Direct Photocleavage of HIV−DNA by Quinacridine Derivatives Triggered by Triplex Formation

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Abstract: Amino-*p*-quinacridine compounds (**PQs**) have been shown to stabilize strongly and specifically triple-helical DNA. Moreover, these derivatives display photoactive properties that make them efficient DNA cleavage agents. We exploited these two properties (triplex-specific binding and photoactivity) to selectively cleave a double-stranded (ds)DNA sequence present in the HIV-1 genome. Cleavage was first carried out on a linearized plasmid (3300 bp) containing the HIV polypurine tract (PPT) that allowed targeting by a triplex-forming oligonucleotide (TFO). **PQ_3**, the most active compound of the series, efficiently cleaved double-stranded DNA in the vicinity of the PPT when this sequence had formed a triplex with a 16-mer TFO. Investigation of the cleavage at the molecular level was addressed on a short DNA fragment (56 bp); the photoinduced cleavage by **PQ_3** occurred only in the presence of the triple helix. Nevertheless, unusual cleavage patterns were observed: damage was observed at guanines located 6−9 bp *away* from the end of the triple helical site. This cleavage is very efficient (up to 60%), does not require alkaline treatment, and is observed on both strands. A quinacridine−TFO conjugate produced the same cleavage pattern. This observation, along with others, excludes the hypothesis of a triplex-induced allosteric binding site of **PQ_3** adjacent to the damaged sequence and indicates that **PQ_3** preferentially binds in the vicinity of the 5′-triplex junction. Irradiation in the presence of TFO-conjugates with acridine (an intercalative agent) and with the tripeptide lys-tryp-lys led to a complete inhibition of the photocleavage reaction. These results are interpreted in terms of competitive binding and of electron-transfer quenching. Together with the findings of simple mechanistic investigations, they led to the conclusion that the photoinduced damage proceeds through a direct electron transfer between the quinacridine and the guanines. This study addresses the chemical mechanism leading to strand breakage and characterizes the particular photosensitivity of the HIV−DNA target sequence which could be an oxidative hot spot for addressed photoinduced strand scission by photosensitizers.

Introduction

Photosensitization of nucleic acids has found successful applications in several skin disease treatments and is also a promising approach for the development of new antitumor and antiviral therapeutic strategies.[1] A large number of compounds either of synthetic or natural origin have been described for their ability to damage DNA upon photochemical activation.[2] Some of them have proven to be valuable tools in biotechnology as structural probes or photofootprinting reagents.[3] Currently, however, there are still several limitations to the application of photocleavers as cytotoxic agents. One major limitation is the absence of specificity of the cleavage that prevents targeting of a unique DNA sequence or structure. Indeed, even though photoactive compounds can cause selective damage, for example, at G or T bases and/or can display preferential binding for DNA secondary structures,[4] unless they are covalently tethered to a DNA-binding ligand, they exhibit little if any sequence specificity for DNA.

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A very efficient approach to direct the damage to a unique site is to tether the photoactive molecule to an oligonucleotide able to recognize the double-stranded DNA sequence via triple helix formation.[5] However, the various methods to functionalize oligonucleotides are somewhat tedious and until recently[6] did not permit preparation of large amounts of the conjugate. Another severe limitation for the use of photocleavers is the "damage efficiency" which strongly depends on the mechanism that initiates the cleavage.[7] Furthermore, with regard to cell toxicity, the preference would be given to compounds that are capable of generating double-strand breaks (DSB) since such lesions are potentially a catastrophic event which is more difficult to repair than single-strand breaks (SSB).[8] Finally, a primordial necessity is that the wavelength used for selective excitation of the photocleaver must be above 300 nm to avoid light absorption and damage by nucleic acid or protein components.[9]

Here we describe the photoinduced DNA cleaving properties of a family of triple helix specific ligands, the quinacridines (**PQs**, Figure 1A). These molecules possess a crescent-shaped dibenzophenanthroline skeleton and strongly stabilize triple helices with both T·AxT and C·GxC$^+$ base triplets.[10] The stabilizing effect of **PQs** is highly selective for triplex DNA.[10] By analogy with structurally related triplex-specific ligands such as benzopyridoindoles,[11] it is likely that quinacridines bind triple-helical DNA via intercalation between base triplets. Indeed, molecular modeling of the complex **PQ$_3$**/triple helix showed that the quinacridine ring is stacked between the base triplets with the positively charged side chain anchored in the minor groove (Figure 1B).[12a] Furthermore, **PQs** exhibit interesting photochemical properties*:* not only are they triplex-specific as previously established by classical melting studies[10] but their specific interaction with DNA can be monitored by both their fluorescence (shown qualitatively herein) and by their ability to initiate direct photocleavage of plasmid DNA.[12a,b] This prompted us to investigate the ability of amino-quinacridines

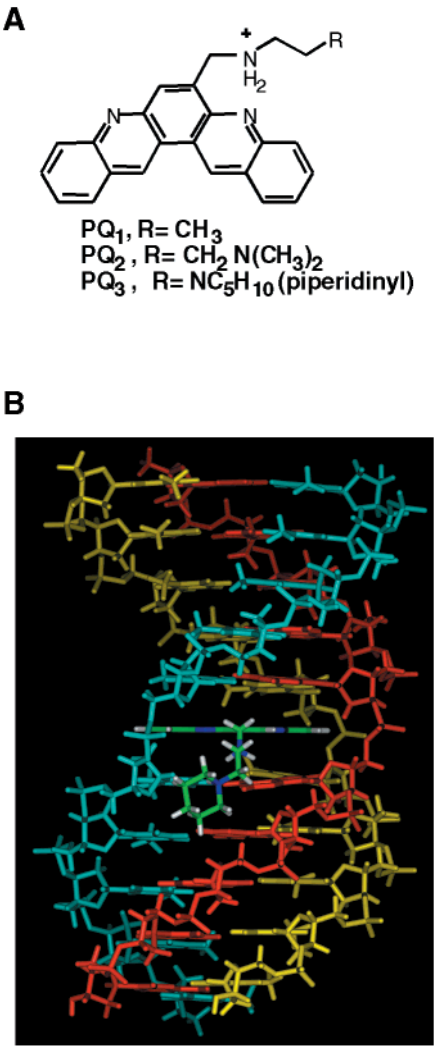

Figure 1. (A) General formula of aminoquinacridine derivatives **PQs** and (B) energy-minimized model of **PQ$_3$** intercalated within a triple helix composed of T·AxT base triplets. The oligopyrimidine and oligopurine strands of Watson−Crick double helix are blue and red, respectively, and the third strand oligonucleotide is yellow. The hydrogen atoms in the triple helix are omitted for clarity (Jumna package).

to perform triplex-directed photocleavage of DNA. This study was also stimulated by the observation that there may exist quite a number of related chemical compounds available for detection of triplex conformation.[13]

The HIV-proviral DNA sequence contains two copies of a 16 bp polypurine tract (PPT), which can be recognized by triple helix formation. This sequence was proposed as a potential target in a triplex-based antigene strategy. This choice was also determined by the numerous studies that emphasized the interest of the PPT/HIV−DNA system for specific transcriptional inhibition of gene expression through triple helix formation.[14]

Results and Discussion

Detection of Triple Helix Formation by PQ$_3$ Fluorescence on Agarose Gel. PQ$_3$ has previously been shown to be the best

