RNA Is More UV Resistant than DNA: The Formation of UV-Induced DNA Lesions is Strongly Sequence and Conformation Dependent

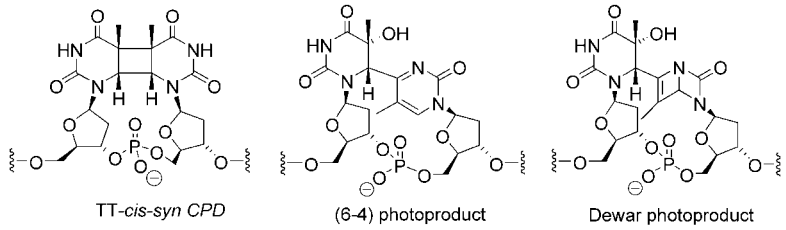
Lal Mohan Kundu,[a] Uwe Linne,[b] Mohamed Marahiel,[b] and Thomas Carell*[a]

Abstract: DNA and RNA hairpins, which represent well-folded oligonucleotide structures, were irradiated and the amount of damaged hairpins was directly quantified by using ion-exchange HPLC. The types of photoproducts formed in the hairpins were determined by ESI-HPLC-MS/MS experiments. Irradiation of hairpins with systematically varied sequences and conformations (A versus B) revealed remarkable differences regarding the amount of photolesions formed. UV-damage formation is, therefore, a strongly sequence and conformation dependent process.

Keywords: DNA damage · DNA repair · DNA · hairpin structures · RNA · UV irradiation

Introduction

Ultraviolet C (UVC) irradiation of cells induces the formation of well-studied DNA lesions.[1,2] The most prominent ones are *cis–syn* cyclobutane pyrimidine dimers (CPD), pyrimidine(6–4)pyrimidone ((6–4)PP) photolesions, and Dewar valence isomers of (6–4) lesions.[3–7]. The formation of these three lesions in sunlight-exposed skin is responsible for the development of a large fraction of non-melanoma skin cancers.[8–12] The *cis–syn* cyclobutane dimers are formed in a $[2\pi+2\pi]$ cycloaddition reaction. The almost exclusive formation of *cis–syn* dimers in double-stranded DNA is caused by the geometrical constraints imposed by the double helix. (6–4)PP lesions are believed to arise from a Paternó–Büchi reaction followed by an opening of the oxetane/azetidine intermediate, which takes place above −80 °C.[13] The Dewar valence isomer is formed upon further irradiation of the (6–4)PP lesion with light around 320 nm.[14] An extensive amount of biochemical and structural information has been collected in the last decades.[15–21] The effect of UV-induced lesions on the DNA double-helix structure and stability was studied using NMR spectroscopy,[17,22–28] X-ray diffraction,[19,20,29] CD spectroscopy,[30] gel-mobility experiments,[31] and theoretical calculations.[32–35] Systematic melting-point measurements revealed how these lesions destabilize the duplex structure.[21,36,37] Ongoing biophysical, biochemical, and cell biological analyses are addressing how these lesions are recognized and repaired in our densely packed genome.[38–45]



In previous experiments designed to determine the reactivity of the two pyrimidines dT and dC, DNA was irradiated with UVC light and then was completely digested; the photoproducts were quantified by using HPLC-MS and HPLC-MS/MS.[46–52]

It was reported that 2′-deoxythymidines react most efficiently in the presence of UVC light to give mainly *cis–syn* cyclobutane 2′-deoxythymidine dimers. In addition, the dCpdT and dTpdC sequences were found to give (6–4)PP, which further isomerize to the Dewar valence photoproducts. Overall, the (6–4)PP lesions are formed less frequently. The ratio between CPD and (6–4)PP lesions was found to

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be around 3:1.[48] Further data about how the nearest neighbors and the duplex conformation influence the reactivity are rare.[53–59]

It is well known that photochemical reactions in crystals (topochemistry) are strongly influenced by the packing of the reaction partners in the crystal lattice.[60] If we consider DNA to be similar to a one-dimensional crystal, we can expect that the local DNA conformation and the local stiffness of the duplex will strongly influence the damage formation process. If this is true, we would anticipate a large modulation of the reactivity of the pyrimidines along a given gene and hence an inhomogeneous mutation frequency.[61,62] This has potentially tremendous impact for our understanding of the process of mutagenesis.

Herein we describe a direct, systematic analysis of how the DNA sequence and the conformation of the DNA duplex influence the UV-damage formation process. We find surprisingly large reactivity differences, which show that long DNA strands will be inhomogenously damaged upon UV exposure.[63]

Results and Discussion

For the investigation we prepared a series of small DNA, RNA, and mixed DNA/RNA hairpins. One advantage of hairpins is that they possess a well-defined structure characterized by high and concentration-independent melting points.[64–67] Also, hairpins are rather small molecules, which enabled us to determine the amount of damaged DNA directly using HPLC. Due to the concentration-independent melting behavior, irradiation experiments performed at very low DNA concentrations still generate a sufficient amount of DNA lesions for direct analysis. Therefore, irradiation of small hairpins with UVC light allows the most direct measurement of the damage formation process. This is important, because previously the amount of DNA damage was determined indirectly from the activities of DNA repair enzymes like T4-endo V[68] or DNA-photolyases.[41,69] However, their activity itself might also be modulated by the sequence and the structural context of the lesion.

All DNA hairpins were constructed from 14 to 16 nucleotides as shown in Figure 1. The DNA hairpins contain a stem region of five to six base pairs; this region is needed to form the necessary duplex structure. The loop region of the hairpins were constructed either from four 2′-deoxycytidines, four 2′-deoxyadenosines, four 2′-deoxythymidines or from a C12 spacer. Labeling of these small hairpin oligonucleotides was performed with a fluorescein tag (6-FAM). In order to exclude that the fluorescence tag interferes with the damage formation process we irradiated one hairpin (**5**) with and without the fluorescence tag and observed exactly the same amount of damage formation. We are therefore sure that the fluorescein molecule does not influence the damage formation process. However, it allows detection of damaged oligonucleotides even in very small quantities. In order to quantify the amount of damaged hairpins after irradiation, we separated the lesion-containing hairpins by ion-exchange chromatography at pH ~13 at temperature $T = 25\,^{\circ}C$. These harsh conditions are needed to fully denature the hairpins during HPLC analysis, particularly the G:C-rich hairpins. For the experiments we dissolved the hairpins in a buffer composed of 10 mM Tris-HCl and 150 mM sodium chloride (pH 7.4) and irradiated the solutions in fluorescence cuvettes for 10 to 30 min with 254 nm light in a fluorimeter equipped with a single monochromator. During the irradiation, the temperature of the solution was kept constant at about 20 °C. Before and after irradiation a small sample was removed and analyzed by ion-exchange chromatography.

