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Mechanisms of DNA damage by photoexcited 9-methyl-β-carbolines

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It has been well documented that β-carboline alkaloids, particularly the 9-methyl derivatives, are efficient photosensitizers. However, structure–activity relationships are missing and the photochemical mechanisms involved in the DNA photodamage still remain unknown. In the present work, we examined the capability of three 9-methyl-β-carbolines (9-methyl-*nor*harmane, 9-methyl-harmane and 9-methyl-harmine) to induce DNA damage upon UVA excitation at physiological pH. The type and extent of the damage was analyzed together with the photophysical and binding properties of the β-carboline derivatives investigated. The results indicate that even at neutral pH most of the DNA damage is generated from the protonated form of the excited β-carbolines in a type-I reaction. Oxidized purine residues are produced in high excess over oxidized pyrimidines, single-strand breaks and sites of base loss. In addition, the excited neutral form of the β-carbolines is responsible for significant generation of cyclobutane pyrimidine dimers (CPDs) by triplet–triplet-energy transfer. In the case of 9-methyl-*nor*harmane, the yield of CPDs is increased in D_2O, probably due to less rapid protonation in the deuterated solvent.

Introduction

β-Carboline alkaloids are a group of heterocyclic compounds with the structural unit 9*H*-pyrido[3,4-*b*]indole. The structures of several β-carboline derivatives are shown in Scheme 1. Derivatives of β-carbolines were found in a wide range of species. So far, they have been identified in many plants,[1–4] arthropods[5] and insects.[6] In mammals, these alkaloids are endogenously synthesized from tryptophan or tryptophan-like indolamines[7] and they were also detected in plasma, platelets and urine (with basal levels of ~0.1 nM).[8–10] Moreover, it was found that after alcohol intake[11] and smoking, the concentrations of β-carbolines are considerably increased (*i.e.*, ~1 nM).[10,12] It has been established that some photoexcited β-carbolines are able to induce chromosome damage in mammalian cells,[13,14] and to inactivate bacteria[15,16] and viruses.[17]

*Nor*harmane, the unsubstituted fully aromatic β-carboline, crosses the blood–brain barrier penetrating into the brain.[18,19] Inside the brain *nor*harmane is converted, by certain methyl-

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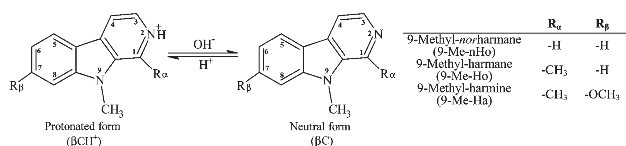

Scheme 1 Structures of β-carboline derivatives investigated in this work.

transferases, into methyl derivatives.[20–22] The effect of these β-carbolines is not yet established. 2,9-Dimethyl-β-carbolines generate neurotoxic effects due to damage of mitochondria.[23,24] But, on the other hand, it has been found that 9-methyl-harmine has a neuroprotective effect.[25] Despite their established importance, the main biological role of these alkaloids and many fundamental aspects of the mechanisms involved still remain poorly understood.

It has been well documented that, upon UVA excitation, β-carboline alkaloids are efficient photosensitizers.[26–28] In this work, we have analyzed in detail the capability of the three β-carboline derivatives listed in Scheme 1 to act as photosensitizers. In particular, the spectrum of photoinduced DNA modifications was investigated by means of repair enzymes and the underlying photochemical mechanisms were elucidated.

Experimental

General

β-Carbolines. Harmine (>98%) from Sigma-Aldrich was used without further purification. The method used for

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synthesizing and purifying 9-methyl-derivatives has been published elsewhere.[29]

DNA material. DNA from bacteriophage PM2 (PM2 DNA, 10 000 bp) was prepared according to the method of Salditt *et al.*[30] Calf thymus DNA (ctDNA) from Sigma-Aldrich was dissolved in 10 mM Tris with 1 mM EDTA at pH 7.5–8.0.

