The influence of the phosphorothioate diester bond on the DNA oxidation process

1 Introduction

Genetic information is stored in the cellular DNA of every living organism [1]. However, this crucial macromolecule is continuously exposed to the oxidative stresses, which can lead to various types of damage. Until now, more than 80 DNA lesions have been identified [2-4]. One of the main sources of their formation is the activity of reactive oxygen species (ROS). These molecules may be generated by endocellular processes such as mitochondria-catalyzed electron transport reactions, metal catalysed reactions, during inflammation by neutrophils and macrophages, and/or physical external factors such as ionisation radiation [5]. It is important to mention that ionisation radiation (alpha, heavy ions, beta, gamma, X-ray, UV) can cause damage to DNA both directly and indirectly, depending on the source and energy. Such damage includes a single/double-strand break, nucleobase damage, and hydrogen atom abstraction from nucleobase moiety [3]. Among all ROS, the hydroxyl radical (•OH) has been found to be the most harmful one (for example, in cell-produced reactions, via the Haber-Weiss reaction), while the hydrogen peroxide (H₂O₂) and superoxide radical (O₂•-) can be recognized as low-active species [5]. In a cell, O₂•- formed as a by-product of respiratory cycles, is rapidly converted into oxygen and H₂O₂, by a superoxide dismutase (SOD) or in a self-dismutation process [6]. Subsequently, catalase converts hydrogen peroxide to a “safe” water molecule [2]. On the other hand, once ds-DNA has been one-electron oxidized by a variety of photo-oxidants, i.e. antraquinone, the generated radical cation hole can hop reversibly through the double-helix until it is trapped, usually by the reaction with H₂O of the formed guanine radical cation (G•+) [7], Fig. 1. Alternatively, a generated G•+ can be rearranged by a proton loss to a neutral radical, which can react with O₂•- [8,9]. The one-electron oxidation process, described above, is discussed further in the Results section of this article. As a result of this, two reaction paths are possible: either one with a water molecule addition, or one without [10,11]. The first path leads to an 8-oxo-7,8-dihydroguanine (8-oxoG) formation, while the second finally leads to a 2,2,4-triamino-5(2H)-...
oxazolone. The mechanisms of the these reactions are presented in Fig. 2 [9]. It is worth pointing out that of all the nucleobases, 2’-deoxyguanosine possesses the lowest ionisation potential [7]. Moreover, in an oxidative condition, guanine can be converted to other “lesions”, such as dehydro-guanidinohydantoin, oxaluric acid, spirominohydantoin, etc. [11,13]. Most of the generated DNA lesions are removed from the genome by a “cell defence machinery”, i.e. a base or nucleotide repair system (BER, NER, etc.) [12-15]. However, the first forces against ROS/radicals in cells comprise of antioxidants and enzymes [16]. These relatively small molecules protect the cell against toxic species formation. Therefore, the discovery by Wang et al. in 2007 that a phosphorothiate (PT) internucleotide bond can naturally appear in the bacterial DNA [17] raises the question of whether their role in the genome is protective or not. This problem is supported by the observation that spermine disulphide can protect ds-DNA from the one-electron oxidative/charge migration process that can lead to its damage [18]. In 2013, the one-electron oxidation of DNA containing the PT moiety was investigated for the first time by Sevilla et al. [19]. In their work, the authors postulated that the backbone-to-base hole-transfer mechanism was induced by the Cl₂●. However, due to the nature of the chlorine anion radical, the authors did not consider the influence of PT on the long charge migration process through ds-DNA, induced by photosensitizers (e.g. antraquinone). Therefore, in this work, the influence of the phosphorothioate internucleotide bond
on the light-induced oxidation/charge migration process was investigated using the Schuster strategy in which the anthraquinone moiety was covalently linked to one strand of ds-DNA [20].