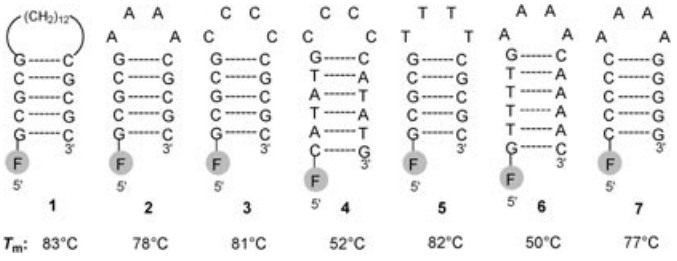

Figure 1. Depiction of the hairpins **1**–**7** prepared to study the reactivity of the various bases in the presence of UVC-light. F (6-FAM) = 5′-Fluorescein. Melting point (T_m) condition: $c_{hairpin} = 3\,\mu M$ in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4).

Lesion formation depending on the kind of nucleobase: We first re-investigated, using our direct method, which nucleobases are most efficiently damaged by UVC irradiation. To this end, hairpins **1**–**7** (Figure 1) were irradiated. Irradiation of hairpins **1**–**4** for over an hour gave no detectable damage. The chromatograms of all four hairpins show a sharp, single peak before and after irradiation (data not shown). In order to exclude that certain lesion-containing hairpins co-elute with the main peak, we digested all the irradiated hairpins **1**–**4**. For this, the irradiated DNA was treated with an enzyme mixture containing nuclease P1 (from penicillium citrinum), phosphodiesterase II (from calf spleen), alkaline phosphatase (CIP), and phosphodiesterase I (from snake venom).[49,51] In the HPL chromatogram of the digested solutions only the four peaks of the four canonical nucleobases could be detected. This result that no lesions were found was confirmed for the hairpins **1**–**3** by HPLC-MS followed by ion extraction, whereby we failed to detect any photoproducts using this very sensitive method. For hairpin **4**, we observed in the HPLC-MS (followed by ion extraction), formation of new peaks with only very small intensity. More detailed analysis of these peaks by HPLC-MS/MS showed that the peaks are formed by dApdT photoproducts, in accordance with previous observations by Zhao et al.[70] In contrast, hairpins **5**–**7** are efficiently degraded by UVC light in comparison to hairpins **1**–**4**. Figure 2 shows the HPLC profiles of all three hairpins before (bottom) and after (top) irradiation. Clearly evident is that hairpins **5** and **6**, which contain short homo 2′-deoxythymidine sequences, are degraded most strongly (32% and 25%, respectively, after 20 min of light exposure) confirming that 2′-deoxythymidines possess the highest UVC sensitivity. Hairpin **7** is degraded much less efficiently to only about 12% (20 min of light exposure), showing that homo 2′-deoxycytidine stretch-

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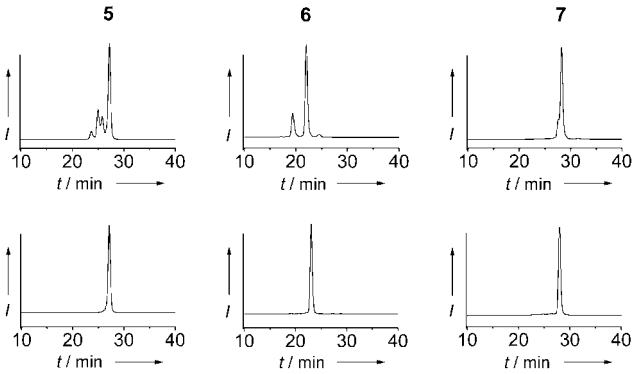

Figure 2. Depiction of the HPL chromatograms before (bottom) and after (top) irradiation of hairpins **5**, **6**, and **7** with UVC light for 20 min. Assay solution: hairpin concentration = 0.2 μM in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). λ_{irr} = 254 nm (band gap = ± 10 nm), T = 20 °C. HPLC conditions: Nucleogel-SAX column (1000–8); eluting buffers (buffer A: 0.2 M NaCl/0.01 M NaOH in H_2O, pH 13; buffer B: 1 M NaCl/ 0.01 M NaOH in H_2O; pH 13); Gradient: 0–75 % B in 25 min and then up to 85 % B in 35 min at a flow of 0.7 mL min^{-1}. t = retention time; I = relative fluorescence intensity.

es are much more UV resistant. A comparison of **7** with **3** and **4**, which both give no HPLC detectable UV degradation although the loop was constructed from dC, proves that the proper alignment of the 2′-deoxycytidines in a double helix structure is a prerequisite for the UV-induced reaction at cytosine sites. This strict pre-organization is seemingly unnecessary for 2′-deoxythymidines, which give large amounts of UV-induced lesions even if the 2′-deoxythymidines are flexibly arranged in the loop region of the hairpin, such as in **5**. Interesting is the observation that hairpin **4** gives only a small amount of dApdT photoproducts. Under our irradiation conditions, formation of dTpdT lesions is clearly not observed, indicating that non-adjacent 2′-deoxythymidines, separated by one A:T base pair can not form dimer lesions; this is different to what has been observed with single strands.[71] We were also unable to detect any cross-link photoproducts between 2′-deoxythymidines present in opposite strands.[72] If at all, the formation of dTpdT photoproducts of non-adjacent 2′-deoxythymidines is of minor chemical significance.

