\Delta T_m$. In contrast, Ru.2Nap was shown to bind to DNA in a more complex fashion; this being evident from the small LD signal of the naphthalimide moiety when bound to DNA and a small $\Delta T_m$. This is an extremely important result as it clearly demonstrates that a strong association with the DNA is maintained between the naphthalimide and the Ru(II)-polypyridyl unit in the case of Ru.Nap. However, in the case of Ru.2Nap, the picture is more complicated, as here, both the naphthalimide units are bound to DNA causing a change in the position of the Ru(II)-polypyridyl centre at the DNA backbone. The result of this change in binding manifests in the difference in photoreactivity between the two complexes in the presence of DNA. Upon light activation Ru.Nap was shown to effectively cleave plasmid DNA while Ru.2Nap showed poor cleavage. Moreover, this difference in photoreactivity in vitro was also observed in cellulo. While both complexes are readily taken up by HeLa cells and emission from both complexes was observed within the cells after 4 hours incubation (the complexes were observed to cluster next to the nucleus), Ru.Nap was found to reduce HeLa cell viability upon photoactivation, whereas Ru.2Nap was nontoxic under identical conditions. The results presented herein demonstrate different activity observed for two closely related conjugates Ru.Nap and Ru.2Nap, where the structural difference is the presence of a second naphthalimide unit in Ru.2Nap, does lead to cooperativity in DNA binding. Such modification however, can have a detrimental effect on their biological activity when such structures are considered as potential therapeutic agents. To the best of our knowledge this has not previously been observed or postulated for such systems when designing DNA targeting and responsive therapeutics. Such properties are highly desirable when one considers the applications of such structures as imaging agents for use in molecular biology, as Ru.2Nap is not particularly toxic upon light irradiation. Hence, while Ru.Nap is a potential candidate for use in therapy, Ru.2Nap, is a potential candidate for use in florescence imaging. We are currently investigating this and such functions of other Ru(II) based polypyridyl complexes in greater detail.

**Experimental**

**Materials and Instrumentation**

All reagents and solvents were purchased commercially and used without further purification unless otherwise stated. Anhydrous solvents were prepared using standard procedures, according to Vögel, with distillation under argon prior to each use. Solutions of DNA in 10 mM phosphate buffer (pH 7) gave a ratio of UV absorbance at 260 and 280 nm of 1.86:1, indicating that the DNA was sufficiently free of protein. Its concentration was determined spectrophotometrically using the molar absorptivity of 6600 M$^{-1}$ cm$^{-1}$ (260 nm).

All NMR spectra were recorded using a Bruker DPX-400 Avance spectrometer, operating at 400.13 MHz for $^1$H NMR and 100.6 MHz for $^{13}$C NMR, or a Bruker AV-600 spectrometer, operating at 600.1 MHz for $^1$H NMR and 150.2 MHz for $^{13}$C NMR. Shifts are referenced relative to the internal solvent signals. Electrospray mass spectra were recorded on a Micromass LCT spectrometer, running Mass Lynx NT V 3.4 on a Waters 600 controller connected to a 996 photodiode array detector with HPLC-grade methanol or acetonitrile. High resolution mass spectra were determined by a peak matching method, using leucine Enkephalin, (Tyr-Gly-Gly-Phe-Leu), as the standard reference (m/z = 556.2771). Melting points were determined using an IA9000 digital melting point apparatus. Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer fitted with a Universal ATR Sampling Accessory. Elemental analysis was conducted at the Microanalytical Laboratory, School of Chemistry and Chemical Biology, University College Dublin.

**Biological Investigations**

**Cell culture:** HeLa cells were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 50 µg/ml penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2.

**Viability assay:** 5×10³ cells/well were seeded in a 96-well plate and treated with the respective compound for 24 h + irradiation with 18 J/cm² of light using a Hamamatsu L2570 200 Watt HgXe Arc Lamp equipped with a NaNO2 filter for 60 mins. After 24h, each well was then treated with 20 µl of Alamar Blue (BioSource) (pre-warmed to 37 °C) and left to incubate at 37 °C in the dark for 4-6 h. Fluorescence was read using at 590 nm (excitation 544 nm). The control untreated cells represented 100 % cell viability. All data points (expressed as means ± S.E.M.) were analysed using GRAPHPAD Prism (version 4) software (Graphpad software Inc., San Diego, CA).