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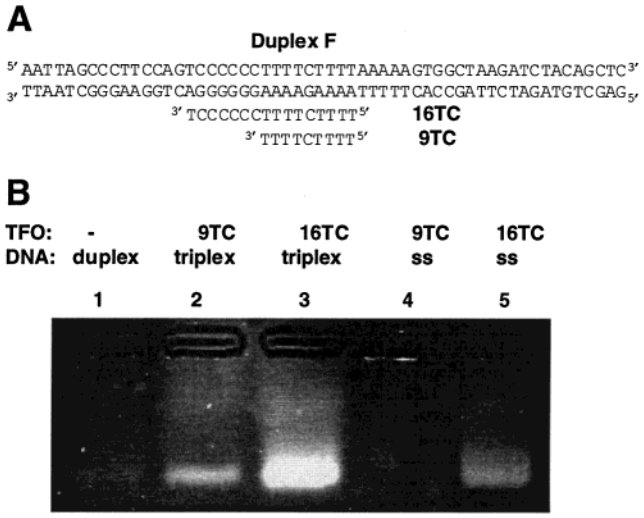


Figure 2. (A) sequences of duplex F and of TFOs 16-TC and 9-TC. (B) Agarose gel electrophoresis of the 56-bp fragment F (0.12 μM) in the presence of **PQ$_3$** (30 μM) and of TFOs (10 μM): lane 1, duplex F; lane 2, duplex F + 9-TC; lane 3, duplex F +16-TC; lane 4, 9-TC alone; lane 5, 16-TC alone.

triplex-stabilizing ligand of a family of related quinacridines (ΔT_m up to +49 °C),[10,12b] and thus it was selected for use throughout the present study. Since **PQs** are both highly fluorescent compounds[12b] and triplex-specific ligands, they could be used as tools to detect triplex formation. This hypothesis has been demonstrated by agarose gel electrophoresis in the presence of **PQ$_3$** and various compositions of duplex and triplex DNA. The 56 bp fragment (F) containing the polypurine tract (PPT) has been used first as the target (Figure 2A). This fragment was incubated for 12 h with a large excess of TFOs (triplex-forming oligonucleotides) of two different lengths, respectively 9-TC [5′-$(T)_4C(T)_4$-3′] and 16-TC [5′-$(T)_4C(T)_4$-$(C)_6$T-3′]; **PQ$_3$** was then added to the duplex, triplex, and also single-stranded TFOs (ss). Following incubation for a few hours, the analysis of the different mixtures was carried out by agarose gel electrophoresis. As can be seen from Figure 2B, the duplex alone is not labeled (lane 1), whereas a bright fluorescent band is detected for the triplex DNA formed with 16-TC (lane 3). A band of diminished luminescence is also seen in lane 2 corresponding to the triplex formed with the shorter oligonucleotide 9-TC. In this case, the weaker staining of the 9-TC triplex can be attributed either to the lower stability of the short triplex that may partially dissociate during migration or to a decrease in the number of triplet sites available for the fixation of the ligand. The 9-TC and 16-TC single-stranded TFOs have been loaded as controls in lanes 4 and 5; 9-TC alone is not revealed (lane 4) whereas a weak fluorescent smeared band is observed for 16-TC (lane 5). The weak intensity of the smeared fluorescence in lane 5 indicates that the cationic quinacridine forms stable associations with the 16-mer oligonucleotide that is present at a high concentration. Qualitatively, it is clear that the fluorescent staining of the mixture 16-TC/**PQ$_3$** is *much* weaker than that observed in lane 3 and thus should contribute weakly to the bright fluorescence detected for the triplex.[15] These observations confirm that there is a poor binding of **PQ$_3$** on duplex DNA at 0.12 μM whereas the ligand is retained when the triplex is formed. The selective staining of the DNA in the presence of the TFO highlights the selectivity of this interaction and suggests that **PQ$_3$** could be used qualitatively for fluorescent detection of stable triple helices in agarose gels and that this technique may be used for rapid screening of other triplex-specific ligands. In contrast, DNA staining by ethidium bromide under these conditions showed little selectivity for duplexes vs triplexes.

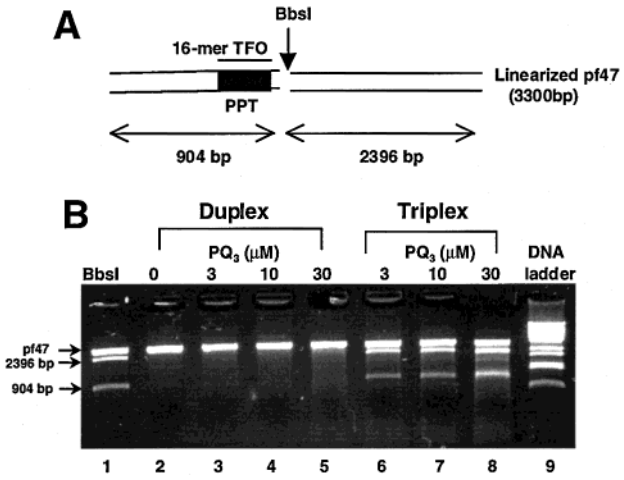


Figure 3. (A) Representation of plasmid pf 47 linearized by the restriction enzyme SspI; the arrow indicates the cleavage site of BbsI. The fragments are shown below. (B) Agarose gel electrophoresis after irradiation of linear pf 47 in the presence of 16-TC (10 μM) and increasing concentrations of **PQ$_3$**: lane 1, cleavage of pf 47 by BbsI; lane 2, pf 47 alone; lanes 3−5, pf 47 + **PQ$_3$** (3, 10, 30 μM); lanes 6−8, pf 47 + 16-TC + **PQ$_3$** (3, 10, 30 μM). Irradiation: 30 min, $\lambda >$ 305 nm, 4 °C.

Cleavage of Double-Stranded Plasmid pf 47 in the Vicinity of the PPT. We first investigated the ability of **PQ$_3$** to induce damage in triplex DNA using a plasmid linearized with SspI, engineered to contain the PPT tract for binding the simple TFO 16-TC[14] (Figure 3A). Triplex-directed specific cleavage of the linear plasmid should produce two fragments of 904 and 2396 bp. These fragments migrate with an electrophoretic mobility that is virtually identical to that of the fragments generated by BbsI digestion given the resolving power of a 0.8% agarose gel (a unique site BbsI is found 33 bp downstream from the PPT) (Figure 3A). The samples were incubated with increasing concentrations of **PQ$_3$** (3−30 μM) and then irradiated for 30 min at 4 °C (320 nm). As shown from Figure 3, the duplex alone remained intact (lanes 3−4) except for some nonspecific degradation that appeared at the highest concentration in ligand (lane 5). In the presence of the triplex 16-TC, a concentration-dependent double-strand cleavage of the plasmid was observed (lanes 6−8). The photoinduced cleavage produced mainly two bands that migrated very similarly to the products of the BbsI digest (compare lane 1 and lanes 6−8). This result clearly indicates that the double-stranded, direct photocleavage induced by **PQ$_3$** occurs in the vicinity of the PPT as would be expected from specific-triplex intercalation of the compound. Therefore, we decided to investigate the DNA cleavage by **PQ$_3$** at single nucleotide resolution to further confirm this hypothesis.

