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Targeting of photooxidative damage on single-stranded DNA representing the *bcr-abl* chimeric gene using oligonucleotide-conjugates containing $[Ru(phen)_3]^{2+}$-like photosensitiser groups

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Photooxidative damage was induced predominantly at a single guanine base in a target DNA by irradiation ($\lambda > 330$ nm) in the presence of complementary oligodeoxynucleotide conjugates (ODN-5′-linker-$[Ru(phen)_3]^{2+}$) (phen = 1,10-phenanthroline). The target DNA represents the b_2a_2 variant of the chimeric *bcr-abl* gene implicated in the pathogenesis of chronic myeloid leukaemia, and the sequence of the 17mer ODN component of the conjugate ($^{3'}$ G G T A G T T A T T C C T T C T T $^{5'}$) was complementary to the junction region of the sense strand sequence of this oncogene. Two different conjugates were prepared, both of them by reaction of the appropriate succinimide ester with 5′-hexylamino-derivatised 17mer ODN. In Ru–ODN-1 (**7**) the linker was $-(CH_2)_6$-NHCO-bpyMe (-bpyMe = 4′-[4-methyl-2,2′-bipyridyl]), whereas in Ru–ODN-2 (**13**) it was $-(CH_2)_6$-NHCO–$(CH_2)_3$-CONH-phen. Photoexcitation of either of the conjugates when hybridised with the ^{32}P-5'-end-labelled target 34mer $^{5'}$T G A C C A T C A A T A A G G A A G A A G^{21} C C C T T C A G C G G C C $^{3'}$ (ODN binding site underlined) led to an alkali-labile site predominantly (> 90%) at the G^{21} base, which is at the junction of double-stranded and single-stranded regions of the hybrid. Greater yields were found with Ru–ODN-1 (**7**) than with Ru–ODN-2 (**13**). In contrast to this specific cleavage with Ru–ODN-1 (**7**) or Ru–ODN-2 (**13**), alkali-labile sites were generated at all guanines when the 34mer was photolysed in the presence of the free sensitiser $[Ru(phen)_3]^{2+}$. Since $[Ru(phen)_3]^{2+}$ was shown to react with 2′-deoxyguanosine to form the diastereomers of a spiroiminodihydantoin derivative (the product from $^1O_2$ reaction), $^1O_2$ might also be an oxidizing species in the case of Ru–ODN-1 (**7**) and Ru–ODN-2 (**13**). Therefore to determine the range of reaction, a series of 'variant' targets was prepared, in which G^{21} was replaced with a cytosine and a guanine substituted for a base further towards the 3′-end (*e.g.* Variant 3; $^{5'}$T G A C C A T C A A T A A G G A A G A A C C G^{23} C T T C A G C G G^{32} C C $^{3'}$). While it was noted that efficient reaction took place at distances apparently remote from the photosensitiser (*e.g.* at G^{32}, but not G^{23} for Variant 3), this effect could be attributed to hairpinning of the single-stranded region of the target. These results are therefore consistent with the photooxidative damage being induced by a reaction close to the photosensitiser rather than by a diffusible species such as $^1O_2$.

1 Introduction

Ruthenium polypyridyl complexes have been widely used as photochemical and photophysical probes for DNA.[1–5] These applications exploit both the convenient luminescence properties of their lowest-lying metal-to-ligand charge transfer (MLCT) excited states (orange–red emission, lifetime in sub-microsecond region) and the fact that their excited states are able to act as both strong reductants or oxidants as well as being excellent sensitisers of singlet oxygen. It has been known for many years that these complexes can sensitise photodamage to DNA (inducing single-strand cleavage,[6–8] photooxidation[9,10] and adduct formation[11,12]), the nature of the process depending on the polypyridyl ligand employed.

The use of oligonucleotide-conjugates to direct a probe molecule or reactive chemical entity to a particular sequence in either single- or double-stranded DNA is now well established and indeed there have been a number of studies with oligonucleotide–ruthenium polypyridyl conjugates. Most of these studies have focussed on the photophysical properties of these materials,[13–23] although photooxidative damage and photoadduct formation have also been investigated.[9,10,24,25]

In this paper we describe the preparation of oligonucleotide–ruthenium polypyridyl complexes and their use in targeting a sequence contained in the *bcr-abl* fusion gene that is detected in >95% of patients with chronic myeloid leukaemia (CML) (Fig. 1). This chimeric gene is formed as a result of a reciprocal translocation of genetic material from chromosomes 9 and 22.

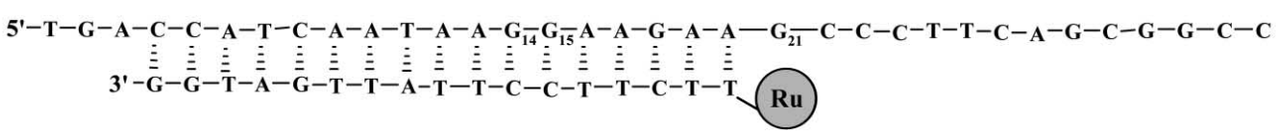

Fig. 1 17mer Ru conjugate hybridised to the target 34mer.

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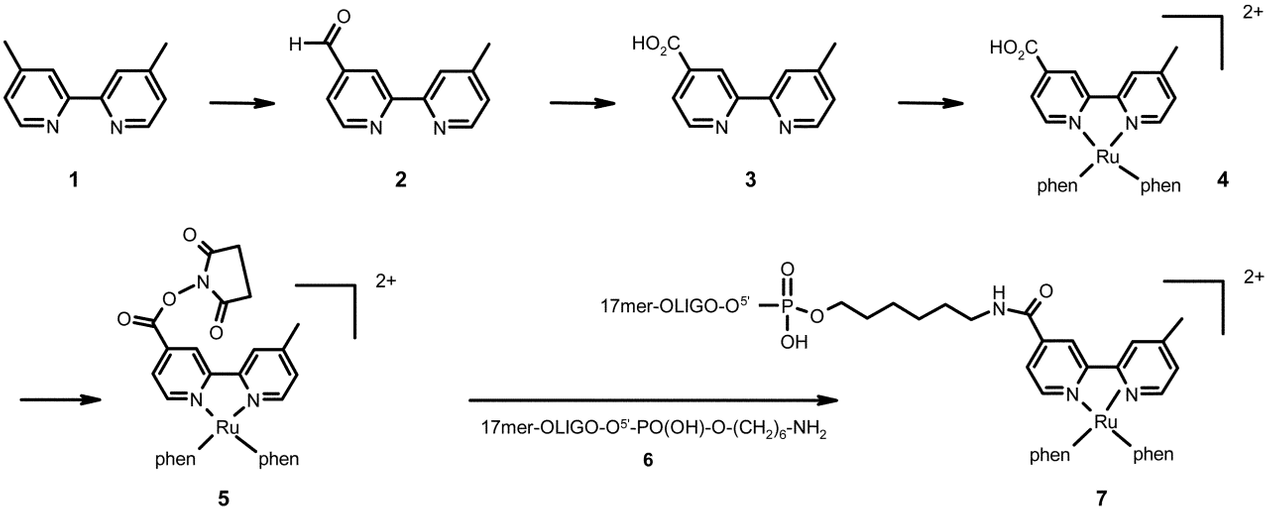


Scheme 1

The *abl* proto-oncogene on chromosome 9 is juxtaposed so that it is under the control of the *bcr* gene on chromosome 22. The resulting rearrangement of genetic information creates a chimeric leukaemia specific gene that codes for a protein (P210) with enhanced tyrosine kinase activity.[26] Its base sequence is therefore an appropriate target for 'anti-sense' control of the messenger RNA and a number of studies have investigated the use of anti-sense approaches in CML, both *in vitro* and *in vivo*.