**Confocal microscopy:** HeLa cells were seeded at a density of 1 X 105 cells /2 ml, left for 24 h before the required treatment. Cells were then washed twice, stained with and analysed by live confocal microscopy using an Olympus FV1000 point scanning microscope with a 60x oil immersion lens with an NA (numerical aperture) of 1.42. The software used to collect images was FluoView Version 7.1 software. Compounds were excited by a 488nm argon laser, emission 600-700 nm. DAPI was excited by a 405 nm diode laser, emission 410-450nm.
N-[(tert-Butyloxycarbonyl)-5-aminopentyl]-1,8-naphthalimide (1): 1,8-Naphthalic anhydride (0.80 g, 4.04 mmol, 1 eq.), N-(tert-butyloxycarbonyl)-1,5-diaminopropene (0.90 g, 4.45 mmol, 1.1 eq.), and Et₂N (1.23 g, 1.69 ml, 12.12 mmol, 3 eq.) were added to anhydrous toluene (60 ml) and the mixture heated at reflux for 24 hrs. The mixture was filtered hot through celite and concentrated under vacuum. The residue was dissolved in CH₂Cl₂ (100 ml), washed with 0.1 M HCl (2×20 ml), water (20 ml), dried over MgSO₄ and concentrated under vacuum. The product was obtained as an orange oily solid without need for further purification (0.57 g, 82%). ¹H NMR (DMSO-d₆, 400 MHz, δ): 8.53 (2H, d, J = 7.6 Hz), 8.49 (2H, d, J = 8.5 Hz), 7.91 (2H, m), 6.82 (1H, s), 4.07 (2H, t, J = 7.0 Hz), 2.94 (2H, d, J = 6.0 Hz), 1.65 (2H, m), 1.40 (13H, m). ¹³C NMR (DMSO-d₆, 100 MHz, δ): 163.4, 155.6, 134.3, 131.3, 130.7, 127.4, 122.1, 77.3, 54.9, 50.8, 29.2, 28.2, 27.3, 23.8; ESI-MS m/z 405.1809 (M+Na)+; IR (ATR, cm⁻¹): 1698, 1656, 1513, 1270, 1263, 1215, 39.0, 38.8, 26.6, 26.3, 23.0; ESI-MS m/z 283.1456 (M)+; IR (ATR, cm⁻¹): 3179 (w, +NH+Bpy+H), 8.57 (1H, d, J = 4.5 Hz, Bpy+H), 4.07 (2H, t, J = 7.6 Hz, CH₂), 3.29 (2H, m, CH₂), 2.44 (3H, s, CH₃), 1.71 (2H, m, CH₂), 1.63 (2H, m, CH₂), 1.41 (2H, m, CH₂); IR (ATR, cm⁻¹): 1698, (m, -CO-N-CO-), 1658 (m, -CONH-); m.p 151 – 152 °C.

4-[N-(Pentylcarboxamide)-1,8-naphthalimide]-4'-methyl-2,2'-bipyridine (3a): Compound 1 (0.15 g, 0.38 mmol, 1.1 eq.) and Et₂N (0.10 g, 0.14 ml, 1.02 mmol, 3 eq.) were dissolved in dry CH₂Cl₂ and 4-(carbonylchlordie)-2,2'-bipyridine (0.08 g, 0.34 mmol, 1 eq.) dissolved in dry CH₂Cl₂ was added dropwise. The solvent was removed under reduced pressure. The resulting residue was stirred with 0.1 M HCl, filtered and washed with water. Purification was performed by silica column chromatography eluting with CH₂Cl₂/MeOH 10 %. The product was obtained as a pale brown solid (0.14 g, 83 %); Calculated for C₂₀H₂₀N₂O₃, C, 50.8, 29.2, 28.2, 27.3, 23.8; ESI+MS m/z 283.1456 (M)+; IR (ATR, cm⁻¹): 1698, 1656, 1508, 1427, 1377, 1339, 1316, 1305, 1285, 1279, 1275, 1269, 1255, 1244, 1224, 1217, 1215, 39.7, 39.6, 28.5, 27.3, 24.0, 20.1; IR (ATR, cm⁻¹): 1698, (m, -CO-N-CO-), 1658 (m, -CONH-).

4,4'-Bis-[N-pentylcarboxamide]-1,8-naphthalimide)-2,2'-bipyridine (3b): It was prepared as above with compound 2 (0.40 g, 1.01 mmol, 2.1 eq.) and 4,4'-bis(carbonylchlordie)-2,2'-bipyridine (0.14 g, 0.48 mmol, 1 eq.). The product was obtained as a purple solid (0.35 g, 95 %). ¹H NMR (DMSO-d₆, 400 MHz, δ): 8.97 (1H, br s, NH), 8.80 (1H, m, Bpy-H), 8.75 (1H, m, Bpy-H), 8.46 (2H, d, J = 7.0 Hz, Naph-H), 8.42 (2H, d, J = 8.0 Hz, Naph-H), 7.83 (2H, m, Naph-H), 7.77 (1H, m, Bpy-H), 4.06 (2H, m, CH₂), ~3.30 (CH₂, under solvent peak – from CH COSY), 1.69 (2H, m, CH₂), 1.65 (2H, m, CH₂), 1.41 (2H, m, CH₂). ¹³C NMR (DMSO-d₆, 100 MHz, δ): 164.8, 163.7, 155.8, 150.2, 143.3, 134.5, 131.6, 131.0, 127.7, 127.5, 122.4, 118.5, 40.1, 39.7, 28.9, 27.6, 24.3; IR (ATR, cm⁻¹): 1698 (w, -CO-N-CO-), 1658 (m, -CONH-); m.p 210 – 211 °C.

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Notes and References


Unexpected DNA binding properties with correlated downstream biological applications in mono vs. bis-1,8-naphthalimide Ru(II)-polypyridyl conjugates

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The development of two structurally related 1,8-naphthalimide-conjugated Ru(II)-polypyridyl complexes, Ru.Nap and Ru.2Nap, which exhibit dramatically different DNA binding and photocleavage behaviour is presented. Moreover this difference in behaviour in vitro is conserved in cellulo, where Ru.Nap exhibits photoinduced toxicity, whereas Ru.2Nap does not. This demonstrates that subtle structural modification within two probes can have a cascading effect on their mechanistic and biological properties.