Investigation of the Cleavage with Nucleotide Resolution on the 56 bp Fragment F. To characterize the cleavage, the 56 bp fragment F previously used in the fluorescent visualization was 5′ end-radiolabeled on each strand alternatively (Figure

(15) The same samples were also run in a 10% acrylamide gel (data not shown). Only the triplex lanes exhibited fluorescence, albeit with a great degree of quenching which was due to the acrylamide matrix. The weak fluorescence observed in the acrylamide gel could be positioned over an autoradiogram that revealed where the radiolabeled duplex migrated. Although the gel was not run sufficiently long to resolve duplex from triplex, a 10% acrylamide gel does resolve the free TFO from the duplex/triplex structures. No fluorescence was observed in the lanes that contained only the TFO in the absence of the duplex target.

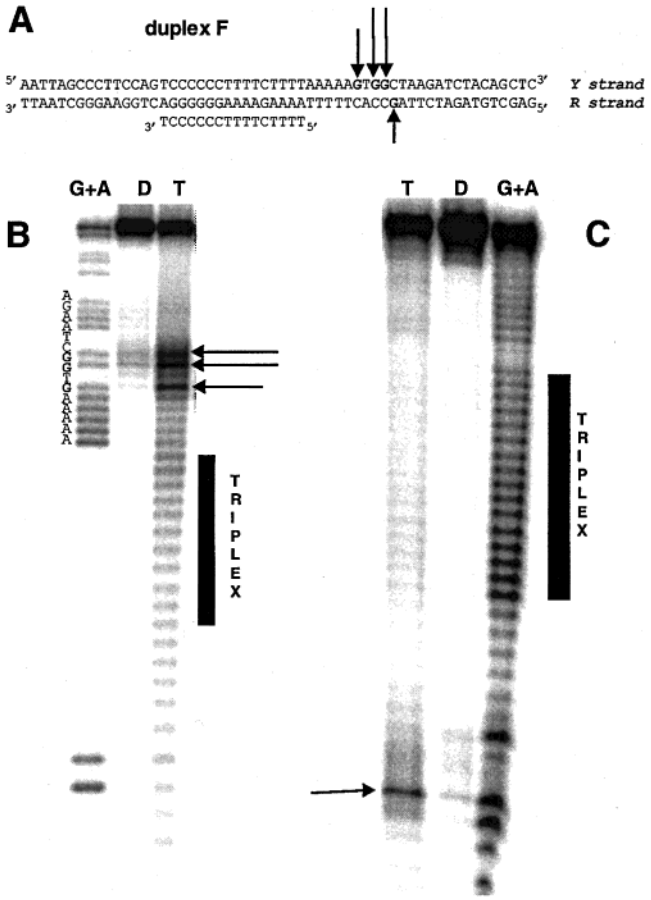


Figure 4. PAGE analysis of the photocleavage of the 56-bp fragment F by **PQ3**. (A) Positions of the photocleavage sites induced by **PQ3** on the two strands of duplex F. (B) Cleavage pattern when the oligopyrimidine-containing strand (Y strand) was 5′ radiolabeled. (C) Cleavage pattern when the oligopurine-containing strand (R strand) was 5′ radiolabeled. In both cases the duplex was irradiated in the absence (lane D) or in the presence of 16-TC (lane T). G + A sequences are shown on the left and right sides of the autoradiograms in B and C, respectively. The triple helix site is indicated. [16-TC] = 10 μM; [**PQ3**] = 5 μM; cacodylate buffer 10 mM (pH = 6.0); NaCl (10 mM); irradiation λ > 305 nm, 10 min, 4°C; no piperidine treatment.

4A: Y, pyrimidine strand; R, purine strand), and irradiation experiments were carried out on the duplex and triplex conformations. When the Y strand was labeled, examination of the autoradiogram (Figure 4B) revealed that DNA was cleaved strongly when 16-TC was present whereas only a minor background cut was detected on the duplex alone (compare lanes D and T). Surprisingly, the cleavage was observed at guanines located at 6, 8, and 9 base pairs away from the triple-helical region. A similar cleavage pattern was observed when labeling the R strand: prominent cleavage was seen 10 bp upstream of the 5′ triple-helical junction at a single G facing the GTGG sequence damaged on the Y strand (Figure 4C). Neither the guanines located downstream of the triple helix nor those within the triple helix (G_6) were modified, and little if any cleavage was observed at these sites (Figure 4C). Remarkably, piperidine treatment did not significantly enhance the cleavage, although there was more general degradation observed throughout, making this assertion difficult to quantify (data not shown).

The quantitative analysis of the gels indicates a high yield for *direct* (piperidine independent) cleavage on the Y strand (up to 60% for the sum of the 3 G bands related to the total radioactivity in the well) with the GG base pair cleaved preferentially (5′-G predominantly) compared to the adjacent single G. The R strand was cleaved less efficiently with maximal cleavage, attaining a fraction as high as 40% (range 10−40%) (in the cases of both the R and Y strands, ranges are reported from at least four experiments). The simultaneous lesions of the R and Y strands at G bases almost facing each other could also explain the double strand break observed on the linear plasmid (Figure 3).

Variation of the excitation wavelength confirmed that the cleavage was induced by excitation of the ligand as there was no cleavage when irradiation was performed above 400 nm, a wavelength above which quinacridine does not absorb light. The intensity of the cleavage was dose-dependent and was slightly enhanced when the concentration in **PQ3** increased from 0.5 to 5 μM (data not shown). This concentration seems to correspond to the saturation level and was used for further experiments. Beyond 5 μM, no significant increase in triplex-dependent cleavage was observed and by comparison more general degradation of the duplex was observed. Increasing ligand concentration (up to 30 μM) only diminished the triplex-selectivity as cleavage was detected on the duplex and even within the PPT (data not shown).

However, the weak cleavage of the duplex observed at 5 μM in ligand (Figure 4, lane D) requires some comments. First, this cleavage which results likely from a nonspecific association of **PQ3** to the duplex was not systematically observed throughout the experiments (see gels included in the Supporting Information section). Second, it is observed at the GG doublet of the GTGG sequence, which could indicate a particular sensitivity of this region resulting from local sequence-specific redox potential (see discussion). However, this activity, when detected, is extremely weak (1−2%) and cannot contribute significantly to the strong cleavage observed when the TFO is present. Furthermore, this is not entirely surprising as any ligand that intercalates with triplexes will necessarily have some affinity for the duplex as the duplex contributes 66% of the π-orbital surface.

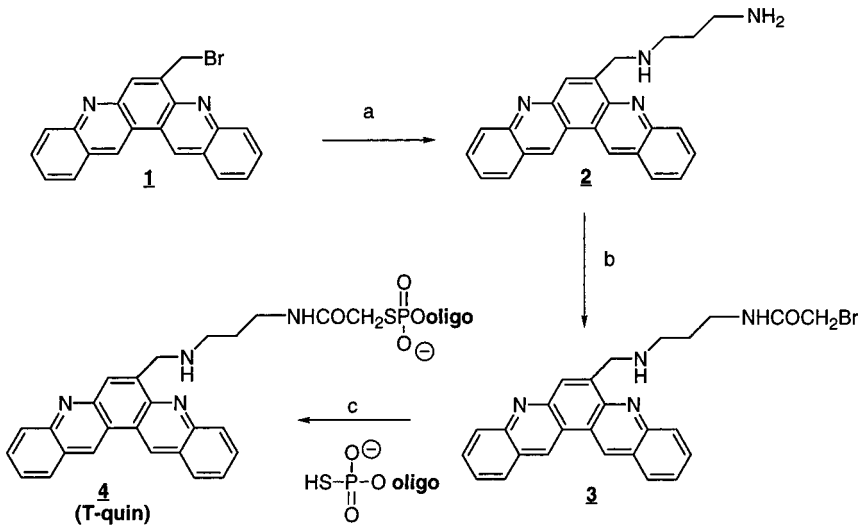
Therefore, the photocleavage of DNA by **PQ3** at the specific site seen on Figure 4A seems to be predominantly induced by triplex formation. This observation is in accordance with the triplex-selective binding of the ligand, and it is likely that the formation of the triplex considerably increases the local concentration in bound ligand, resulting in the strong activity observed in these conditions.