We wished to investigate the properties of Ru–ODN (oligodeoxynucleotide) conjugates as potential antisense agents, focussing on the ability to specifically target the *bcr-abl* junction and promote nucleobase damage. The specificity of the damage to the target nucleic acid may depend on whether the lesions are caused by the excited state reacting directly with the target (Type I processes) or by generating a reactive species (such as singlet oxygen – Type II processes). While many authors assume that the latter is the dominant process for the photosensitised damage caused by ruthenium complexes,[27] it is by no means certain that this will be the case for the oligonucleotide complexes, where the sensitiser and target are positioned in close proximity.

We have prepared two different $Ru(phen)_3^{2+}$ analogues, each of which is attached *via* different linkers to a 17mer oligodeoxynucleotide (17mer ODN). The base sequence of this 17mer is complementary to a segment of 17 bases occurring in the target 34mer ODN whose entire sequence is specific to the *bcr/abl* fusion junction region of the *bcr-abl* mRNA. While *in vivo* the target strand would be RNA, for the purposes of our model system we have used a single-stranded DNA ODN of equivalent sequence. In our photochemical experiments we were able to direct damage on to a single guanine residue (G^{21}) at the junction of the single- and double-stranded regions of the hybridised complex (Fig. 1). Use of the two different linkers has allowed us to gauge proximity effects, and we have also examined the effect of reagents that are known to affect the yield of singlet oxygen-mediated reactions. In addition, we have synthesised and studied a number of variants of the 34mer target ODN, and have been able to demonstrate that the oxidative process is very short range, but can be targeted to more distal guanines in hairpinned structures.

2 Results and discussion

2.1 Synthesis of ruthenium–oligodeoxynucleotide conjugates

In the present investigation, two ruthenium complexes were prepared, (**4**) and (**11**), and each was then linked *via* an aminohexyl spacer to the 5′ end of a 17mer ODN strand. Initial attempts at this coupling used methods originally developed by Barton and Jenkins[13] and by Schubert and co-workers,[28] but difficulties were encountered. Coupling was eventually achieved using an approach similar to that pioneered by Bannwarth and co-workers,[18] and the two new ruthenium–ODN conjugates, (**7**) and (**13**), were obtained successfully. The base sequence in (**7**) and in (**13**) was complementary to the sequence in the synthetic target 34mer ODN (Fig. 1) which, as described above, was designed as a model system to represent the mRNA sequence transcribed from the breakpoint region of the *bcr/abl* oncogene on the Philadelphia chromosome.

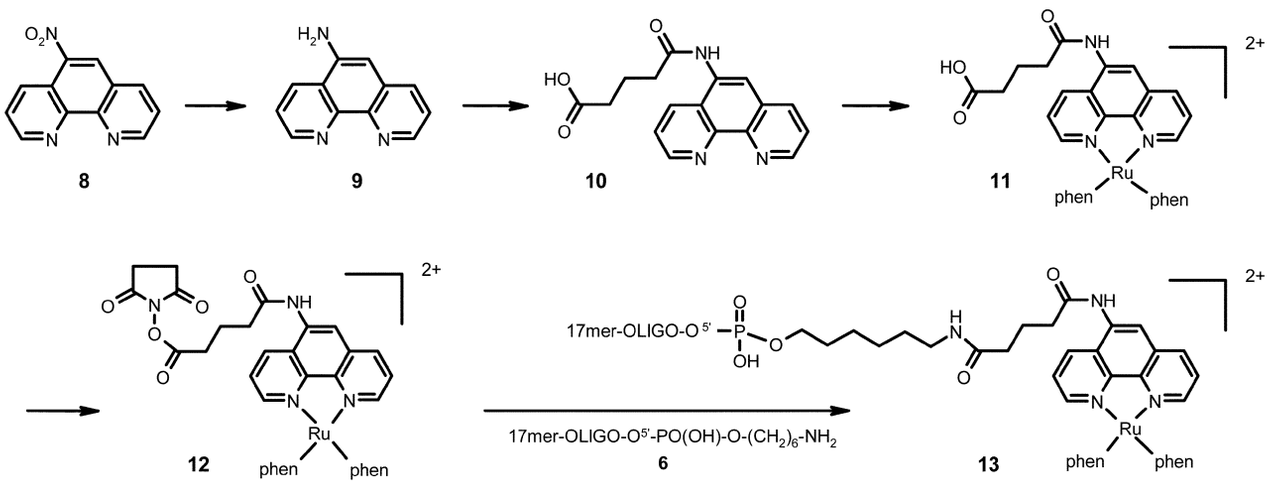
Ruthenium complex (**4**) was prepared (Scheme 1) from 4,4′-dimethyl-2,2′-bipyridine (**1**) by selective oxidation of one of the methyl groups with selenium dioxide to give aldehyde (**2**),[29] which in turn was oxidised to carboxylic acid (**3**)[29] with silver nitrate. The $Ru(phen)_2$ complex of this, (**4**), was formed by reaction of (**3**) with $Ru(phen_2)Cl_2$ and isolated as its hexafluorophosphate salt. The carboxyl group of (**4**) was then converted into its *N*-hydroxysuccinimido ester derivative (**5**) for coupling to the aminohexyl–ODN (**6**). This afforded the desired Ru–ODN (**7**), as shown in Scheme 1. The aminohexyl ODN (**6**) was synthesised using standard phosphoramidite chemistry on an automated DNA synthesiser.

The second ruthenium complex (**13**) was prepared (Scheme 2) from 5-nitro-1,10-phenanthroline (**8**). This was reduced in 69% yield to the corresponding 5-aminophenanthroline (**9**) using tin(II) chloride and ultrasonication, a method first reported for the reduction of nitrobenzodiazepine derivatives.[30] Alternatively, reduction could be achieved in 63% yield using a graphite–hydrazine procedure,[31] but with activated charcoal instead of graphite. The 4-carboxybutanoyl derivative (**10**), prepared from (**9**) with glutaric anhydride,[32] was used to prepare the ruthenium complex (**11**) as its hexafluorophosphate salt. This was converted to its activated ester (**12**), which was in turn coupled to the aminohexyl oligonucleotide (**6**), thus affording the desired Ru–ODN (**13**).