To investigate if somehow the high pH-value of the chromatographic system interferes with our analysis, we irradiated a very small piece of DNA [5′-d(TTTT)-3′] and analyzed lesion formation with ion exchange chromatography at pH 13 and by reversed phase chromatography (C18-column, Nucleosil 250 × 4 mm, 3 μm; buffer A: 0.1 M AcOH/NET_3 in water pH 7.0, buffer B: 0.1 M AcOH/NEt_3 in 80 % acetonitrile pH 7.0; Gradient: 0–30 % B in 90 min). Both analytical methods gave the same result. To fully exclude that the DNA lesions degrade during HPLC analysis we irradiated hairpin **6**, analyzed one fraction immediately and another one after stirring for 30 min at pH 13. The obtained HPLC profiles were identical within an error margin of 1 %, showing that degradation is not an issue during analysis under our condition.

Dose dependence: Because we were comparing the amount of photo damage formed in various hairpins after a certain time of irradiation and since photoproduct formation is known to be a reversible process at 254 nm,[73] we had to make sure that photoproduct formation under our irradiation conditions is linearly dose dependent. This was confirmed by irradiation of the UVC vulnerable hairpin **6** for increasing amounts of time. A plot of the amount of degradation, quantified by HPLC, against the irradiation time is

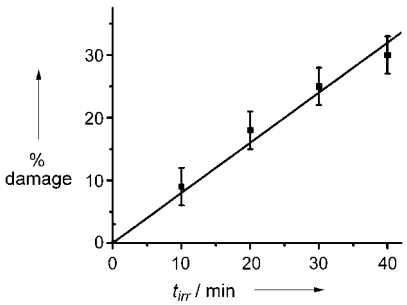

Figure 3. Dose dependence of the lesion formation process measured by irradiation of hairpin **6** at 254 nm. Assay solution: $c_{hairpin}$ = 0.2 μM in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). λ_{irr} = 254 nm (band gap = ± 10 nm), T = 20 °C. HPLC conditions: Nucleogel-SAX column (1000–8); eluting buffers (buffer A: 0.2 M NaCl/0.01 M NaOH in H_2O, pH 13; buffer B: 1 M NaCl/0.01 M NaOH in H_2O; pH 13); Gradient: 0–75 % B in 25 min and then up to 85 % B in 35 min at a flow of 0.7 mL min^{-1}; t_{irr} = irradiation time.

shown in Figure 3. Even up to 30 min of irradiation we observed that the damage formation process depends linearly on the dose of UVC light under our conditions (see Experimental Section). At and above 40 min the damage formation starts to deviate from linearity (not shown in Figure 3) due to the reversibility of the UV-damage formation process. We therefore compare in this study only the amount of DNA damage formed up to 30 min of irradiation.

Lesion analysis: In order to analyze which lesions are predominately formed, we irradiated a small all-dT containing tetranucleotide 5′-d(TTTT)-3′ and analyzed the irradiated solution by reversed phase HPLC coupled to electrospray mass spectrometry. However, all the new peaks detected in the HPL chromatogram after irradiation had the same molecular weight when compared to the unirradiated oligonucleotide (m/z = 1154). This result is in full agreement with the formation of mainly CPD, (6–4)PP, and Dewar valence isomer lesions, which all have a molecular weight indistinguishable from a dTpdT dinucleotide. Further proof for the formation of these three lesions was obtained after a complete digestion of the irradiated hairpin **6** and subsequent HPLC-MS/MS analysis of the resulting solution. A part of the chromatogram obtained from the HPLC-MS/MS analysis is depicted in Figure 4. For the experiment we set the detector to a mass (m/z) of 545, which is the mass of a dTpdT dinucleotide and of the photoproducts. The insets next to the chromatogram show the fragmentation pattern of every detected lesion. At 19 min a compound (signal a) was detected, which has a fragmentation pattern the same as that reported for the Dewar valence isomer.[51] The fragmentation pattern of the signal at 20 min (signal b) is identical to the reported fragmentation of the CPD lesion. At 24.5 min

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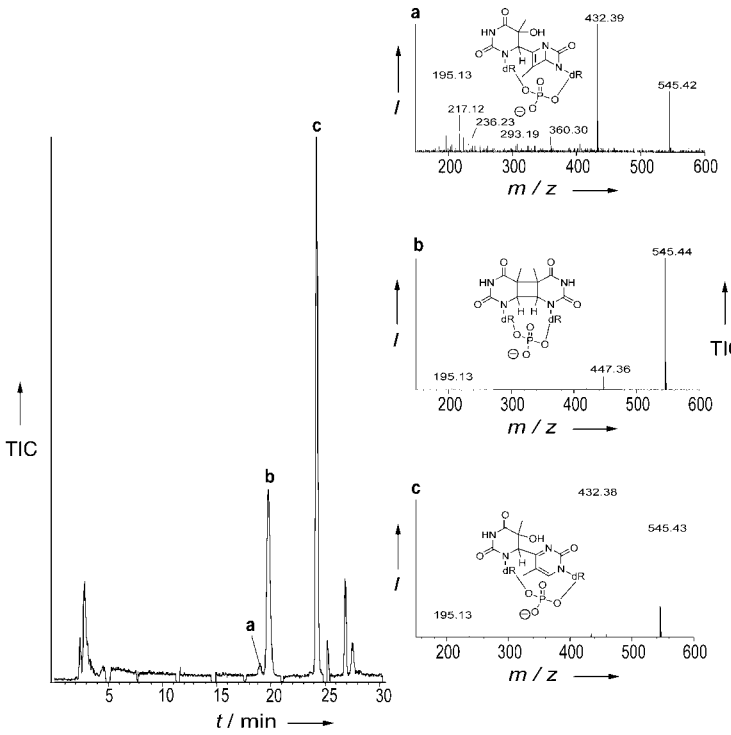

Figure 4. Depiction of the HPLC-MS/MS data obtained for hairpin **6** after irradiation and complete digestion. The insets show the fragmentation pattern of the detected lesions. The first quadrupole (Q1) was set to $m/z = 545$, and the fragmentation was measured in a mass range of 150–600 amu. The polarity was set to the negative ion mode. a) dTpdT Dewar photoproduct, b) dTpdT *cis–syn*-CPD, and c) dTpdT-(6–4)PP lesion; dR: 2-deoxyribose, t = retention time, TIC = total ion current, I = relative signal intensity.