Finally, it is striking that the cleavage was observed at 6−9 base pairs *upstream* of the TFO recognition sequence (PPT), which is the presumed binding site of the ligand.[16] Preferential cleavage at particular sequences could be due either to preferential binding or to preferential reactivity, and consequently our observations raise interesting structural and mechanistic questions.

Question Relating to the Ligand Position on the DNA Target. Answering questions regarding this preferential cleavage is paramount to addressing other questions related to mechanism. Indeed, despite the fact that there are several independent indications for triplex-specific binding, the data presented here thus far afford no definite proof as to the exact position of the ligand. First, it is likely that **PQ3** is not uniformly distributed all along the triplex due to the high content in C·GxC$^+$ triplets positively charged at pH 6.0.[5,10] The fixation of **PQ3** might thus be confined to the T·AxT triplet tracts due to a strong electrostatic repulsion between the 3′-side of the triplex

(16) The ability of **PQ3** to selectively photocleave triplex HIV−DNA was also examined on a radiolabeled 105bp, plasmid-derived restriction fragment and similarly, a unique cleavage at the GTGG site was induced in the presence of 16-TC.

Scheme 1. Synthesis of the Conjugate 16-TC^{me}-quinacridine (T-quin)



a) $H_2N(CH_2)_3NH_2$, CH_2Cl_2/MeOH , 60°C, 1h. b) $BrCH_2COOH$, DCC, $CHCl_3$, r.t. 2h. c) TCEP, ET_3N, DMSO/H_2O, 12h, r.t., **oligo**= 16TC^{me}.

($C{\cdot}GxC^+$ triplets) and the polyammonium side chain of the quinacridine. This might explain why cleavage occurs quite predominantly on the 5′-side of the triple helix and also why the guanines inside the triplex are protected. Second, the 5′-triplex junction was previously shown to be the preferential binding site of several intercalators[17a,b] and minor groove binders[17c] due to alleged local distortion induced by the hybridization of the third strand. Therefore, binding of **PQ$_3$** at the 5′-triplex junction is a likely possibility.

In addressing this question, we especially wanted to exclude the possibility that triplex formation resulted in a localized deformation of the DNA which in turn provided for an allosteric ligand binding site 6−9 base pairs away. Such localized triplex distortion that would consequently result in altered chemical reactivity of upstream bases has been previously observed.[17b] It was thus crucial to obtain more information on the binding site of **PQ$_3$**, and we carried out a series of experiments designed to resolve this question.

(a) Coupling the Quinacridine to the TFO. The quinacridine ring was covalently attached to the 5′-terminus of 16-TC so as to restrict the binding to a well-defined site. To ensure proper stacking in the proximity of the triplex junction, a short linker was chosen. The synthesis was achieved according to the strategy depicted on Scheme 1. The (3-aminopropyl)-aminomethyl-substituted quinacridine **2** was prepared by substitution of the bromomethyl derivative **1** by 1,3-diaminopropane (step 1). **2** was then activated by bromoacetylation and condensed on the phosphorothioate-terminated oligonucleotide 16-TC^{me} to give T-quin, according to a procedure already described.[6d,18] (16-TC^{me} corresponds to 16-TC 5′-substituted by a terminal phosphorothioate and where C^{me} refers to 5-methylcytosine which has been introduced to provide added triplex stability).[19] The modified product was purified by a 15% denaturing PAGE, and the oligonucleotides were detected by UV-shadowing. The quinacridine-derivatized product T-quin is fluorescent and migrates more slowly than the free oligonucleotide, two features that allowed an easy separation from the unreacted material. The hybrid T-quin was then used without further purification. Irradiation was performed on duplex F in the presence of the hybrid T-quin. It appears from this experiment[20] that the cleavage occurs similarly when the quinacridine is covalently linked to the TFO or added free to the solution where the triplex is formed with the unmodified TFO. This tends to demonstrate that the binding of **PQ$_3$** might take place in the vicinity of the 5′-triplex junction rather that at a supposed allosteric site that we have nevertheless considered. The distance between the **PQ$_3$** ligand and the damaged sites can thus be estimated to be approximately 6−9 base pairs, a distance that is too great to be spanned by the short linker arm used in the experiment.

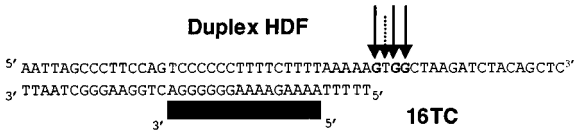


Figure 5. Sequence of heteroduplex HDF and position of the cleavages (indicated by arrows).

(b) Changing the Conformation of the GTGG Site. To test the hypothesis of a triplex-induced allosteric binding site in the vicinity of the damaged site, the conformation of the GTGG sequence was altered from double-stranded to single-stranded. To this end, the R strand was shortened in order to leave the GTGG sequence in a single-stranded conformation (Figure 5). Irradiation of the resulting heteroduplex HDF in the presence of 16-TC and **PQ$_3$** revealed that the cleavage was still triggered by triplex formation and that it occurred at the same position (Figure 5).[20] Although the reaction is less efficient than with the regular duplex F (10% cleavage), this result supports the conclusion that the specificity of the cleavage is *not* related to recognition of a particular dsDNA conformation induced by triplex formation as the target Gs in the heteroduplex are rendered flexible and will not suffer distortion from local unwinding at the PPT. Clearly, the single-stranded nature of the target Gs influences the yield of cleavage but not the basic

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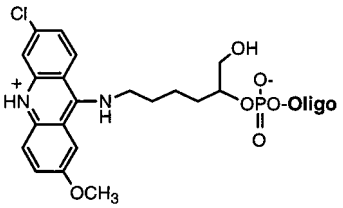
(20) PAGE analysis is included in the Supporting Information section.

Chart 1. Structure of the TFO−Acridine Conjugate (T-acr)



features of the damage. The relatively lower yield of the cleavage might be attributed to the lower stability of the heteroduplex. It also can be interpreted in terms of weaker stacking of the dangling G bases resulting in a less favorable electron transfer[21] (see discussion).

(c) Competition Experiment between PQ_3 and a 16TC−Acridine Conjugate (T-acr). In order to gain more information regarding the binding of **PQ_3**, an acridine−TFO conjugate (T-acr) was examined as the third strand for triplex formation. Acridine-modified oligonucleotides are commercially available and have been widely used for enhancing triplex stability.[22] Numerous studies have demonstrated that the acridine ring intercalates at the triplex-junction above the first base triplet when the linker is properly chosen.[22] Irradiation of duplex F was carried out in the presence of T-acr (Chart 1) and **PQ_3**. When the triplex was formed with the conjugated oligonucleotide T-acr, a quasi-total inhibition of the cleavage was observed.[20] These results are consistent with a strong competitive binding between the acridine introduced by the triplex-forming oligonucleotide and the free quinacridine. The data suggest that the acridine competes with **PQ_3** for binding at the junction. It is interesting to note that the acridine nucleus itself is unable to induce damage although it strongly absorbs light in our experimental conditions and although the same acridine derivative (9-amino-6-chloro-2-methoxyacridine) has been used recently for photooxidation of DNA.[23] These results further underscore the novelty of the quinacridine family of triplex-specific ligands.