Isolation and purification of the two new ruthenium oligonucleotide derivatives (**7**) and (**13**) presented special difficulties. After the final condensations of (**5**) or (**12**) with 17mer (**6**), a large excess of unconjugated ruthenium complex had to be removed. Initially we tried a solvent extraction method such as that used by Bannwarth and co-workers,[18] but the free complex proved to be too hydrophilic for this to be successful. The use of electroelution, however, was very effective as the positively charged reagent was readily separated from the negatively charged ODN–conjugate. Subsequent PAGE (12%) analysis of the products revealed bands of reduced mobility with respect to the free 17mer ODN, and these bands were excised and re-electroeluted in order to isolate the desired conjugated species from the gel matrix. The final Ru–ODN conjugates thus

Table 1 Comparison of extent of cleavage in 5′-^{32}P-labelled 34mer (1μM induced by $[Ru(phen)_3]^2$ + (1μM) under high salt (8 mM potassium phosphate buffer/80 mM NaCl) and low salt (8 mM potassium phosphate buffer) conditions

Position cleaved	% Cleavage under high salt conditions	% Cleavage under low salt conditions
G31	3.3	2.7
G29	2.4	2.2
G21	2.0	2.3
G18	2.3	3.5
G15	4.8	4.5
G14	11	7.6



Scheme 2

obtained, (**7**) and (**13**), were characterised by HPLC, ESMS or UV/vis spectroscopic methods.

2.2 Photochemical damage sensitised on the target 34mer ODN and on 2′-deoxyguanosine by $[Ru(phen)_3]^{2+}$

Before starting the experiments with Ru–ODN-1 (**7**) and Ru–ODN-2 (**13**), a study was made of the photochemical reactions induced by the prototype complex $[Ru(phen)_3]^{2+}$ with single-stranded target 34mer ODN. This was carried out both at low ionic strength in 8 mM phosphate buffer, pH 6.9, and at higher ionic strength in the same buffer containing 80 mM NaCl. The former conditions are expected to favour electrostatic binding of the metal complex to the single-stranded oligonucleotide, whereas the addition of salt should suppress this, allowing the sensitiser to be free in solution. Experiments were performed with ^{32}P-5′-end labelled target 34mer ODN in the presence of $[Ru(phen)_3]^{2+}$ at both a 10 : 1 and a 1 : 1 ruthenium : ODN ratio. Samples were irradiated with UV/visible light ($\lambda > 330$ nm) for a range of times, then treated with hot piperidine to develop alkali-labile sites (a consequence of nucleobase oxidation). Analysis of the resulting cleavage products was achieved by denaturing polyacrylamide gel electrophoresis (PAGE). The results were visualised by autoradiography and quantified by phospho-imagery. Cleavage sites were identified by comparison with a control purine-specific G + A chemical sequencing ladder of the target strand.

Photosensitised cleavage of the target 34mer was found to occur at all guanine bases and there was no piperidine-induced scission at adenine or any of the pyrimidine sites. However, as shown in Table 1, there is a marked preference for the doublet guanine site G^{14} G^{15} (see Fig. 1) under both solution conditions. Interestingly, both the yield and the preference for this site are enhanced in the buffer solution containing 80 mM salt.

Preference for cleavage at the 5′-base of a GG doublet (in double-stranded DNA) has previously been ascribed to a greater ease of one-electron oxidation at this base in Type I processes.[33] It is striking, however, that the yield of this process should increase when salt is added as this would be expected to reduce electrostatic binding and hence diminish any Type I process which required close contact between the sensitiser and the target ODN. A further consequence of the release of the sensitiser into solution is expected to be a greater likelihood of singlet oxygen production.

In order to investigate the role of singlet oxygen we carried out the reaction in the presence of a $^1O_2$ quencher (sodium azide). Inclusion of this in the $[Ru(phen)_3]^{2+}$–34mer ODN sample caused a substantial reduction in the overall yield of guanine-specific cleavage in the target 34mer ODN (Fig. 2).

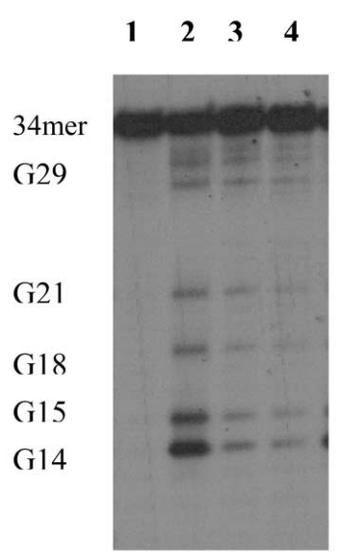


Fig. 2 Autoradiogram of a 20% denaturing polyacrylamide gel showing the irradiation of 5′-^{32}P-end labelled 34mer target (1 μM, in the presence of $Ru(phen)_3^{2+}$ (1 μM) in high salt buffer (8 mM potassium phosphate/80 mM NaCl) under various conditions.. All samples were piperidine treated prior to electrophoresis. **Lane 1** 0 min; **Lane 2** 10 min; **Lane 3** 10 min, azide (10 mM); **Lane 4** 10 min, argon degassed.

An even greater reduction was seen in the overall cleavage by the complex in samples initially purged with argon and maintained under an atmosphere of argon during the course of the irradiations. The data obtained with sodium azide suggest that the guanine photo-damage sensitised by $[Ru(phen)_3]^{2+}$ is largely dependent on singlet oxygen production – *i.e.* a Type II mechanism. Experiments carried out in D_2O (data not shown) showed a slight increase in overall cleavage, possibly due to the increased lifetime of singlet oxygen in the D_2O environment[34] or to an increase in lifetime of excited $[Ru(phen)_3]^{2+}$ in the deuterated solvent.[35] However, the incomplete effect of azide suggests that Type I pathways are also non-negligible. The fact that there is a small amount of reaction even in argon-flushed solutions indicates that some of this pathway may involve oxygen independent processes. (However this result should be viewed with caution, as complete removal of air from the tiny (10 μL) samples is difficult to achieve.)

In order to gain more information about the mechanism of the photodynamic action of the $[Ru(phen)_3]^{2+}$ photosensitiser on guanine derivatives, we studied its reaction with 2′-deoxyguanosine, since Cadet and co-workers,[36,37] have shown that the products formed by Type I processes (*e.g.* **14**) and Type II reactions (*e.g.* **15**) can be clearly resolved by HPLC. (The spiro product (**15**)[38,39] was formerly assumed to be 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine[36]).

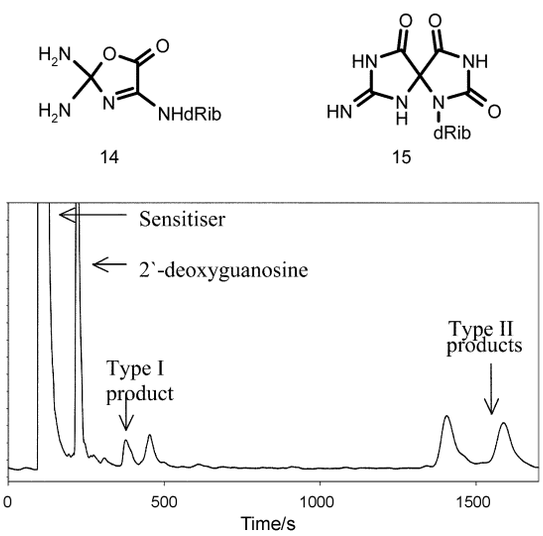


Fig. 3 HPLC trace showing photooxidation products of 2′-deoxyguanosine (1 mM) produced from irradiation in the presence of $[Ru(phen)_3]Cl_2$ (0.5 mM).

