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Photoinduced DNA damage efficiency and cytotoxicity of novel viologen linked pyrene conjugates†

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Novel viologen linked pyrene conjugates permeate cells efficiently and exhibit spacer length dependent DNA damage and cytotoxicity upon photoexcitation.

The design of molecules that can efficiently interact and cleave DNA has been the subject of enormous interest from the point of developing new drugs and for studies of the electron and energy transfer properties of DNA.[1–3] Several photosensitizers such as porphyrins, rhodamine, pyrenes, acridines *etc.* have been extensively investigated, incorporating suitable chemical modifications and encapsulating them in biocompatible nanocontainers to achieve solubility, stability, permeability and selectivity towards the cell.[4–7] In this context, the design of water soluble sensitizers based on the pyrene chromophore that exhibit efficient cytotoxicity and the guanine selective DNA damage[8] through an electron transfer mechanism has been challenging.

Recently, we reported the synthesis and investigation of the photophysical properties of a few novel viologen linked pyrene conjugates **1–3** (Chart 1).[9] These conjugates exhibited DNA intercalative interactions and on photoactivation showed the formation of radical cation of DNA and reduced viologen moiety. Therefore, it was of interest to us to evaluate how these conjugates permeate cells and induce DNA damage and cytotoxicity in the presence of light. Our results demonstrate that the subtle modification of the pyrene chromophore through covalent linkage to a viologen moiety changes the mechanism by which these conjugates induced DNA damage from a singlet oxygen mediated process to an electron transfer mediated process.

In order to evaluate the stabilisation of calf thymus DNA in the presence of the conjugates **1–3**, we have determined the melting temperature (T_m) of the DNA duplex (Fig. 1). The DNA duplex alone showed a T_m value of 53 °C, while T_m values of 60, 63 and 66 °C, respectively, were observed in the presence of **1–3**, indicating thereby the significant stabilisation (*ca.* 7–13 °C) of the DNA duplex by these systems.

To understand the efficiency and nature of the oxidatively generated DNA damage induced by the conjugates **1–3**, we investigated the damage of the supercoiled DNA from bacteriophage PM2 (PM2 DNA, 10^4 bp) in the presence and absence of various repair endonucleases.[10] Phosphate-buffered solutions of DNA (10 μg mL^{-1}) were exposed at 0 °C to near-UV irradiation (360 nm) in the presence of different concentrations of **1–3**, and the model compound pyrene. Subsequently, DNA was analysed for the following types of modifications: (i) DNA single strand breaks (SSBs); (ii) sites of base loss (AP sites) recognised by exonuclease III; (iii) AP sites plus oxidatively generated pyrimidine modifications sensitive to endonuclease III; (iv) AP sites plus cyclobutane pyrimidine dimers sensitive to T4 endonuclease V; and (v) AP sites plus oxidatively generated purine modifications sensitive to Fpg protein.[11]

The results of various modifications induced by the photoactivated conjugates **1** and **2** in the form of damage profiles are shown in Fig. 2A. It is evident from the damage profiles, that both these conjugates induce negligible SSBs, very few AP sites and pyrimidine modifications sensitive to endonuclease III. But importantly, we observed a large number of purine modifications sensitive to Fpg protein. The conjugate **1** (n = 1) was found to be more effective (*ca.* 2-fold) in inducing the DNA damage compared to **2** (n = 5). This is in good agreement with the observed higher efficiency of the rate of photoinduced electron transfer K_{ET} for **1** (3.2×10^9 s^{-1}), when compared to **2** (1.5×10^9 s^{-1}).[9] Further, no DNA damage was observed either by irradiation of PM2 DNA alone or in the dark in the presence of **1** and **2** at the highest concentrations, indicating that the damage observed is purely initiated by the photoactivated viologen linked pyrene conjugates.

Fig. 2B shows the irradiation time dependent formation of SSBs and Fpg sensitive damage induced by the conjugate **1**. The damage sensitive to Fpg protein induced by **1** increased with increasing time of irradiation. No significant increase in SSBs was observed, even after irradiation for 22.5 min. The concentration dependent SSBs and Fpg sensitive modifications induced by the conjugates **1–3** and for comparison, the model compound pyrene, are shown in Fig. 3. We observed an increase in Fpg-sensitive modifications with increasing concentration of the conjugate **1** (n = 1). Similar observations have been made with the higher conjugates **2** (n = 5) and **3**

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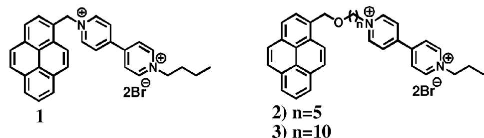

Chart 1

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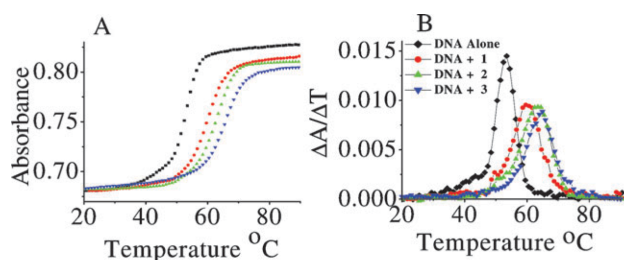

Fig. 1 The differential thermal denaturation curves (A) and first derivative plots (B) of the DNA duplex (5′-CAC TGG CTT TTC GGT GCA T-3′: 5′-ATG CAC CGA AAA GCC AGT G-3′) in the presence and absence of the viologen linked pyrene conjugates **1**, **2** and **3** (20 μM each).