Question Relating to Cleavage Mechanism. This question concerns the primary process responsible for the G selectivity of the damage. It is well established that guanine bases are a target for oxidative damage in DNA which can be initiated by one-electron oxidation or by reaction of singlet oxygen.[2,24] These two pathways should thus be taken into account to explain the G selectivity of the quinacridine-induced cleavage. Recently, long-range charge transport in DNA has attracted considerable attention[25−27] and it has been established from several studies that oxidative damage can occur at G bases that are far away from the oxidant.[28] If **PQ_3** is indeed associated within the triple helix or at the 5′-junction, charge migration within the duplex from the excited ligand to the Gs would explain how damage could occur outside the immediate vicinity of the binding site of the quinacridine.

(a) The Argument Against Singlet Oxygen. We began the investigation by supposing that singlet oxygen might be operative. Singlet oxygen could be generated by photoexcitation of the quinacridine excited either in the unbound state given its large excess in the bulk media or by the quinacridine associated at the triple helix junction. In both cases, the singlet oxygen would have to be diffusible in order to reach the GTGG site. Therefore, the yield of cleavage should be significantly increased by changing H_2O to D_2O since the lifetime of $^1O_2$ is greatly enhanced in D_2O (from 4 μs in H_2O to 60 μs in D_2O).[9b] Photocleavage of duplex and triplex DNA by **PQ_3** was carried out in the presence of D_2O. It appeared from the experiment that the yield of cleavage of triplex DNA in D_2O is identical to that observed in H_2O.[20] The absence of a solvent isotope effect can be interpreted in two ways: *either* singlet oxygen is simply not involved in the reaction *or* singlet oxygen *is* nevertheless operative but is not diffusible. In this latter case, one must accept the condition that **PQ_3** and the GTGG sequence are positioned in close contact and that the singlet oxygen reacted in the solvent cage around the quinacridine. This latter hypothesis has been ruled out by the data presented above where we have excluded a triplex-induced allosteric site and it can be concluded that cleavage is most likely *not* mediated by singlet oxygen.

These considerations provide a useful starting point for the implication of a direct electron transfer from guanine to **PQ_3**. Indeed, a first examination of the cleavage profile would suggest that the GG vs G selectivity of the cleavage is consistent with the formation of a guanine radical rather than singlet oxygen attack.[2,29] Two complementary experiments were designed in an attempt to demonstrate the implication of an electron transfer.

(b) Photocleavage by PQ_3 in the Presence of a 16-TC/Peptide Conjugate (T-KWK). Irradiation was conducted in the presence of **PQ_3** and with a TFO conjugated to the tripeptide lysine-tryptophane-lysine (KWK). The tripeptide KWK has been widely used as a model compound for DNA-binding proteins, and it is known to bind duplex DNA via partial intercalation of the aromatic tryptophan ring coupled with electrostatic interac-

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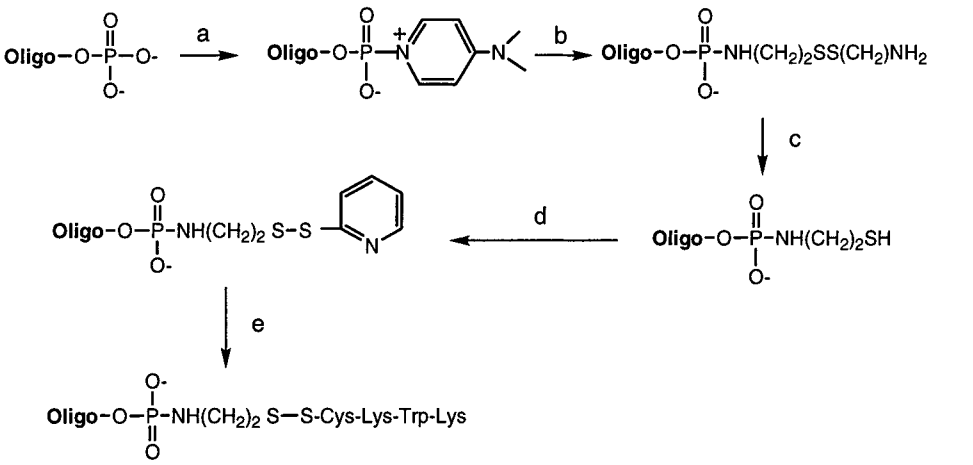
Scheme 2. Synthesis of the Conjugate 16-TC^{me}-tripeptide (T-KWK)



a) PPh_3, $DiPy_2S_2$, DMAP, DMSO, 20min, r.t. b) $H_2N(CH_2)_2SS(CH_2)_2NH_2$, ET_3N,DMSO, 20min, r.t..c) DTT, N_2, 1h, r.t. d) $DiPy_2S_2$, DMSO/H_2O, overnight, r.t. e) NH_2LysTrpLysCysCONH_2, 1h, r.t., **Oligo**= 16TC^{me}.

tions of the cationic lysine with the phosphate groups.[30] Barton and co-workers demonstrated that the radical cation of guanine was able to oxidize tryptophan due to the low oxidation potential of this amino acid.[31a] Moreover, the KWK peptide has recently been used as a quencher for radical cation formation/migration in DNA.[31b] The possibility of positioning the KWK peptide at the triplex junction so as to interfere with a potential charge transfer between the quinacridine and the GTGG site was thus an attractive idea. To this end, the tripeptide was linked to the 16-TC TFO following a reaction scheme adapted from standard coupling methods recently optimized[6d] (Scheme 2). The coupling was achieved with the 16-mer oligonucleotide 16-TC^{me} which is identical in sequence to the oligonucleotide previously used for the synthesis of the conjugate T-quin. The activation of the 5′ terminal phosphate of the oligonucleotide 16-TC^{me} was carried out with Mukaiyama reagents, and then condensation with cystamine was performed. The resulting product was then submitted to reduction with DTT followed by protection/activation of the free thiol with dipyridyl disulfide. Subsequent coupling with the tretrapeptide Lys-Tryp-Lys-Cys* (Cys* is amidocysteine chosen as the linker) afforded the derivatized compound T-KWK which was then desalted on HPLC. The conjugate T-KWK was tested by nondenaturing gel retardation to confirm its ability to complex with the target duplex (data not shown). Subsequently, the conjugate was used in irradiation experiments with **PQ$_3$**. The experiment was analyzed by denaturing PAGE, and it revealed that the **PQ$_3$**-induced photocleavage was completely inhibited when T-KWK was hybridized to the duplex.[20] This result can be interpreted from two points: (1) the indole ring simply intercalates at the 5′-triplex junction to compete with the quinacridine provided that the activity of the quinacridine is localized to the junction or (2) the tryptophan intercalates *and* is oxidized preferentially by the photoexcited quinacridine which prevents reaction at guanine.[31b] Certainly, further investigations will be required to prove that the tryptophan residue is indeed oxidized by the quinacridine. However, these results, along with recent observations that the tripeptide will protect Gs from damage,[31b] might implicate a direct redox process between the quinacridine and the guanine.