Fig. 3 shows the HPLC trace for the products produced from the irradiation of 2′-deoxyguanosine in the presence of $[Ru(phen)_3]^{2+}$.Compounds **14** and **15** were identified by comparison with the products sensitised by methylene blue.[36] Even though it has been shown very recently[39] that **15** is also produced by some Type I sensitisers, this experiment is consistent with the involvement of $^1O_2$ in photoxidation reactions between $Ru(phen)_3^{2+}$ and guanine as its deoxyribonucleoside, although indicating that even with the nucleoside Type I processes are also present. Of course, caution must be exercised when extrapolating these results to the oligonucleotides, as it is known that different products are formed in DNA.[40] Nevertheless, these findings show that free $[Ru(phen)_3]^{2+}$ can act as both a Type I and Type II photosensitiser with either deoxyguanosine or the 34mer oligonucleotide.

2.3 Photochemical damage sensitised on the target 34mer ODN by Ru–ODN conjugates, (7) and (13)

Photochemical targeting of the target 34mer was investigated using both Ru–ODN conjugates (**7**) and (**13**). Binding of the conjugate to the 34mer ODN (Fig. 1) will bring the ruthenium centre close to the guanine residue situated 21 bases from its 5′ end (G^{21}). Each conjugate was therefore allowed to hybridise with the ^{32}P-5′-end-labelled target 34mer strand in 8 mM phosphate buffer containing 80 mM NaCl. The samples were then irradiated (λ > 330 nm) and subsequently treated with hot piperidine to reveal sites of nucleobase modification. The autoradiogram in Fig. 4 compares the cleavage patterns induced by

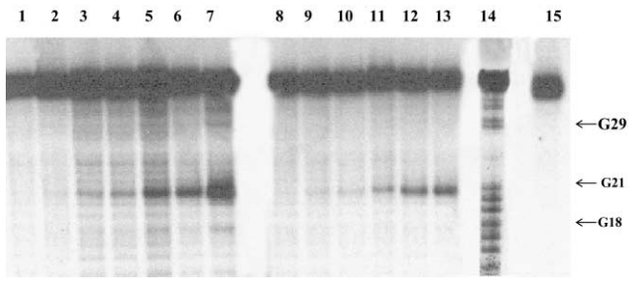


Fig. 4 Autoradiogram of 20% denaturing polyacrylamide gel showing a time course experiment for irradiation of 5′-^{32}P-end labelled 34mer target (1 μM) in the presence of Ru–ODN-1 (1 μM), (lanes 1–7) and Ru–ODN-2 (1 μM) (lanes 8–13) in high salt buffer (8 mM potassium phosphate–80 mM NaCl). Unless stated, all samples are piperidine treated. **Lane 1** 0 min; **Lane 2** 5 min, no piperidine; **Lane 3** 1 min; **Lane 4** 2 min; **Lane 5** 5 min; **Lane 6** 10 min; **Lane 7** 20 min; **Lane 8** 0 min; **Lane 9** 1 min; **Lane 10** 2 min; **Lane 11** 5 min; **Lane 12** 10 min; **Lane 13** 20 min; **Lane 14** G + A lane; **Lane 15** 34mer, no Ru–ODN conjugate, 5 min, no piperidine.

both ruthenium-ODN conjugates upon irradiation for various time intervals from 0 to 20 minutes. Site-selective cleavage at G^{21} was observed for both Ru–ODN-1 (**7**), (lanes 1–7) and Ru–ODN-2 (**13**), (lanes 8–13). No cleavage was observed for either conjugate in the absence of irradiation (lanes 1 and 8) or upon irradiation in the absence of Ru–ODN conjugate (lane 15). Increased cleavage at the target base was observed with increasing irradiation times. For both Ru–ODN conjugates no cleavage was observed in either the rest of the single-stranded (*i.e.* G^{32}, G^{31}, G^{29}) or double-stranded regions (*i.e.* G^{18}, G^{15}, G^{14}) of the target 34mer. A quantitative comparison of G^{21} cleavage by each conjugate is shown in Fig. 5. After 10 minutes

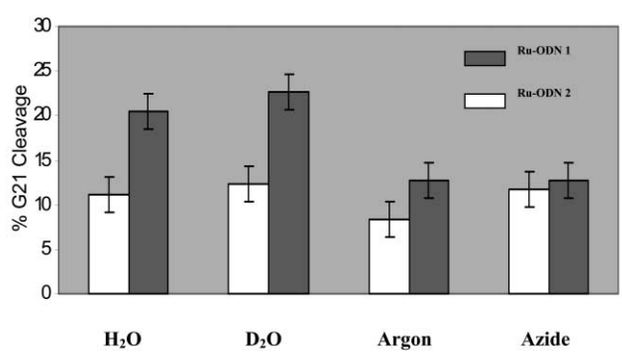


Fig. 5 Quantification of a 20% denaturing polyacrylamide gel showing the irradiation (10 min) of 5′-^{32}P-end-labelled 34mer target (1 μM) in the presence of Ru–ODN-1 (**7**) or Ru–ODN-2 (**13**) (1 μM) in high salt buffer (8 mM potassium phosphate–80 mM NaCl, pH 6.9, prepared in (a) water, (b) D_2O, (c) argon–degassed water or (d) in the presence of azide (10 mM).

irradiation in the presence of Ru–ODN-1 (**7**), 20% of the target 34mer was cleaved at G^{21} compared with only 10.6% cleavage with Ru–ODN-2 (**13**). This result may be due to the shorter linker chain between the ruthenium complex and the 17mer in Ru–ODN-1 (**7**) compared to the longer linker chain present in Ru–ODN-2 (**13**). That hybridisation of the 17mer conjugate to the 34mer is essential for the specificity of the reaction was further demonstrated by the fact that photolysis of the conju-

34mer ODN $^{5'}$ TGA**CCATCAATAAG^{14}G^{15}AAGAA**G^{21}CCCTTCAG^{29}CGG^{32}CC $^{3'}$

Variant 1 (**no G21**) $^{5'}$ TGA**CCATCAATAAGGAAGAA**TCCCTTCAGCGGCC $^{3'}$

Variant 2 $^{5'}$ TGA**CCATCAATAAGGAAGAA**CG^{22}CCTTCAGCGGCC $^{3'}$

Variant 3 $^{5'}$ TGA**CCATCAATAAGGAAGAA**CCG^{23}CTTCAGCGGCC $^{3'}$

Variant 4 $^{5'}$ TGA**CCATCAATAAGGAAGAA**TAG^{23}CTTCAGCGGCC $^{3'}$

Nonsense 34mer $^{5'}$ TGATACAACCACTGGCCGCTG^{21}AACTACAGAGGCA $^{3'}$

Fig. 6 Sequence of target 34mer, nonsense strand and variant strands 1–4 with complementary regions within each strand underlined and double-stranded region shown in bold.

gates in the presence of the 'nonsense' ODN (Fig. 6) led to no cleavage in this 34mer.