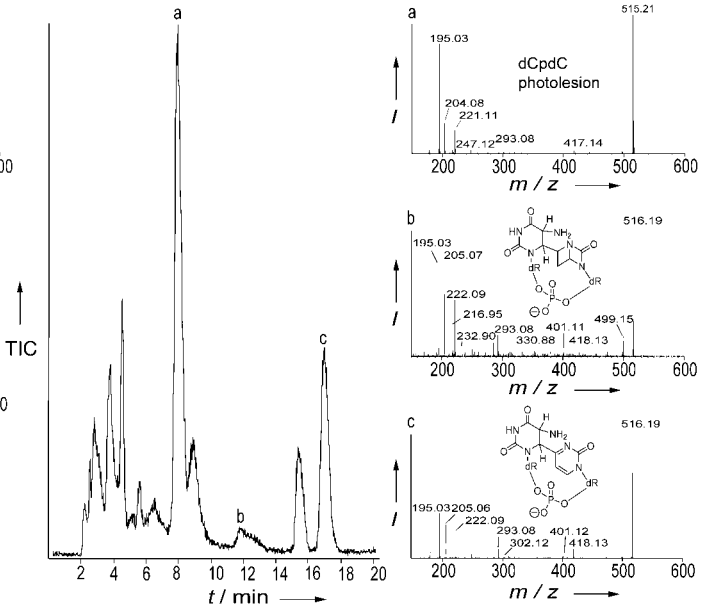

Figure 5. Depiction of the HPLC-MS/MS data of hairpin **7** after irradiation and complete digestion. The insets show the fragmentation pattern of the detected lesions. The first quadrupole (Q1) was set to $m/z = 515$, and the TOF range was chosen from 150–600 amu. The polarity was set to the negative ion mode. a) dCpdC photolesion, b) dUpdC Dewar valence isomer, c) dUpdC-(6–4) lesion; dR: 2-deoxyribose, t = retention time, TIC = total ion current, I = relative signal intensity. (The structures of the photoproducts were drawn in accordance with reference [59]). Unassigned peaks are also present in the control. They are non-specific.

a new peak was detected, whose fragmentation pattern is indicative for a (6–4)PP lesion (signal c).

We conclude that the observed UVC-induced degradation of the oligonucleotide hairpins **5** and **6** (Figure 2) is caused by formation of the usual dTpdT-dinucleotide-derived UV lesions. This is further supported by two experiments: first we collected the fraction containing the damaged hairpins from the ion exchange chromatography after irradiation and performed the digest with just this fraction. HPLC-MS (followed by ion extraction) and HPLC-MS/MS confirmed that the usual dTpdT dinucleotide lesions are responsible for the hairpin degradation. The collected undamaged peak was also digested. Here, however, no lesions were detected by HPLC-MS and HPLC-MS/MS analysis, which again excludes co-elution and it confirms that quantification of the new peaks within the HPLC profiles of the irradiated hairpins is indeed a good measure of the total amount of photoproducts.

To analyze the lesions formed in the C-rich hairpin **7**, we also digested this hairpin after irradiation and analyzed the digest by HPLC-MS/MS. The obtained chromatogram together with the fragmentation pattern of the detected peaks is shown in Figure 5. We detected at a retention time of 8 min and 9 min peaks (signal a) with a mass (m/z) of 515, which is the mass of a dCpdC dinucleotide and of the corresponding photoproducts. These peaks arise most likely from a dCpdC–CPD dimer, a dCpdC–(6–4)PP photoadduct, or a dCpdC Dewar valence isomer. At 12 min and 17 min peaks are observed with a mass of 516. These lesions are likely to be dU-containing photoproducts that are formed by deamination of one dC subunit. We think that the peak at 12 min (signal b) is due to dUpdC Dewar valence isomer and that at 17 min (signal c) due to dUpdC–(6–4)PP lesion. The tentative assignment is based on the detected eluting times, the m/z value of 516 and the fragmentation patterns, which are comparable to an assignment by Cadet et al.[48]

We finally looked for hydration and oxidation products of, in particular, thymine and cytosine by HPLC-MS followed by ion extraction. However, these reported lesions could not be detected, indicating that they are not formed under our soft irradiation conditions.

Sequence context: The DNA hairpins **8**–**12** (Figure 6) all contain a UV reactive dTpdT dinucleotide embedded in different sequence contexts in the middle of the stem structure of DNA hairpins. The chromatograms obtained before and after 30 min of irradiation of the hairpins **8**, **9**, and **10** are depicted in Figure 7. Clearly evident is a large modulation of the reactivity of the dTpdT dinucleotide by the neighboring base sequence. Most damage is observed in a stem structure such as **8**, in which the dTpdT-sequence is flanked by two 2′-deoxycytidines. Here we detected about 40% degradation after 30 min of irradiation. This is not surprising, because in such a sequence the 2′-deoxythymidines can react not only with each other, but also form dTpdC photoproducts with the neighboring cytosine bases. If we change the

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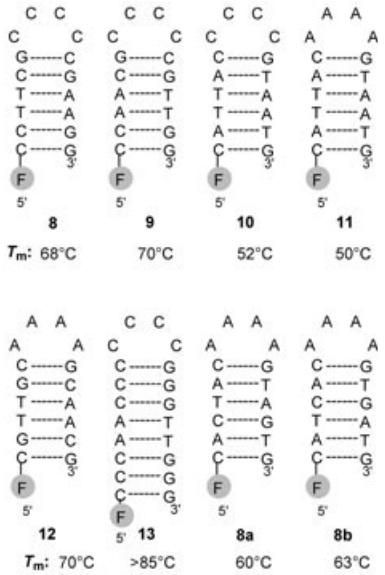

Figure 6. Depiction of the eight hairpin molecules **8**–**13**, **8a**, and **8b** analyzed in order to assess how the nearest neighbors influence the UV lesion formation process in a dTpdT-dinucleotide sequence. F: (6-FAM) = 5′-Fluorescein phosphoramidite. Melting point (T_m) condition: $c_{hairpin} = 3\,\mu M$ in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4).