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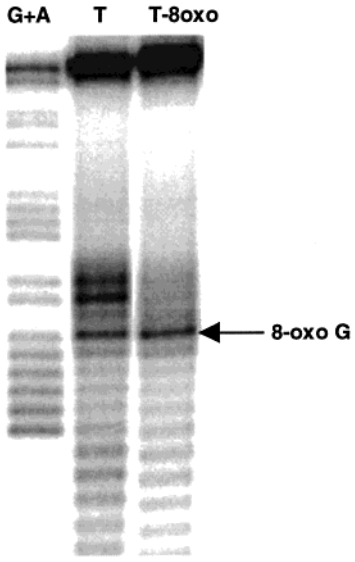

Figure 6. PAGE analysis of the photocleavage of duplex F containing 8-oxo-G by **PQ$_3$** (5μM): lane T, duplex F + 16-TC (10 μM); lane T-8oxo, duplex F containing 8-oxo-G + 16-TC (10 μM). Irradiation was carried out in the same conditions as those of Figure 4.

(c) Inhibition of the Cleavage by 8-Oxoguanine. A last control experiment was conducted in order to confirm the G radical cation formation. The radical cation might be trapped by 8-oxoguanine, a highly oxidizable residue.[28e] Thus, when 8-oxo-G is placed before a GG step and if the radical cation must pass through the trap, inhibition of the cleavage at GG is seen. Therefore, the first single G of the damaged sequence GTGG was substituted by 8-oxo-G and irradiation performed in the presence of **PQ$_3$**. Examination of the autoradiogram (Figure 6) shows that the cleavage at GG step is strongly diminished when the 8-oxo-G is present (compare lanes 2 and 3) and mainly focused on the modified base. This result demonstrates unambiguously that the cleavage is initiated by the formation of a radical cation and that the charge migration proceeds from the quinacridine toward the GG step.

In summary, several conclusions can be drawn from the above experiments:

(1) Binding Site of the Quinacridine and Triplex Specificity of the Damage. We demonstrated that cleavage by **PQ$_3$** is triggered by triplex formation and is both site and sequence specific. The similar behaviors of the quinacridine whether it

is free or linked to the 5′ end of the TFO along with examination of the heteroduplex conclusively exclude the hypothesis that the triplex and the associated **PQ_3** provokes an allosteric site in the vicinity of the damaged sequence GTGG or that a particularly altered double-stranded conformation is recognized by **PQ_3**. The "regioselectivity" of the cleavage and the competitive binding with the TFO−acridine further corroborate the assertion that **PQ_3** binds in the vicinity of the 5′-triplex junction. The triplex−duplex junction has been a reliable locus for targeting DNA damaging ligands of numerous types. Site-selective photocleavage of triplex DNA was observed with riboflavin and anthraquinone derivatives when conjugated to the 5′-terminus of TFOs.[32,33] In both cases, preferential reaction at a GG step upstream of the intercalation site (i.e., the 5′-triplex junction) was observed and the authors suggested that the site selectivity may be controlled by local structural changes of the DNA such as unwinding at the triplex−duplex junction. The report about the riboflavin−TFO hybrid[32] was particularly stimulating during our investigation since it was conducted on the same HIV−DNA/PPT system as the one used in this work. However, in contrast with the triplex-specific quinacridine, neither free riboflavin nor amino-anthraquinones induce sequence-selective damage since they bind randomly to DNA. The only antecedent photocleavage by a triplex-specific ligand has been demonstrated with ellipticine.[17a]

(2) Cleavage Initiation and Process. The absence of solvent isotope effect indicates that singlet oxygen is not sensitized by the quinacridine excited state. In addition, the possible inhibition by tryptophan and especially the trapping by 8-oxoguanine are arguments in favor of an electron transfer from guanine to quinacridine. In this hypothesis, we have to assume that the elementary process of electron transfer operates via oxidation of an adenine adjacent to the photoexcited quinacridine which is then followed by charge migration to the first G. The A-tract bridging the binding site and the GTGG site might be a structural characteristic favorable to the redox exchange since repetitive AA regions have been described to provide a better electronic coupling than do TT and AT sequences.[26c,28c] This is furthermore in accordance with the recent finding that both G and A bases are able to act as charge carriers and that the radical cation can travel through quite long AT stretches (up to 8 base pairs) via an "A hopping" mechanism.[32] The distance between the quinacridine (electron acceptor) and the first G (electron donor) was estimated to lie within 18−21 Å (5−6 AT bp), and it is compatible with such a mechanism. In addition, the high efficiency of the damage could be related to the particular structure/sequence of the dsDNA target[35] or to the formation of the triplex that is known to cause a significant rigidification of the template duplex, which in turn can be favorable to charge transport.[26b] However, further experimental measurements are required to elucidate this point and in particular the photochemical properties of amino-quinacridines should be studied in more detail to understand the mechanism and the high efficiency of the cleavage.[25e,28d] Finally, the "side selectivity" of the damage addresses the question of the triplex influence on the ET process. This point has been thoroughly discussed in photooxidation studies by anthraquinones,[33] and it was shown that the particular triplex conformation which displays an important distortion from the B-form helix could lower the reaction of ET. Furthermore, in the case of 16-TC, increased positive charge due to cytosine protonation in the triplex may also impact the process with the C·GxC$^+$ triplet acting as an electrostatic barrier to charge transfer. It thus would be informative to test triple helices with C·GxG triplets instead of C·GxC$^+$ to avoid the creation of a region containing a high concentration in positive charges. Also, a more comprehensive examination of numerous triplex targets and vicinal duplex sequences will help further substantiate the full sequence determinants of this cleavage. Finally, sequence modifications such as the positioning of GG steps regularly on the dsDNA can be pertinent to further probe the ability of quinacridine to induce electron transfer at long distance.[24,28]

(3) Mechanism of Strand Scission. The insensitivity to the alkaline treatment is also a surprising feature of the photoinduced cleavage by quinacridines. Generally, oxidative damage of nucleic bases does not lead to spontaneous strand scission in the absence of piperidine treatment.[24] Direct strand breakage is thus a hallmark of H-abstraction from deoxyribose that occurs generally in the immediate vicinity of the ligand insertion site. However, studies on sequencing gels are subject to caveats concerning the molecular events that occur between the initial step and the realization of strand scissions since samples are submitted to heating in concentrated urea or formamide before loading, a drastic treatment that can be deleterious for oxidized DNA. The mechanistic investigations described here are not sufficient to determine the exact nature of the cleavage products. However, the direct strand breaks observed on the linear plasmid which did not suffer heat treatment just prior to analysis would exclude the argument that scission is occurring following workup and prior to gel loading.