2 Experimental Procedure

Substrate oligonucleotides [21]. Oligonucleotides were purchased as HPLC-purified compounds from the Bioorganic Chemistry Department, Polish Academy of Science, Lodz, Poland (Geneworld synthesizer, K&A Laborgeraete GbR) using nucleotide phosphoroamidites synthons as substrates (ChemGenes Corporation). The anthraquinone phosphoramide derivative was synthesized according to Schuster’s method [22]. The purchased oligonucleotides were characterised by a Time of Flight Mass Spectrometry, namely a MALDI-TOF, performed in a negative ion mode (Voyager-Elite, PerSeptive Biosystems Inc., Framingham, MA, USA), Table 1SM (SM-supplementary materials). The oligonucleotide concentrations were determined from a maximum of absorbance at λ-260 nm (Hitachi U-2800 double beam UV/VIS spectrophotometer), Fig. 1SM. The thermal stabilities of oligonucleotides (melting temperatures – Tm) were assigned using a Cary 1.3E spectrophotometer, equipped with a multilcell block and temperature controller, Table 2SM. Oligonucleotide circular dichroism spectra were obtained by CD dichrograph (Instruments SA JobinYvon, Longjumeau, France) according to previous experiments [21], Fig. 2SM. The purified oligonucleotides were 5'-end-labelled, as described previously, using T4 polynucleotide kinase (New England BioLabs, USA) and [γ-32P]ATP (PerkinElmer, Poland) [21], Fig. 3SM.

Hybridisation, UVA duplex irradiation and cleavage analysis. The labelled oligonucleotides were hybridized, as described previously [21], with a 2-fold excess of a purified non-radiolabelled complementary strand in 100 μL of 0.1 M NaCl with a 0.01 M MgCl2 buffer solution at pH 6.7. For the experiments with enzymes, the following amounts of enzymes (concentrations) were used: 10 μL of catalase (250 μg mL–1) and 10 μL of superoxide dismutase (200 μg mL–1). After hybridization, the total amount of oligonucleotide solution was divided into 10 μL1 samples and irradiated for 0, 30, 60 and 120 minutes at ~35°C in a Rayonet Photoreactor RMR-600 (Southern New England Ultraviolet Co., Bradford, CT) equipped with two 4W, 350 nm lamps. After each irradiation, the oligonucleotide was precipitated with cold ethanol (100 μL) and 2 μL of glycogen, vortexed, placed on dry ice for 30 min, and subsequently centrifuged at 12000 rpm for 30 min at 4°C[21]. The ethanol was removed and the residue then dried under air conditioning at room temperature. Fig. 4aSM shows the stability of the oligonucleotide after 120 minutes of UVA irradiation compared with 120 minutes of un-irradiated samples. To reveal the DNA lesions, the dry samples of each investigated oligonucleotide were treated with 100 μL of 1 M piperidine solution at 90°C for 30 min. The piperidine was removed by evaporation under reduced pressure. The residue of the investigated oligonucleotides was dissolved in 7 μL of denaturing loading dye (98% formamide, 2 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). The samples were then subjected to electrophoresis on a 20% denaturing polyacrylamide gel containing 7 M urea in 1X TBE (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.3) for 180 min at a constant power of 24 W. The results of the PAGE electrophoresis analysis were visualized by autoradiography. Quantity One 1-D analysis software (Bio-Rad) was used to estimate the cleavage band and autoradiogram analysis [23]. For the experiment in the presence of superoxide dismutase (SOD), quantities of ds-oligonucleotides the same as previously were taken. Following the work of Brinboim [24], 10 μL of SOD (concentration of stock solution – 200 μg mL–1) was added to each sample of ds-oligo A-H. The investigated samples subsequently were UVA irradiated for 120 min with the following precipitation and piperidine treatment: superoxide dismutase from bovine erythrocytes (CAS: 9054-89-1), Sigma, 5673 units mg–1, Lot SLBH4934V. The experiment was performed in the absence of oxygen, in an equipped glove box (photoreactor, centrifuge, vortex, pipettes, tips, etc.). The results are presented in Figs. 4b, cSM. The water for the experiment was saturated with argon for 60 min and subsequently vacuumed, frozen, argonated and defrosted and all operations were repeated four times. The ds-oligonucleotide samples for these experiments were dried under highly reduced pressure in an exicator overnight and placed in the glove box. Before the experiment, all the instruments had been placed in the vacuum bag [the vacuum was replaced four times by argon (5*10–6 level of O2), and the bags were opened under argon in the glove box], the free volume of glove box was replaced four times by argon-reduced pressure. During the experiment, a slow flow of argon was applied to avoid any oxygen contamination.