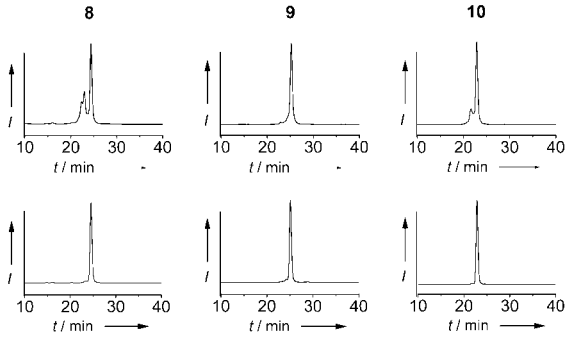

Figure 7. HPL chromatograms of the hairpin molecules **8**, **9**, and **10** before (bottom) and after (top) irradiation with UVC light (254 nm) for about 30 min. Assay solution: $c_{hairpin} = 0.2\,\mu M$ in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). $\lambda_{irr} = 254$ nm (±10 nm), $T = 20\,°C$. HPLC conditions: Nucleogel-SAX column (1000–8); eluting buffers (buffer A: 0.2 M NaCl/0.01 M NaOH in H_2O, pH 13; buffer B: 1 M NaCl/0.01 M NaOH in H_2O; pH 13); Gradient: 0–75 % B in 25 min and then up to 85 % B in 35 min at a flow of 0.7 mL min^{-1}. t = retention time; I = relative fluorescence intensity.

base sequence so that the dTpdT sequence is flanked by 2′-deoxyadenosines as in hairpin **10**, the amount of UV-damage is strongly reduced to only about 20–25 % (30 min of irradiation) relative to **8**. Here only dTpdT photoproducts can form (the amount of dApdT photoproducts is negligible in this context). This result is independent of the loop sequence, because the same amount of UV degradation is observed in hairpin **11** in which the homo-dC loop was replaced by a homo-dA loop. The observed DNA damage is consequently formed only in the hairpin stem. Surprising is the fact that a dTpdT dinucleotide, sandwiched between 2′-deoxyguanosines as in the hairpins **9** and **12**, shows after 30 min of UV irradiation almost no UV damage (only the chromatogram of **9** is shown in Figure 7). If we enlarge the number of G:C base pairs in 3′ and 5′ direction of the dTpdT sequence as in hairpin **13**, the large protective role of the guanines is manifested. The dTpdT-dinucleotide sequences embedded between 2′-deoxyguanosine bases are, as a result, significantly more stable under UVC irradiation than in a mixed sequence context.

All these irradiated hairpins were also digested and HPLC-MS and HPLC-MS/MS measurements were performed. Hairpin **8** showed all the usual dTpdT and dTpdC photoproducts, whereas almost no lesions were found in the hairpins **9**, **12**, and **13**, which again excludes co-elution of any photoproducts in the ion exchange HPLC. However, a very small amount of dApdT photoproducts were obtained along with dTpdT lesions in the HPLC-MS followed by ion extraction for hairpins **10** and **11**. These dApdT photoproducts were not detectable by UV during HPLC analysis.

In order to analyze the UV reactivity of dTpdC dinucleotides using our direct approach, we prepared the two additional hairpins **8a** and **8b** (Figure 6) containing either a 5′-dCpdT-3′ (**8a**) or a 5′-dTpdC-3′ (**8b**) dinucleotide sequence. The hairpins were irradiated for 35 min. Here, for **8a** (5′-dCpdT-3′) we detected 25 % degradation. For **8b** (5′-dTpdC-3′) the degradation was determined to 35 %, in accord with previous studies that 5′-dTpdC-3′ sequences are a little more UVC vulnerable.[48]

Analysis of how the DNA conformation influences UVC-damage formation: To analyze how the damage formation process is influenced by the DNA conformation,[63,74] we prepared the DNA/RNA hairpins **14**–**17** (Figure 8), which

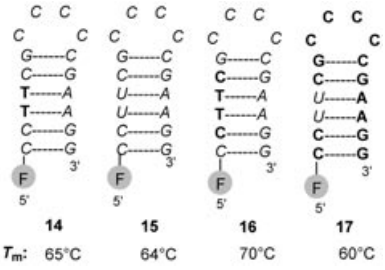

Figure 8. DNA/RNA hairpins **14**–**17** prepared for the investigation of how strongly the duplex conformation influences the UVC damage formation process. RNA bases are shown in italics, DNA bases are printed in bold. F: (6-FAM) = 5′-Fluorescein. Melting point (T_m) condition: $c_{hairpin} = 3\,\mu M$ in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4).

contain a varying number of RNA nucleosides. In contrast to DNA, which forms a B-type double strand, RNA double strands adopt an A-type conformation.[75] RNA/DNA heteroduplexes are known to possess a more A-like helix conformation.[36] The RNA hairpin **14** contains an isolated dTpdT dinucleotide embedded in the stem structure. In hairpin **16** we increased the DNA stretch, containing the vulnerable dTpdT to a total of four DNA nucleotides. Hairpin **15** is a pure RNA hairpin containing two uridines instead of a dTpdT dinucleotide sequence. In order to analyze how strongly two adjacent uridines UpU form UVC-induced lesions, we also prepared hairpin **17** containing a UpU dinucleotide in a pure DNA environment.