Though direct photolesions at G bases have been already described in the literature,[2,36] there is currently no firm mechanistic evidence available for the occurrence of such a process.[24,37] Direct strand breaks of DNA have been described in some cases with photosensitizers,[2,36b] or after photoionization by laser.[36,37] They have also been observed after irradiation of RNA substrates.[37b] Generally, however, the factors that determine whether the break will occur prior to or following piperidine treatment are not clearly understood. Also, the decomposition of the guanine radical cation G$^{\bullet+}$ formed from one-electron oxidation of DNA might take two competitive pathways: deprotonation followed by reaction with O_2 leading to alkali-labile products (imidazolone, oxazolone)[38,39] or hydration leading to 8-oxo-G, the latter being less efficiently cleaved by hot piperidine.[39,37a] The relative probability of hydration vs deprotonation of the radical cation is modulated by the local helical conformation and dynamics variations of DNA.[39] However, none of these two reactions are likely to give rise to direct strand breaks.[24] Alternative degradation pathways have been proposed to explain direct strand scissions, for example, the possibility for the neutral G radical to abstract a proton from the neighboring sugar residue.[37b,c] Radical transfer from base

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to sugar was previously shown to occur with pyrimidine nucleosides,[24c,40] but there is no experimental evidence for this type of mechanism naturally occurring in DNA sequences. Furthermore, the influence of heating required before sequencing gel on the various degradation pathways has never been systematically evaluated; thus during treatment one chemical reaction could be favored over the others whereas others may be obscured by secondary processes ensuing during electrophoresis workup. Finally, although most of the photosensitizers cleave DNA via a predominant mechanism, one must consider that several mechanisms might be operative at the same time which significantly complicates interpretation of the experimental data.[2,24,36b] In particular, the probability to induce over-oxidation which may generate heat-labile products should be to taken into account when photooxidation is performed.[24c]

Elucidation of the cleavage mechanism is the next step of this study which is currently under investigation through the identification of end products.

Conclusion

This high-yielding, site-specific scission is the result of a photodynamic process ensuing from a synergistic interaction between duplex B-DNA, a pyrimidine triplex-forming oligonucleotide, and a cationic quinacridine. The direct cleavage and the mechanism of strand scission distinguish quinacridine compounds **PQs** as a new class of DNA photocleavers that probably operate via direct electron transfer from guanines bases. The high yield of cleavage, the G selectivity, and the possibility to trigger the damage via triplex formation *without* necessarily coupling the ligand to the TFO make these compounds very attractive tools for inducing site-directed double-strand breaks at DNA. The ability of amino-quinacridines to induce efficient strand scission in other DNA targets is currently under investigation and particularly in G-rich sequences of double-, triple-, or quadruple-stranded conformations.

The fluorescent properties of quinacridines represent a very interesting feature that beckons further study given that fluorescent probes for noncanonical B-DNA structures will likely find increased use in understanding the intricacies of the chromatin−DNA structure. Regarding the fluorescent detection of triple-helical regions by amino-quinacridines, it might be interesting to test these compounds for detection of intramolecular triplexes either of synthetic or native origin (H-DNA). In addition, chemical substitutions at quinacridine ring (chlorination, nitration) are potentially feasible[41] with the goal of red-shifting their optical absorption and/or to modulate their photochemical characteristics and hence their DNA cleavage ability.

Finally, the present results address the question of the particular sensitivity of the HIV-1 DNA target that has been used. Does this DNA fragment inherently exhibit a high sensitivity to irradiation due to its particular primary sequence and/or local helical conformation? Or does the hybridization of the third strand in the PPT region emphasize or create this sensitivity by inducing structural or dynamic variations of the duplex template? The unique damaged site GTGG could thus display a relatively low redox potential and might be a "hot spot" for oxidative damage by photosensitizers.

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Experimental Section

Materials and Methods. All the reagents and solvents were purchased from Sigma-Aldrich-Fluka and used without further purification. [γ-^{32}P]ATP was purchased from ICN. Oligonucleotides 56-mer Y and R strands (duplex F), 36-mer R strand (duplex HDF), 56-mer Y-8oxo-G, 16TC, 9TC, 16TC^{me}, and T-acr were synthesized by Eurogentec and were used after ethanol precipitation and G-10 (BioRad) filtration (sequences are shown on figures and schemes). 5′-Phosphorylation was carried out with T4 polynucleotide kinase purchased from Biolabs. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer. Electrospray mass spectra were recorded on an Esquire ion trap (Bruker-Franzen Analytic Gmbh) at the Laboratoire de Chimie Structurale Organique et Biologique, Université Pierre & Marie Curie, Paris. The microanalyses were performed at the Service Regional de Microanalyse de l'Universite Pierre & Marie Curie.

Synthesis of Conjugate T-quin. 6-Bromomethyldibenzo[*b,j*][4,7]-phenanthroline (1) was synthesized as previously described.[10] **6-[(3-Aminopropyl)aminomethyl]dibenzo[*b,j*][4,7]phenanthroline (2).** A solution of **1** (100 mg, 0.27 mmol) in CH_2Cl_2/CH_3OH (1/1) was added dropwise to a large excess of 1,3-diaminopropane (2 g, 27 mmol) dissolved in CH_2Cl_2/CH_3OH (50 mL). The mixture was then stirred at 60 °C for 2 h. The mixture was cooled and the solvent evaporated. The crude residue was dissolved in $CHCl_3$, washed with water, and dried on Na_2SO_4. After evaporation of the solvent under vacuum, the oily residue was triturated in ether and filtered, yielding 45 mg (45%) of a yellow brown powder: mp >260 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.75 (dt, 2H); 2.70 (m, 4H); 4.47 (s, 2H); 7.6 (t, 2H). 7.9 (t, 2H); 8.25 (m, 5H); 9.89 (s, 1H); 9.98 (s, 1H). MS (electrospray, CH_3OH) *m*/*z* 367.3 ($[C_{22}H_{22}N_4 + H]^+$).

6- [3-((Bromoacetyl)aminopropyl)aminomethyl]dibenzo[*b,j*][4,7]-phenanthroline (3). 2 (20 mg, 0.05 mmol), bromoacetic acid (9.8 mg, 0.08 mmol), and DCC (18 mg, 0.06 mmol) were mixed in 4 mL of $CDCl_3$ and stirred in the dark at room temperature for 3 h. Completion of the reaction was controlled by direct NMR analysis of the reaction mixture. The solution was filtered to eliminate dicyclohexylurea, washed with a solution of Na_2CO_3 in D_2O, and dried. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.7 (m, 2H); 3.3 (t, 2H); 3.67 (m, 2H); 4.35 (s, 2H); 5.36 (s, 2H); 7.71 (m, 2H). 7.91 (t, 2H); 8.07 (s, 1H); 8.17 (d, 2H); 8.30 (m, 2H); 9.50 (d, 2H). The product is stable for 2 weeks in chloroform solution if stored at −20 °C. MS (electrospray, CH_3OH) *m*/*z* 488 ($[C_{26}H_{23}N_4OBr + H]^+$).

Coupling of 3 to 16-TC^{me} Oligonucleotide; Obtention of the Conjugate T-quin (4). A total of 5 μL of triethylcarboxyethylphosphine (TCEP) (10 mM in water adjusted to pH 7 with triethylamine) was added to 20 μL of a 1 mM solution of 5′ thiophosphorylated 16-TC^{me} oligonucleotide and incubated for 20 min prior to addition of 60 μL of **3** (6 mM) and triethylamine (69 mM) in DMSO. The alkylation was continued overnight in the dark, and the oligonucleotide was precipitated with 2.5 mL of 3% $LiClO_4$ in acetone, washed with ethanol, resuspended in formamide, heated briefly at 65 °C, and loaded into a 20% 7 M urea 29-1 monomer:bispolyacrylamide gel. The bromophenol blue dye was migrated 90%. The conjugate was localized by UV shadowing at low wavelength (265 nm) (estimated >90% conversion) and by UV fluorescence (brilliant light-blue) at high wavelength (315 nm). The conjugate migrated approximately 2 cm more slowly than the parent thiophosphorylated 16-TC^{me}. It was eluted from the gel into 2 × 1 mL of 1% $LiClO_4$ in aqueous 10 mM Tris-HCl pH 7.9, lyophilized, precipitated with 2 × 1 mL of ethanol, resuspended in 10 mM Tris-HCl pH 7.9, and desalted on a G10 spin column (BioRad). The concentration was measured by UV−vis (λ_{max}: 265 nm, $\epsilon = 2 \times 10^5$ M^{-1} cm^{-1}).