RP-HPLC analysis of UVA (λ=350 nm) irradiated ds-DNA. The stability/integrity of UVA radiated double-stranded oligonucleotide, i.e. oligo-E/AQ-PO-29, was analysed by RP-HPLC using the following: a Varian analytical system UV/VIS spectrophotometer, with UV/VIS detection in dual wavelengths A: 260 nm and B: 260 nm, C-18 column: Phenomenex, Synergi 4u Fusion-RP 80A, 250 mm × 4,6 mm. The elution was achieved using
a 0.1 M ammonium acetate solution in water as a buffer “A” at pH-7, with a gradient from 0% to 100% of buffer “B” (40% of acetonitrile HPLC grade in 0.1 M ammonium acetate water solution) over 40 min, then from 100% to 0% of buffer “B” over 5 min, followed by 0% of buffer “B” over 5 min (Fig. 5SM).

3 Results and discussion

Among the variety of photo-oxidants used in artificial systems, an anthraquinone (AQ) derivative, covalently linked to the 5’-end was used to initiate the cation radical migration through ds-DNA [7,25] in these studies (Fig. 3). The series of five ds-DNA oligonucleotides (Table 1) was synthesized, purified and characterized according to the commonly accepted procedure [26] (Supplementary Material). Each of the duplexes contained an AQ moiety on the 5’-end of one strand (unmodified), and a 32P on the 5’-end of the complementary one (containing phosphorothioate internucleotide bond and 2’-deoxyguanosines). Irradiation of the discussed double-stranded oligonucleotides by UVA (λ-350 nm) rapidly formed a triplet excited state of anthraquinone (AQ*3) from the singlet state, which was formed via one-electron ds-DNA oxidation. This process is in competition with the direct AQ*1 singlet state regeneration of the contact radical ion pair initially formed in the singlet spin state. A generated radical ion pair AQ*•-B*• can lead to the appearance of an O2•- by the reaction of the radical anion with O2. A radical cation (B*•) from the individual nucleobase was able to migrate through ds-DNA until it was annihilated [25]. Each of the investigated duplexes presented in Table 1 contained in the central part two G:::C base pairs isolated by a 5’-TATT-3’ sequence. To investigate the influence of the PT internucleotide bond on the one-electron oxidation process, their different positions were examined. For this purpose, the following distribution of PT was chosen in ds-oligonucleotides: A and B on the 3’ or 5’ site of G11 respectively, C on the 3’-site of G16, D on the 3’ site of G11 and G16, E first control (PT not present), and H second control (without AQ and PT moieties) (Table 1). All the double-stranded oligonucleotides were irradiated at λ-350 nm for: 0, 30, 60 and 120 minutes. The DNA lesions were detected by an autoradiogram of PAGE analysis with the previous piperidine sample treatment and quantified by Quantity One 1-D software.

In this study, based on available literature data, the formation of guanine (G) damage was expected to occur with its potential inhibition by phosphorothioate [21]. The distance between the AQ moiety and first guanine (G16) was observed here at approximately 54 Å (i.e. 14 bases), while in Schuster’s previous experiments, this distance extended up to 22 bases corresponding to ~84 Å [27]. Therefore, in the case of unmodified ds-oligonucleotide E, it was expected that 8-oxoG formation induced by hole migration should be well observed.

Surprisingly, as depicted in Fig. 4, in every case, no strand cleavages were observed at the position corresponding to G16 and hardly any at G11. Instead, strand breaks were noted at thymidines (T), corresponding to the T19, T21 and T26 locations (Table 1). Moreover, for the investigated ds-oligo A-E,
Table 1: Structure of ds-oligonucleotides indicating the position of the phosphorothioate internucleotide bond.