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First we investigated the conformations of the prepared hairpins **14**–**17** by CD spectroscopy. A selection of CD spectra is depicted in Figure 9. The pure DNA hairpin **12** adopts

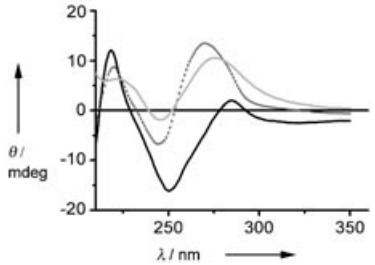

Figure 9. CD spectra of the hairpins **12** (solid black), **15** (dotted black) and **16** (solid gray). $c_{hairpin}=3\,\mu M$ in 150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4. $T=20\,°C$.

clearly a B-duplex structure as evident from the two maxima at 284 and 218 nm and the minimum at 250 nm. The pure RNA hairpin **15** in contrast takes on an A-type conformation. Here, the maxima are shifted to 271 and 221 nm; the minimum was found at 242 nm, in full agreement with literature data expected for oligonucleotide duplexes in the A-type conformation.[76,77] The mixed hairpins such as **16** should have a conformation between A and B. The CD spectra prove a globally more A-like conformation. The obtained CD spectra of **14**, **16**, and **17** (only the spectrum of **16** is depicted in Figure 9 for clarity reasons) are almost identical with the measured spectrum of the pure RNA hairpin **15**. We can conclude that all the prepared RNA-containing hairpins adopt, in accord with literature, a more A-like conformation.

The results of the irradiation experiments are presented in Figure 10. We first irradiated the RNA-hairpin **14**, containing a dTpdT sequence, for 30 min. Analysis of the assay solution revealed, to our surprise, no formation of UV lesions at all. This hairpin did not degrade on exposure to UVC light. The same unusual UVC resistance was also detected for RNA hairpin **15**, which contains a UpU instead of a dTpdT dinucleotide. Even after 30 min of UV irradiation we were unable to detect any UV-induced UpU lesions, revealing a very strong UV protection of both dTpdT and UpU sequences by the A-like environment. This observation was supported by HPLC-MS/MS analysis of the irradiated and subsequently digested hairpins **14** and **15**, which also gave no detectable lesions. The fact that a UpU sequence is, in principle, able to form UV lesions upon irradiation was proven with hairpin **17**, containing the UpU sequence within a DNA hairpin. Here we measured about 10% degradation already after 20 min of irradiation.

If the dTpdT-containing DNA stretch was enlarged within the RNA hairpin as in **16**, we still observe a strong reduction of the amount of UV lesions, proving that it is indeed the A-conformation of the hairpin stem and not the contact of the dTpdT sequence with RNA nucleotides that is responsible for the UV protective effect.

To analyze which lesions are formed in these A-like structures, we irradiated hairpin **16** for a rather long time

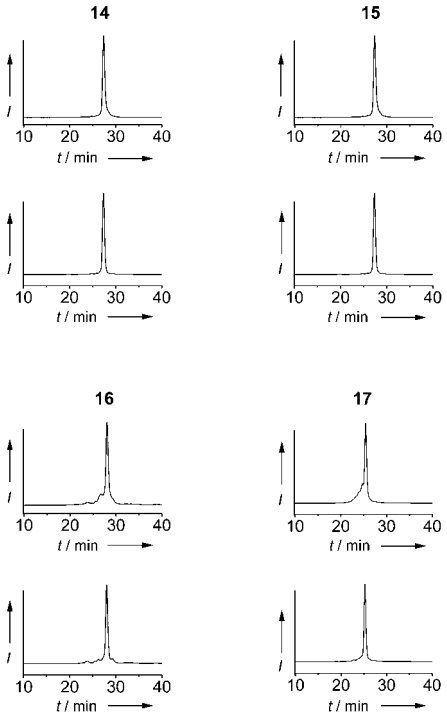

Figure 10. HPL chromatograms of the four hairpin molecules **14**–**17** before (bottom) and after (top) 20 min of irradiation with UVC light. Assay solution: $c_{hairpin}=0.2\,\mu M$ in buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). $\lambda_{irr}=254$ nm (±10 nm), $T=20\,°C$. HPLC conditions: Nucleogel-SAX column (1000–8); eluting buffers (buffer A: 0.2 M NaCl/0.01 M NaOH in H_2O, pH 13; buffer B: 1 M NaCl/0.01 M NaOH in H_2O; pH 13); Gradient: 0–75% B in 25 min and then up to 85% B in 35 min at a flow of 0.7 mL min^{-1}. t = retention time; I = relative fluorescence intensity.

(60 min) to produce enough lesions for HPLC-MS/MS analysis. We then digested the RNA/DNA hairpin **16** and analyzed the digest by HPLC-MS/MS (the enzyme mixture digests RNA as well). The chromatogram is depicted in Figure 11, together with the fragmentation pattern of the photoproducts (insets). At a detection mass of $m/z=530$, indicative for dTpdC photoproducts after deamination, we observed two photolesions. The mass and the fragmentation shows clearly that the lesions are either dTpdC–(6–4)PP or dTpdC Dewar lesions. Since the Dewar lesions are generally more polar, we speculate that peak a may be the Dewar lesion and peak b the (6–4) lesion. However, the data are not sufficient for a clear assignment.[48] At a detection mass of $m/z=545$ (produced by dTpdT dinucleotides) we detected again all the usual photoproducts, the dTpdT–CPD, the dTpdT–(6–4)PP and its Dewar valence isomer (Figure 12).

We can conclude that an A-like double helix structure dramatically reduces the reactivity of dTpdT and UpU dinucleotides in the presence of UVC light. RNA is in summary, much more UV stable than DNA if exposed to UVC light.

Conclusion

UV irradiation leads to severe genome damage. The DNA lesions formed upon UV irradiation are mainly cyclobutane pyrimidine dimers, (6–4)PP photolesions, and Dewar va-

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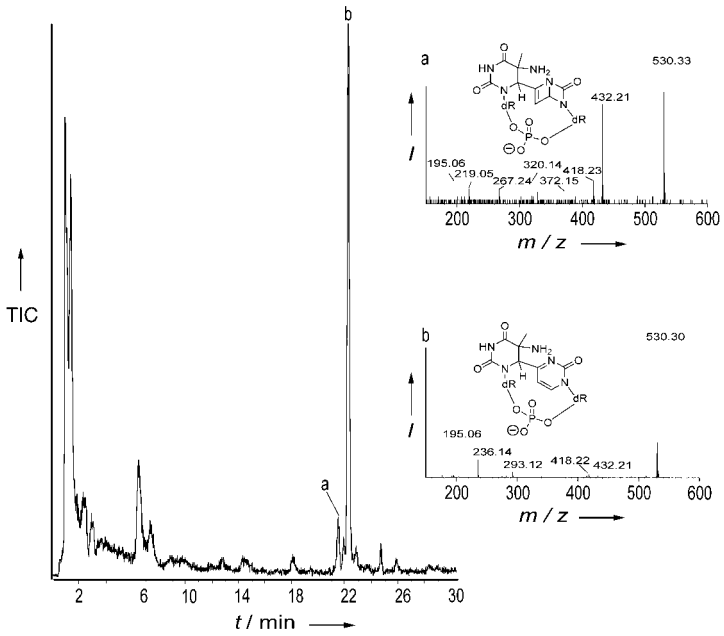