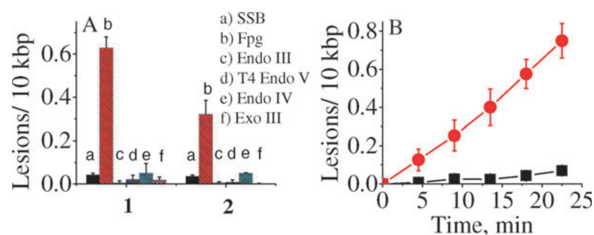

Fig. 2 (A) DNA damage profiles showing SSBs and various endonuclease sensitive modifications induced in PM2 DNA by photo-activated conjugates **1** and **2** (10 μM). (B) Time dependence of (■) SSBs and (●) Fpg-sensitive modifications induced in PM2 DNA by **1** (10 μM, 0 °C). UV irradiation: 360 nm light source, 90 kJ m^{-2}.

(n = 10), but with significantly reduced efficiency when compared to **1** (n = 1). The Fpg modifications induced by **2** and **3** are found to be lower by a factor of *ca.* 2 and 10, respectively, as compared to the conjugate **1** (n = 1). In contrast, the model compound pyrene (inset of Fig. 3B) induced negligible DNA modifications even at high concentrations (20 μM), indicating its inefficiency in inducing the DNA damage at this concentration range.

To understand the mechanism of DNA damage induced by the conjugates **1–3**, we estimated their singlet oxygen generation efficiency and have investigated the generation of Fpg sensitive damage in the presence of various additives and scavengers. These include catalase, superoxide dismutase (SOD), and by replacing H_2O in the buffer with D_2O. When we monitored the absorbance of diphenylisobenzofuran, a known singlet oxygen scavenger,[12] we observed negligible changes with the pyrene conjugates, whereas the model derivative pyrene showed the

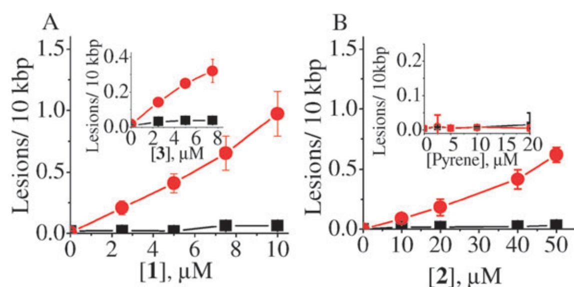

Fig. 3 (A) Concentration dependence of (■) SSBs and (●) Fpg-sensitive modifications, induced in PM2 DNA by (A) **1** and **3** (inset); (B) **2** and pyrene (inset). UV irradiation: 360 nm light source, 90 kJ m^{-2}, 0 °C.

formation of singlet oxygen in good yields (Fig. S1, ESI†). Expectedly, no significant increase in the ratio of Fpg sensitive damage in D_2O and normal buffer media was observed in the case of the conjugates **1–3** (Fig. S2, ESI†). In contrast, the model compound pyrene showed *ca.* 3-fold increase in Fpg sensitive damage in D_2O as compared to H_2O buffer, indicating the involvement of singlet oxygen in the latter case. Further, we observed negligible changes in the Fpg sensitive damage in the presence of catalase and SOD, indicating that neither the superoxide radical anion nor hydrogen peroxide is involved in the DNA damage induced by the conjugates **1–3**.

The efficacy of photosensitization depends on the close proximity of sensitizers and their targets.[3a,13] To understand the cellular targets of the conjugates **1–3**, we have investigated their localisation in L1210 murine leukemia cells employing fluorescence microscopy. Fig. 4 shows fluorescence microscopic images of L1210 cells in the presence of the conjugate **1** incubated for different time intervals. Incubation of these cells with **1** (10 μM) for 1 min at 37 °C followed by the fluorescence microscopy analysis clearly showed the observation of fluorescence of the pyrene chromophore in the nuclei of the cells. Similar observations have been made with **2** and **3**. Further, with increasing time of incubation of the cells with the conjugates, we observed an increase in the fluorescence intensity of the pyrene chromophore and reached saturation at around 30 min.