- **A**: 3'-P. A'A'TA'TA'A'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'-end
- **B**: 3'-P. A'A'TA'TA'A'TA'TA'TA'TA'TAT-5'
- **C**: 3'-P. A'A'TA'TA'A'TA'TA'TA'TA'TA'TA'TA'TA'TA'TA'-end
- **D**: 3'-P. A'A'TA'TA'A'TA'TA'TA'TA'TA'TA'TA'TA'-end
- **E**: 3'-P. A'A'TA'TA'A'TA'TA'TA'-end
- **F**: 3'-P. A'A'TA'TA'-end
- **G**: 3'-P. A'A'TA'-end
- **H**: 3'-P. A'A'TA'-end

**Figure 4**: Autoradiogram of the results of irradiation of AQ-linked ds-DNA: A–H, indicating the positions of the main lesions after piperidine treatment. Radiation time is given in minutes.

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The thymidine (T21) was observed as a more likely oxidized place/position, irrespective of the presence or absence of PT in ds-DNA. It should be pointed out here that the distance between T21 and the AQ moieties in the discussed cases was assigned at approximately 35 Å. As presented in Table 2, Fig. 6SM, for all DNAs, the following order of reactivity was found: T21 > T26 > T19 > G16. To avoid the influence of unexpected environmental conditions, a control experiment was performed. Oligo-H, which was deprived of the PT linkages as well as the anthraquinone moiety, was irradiated in the same conditions as the other samples. As expected, no lesions were detected for oligo-H, including 8-oxoG or any types of crosslinks. Therefore, for the investigated ds-oligonucleotides A–E, the observed single-strand breaks formed after piperidine treatment are not the result of any impurities or other artificial environmental contamination (Fig. 4). These observations are in contrast to previous data published by Kanvah, which indicated that if in an A- and T- rich sequence only one dG appears, it would be the main place of oxidation [27]. On the other hand, Geise and Wasilewski independently showed that an A::T base pair is not equal to T::A for the hole transport [28,29]. The T::A base pair affected the charge migration by a factor of 10 or 40, compared
to A::T, depending on the cited author. These data are in good agreement with the work of Schuster, who showed that the introduced TTTT sequence induced a sufficiently high barrier to the hole hopping [28]. Here, the distance between the last G16 and AQ moiety was composed of a 5'-TATATAAATTATT-3' sequence. In this part of the 5'-end 32P labelled oligonucleotide strand (containing Gs), a shutter, charge trap and barrier should be expected. According to Schuster and Giese, the sequence AAA can be recognized as a shutter, TT as a barrier and AT as a trap [25,28]. In the case of the last TT the proximity of AQ in the complementary strand effectively forces the hole hopping. The observed pattern of lesion distribution presented in Fig. 4 may indicate that the nucleic base sequence can play a crucial role in the hole hopping mechanism.

This was confirmed by the fact that the lesion was mainly detected at the location of T26. This observation may be supported by previous data, however, for the induction of a T lesion, a TT sequence is necessary [31].