As we showed earlier that free $[Ru(phen)_3]^{2+}$ causes G-selective damage mediated partly by $^1O_2$, we have studied the effects of carrying out reactions with the Ru–ODN conjugates in D_2O buffer, with the inclusion of sodium azide, or under an atmosphere of argon (Fig. 5). In D_2O there was a slight increase in extent of alkali-labile sites at G^{21} for both ruthenium-ODN conjugates. This effect may be attributed to an increased lifetime of the excited state of $^1O_2$ and/or the excited state. With the inclusion of azide, a noticeable decrease in G^{21} cleavage was observed with Ru–ODN-1 (**7**) whereas with Ru–ODN-2 (**13**) no significant difference was found between the yield of G^{21} cleavage in the presence or absence of azide. These results indicate a very marked decrease in the role of $^1O_2$ for reactions sensitised by the Ru–ODN conjugates compared to those of the free complex. Similarly it was found that while removal of oxygen by argon flushing reduced the amount of guanine photodamage, the extent of this reduction was much less than with $[Ru(phen)_3]^{2+}$. From these effects it may be concluded that the damage at G^{21} probably proceeds by several competing mechanisms, but that Type I processes are more important than they are with the free sensitiser.

2.4 Photochemical damage sensitised on the variant 34mer ODN by Ru–ODN conjugates, (7) and (13)

In order to investigate whether the agent primarily responsible for causing the oxidative damage was able to diffuse, it was decided to replace the target 34mer ODN with variants in which the guanine target at position 21 was moved in single base increments towards the 3′ end of the 34mer and away from the site of photosensitiser attachment (see Fig. 6). Three variant strands were initially designed; variant 1 with no G^{21} target, variant 2 with the G^{21} target moved one base towards the 3′-end of the target sequence (*i.e.* $G^{21} \longrightarrow G^{22}$) and variant 3 with the G^{21} target moved two bases towards the 3′-end of the target sequence (*i.e.* $G^{21} \longrightarrow G^{23}$). Based on initial results with variants 1–3, a fourth variant was introduced. Variant 4 was a modification of variant 3, designed so as to minimise hairpinning (self-complementary binding) at the 3′-end of variant 3. The sequence of the region complementary to the 17mer of the ruthenium–ODN conjugates remained unchanged in each case, thus allowing normal hybridisation between each variant strand and the particular ruthenium–ODN conjugate.

The results of experiments with the variant strands in the presence of Ru–ODN-1 (**7**) and Ru–ODN-2 (**13**) are shown in Fig. 7 and Table 2 respectively. A higher yield of cleavage was seen in each case with Ru–ODN-1 (**7**), a result comparable to the original experiment with the target 34mer ODN. As expected variant 1 showed no cleavage at position 21 by either conjugate as expected as the guanine at this position was replaced with a thymine. Interestingly, with variants 2 and 3 where guanines were located at positions 22 and 23 respectively, only very slight damage was induced at these sites. Instead in both these variant 34mer ODNs, damage was observed at remote G-bases. Thus variant 2 showed strong cleavage at G^{32} and G^{31} for both Ru–ODN conjugates, while variant 3 showed the most dramatic result with strong cleavage observed at G^{32} for both Ru–ODN conjugates. In no case was damage observed in the double-stranded section of the ODN (*e.g.* G^{18}, G^{15}, G^{14}).

Table 2 Extent of cleavage induced in variant strands caused by irradiation (10 min) of 5′-^{32}P-labelled targets (1μM) in presence of Ru–ODN-2 (**13**) (1 μM) in high salt buffer (8 mM potassium phosphate–80 mM NaCl, pH 6.9). all samples were piperidine treated prior to electrophoresis

Target	% Cleavage at Guanines
34mer	G21 (6.8)
Variant 1	G32 (3.8), G29 (1.4)
Variant 2	G32 (7.0), G31 (7.2)
Variant 3	G32 (9.5)
Variant 4	G32 (2.9)

The lack of damage at the guanine bases close to the expected location of the sensitiser suggests that if the species causing the damage to the guanine is diffusible then it must be highly reactive or be very rapidly deactivated. This explanation would apparently conflict with our finding of damage to remote sites in the variant ODNs. However, consideration of the structures of these ODNs reveals that under the high salt experimental conditions used they are capable of hairpinning *i.e.* self-complementary binding within a strand. The various possible hairpin conformations and their relative stability, as calculated from their free energy of formation,[41] were determined for each variant. Of variant strands 1–3, the proposed hairpin in variant 3 (Fig. 8) has the most negative free energy of formation (*ca.* 16 kJ mol^{-1}). The pattern of hairpin formation was in agreement with the observed cleavage pattern for both Ru–ODN conjugates. As variant 3 formed the most stable hairpin structure, a new 34mer, variant 4, was synthesised, which would be unlikely to hairpin. Analysis showed a large decrease in cleavage by both Ru–ODN conjugates at G^{32} in variant 4 compared to variant 3 (Fig. 7). Despite the fact that variant 4 was unlikely to hairpin, no cleavage was induced by either Ru–ODN conjugate at the G^{23} target. These results confirm that both conjugates are unable to induce damage in a target guanine two bases removed from the original G^{21} target and suggest that the ruthenium complexes are only capable of inducing damage in their immediate vicinity.