The pyrene conjugates under investigation showed efficient DNA damage and cell permeability and hence it was of interest to us to investigate their cytotoxicity in the presence and absence of light. We carried out the cytotoxicity studies employing murine hematopoietic cancer L1210 cells. Experiments were carried out by exposing cells to various concentrations of the conjugates with and without irradiation, and the percentage survival of the cells in full medium was determined subsequently after extensive washing. Fig. 5 shows the cytotoxicity induced by the conjugates **1** and **2**. In the presence of light, the conjugate **1** reduced the number of cells, counted after 48 h, to less than 20% at concentrations as low as 20 μM. In the dark, the cell survival was found to be almost 100% at the same concentration, indicating its dark non-toxicity. Interestingly, the higher homologue **2** (n = 5), showed high cytotoxicity upon excitation (*ca.* 20% survival) even at half the concentration, when compared to **1** (n = 1). These results clearly indicate that these molecules show significant cellular toxicity only upon excitation and that the spacer length and substituents play a major role in the cell permeability and cytotoxicity of these conjugates.

The spacer length dependent increase in the thermal denaturation temperature of CT DNA in the presence of the conjugates **1–3** confirms that these systems undergo primarily intercalative interactions with DNA. Further, the observation of predominantly Fpg sensitive modifications in DNA indicate that the excited state of the pyrene chromophore of these conjugates first transfers an electron to the viologen moiety resulting in the formation of the charge separated species, *i.e.*, radical cation of the pyrene and reduced viologen moieties (Fig. S3, ESI†). In the second step, the radical cation of the pyrene moiety oxidises DNA, resulting in the formation of a radical cation of DNA.[14] The evidence for the involvement of

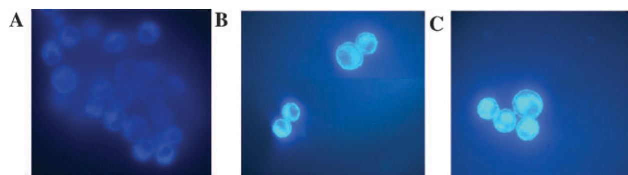

Fig. 4 Intracellular localisation of the conjugate **1** (10 μM) in L1210 murine leukemia cells incubated for different time intervals at 37 °C; (A) 5, (B) 15 and (C) 30 min. Images were obtained using an inverted fluorescence microscope (400×).

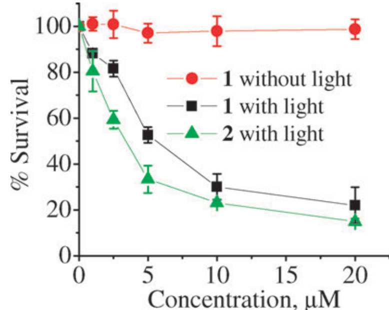

Fig. 5 Cytotoxicity of **1** and **2** in L1210 murine leukemia cells. Data give the percentage cell survival counted 48 h after the treatment for 22.5 min at 0 °C without and with UV irradiation (360 nm, light dose, 90 kJ m^{-2}). Data points represent the mean of 3 independent experiments (±SD).

the reduced viologen and radical cation of DNA was obtained through laser flash photolysis studies.[9] This leads to the formation of purine oxidised products like 8-oxo-7,8-dihydroguanine (8-oxoGua) and formamido-pyrimidines, which are recognized by the Fpg protein.[10,15] In contrast, the model compound pyrene showed mainly singlet oxygen ($^1O_2$) mediated DNA damage in agreement with literature reports.[7] Expectedly, when H_2O in buffer is replaced by D_2O, we observed an increase in Fpg sensitive modifications with pyrene, while **1–3** showed negligible changes, indicating that $^1O_2$ is not responsible in these systems.

The possibility of involvement of hydroxyl radicals in the DNA damage induced by **1–3** can be ruled out, since the damage profiles observed are quite different from those generated by ionizing radiation, which is a source of hydroxyl radicals.[10] Furthermore, neither hydrogen peroxide nor superoxide radical anion is involved in the damage induced by these systems, since the numbers of Fpg modifications were not altered in the presence of superoxide dismutase and catalase or in the presence of both these enzymes. These results clearly indicate the fact that the DNA modifications induced by the conjugates **1–3** originate from the oxidation of DNA, particularly guanine, since it has the lowest ionization potential.[14,15] Moreover, the selective oxidation of guanine in DNA can also be rationalised by the mechanism of fast hole hopping even if the initial electron transfer caused by the photoexcited conjugates occurs at a remote site in the DNA duplex.[2] In agreement with the electron transfer rates observed,[9] the conjugate **1** having the spacer $n = 1$, induced *ca.* 2- and 10-fold higher DNA damage as compared to **2** ($n = 5$) and **3** ($n = 10$). In contrast, the cytotoxicity showed by these conjugates was found to be in the reverse order, which could be attributed to the differences in their hydrophobicity and cellular localisation.

In conclusion, the viologen linked pyrene conjugates **1–3** undergo predominantly intercalative interactions with DNA, induce spacer length dependent DNA damage through a photoinduced electron transfer mechanism, localise inside the nucleus and exhibit cytotoxicity only upon photoexcitation. Further studies are in progress to understand the effect of spacer and bridging units and the potential use of such conjugates as phototherapeutic agents.

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