In the case of the two other thymidine lesions located at positions T21 and T19, the neighbouring thymine was not present, which is in opposition to the above-mentioned requirement [31]. The next section of discussed strand consists of AAA accomplishing the role of shutter, which has been previously proven [30,32]. In this situation, the undisturbed flow of charge to G16, a potential trap, should be expected. However, the analysis of the PAGE autoradiogram (Fig. 4) shows an absence of the expected lesion. It can be postulated that the appearance of two T:A base pairs separated by a single A::T pair effectively trap the hole hopping, almost completely protecting G16 before the oxidation process (Fig. 4). Moreover, all the oligonucleotides showed their initial integrity before piperidine treatment (PAGE analysis). Almost none of the single-strand breaks were noted, as shown in Fig. 3aSM. The abstraction of the hydrogen atom from the sugar moiety by a hydroxyl radical, for example, is not involved in the lesion formation process in the cases of the systems discussed. Therefore, the effect of the neighbouring base next to G should be taken into consideration. Senthilkumar has shown that the effective energy of the positive charge localised in the middle of G in the trimer (5'-XGY-3' sequence) was strongly dependent on the adjacent nucleobases. The energy adopts values in the range of 7.89 to 8.85 eV [33]. The lowest value was observed for GGG while the highest was noted for 5'-TGC-3' and 5'-TGT-3'. As shown in Table 1, in the investigated oligonucleotides, both isolated Gs are flanked by thymidine. Hypothetically, this can slow down the charge migration process. Moreover, following Giese’s observation, the charge transfer from G16 to G11 (separated by a tetramer) occurs by a hopping and not tunnelling mechanism [34]. Therefore, the oxidation efficiency of G11 should be reduced. On the other hand, Schuster has shown that the G nearest to to antrachinone is mainly oxidised [35]. It should be pointed out that Cadet et al. investigated the A/T oligonucleotides with and without the GG segment [31]. In the first case, the hole trapping occurred at T, while in the second, as expected, G was the most reactive site. These observations are partially coincident with the results presented in this work – irreversible charge trapping occurs at Ts. However, the lack of 8-oxoG formation is in opposition to the widely accepted theory. In the currently studied oligonucleotides only two isolated Gs are present, instead of GG [31]. Moreover, in 5'-TGT-3', the unfavourable neighbourhood of G possesses 0.48/0.09 eV higher effective energy of the positive charge than 5'-AGG-3' or 5'-GGT-3', respectively [33]. The above-mentioned data indicates that for the used oligonucleotides the nature of the nucleic base sequence may prefer thymidines as a more reactive place in the hole migration process. This postulation is indirectly confirmed by the crosslink formation. As shown at Fig. 4 and 4a-cSM, bands corresponding to the crosslink area were noted in every case. The inter-strand junction was not present in the control experiment, i.e. oligo-H without antrachinone and PT moiety. Greenberg has shown that the crosslink was formed between the adenine N6 function and CH3 of thymidine after allyl radical formation [36]. Based on the above, it can be suggested that the main incidences of DNA damage formed in the mentioned experiments are thymine glycol, 5-FormUrd, 5-HMdUrd, and A^T crosslink. Fig. 7SM presents the lesion structures and proposed mechanisms of their formation. It should be pointed out that the yield of the crosslink is higher in the presence of oxygen than when it is absent. This indicates that oxygen can play a special

| oligo | Strand cleavage [%] |
|-------|---------------------|
|       | T^a | T^b | T^o | G^o |
| A     | 10  | 17  | 7   | 2   |
| B     | 13  | 20  | 11  | 3   |
| C     | 17  | 21  | 13  | 6   |
| D     | 10  | 15  | 9   | 5   |
| E     | 14  | 23  | 16  | 5   |
role. Under radiation (UVA) conditions, antraquinone loses an electron, and a superoxide anion radical can be produced. To elucidate the phenomenon of Ts oxidising instead of G, additional experiments without oxygen as well as with SOD and O₂ were performed (Figs. 4b, cSM). In both cases the lack of single-strand breaks was noted, as well as other DNA lesions before and after piperidine treatment. Moreover, in the presence of SOD and in the absence of O₂, only a negligible amount of interstrand crosslinking was noted. The first of these results could be expected, as superoxide dismutase converts the superoxide radical anion to H₂O₂ very effectively; in the second, likely the lack of O₂ effectively inhibited the inter-strand crosslink formation. The following order of crosslink formation efficiency, given in %, was assigned by radiogram densitometry: A) 4.0, 3.2, 5.9, 5.6, 9.9 (in the presence of oxygen and SOD); B) 1.9, 0.3, 2.0, 1.1, 3.2 (in the absence of oxygen and SOD); C) 18.0, 15.0, 18.4, 15.6, 25.4 (in the presence of oxygen and in the absence of SOD) for ds-oligos A, B, C, D, E respectively (Fig. 5). The results of these experiments indicate that the returning of the electron to the antraquinone moiety is privileged in this system without any possibility of being trapped, which is in good agreement with Schuster’s previous work [35].

Due to the energetically unfavourable barrier conditions, the densitometry of the autoradiogram PAGE analysis showed little influence of the PT internucleotide bond on charge migration. This observation is in a good agreement with recent theoretical data, which has shown almost the same value of ionisation potential for model single and double-stranded DNA in which the phosphate and phosphorothioate internucleotide bonds were present [37]. Moreover, the shift to PT linkage next to the AQ moiety did not change the pattern of DNA lesion. However, due to the steric hindrance forced by the sulphur atom in the PT bond, a slower charge migration was observed [21].