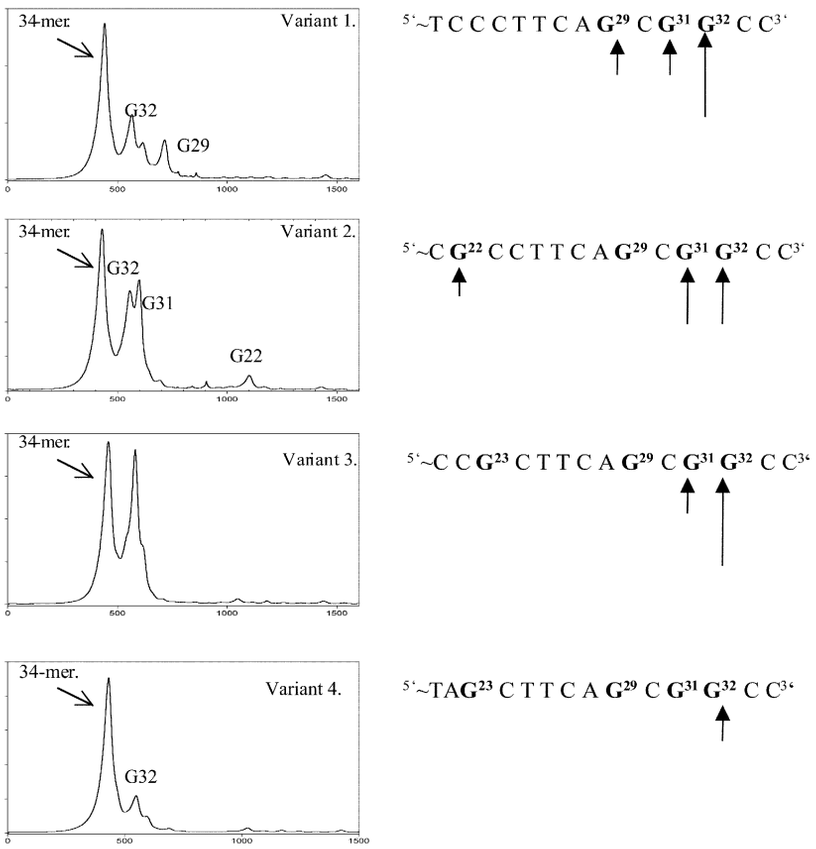


Fig. 7 Densitometry plots of sites of base damage in variant target strands. All experiments involved irradiation (10 min) of 5′-^{32}P-labelled targets (1 μM) in the presence of Ru–ODN-1 (**7**) (1 μM) in high salt buffer (8 mM potassium phosphate–80 mM NaCl, pH 6.9). All samples were piperidine treated prior to electrophoresis. (Relevant portions of target strands are shown beside plots with arrows showing the sites and approximate extent of base damage.)

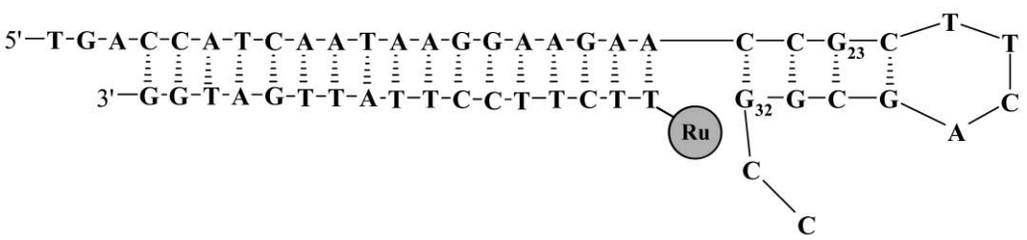


Fig. 8 Variant 3 showing the hairpinning structure of the target strand that brings G^{32} into close proximity to the photoactive ruthenium complex.

2.5 Photochemical induced reactions with 3′-end-labelled ruthenium-ODN conjugates

In the above experiments only the migration of the 5′-end labelled target strand is visualised and the effect of photolysis on the Ru–ODN conjugate while hybridised to the target strand is not observed. In order to monitor what was happening to the Ru–ODN conjugate itself during the course of an experiment it is necessary to label each Ru–ODN conjugate at its 3′-end. This allowed us to assess their relative stability during the course of the irradiations. Results of 3′-end labelling experiments with each conjugate showed that relative to Ru–ODN-2 (**13**), much less of Ru–ODN-1 (**7**) was damaged after 20 minutes irradiation in the presence of the unlabeled 34mer ODN target (11.2% for Ru–ODN-1 (**7**) compared with 23.2% for Ru–ODN-2 (**13**)). This result is consistent with the increased yield of cleavage observed at G^{21} in the 34mer (Fig. 5) for Ru–ODN-1 (**7**). A possible explanation for this result may be that the shorter linker chain in Ru–ODN-1 (**7**) may have been more stable to degradation during the course of the irradiations, thus resulting in a system capable of higher yields of guanine-specific cleavage due to a higher overall level of Ru–ODN conjugate in the experimental samples.

3 Conclusions

The experiments described above show that it is possible to target the sequence of the nucleic acid corresponding to the *bcr-abl* translocation that is characteristic of CML and to direct the photodamage caused on irradiation overwhelmingly to a sole guanine residue (G^{21}). This residue, which is at the junction of the single- and double-stranded sections of the target 34mer–Ru–ODN conjugate ensemble, is also presumably closest to the sensitiser moiety of the Ru–ODN conjugate. It may be argued that it is this proximity that favours the oxidation at this guanine. The short-range nature of this interaction is further demonstrated by the fact that there is only very weak cleavage at G^{22} in variant 2. This is perhaps surprising as it might be expected that the linker between the ODN and the attached ruthenium centre would be sufficiently flexible to allow it to cause reaction at this guanine base. It may also be noted that no photooxidative damage occurs at G^{18}, which is in the double-stranded portion of the hybrid, presumably because this guanine is protected from the close approach of the ruthenium photosensitiser moiety.

The experiments conducted with *free* [Ru(phen)$_3$]$^{2+}$ and deoxyguanosine or the single-stranded 34mer, imply an import-

ant role for singlet oxygen. However, sodium azide (a known $^1O_2$ quencher) is much less effective at quenching the photooxidation caused by the Ru–ODN conjugate. This, of course, would be expected if Type I processes were to predominate under these conditions. It is also possible that these processes could be $^1O_2$-mediated but that the $^1O_2$ reacted only close to its site of generation. In this case the quencher would be expected to be ineffective. However $^1O_2$ is known to react relatively slowly with guanine[42] and it seems unlikely that the reaction with guanine would compete with diffusion. Our data may be compared to those of Boutorine *et al.*,[43] where photochemical damage was targeted on a single-stranded DNA using a complementary ODN chlorin conjugate. Guanine oxidation was found to be significant over about 7 bases in both the double strand and single strand regions. Azide quenched the reaction by about 50% and it was proposed that $^1O_2$ was the reactive species. This behaviour, therefore differs somewhat from what is observed with Ru–ODN conjugates **7** and **13**. If the reactions of **7** and **13** do indeed involve a Type I process – what could this be? Direct oxidation of guanine is known to be efficient only for oxidising complexes such as $[Ru(TAP)_2L]^{2+}$ (TAP = 1,4,5,8-tetraazaphenanthrene; L = bpy, phen, TAP).[10] The reduction potentials of the excited state for $[Ru(phen)_3]^{2+}$ and for conjugates **7** and **13** are too low to allow reaction (1) to proceed efficiently, although it is conceivable that it might be a relatively low quantum yield process.

$$[Ru(phen)_3]^{2+}* + G \longrightarrow [Ru(phen)_3]^{+} + G^{\bullet +} \qquad (1)$$

Alternatively it is possible that another light-induced reaction might initially convert the sensitising centre to a Ru(III) complex. Such complexes are known to oxidise DNA.[44] This could happen if the complex were to reduce some other species present in solution. Transition metal ions (such as Fe^{3+} or Cu^{2+}) are known to act in this way. To rule out such a process we have carried out the reaction in the presence of desferrioxamine, which would be expected to chelate any such ions present in solution. However it was found that the yield was not decreased by the presence of this scavenger (data not shown). Oxygen itself might also be effective in this regard (reaction 2), although it is known that the overall escape yield is rather small.[45] If such a process does occur it would be consistent with our observations and especially with the short range nature of the guanine oxidation.

$$[Ru(phen)_3]^{2+}* + O_2 \longrightarrow [Ru(phen)_3]^{3+} + O_2^{\bullet -} \qquad (2)$$

A major finding of our study is that in the variants remote bases can be damaged. We attribute this to hairpinning bringing the oxidisable base close to the ruthenium centre. The yield of the process is particularly high for variant 3, where the damage at G^{32} is substantially greater than that found at G^{21} in the parent 34mer. This suggests that this G site is especially reactive (perhaps because it is a doublet G in a double-stranded segment in its hairpinned conformation).