Synthesis of Conjugate T-KWK. The oligonucleotide 16-TC^{me} was precipitated by an aqueous solution of hexadecyltrimethylammonium bromide (CTAB, 8%) until oligonucleotide charge neutralization was achieved and dried. The resulting salt was solubilized in DMSO (50 μL), and a solution containing 5 mg (40 μmol) of (dimethylamino)-pyridine (DMAP), 6.6 mg (30 μmol) of dipyridyl disulfide, and 7.9 mg (30 μmol) of triphenylphosphine in DMSO in a total volume of 50 μL was added. After 20 min of incubation at rom temperature, the activated oligonucleotide was precipitated by a solution of $LiClO_4$ in

acetone (3%), washed with acetone, and resuspended in an aqueous solution (50 μL) containing 5 mg of cystamine-HCl and 10 μL of triethylamine. Incubation was carried out for at least 20 min at room temperature. The cystamine-substituted oligonucleotide 16-TC^{me}-cyst was precipitated in $LiClO_4$/acetone (3%), washed with acetone, dried, and resuspended in water (yield 94%, measured by HPLC). Dithiothreitol (7 mg) and 1 μL of Tris/HCl buffer (2 M, pH 7.5) were added to this solution (90 μL), and the mixture was incubated for 1 h at room temperature. The reduced oligonucleotide-SH was precipitated twice under an inert atmosphere in a degassed solution of EtOH/sodium acetate and then washed with degassed EtOH and resuspended in 20 μL of degassed Tris·HCl (pH, 7.5). Dipyridyl disulfide (16.5 mg) in DMSO (90 μL) were added and the reaction allowed to proceed overnight at room temperature. The resulting product 16-TC^{me}-SS-Pyr was then precipitated in EtOH and washed. An aqueous solution containing 16-TC^{me}-SS-Pyr (90 μL), the peptide LysTrpLysCysCONH$_2$ (10 equiv), NaCl (0.15 M), and Tris·HCl (6 mM, pH 7.5) was incubated for 3 h; then the oligonucleotide was precipitated and washed in EtOH. The conjugate was analyzed by denaturing gel electrophoresis in 20% polyacrylamide gel/tris-borate-EDTA buffer in the presence of 7 M urea and visualized by UV shadowing. HPLC analysis of T-KWK was carried out on a Lichrosorb C-18 column 250 × 4 mm (system 1010, Agilent Technologies). A 30 min linear gradient of acetonitrile 5−40% in 0.02 M ammonium acetate was applied for elution. The product was analyzed by UV−vis spectrophotometry (Kontron Uvikon 923). Mass spectrometry analysis (MALDI-TOF, M = 5493.86, calculated = 5494).

Fluorescent Labeling of Triplex DNA on Agarose Gel. Samples of duplex F (0.12 μM) were incubated overnight at 4 °C in 10 mM cacodylate buffer (pH 6.0), 10 mM NaCl, either alone or in the presence of 10 μM TFO (16-TC or 9-TC). A **PQ$_3$** solution in water was added to each sample and incubation continued for 1 h at 4 °C. Samples were then loaded on agarose gel (4%), 4 °C, migration time 15 min (note that the 2.5% agarose does not permit distinction of various DNA conformations; consequently, a 4% agarose gel was attempted. However, it suffered from overheating at 4 °C; significant reduction of voltage circumvented overheating but resulted in longer migration times that afforded no increased resolution because of diffusion).

Plasmid DNA Cleavage Assay. SspI cleavage of plasmid DNA (pf47) was accomplished by cleaving 10 μg of plasmid with SspI at 37 °C overnight. Specific cleavage (position 2855) was verified by comigration analysis with a molecular weight marker (Sigma). Linear pf47 was incubated overnight at 4 °C in 10 mM cacodylate buffer (pH. 6.0), 100 mM NaCl, either alone or in the presence of 16-TC (10 mM). **PQ$_3$** was added to the samples in order to get the final concentration of 3, 10, and 30 μM, and all cleavage reactions were preequilibrated for 1 h at 4 °C. Irradiation was carried out directly in the tube for 30 min at 4 °C, with a cutoff filter (λ > 305 nm, Xe/Hg Lamp 150 W) and followed immediately by 1% agarose gel electrophoresis (4 °C, in the dark).

Cleavage Assay on the 56-bp DNA (F), Standard Protocol. The oligopurine- and oligopyrimidine-containing strands (R and Y strands) of the duplex were separately 5′-end radiolabeled. Samples of the corresponding duplexes (Y*R and YR*) were incubated overnight at 4 °C in 10 mM cacodylate buffer (pH 6.0), 100 mM NaCl, either alone or in the presence of 20 μM 16-TC oligonucleotide. The same protocol was applied for preparation of triplexes formed with the conjugates (T-quin, T-acr, T-KWK). Samples were incubated with **PQ$_3$** (5−30 μM) in the dark for 1 h at 4 °C and irradiation was carried out for 10 min at 4 °C; the reaction was stopped by adding 2 μL of a stop solution (1 mM EDTA, salmon sperm DNA 10 mg/mL). Each sample was lyophilized and loaded on an acrylamide gel for analysis.

Replacement of H_2O by D_2O. To ensure that any observed effects of D_2O could be attributed solely to its effect on photochemistry, all samples were first lyophilized and then reconstituted with either H_2O or D_2O.

Molecular Modeling. A DNA triplex structure was constructed according to the previously published coordinates that correctly take into account the sugar conformation of (T,C)-motif triple helices.[42] This structure is closer to a B-form DNA consistent with NMR studies[43,44] than the structure previously proposed by Arnott[45] based on fiber X-ray diffraction. The JUMNA program permitted construction DNA structures according to their helical parameters.[46]

An intercalation site was created in the middle of triplex by doubling the rise parameter for two adjacent T•AxT base triplets (rise = 6.8 Å) and subsequently decreasing the twist parameter between these two triplets from 34° to 16° in order to reduce bond distance constraints. The ligand **PQ$_3$** (see Figure 1A) was constructed using the builder module of the Insight II package (MSI, San Diego) and energy minimized using the discover module. **PQ$_3$** was docked into the intercalation site with the charged side chain in the major or minor groove of Watson−Crick double helix. Then, energy minimization was performed in order to obtain the complex with the lowest conformational energy. Energies were also calculated for the free ligand molecules and the triplex structure in the absence of ligand. Solvent and counterions were not explicitly included in these calculations. Instead, a sigmoïdal distance-dependent function of dielectric constant was used, and each phosphate group was assigned half an electronic charge.

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Supporting Information Available: PAGE analysis of the photocleavage experiments conducted with T-quin, duplex HDF, T-acr, D_2O, and T-KWK. This material is available free of charge via the Internet at http://pubs.acs.org.

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