The difference in oligonucleotide resistance to UVA irradiation when oxygen is present might be due to the dynamic fluctuation of double-stranded DNA, which is slightly disturbed by the presence of PT, as previously noted [21]; or it might be because in this specific sequence, the radical cation never reaches G11, or barely reaches G16 (the hole hopping barrier). On the other hand, Wang et al. have postulated that phosphorothioate could be recognized as an antioxidant [38]. This ability is the result

![Crosslink area](image)

Figure 5: Autoradiogram of the results of irradiation of AQ-linked ds-DNA (A–E) after piperidine treatment: A) in the presence of oxygen and SOD, B) in the absence of oxygen and SOD, C) in the presence of oxygen and absence of SOD.
of the PT internucleotide bond desulfurization process, as postulated by the authors. Furthermore, this observation was confirmed by Wu, who investigated the desulfurization process induced by a hydroxyl radical during a negative electrospray ionization mass spectroscopy [39]. It is important to mention that the one-electron oxidation of DNA containing the PT moiety was investigated for the first time by Sevilla et al. [19]. In their work, it was postulated that the backbone-to-base hole-transfer mechanism was induced by the Cl$_2^·$. This process can lead to the formation of a nucleobase radical cation or a disulfide bond between the two adjacent phosphorothioate internucleotide bonds on the intra- or intermolecular path. Due to the fact that the redox potential of the P-S^Cl moiety is higher than guanine but lower than for the remaining nucleobases, only one-electron oxidation of G is induced. The disulfide bond formation by the mentioned mechanism was not observed in this study, as only one PT linkage was present in oligonucleotides A, B, C, or two PT in the case of D (which were relatively far from each other). Moreover, the absence of $^3$P labelled strand cleavage at G16 or G11 positions indicated the lack of backbone-to-base hole-transfer, which theoretically could lead to 8-oxoG formation.

In the presented results the desulfurization process via hole migration is less expected due to the fact that it can be initiated theoretically by a solvated electron or hydroxyl radical [19,37-39]. In the anthraquinone strategy, the ejection of an electron is far enough from the PT bond, therefore it can be trapped by O$_2$ before it reaches the phosphorothioate moiety. Alternatively, the desulfurization process can be forced by a hydroxyl radical, however, this molecule is highly reactive and the single-strand break should be observed on the radiograms for the samples before piperidine treatment. As mentioned previously, in all cases no single strand breaks were noted (Figs. 4SM and 5).

4 Conclusions

Based on the experimental findings presented above, the following conclusions are proposed:

1. The main cation radical trap occurs, in the presence of oxygen, at T26, T21, T19 and just at the limit of detection on G16. The results presented here point to the hole hopping process being strongly sequence-dependent and should be understood from a qualitative potential energy landscape point of view [30].

2. The one or two PT internucleotide bonds present in ds-DNA did not change the charge migration due to the fact that they are located far enough from the source of hole initiation.

3. Oxygen may be playing a special role in the interstrand crosslink formation process as well as in the DNA lesion formation derived from thymidine.

Future studies are necessary to elucidate these unexpected phenomena. The proposed follow up studies include: insertion of GG segments in the oligonucleotide sequence instead of the isolated G; moving the phosphorothioate internucleotide bond close to the AQ moiety; increasing the number of PTs in the oligonucleotide structure; and a theoretical estimate of the ionisation potential of the different tetramer 5'-XGGY-3'.

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Supplementary materials: The electronic Supplementary Materials contain: oligonucleotide characterisation (MALDI ToF analyses, CD and UV/VIS spectra, thermal stability, i.e. Tm values), autoradiograms of purified and the 5'-end $^3$P labelled oligonucleotides used, ds-DNA cleavage analysis after UVA irradiation, and RP-HPLC chromatogram profiles of ds-DNA after UVA (λ-350 nm) irradiation.

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