The demonstrated ability of the Ru–ODN conjugate to target a specific region of a chimeric gene implicated in the development of malignancy, and then to direct base-specific photooxidation merits further study. Most antisense approaches rely on steric hindrance of ribosome translation of the target mRNA and activation of the RNase H pathway. The Ru–ODN complex can bind to its target and damage it photochemically without the requirement for RNase H. With further development, this system may have scope as a new form of antisense approach.

4 Experimental

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded using 300 MHz (Bruker) and 400 MHz (Bruker) instruments and UV/Vis absorption spectra with Unicam UV-4 and Shimadzu UV-2401 spectrometers. Infrared spectra were recorded using Genesis II FTIR and Perkin Elmer Paragon 1000 spectrometers. Mass spectrometry measurements were made using a Micromass LCT electrospray TOF spectrometer with a Shimadzu LC-10AD solvent delivery module and Micromass Mass Lynx software. HPLC was carried out using either Shimadzu SCL-10A equipment (PDA detector, Shimadzu Class V software), or Perkin Elmer equipment (Perkin Elmer series 2000LC pump, 235C diode array detector, Turbochrom 4.1 software). The column used was Nucleosil C18 RP 10 μm 250 × 4 mm (Supelco).

Synthesis

All reagents used in the synthesis of the ruthenium complexes were purchased from Sigma Aldrich and used without further purification unless otherwise stated. The activated charcoal used was Darco G-60, 100 mesh powder from Sigma Aldrich. Reagent grade solvents were purchased from Riedel-de Haen. Solvents used in coupling reactions were extra pure and purchased from Sigma Aldrich in their anhydrous form.

Synthesis of Ru–ODN-1 (7)

4′-Methyl-2,2′-bipyridine-4-carbaldehyde (**2**) and 4′-methyl-2,2′-bipyridine-4-carboxylic acid (bipy′) (**3**) were synthesised according to previously reported procedures.[29]

Bis(1,10-phenanthrolinyl)(4′-methyl-4-carboxy-2,2′-bipyridyl)-ruthenium bis(hexafluorophosphate) (4)

This was synthesised by refluxing bipy′ (**3**) and $[Ru(phen)_2Cl_2]$[46,47] in an ethanol–water mixture according to a previously reported procedure[48] for an analogous complex. The desired complex was precipitated with ammonium hexafluorophosphate. The product (**4**) (316 mg,77%) was isolated as a dark orange solid by filtration. TLC analysis (silica, 1 : 1 : 2 H_2O : DMF : NH_4Cl (2 M)); $\lambda_{max}(CH_3CN)$/nm 262 and 450; δ_H (400.13 MHz, CD_3CN) 2.4 (3H, s, CH_3), 7.3–9.15 (22H, m, bipy, phen H); ESMS: calculated for $C_{36}H_{26}N_6O_2RuP_2F_{12}$, $M - 2PF_6^-/2 = 337.84$, found 338.02.

N-hydroxysuccinimido ester of bis(1,10-phenanthrolinyl)-(4′-methyl-4-carboxy-2,2′-bipyridyl)ruthenium bis-(hexafluorophosphate) (5)

The ruthenium complex (**4**) was converted to its corresponding *N*-hydroxysuccinimido ester (**5**) using a modified version of a reported procedure.[18] Complex (**4**) (75 mg, 0.07 mmol) was dissolved in anhydrous DMF (2 cm^3) and diisopropylethylamine (28 μl, 0.15 mmol). *N,N,N′,N′*-tetramethylsuccinimido uronium tetrafluoroborate (TSU) (35 mg, 0.02 mmol) was added and the solution was stirred in the dark under a nitrogen atmosphere. The reaction mixture was used for the next step without further isolation The reaction was monitored by TLC (silica, 1 : 1 : 2 H_2O : DMF : NH_4Cl (2 M)); ESMS: calculated 386, found [M + H] 387.

Ruthenium–ODN conjugate (7)

Reagents used to prepare buffers, salt solutions and polyacrylamide gels were purchased from Sigma, Aldrich or Merck and used without further purification. Electroelution was carried out using a Biotrap (Schleicher and Schuell) electroelution apparatus. Singly distilled water autoclaved prior to use was used for all ODN work. The 34mer and 17mer ODNs were synthesised using standard phosphoramidite chemistry on an Applied Biosystems 391 DNA synthesiser and the variant strands were synthesised on a Beckman Oligo 1000M DNA synthesiser.

The 5′-aminohexyl modified ODN (**6**) was prepared from the 17mer using 5′-AminoModifier phosphoramidite (Glen Research). All ODNs were cleaved and deprotected in concentrated ammonia (28%). Compound **6** was purified by the Reverse Phase Cartridge technique prior to ruthenium complex conjugation. All other ODNs were purified by denaturing (7 M urea) PAGE, detected with minimal UV shadowing, and eluted by electroelution followed by butan-1-ol concentration and ethanol precipitation.

5′-Aminohexyl modified 17mer ODN (**6**) (16 optical density units, 106 nmol) and (**5**) (250 μl, 2.65 μmol) were added to tris buffer (100 μl). The mixture was vortexed and heated at 40 °C for 1 hour. The material was purified by electroelution (130 V, 45 min) to remove free ruthenium species. The material extracted from the anodic well was lyophilised and purified by 12% PAGE. The appropriate band in the gel was excised, purified by electroelution, extracted with butan-1-ol and lyophilised giving the desired ruthenium–ODN conjugate (**7**). Yield 12.5%; ESMS (negative ion mode): calculated 5994.1, found, 5993.6.

Synthesis of Ru–ODN-2 (13)

5-Amino-1,10-phenanthroline (9). Reduction of 5-nitro-1,10-phenanthroline (**8**) to 5-amino-1,10-phenanthroline (**9**) was best achieved using a method reported for the reduction of nitrobenzodiazepine derivatives.[30] A mixture of 5-nitro-1,10-phenanthroline (**8**) (100 mg, 0.44 mmol) and powdered tin(II) chloride dihydrate (400 mg, 1.77 mmol) in absolute ethanol (6 ml) was ultrasonicated at room temperature for 2 hours. The resulting mixture was added to distilled water (50 ml), rendered alkaline with aqueous ammonia and extracted with dichloromethane (2 × 25 ml). The organic layer was dried with sodium sulfate and filtered. The filtrate was evaporated under reduced pressure to give product (**9**) as a yellow powder (60 mg, 69%), mp 259 °C (lit[49] 259 °C–260 °C); ν_{max}(Nujol)/cm^{-1} 3400 (NH); ESMS: calculated 195, found [M + H] 196.