Enzymes. Formamidopyrimidine-DNA glycosylase (Fpg protein) was obtained from *E. coli* strain JM105 harboring plasmid pFPG230.[31] Endonuclease IV and T4 endonuclease V were partially purified from an inducible overproducer (*E. coli* strain A 32480 carrying the plasmid ptac-denV) provided by L. Mullenders, Leiden. Endonuclease III from *E. coli* was kindly provided by S. Boiteux, Fontenay aux Roses, France. All repair endonucleases were tested for their incision at reference modifications (*i.e.*, thymine glycols induced by OsO_4, AP sites by low pH and CPDs by UV254) under the applied assay conditions (see below) to ensure that the correct substrate modifications are fully recognized and no incision at non-substrate modifications takes place.[32] Superoxide dismutase (SOD) and catalase were from Sigma-Aldrich.

pH adjustment. The pH of β-carboline derivative aqueous solutions was adjusted by adding drops of aqueous NaOH or HCl solutions (concentration ranged from 0.1 M to 2.0 M) with a micropipette. The ionic strength was approximately 10^{-3} M in all the experiments. In experiments using D_2O as a solvent, D_2O (>99.9%; Sigma), DCl (99.5%; Aldrich) in D_2O, and NaOD (Aldrich) in D_2O were used. The pD of the solution was adjusted as described elsewhere,[33] and the final isotope purity was greater than 96%. The β-carboline acidic, neutral and alkaline solutions were prepared in acetic acid–sodium acetate (pH 4.8), KH_2PO_4–NaOH (pH 7.4) and $Na_2B_4O_7$–NaOH (pH 10.0) diluted buffers, respectively.

Absorption and emission spectroscopy

UV-vis analysis. Electronic absorption spectra were recorded on a Perkin-Elmer lambda 25 spectrophotometer, in quartz cells of 1 cm path length, at room temperature.

Fluorescence emission. Steady-state fluorescence emission measurements were performed using a Fluoromax4 (HORIBA Jobin Yvon) and a single-photon-counting equipment FL3 TCSPC-SP (HORIBA Jobin Yvon). Measurements were made in quartz cells of 0.4 × 1 cm path length at room temperature.

Phosphorescence emission at low temperature (77 K). Phosphorescence emission spectra were measured in the same spectrophotometer described above at 77 K. Briefly, a mixture of isopropanol–ethyl ether (1 : 1; v/v) was used as a solid matrix where βC derivatives were dissolved. To record the spectra of the protonated form, perchloric acid ($HClO_4$) was added to the previous solution. Measurements were recorded in NMR tubes using a delay after flash of 0.5 μs, an excitation slit of 10 nm and an emission slit of 5 nm.

Binding studies

The interaction of β-carboline derivatives with ctDNA was studied using UV-vis absorption and fluorescence emission spectroscopy. The equipment and data analysis used were as described elsewhere.[28]

DNA photoproduct characterization

Irradiation set-up. Buffered aqueous solutions (10 mM KH_2PO_4, 50 mM NaCl, pH 7.4) of β-carboline and PM2 DNA (at 10 μg ml^{-1}) were irradiated for 20 min on ice in a 96-well plate with a Philips HPW 125 W lamp emitting at 365 nm (bandwidth ~20 nm), placed at a distance of 10 cm. After treatment, the DNA was precipitated using ethanol–sodium acetate and re-dissolved in BE1 buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) for damage analysis.

Quantification of endonuclease-sensitive modifications in PM2 plasmid. The DNA relaxation assay used to quantify endonuclease-sensitive sites (ESS) and single-strand breaks (SSB) in PM2 DNA has been described earlier[34] and we have recently reviewed its application in characterizing the DNA damage induced by photosensitization.[35] It makes use of the fact that supercoiled PM2 DNA is converted by either a SSB or the incision of a repair endonuclease into a relaxed (nicked) form, which migrates separately from the supercoiled form in agarose gel electrophoresis.