Figure 11. HPLC-MS/MS experiment at $m/z = 530$ after digestion of irradiated hairpin **16**. The insets show the fragmentation pattern. The first quadrupole (Q1) was set to $m/z = 530$, and the TOF range was chosen from 150–600 amu. The polarity was set to the negative ion mode. Peaks a and b: dTpdC-derived photo lesions, t = retention time, TIC = total ion current, I = relative signal intensity.

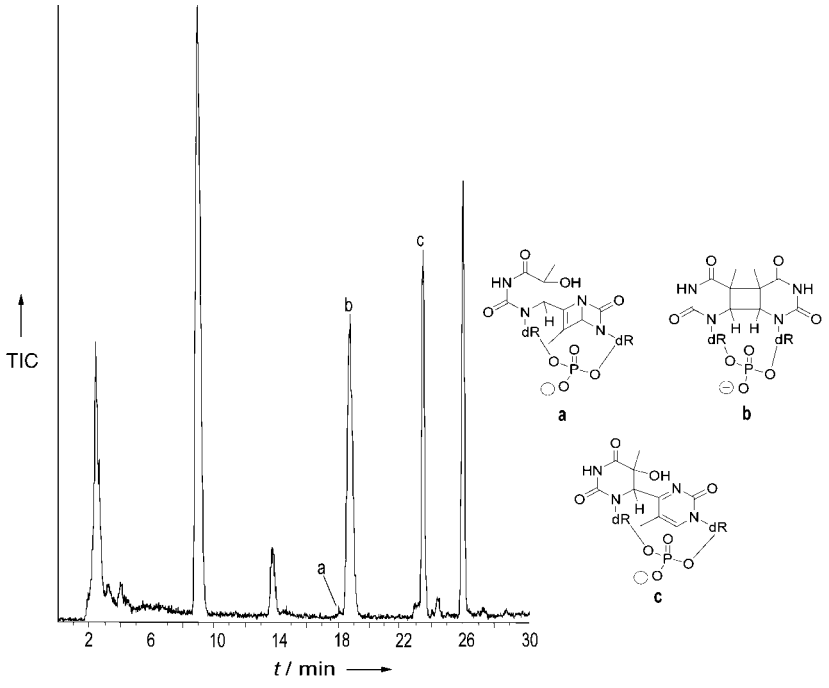

Figure 12. HPLC-MS/MS experiment at $m/z = 545$ after the digestion of irradiated hairpin **16**. The first quadrupole (Q1) was set to $m/z = 545$, and the TOF range was chosen from 150–600 amu. The polarity was set to the negative ion mode. a) dTpdT Dewar photoproduct, b) dTpdT-CPD, and c) dTpdT-(6–4)PP lesion. dR: 2-deoxyribose, t = retention time, TIC = total ion current, I = relative signal intensity. For the fragmentation pattern see Figure 4.

lence isomers. These lesions are predominatly responsible for the development of non-melanoma skin cancers.[78] We have prepared small fluorescein-labeled DNA, RNA, and mixed DNA/RNA hairpins that form stable, folded oligonucleotide duplex structures at room temperature. The concentration-independent melting behavior allows for irradiation at very low concentrations, which produces a significant amount of lesions even under very mild UVC conditions. Due to the small size of the hairpins it was possible to quantify the amount of UVC degradation directly by using ion-exchange chromatography at pH 13 (fluorescence detection) at room temperature. Irradiation of hairpins possessing various sequences once again established that 2′-deoxythymidine is the most vulnerable DNA base in UVC light. Our HPLC studies of the total damage together with MS/MS structure determination of the formed lesions prove that homo 2′-deoxythymidine stretches rapidly form photoinduced lesions in large quantities. The Dewar valence isomer lesion is formed in much smaller amounts. 2′-Deoxythymidines give rise to these lesions in flexible DNA regions as well as in the well-structured, double-helical stem area. 2′-Deoxycytidines, in contrast, react in the presence of UVC light only in the well-organized B-duplex. Flexible homo dC-sequence regions are rather UVC resistant.

Investigation of the reactivity of a dTpdT dinucleotide in various sequence contexts revealed the surprising result that the reactivity is strongly reduced if the 2′-deoxythymidines are flanked by two 2′-deoxyguanosines. We believe that the reason for the protective effect is a reduced flexibility of the 2′-deoxythymidines embedded between 2′-deoxyguanosines. First, the dT's stack on top of large purine bases and second, the flanking sequences are G:C base pairs, which have significantly higher pairing strength than an A:T base pair, making the duplex more rigid which may hinder the reorientation of the duplex upon photolesion formation.

Most surprising is the result that an oligonucleotide duplex, which exists in an A-like conformation is extremely UV resistant. The UV-induced degradation, under our conditions, was almost fully abolished. RNA duplexes are in consequence much more UV resistant than DNA duplexes. Again, the explanation could be an increased duplex stiffness in combination with a more "unfavorable" orientation of the pyrimidine bases. The A-duplex is much more compact. Formation of photolesions may require larger structural rearrangments within the duplex, which could be energetically more costly. This would then raise the energy of the transition state. In the A-type conformation, the dT's of a dTpdT dinucleotide

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may simply not be well enough arranged for the photochemical reaction.