Reduction of (**8**) was also successful using the previously reported graphite–hydrazine monohydrate procedure[31] (45% yield) with better yields being obtained using activated charcoal in place of the graphite (63% yield).

5-(4-carboxybutanamido)-1,10-phenanthroline (phen′) (10). This was prepared from 5-amino-1,10-phenanthroline (**9**) in modest yields using glutaric anhydride according to a previously reported procedure[32] (40%, mp 200 °C).

Bis(1,10-phenanthrolinyl)[5-(4-carboxybutanamido)-1,10-phenanthrolinyl]ruthenium bis(hexafluorophosphate) (11). Product (**11**) was synthesised by refluxing phen′ (**10**) and [Ru(phen)$_2$Cl$_2$][46,47] in an ethanol–water mixture according to a previously reported procedure[48] for an analogous complex. The desired complex was precipitated with ammonium hexafluorophosphate. The product (**11**) (244 mg, 78%) was purified using a Sephadex LH20 column (45 × 2.5 cm) eluting with methanol; TLC analysis (silica, 1 : 1 : 2 H_2O : DMF : NH_4Cl (2 M)); λ_{max}(CH_3CN)/nm 262 and 450; HPLC analysis (70 : 30 H_2O : CH_3CN, 1.5 ml min^{-1}, single peak with retention time of 2.04 min).

***N*-Hydroxysuccinimido ester of bis(1,10-phenanthrolinyl)-[5-(4-carboxybutanamido)-1,10-phenanthrolinyl]ruthenium bis-(hexafluorophosphate) (12).** Activation of (**11**) was carried out in an analogous fashion to (**4**) as described above. λ_{max}(CH_3CN)/nm 262 and 450; HPLC analysis (70 : 30 H_2O : CH_3CN, 1.5 ml min^{-1}, single peak with retention time of 4.80 min).

Ruthenium–ODN conjugate (13). 17mer ODN (**6**) (15 optical denisty units, 0.1 μmol) and (**12**) (100 μl, 5 mmol) were added to water (30 μl) and diisopropylethylamine (DIPEA) (6.8 μl, 0.04 mmol). The mixture was shaken overnight in the dark at room temperature. Water (120 μl) was added followed by electroelution (130 V, 45 min) to remove free ruthenium complex. The material isolated from the anodic well was extracted with butan-1-ol and lyophilised. The material was analysed by PAGE (12%) and orange material of reduced mobility with respect to the free 17mer ODN was excised after UV shadowing. The excised material was electroeluted, extracted with butan-1-ol and lyophilised; λ_{max}(H_2O)/nm 264 and 450; HPLC analysis (85 : 15–95 : 5 H_2O : CH_3CN, 1.5 ml min^{-1}, single peak with retention time of 8.99 min). Ruthenium–ODN conjugate (**13**) was obtained in 10% yield.

Experiments with ^{32}P labelling

[γ-^{32}P]ATP (5000 Ci mmol^{-1}) and [α-^{32}P]ddATP (5000 Ci mmol^{-1}) were purchased from Amersham International plc. Polynucleotide kinase (PNK) enzyme and buffer were purchased from Bio Labs, New England. Terminal deoxynucleotidyl transferase (TdT) enzyme and buffer were purchased from Promega. X-ray films (Curix, 100NIF 35 × 43 cm, Agfa Ltd. and Hyperfilm™ MP 35 × 43 cm Amersham Pharmacia biotech) were developed using a Fuji RG2 X-ray film processor. Phosphoimagery was carried out using a Fujifilm FLA-3000 phosphoimager.

5′-End labelling[50]

The ODNs (synthesised without a 5′-phosphate group) were treated with [γ-^{32}P]ATP and PNK at 37 °C for 40 min and 68 °C for 20 min. The labelled ODNs were purified by precipitation with ethanol (80%) and lyophilised.

3′-End radiollabeling of ruthenium–ODN conjugates[50]

The ODNs (synthesised without a 3′-phosphate group) were treated with [α-^{32}P] ddATP and TdT at 37 °C for 70 min and 68 °C for 20 min. The labelled ODNs were purified by precipitation with ethanol (80%) and lyophilised.

Photolysis experiments

Samples were vertically irradiated in an open Eppendorf™ tube using a 500 W (HBO 500 W) mercury lamp. A glass filter was used to remove wavelengths less than 330 nm and a water filter to remove IR radiation.

Each sample was made up in an Eppendorf™ with the addition of the appropriate buffer (either low salt buffer comprising of 10 mM potassium phosphate, pH 6.9 or high salt buffer comprised 10 mM potassium phosphate + 100 mM NaCl, pH 6.9) to give a final volume of 10 μl as follows: radiolabelled ODN (1 μl of 10 μM stock); photosensitiser (1 μl of 10 μM stock); buffer (8 μl). For azide experiments, 1 μl of 0.1 M sodium azide was added to radiolabelled target (1 μl), photosensitiser (1 μl) and appropriate buffer (7 μl). For D_2O experiments, the buffer was prepared using D_2O in place of H_2O prior to sample preparation and used as normal. For argon experiments, samples were purged with argon for several minutes, vortexed, and centrifuged before irradiation. This was repeated twice. Samples were also maintained under an atmosphere of argon during the course of the irradiations.

Samples for double-stranded experiments were heated at 80 °C for 5 min and allowed to cool slowly to room temperature (typically 2–3 h) to allow for maximum hybridisation. All samples were placed on ice for 1 h prior to irradiation and lyophilised after irradiation.

Following irradiation, base modifications in the target strand were cleaved by piperidine treatment.[50] Piperidine (1 M, 15 μl) was added to the appropriate lyophilised samples, heated at 90 °C for 35 min and lyophilised again. Results were analysed by PAGE (30%),[50] visualised by autoradiography and quantified by phosphoimagery. Sites of cleavage were identified

relative to a control purine-specific G + A chemical sequencing ladder[50] included in each polyacrylamide gel.

HPLC assay for oxidative damage to 2′-deoxyguanosine

To a cuvette (3 cm^3) were added 2′-deoxyguanosine (750 µl of 2 mM solution) and the relevant photosensitiser (750 µl of a 1 mM solution). The cuvette was placed 20 cm from the beam of the 500 W, medium pressure Hg lamp with a filter appropriate to the sensitiser in a cuvette between the beam and the cuvette containing the reactants. The solution was irradiated and 50 µl aliquots were taken at time intervals and evaporated to dryness by vacuum centrifuge. The samples were then dissolved in 100 µl of mobile phase (75 : 25, acetonitrile : 25 mM ammonium formate) and analysed by normal phase HPLC using a Supelco amino propyl silica column (12.5 × 4.6 mm). The flow rate used was 1 ml per minute and the detector was set at 230 nm. For the ruthenium complex and methylene blue a 0.1 M $NaNO_2$ filter was used to remove $\lambda \leq 400$ nm.

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