This observation is particularly noteworthy in a biological context, because DNA in eukaryotes is densely packed in chromatin. The genomic DNA is wound around nucleosomes. Hence, DNA in our cell nuclei is not in an ideal B-type conformation but partially distorted. Our study shows that this distortion will tremendously effect the UV stability of the duplex.[44,79]

The performed HPLC-MS/MS experiments support results from the Cadet group. The major UV-induced lesions in DNA are CPD lesions, (6–4)PP photoproducts, and their corresponding Dewar valence isomers.[48,80]

The fact that RNA is much more UV-resistant is also an important discovery with respect to our current understanding of the origin of life. Due to the lack of an ozone shield at the beginning of life on earth, we can suppose that the UV irradiation doses on the earth's surfaces were significantly higher than today. The RNA-world theory suggests that RNA acted as a precursor of todays DNA/protein world.[81] Our result that RNA is much more UV-resistant than DNA could be one more factor that may have helped RNA to evolve on the primordial earth. The higher UV-resistance of RNA is, hence, one more argument supporting the RNA-world theory.[82,83]

Experimental Procedures

General: All the DNA oligonucleotides were purchased from Metabion (Germany). The DNA was further purified by HPL chromatography by using a reverse phase Machery–Nagel Nucleosil (RP-C18,100–7, 250/10) column. The purified DNA hairpins were characterized by MALDI-TOF mass spectrometry (Brucker Biflex) and subsequently melting temperatures were measured. The RNA and/or mixed RNA hairpins were purchased from IBA (Germany) and were characterized by MALDI-TOF mass spectrometry prior to use. All the hairpins were self-hybridized when heated in a buffer (150 mM NaCl/10 mM Tris-HCl; pH 7.4) to 90 °C and subsequent slow cooling to room temperature. HPLC measurements were performed with a Merck–Hitachi–Lachrom system associated with L-7400 UV and L-7480 fluorescence detector.

The melting points and the concentrations of the hairpins were measured by using a Varian Cary Bio100 UV spectrometer equipped with a Cary temperature controller. The irradiation experiments were performed with a JASCO-FP-750 fluorescence spectrometer.

The MS/MS experiments were carried out with a PE ScieX API Qstar pulsar i mass spectrometer (Applied Biosystems).

Irradiation of hairpins: All irradiations were performed at 254 nm (±10 nm band path) inside a fluorimeter (150 W Xe-lamp, single monochromator; JASCO) connected to a thermostat controller. All oligonucleotide solutions were prepared at a same concentration (0.2 μM) in a buffer containing 150 mM NaCl/10 mM Tris-HCl; pH 7.4. About 200 μL of each solution was taken in a 2 mm fluorescence cuvette and was irradiated for 20–30 min at a constant temperature of 20 °C. After the irradiation 20 μL of solution was injected into the HPLC for the damage formation analysis.

HPLC detection: The damage formations were analyzed at room temperature (25 °C) by using HPLC associated with a fluorescence detector (L-7480). A Machery–Nagel Nucleogel-SAX column (1000–8) was used to separate the damaged products from the undamaged oligonucleotides. Two eluting buffers (buffer A: 0.2 M NaCl/0.01 M NaOH in H_2O, pH 13; buffer B: 1 M NaCl/0.01 M NaOH in H_2O; pH 13) were used in a gradient of 0–75 % B in 25 min and then up to 85 % B in 35 min at a flow of 0.7 mL min^{-1}. A 20 μL aliquot of sample solution was injected into the HPLC through an autosampler (L-7200). The excitation wavelength of the fluorescence detector was set to 495 nm, which is the absorption maxima of fluorescein, and the emission was measured at 520 nm.

Enzymatic digestion: The oligonucleotides to be digested were irradiated in a fluorescence cuvette at 254 nm (±10 nm band path) inside a fluorimeter over a period of 1 h. The oligonucleotide concentration was set to 2 μM to induce sufficient damage formation. About 100 μL of irradiated solution was transferred into an Eppendorff vial and 10 μL of buffer (300 mM ammonium acetate, 100 mM $CaCl_2$ and 1 mM $ZnSO_4$; pH 5.7) was added followed by addition of 22 units nuclease P1 (penicillium citrinum) and 0.05 units calf spleen phosphodiesterase. The solution was incubated at 37 °C for about 3 h. To the resulting solution 12 μL buffer (500 mM Tris-HCl, 1 mM EDTA; pH 8.0), 10 units alkaline phosphatase (CIP) and 0.1 unit snake venom phosphodiesterase were added sequentially followed by incubation at 37 °C for another 3 h. The solution thus obtained was added to 6 μL of 0.1 N HCl to bring the pH down to approximately 7.0. The solution was then centrifuged at 3000 g for about 5 min. A 30 μL sample of the solution was transferred into an HPLC vial and was injected into an HPLC-MS/MS system. As a control, a parallel digestion was always performed with the corresponding unirradiated oligonucleotide.

HPLC-MS/MS analysis: The single nucleotides and the photoproducts after complete enzymatic digestion were separated and analyzed by using HPLC-MS/MS. A highly efficient Uptisphere 3 HDO column (150 × 2.1 mm) was utilized for HPLC separation. Detection of the analytes was carried out by UV and ESI-MS or ESI-MS/MS. The mobile phase contained two buffers (buffer A: 2 mM NEt_3/AcOH in H_2O; buffer B: 2 mM NEt_3/AcOH in 80 % CH_3CN). A mixed gradient was used with 0–3 % B in 12 min and then up to 20 % B in 30 min at a flow rate of 0.2 mL min^{-1}. The UV detector was set at 210 nm to detect the dinucleotide photoproducts. MS and MS/MS experiments were carried out with a Qstar pulsar i mass spectrometer (Applied Biosystems). The polarity was set to the negative ion mode with ion-spray voltage of −4000 V. Ion source gas 1 was set to 40, curtain gas to 25. Other measuring parameters were as follows: DP1 −60 V, FP −225 V, DP2 −10 V.

The TOF range was chosen from 150 to 600 amu for both MS and MS/MS measurements. For ESI-MS a standard CAD-gas value of 3 was used, while it was increased to 7 for MS/MS analysis. For better signal intensities, the product ion scans (MS/MS) were performed in the "Enhance All" modes with a value of −34 V for the collision energy (CE), while the first quadrupol (Q1) was fixed during these experiments to the mass of the ions of interest.

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