An aliquot of 0.2 μg of the modified DNA in 20 μL BE1 buffer was incubated for 30 min at 37 °C with 10 μL of BE1 buffer (for the determination of directly produced SSB) or of one of the following repair endonuclease preparations: (i) Fpg protein, 3 μg ml^{-1} in BE1 buffer, (ii) endonuclease IV, 0.3 μg ml^{-1} in BE1 buffer, and (iii) T4 endonuclease V, 3 μg ml^{-1} in BE15 buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 15 mM EDTA). (iv) endonuclease III, 30 ng ml^{-1} in BE1 buffer. The reactions were ended by addition of 10 μL of stop buffer to 2% sodium dodecyl sulfate and the DNA was applied to an agarose electrophoresis gel. Fluorescence scanning of the relaxed and supercoiled forms of the DNA after staining with ethidium bromide allowed calculating the number of SSB or, if an incubation with a repair endonuclease preceded the gel electrophoresis, the number of SSB plus ESS. To obtain damage profiles, data were corrected for the number of "background" lesions observed in control experiments (*i.e.*, modifications observed in untreated DNA plus the lesions that are induced by UV alone), and the number of ESS was obtained by subtraction of the number of SSB (the number of lesions induced by UVA (365 nm) radiation alone, *i.e.* damage observed in the absence of β-carbolines, is only 0.024 ± 0.021 site per 10 000 bp).

Each repair endonuclease used recognizes different DNA modifications.[36] Fpg protein recognizes oxidized purines such as 8-oxo-7,8-dihydroguanine (8-oxoGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and also sites of base loss (AP sites). In contrast, substrates of endonuclease III are various oxidized pyrimidines, in addition to AP sites. T4 endonuclease V recognizes CPDs together with some types of AP sites, while endonuclease IV recognizes all types of sites of base loss, including all types of oxidized AP sites. The amount of CPDs produced upon photoexcitation therefore is calculated as the difference

between the number of sites recognized in the incubation with both endonuclease IV and T4 endonuclease V and the number of AP sites recognized by endo IV alone. We refer to this difference as "CPDs^{calc}".[35]

Also, specific ROS scavengers such as SOD (2 mg ml^{-1}) and catalase (315 U ml^{-1}) were used to evaluate the role of $O_2^{\cdot -}$ and H_2O_2, respectively.

Results

Photophysical properties of 9-methyl-β-carbolines

In aqueous media, in the pH range 2–12, the 9-methyl-β-carbolines depicted in Scheme 1 show an acid–base equilibrium with a pK_a ~ 6–7 (Table 1). Therefore, a mixture of the protonated and the neutral species is present at physiological pH. From Fig. 1, it is evident that the UV-vis absorption spectrum of protonated (βCH^+) and neutral (βC) forms of each 9-methyl-β-carboline strongly depends on the pH. These changes in the electronic ground state distribution have a strong effect on its photochemical and photophysical properties (see below).[37,38] However, it is worth mentioning that both protonated and neutral species of all compounds investigated have significant absorption at the wavelength (365 ± 20 nm) used in this study (see grey bars in Fig. 1).

Fluorescence spectra of both protonated and neutral forms of each β-carboline derivative were recorded in aqueous solution (Fig. 2a). The corresponding quantum yields of fluorescence (Φ_F) are listed in Table 1. In each case, the values of Φ_F obtained using excitation wavelengths over the entire range of the lowest-energy absorption band were the same within the experimental error (results not shown).

The results show that the protonated forms of all alkaloids have rather large Φ_F values. A quite an intense emission band is observed centered at ~430–460 nm. In alkaline media (pH 10.0) the presence of an extra pH-dependent emission band is observed at ~370–380 nm, in addition to the more intense band at 430–460 nm that is also observed at pH 4.8 (Fig. 2a). Therefore, the intense band is assigned to the excited-state pyridinic protonated β-carboline cation (^{1}[βCH^+]*) and the weaker band, at ~370–380 nm, to the neutral form of the β-carboline (^{1}[βC]*).[38] These results are consistent with the suggestion that, upon excitation, the basicity of the β-carboline pyridinic nitrogen is greatly enhanced.[27] As such, even at pH 10.0 in water, the ^{1}[βC]* species is readily protonated during the lifetime of its S_1 state. In turn, the predominant emitting species is ^{1}[βCH^+]* not ^{1}[βC]*. However, the presence of the 370–380 nm emission band shows that, although in a small proportion, ^{1}[βC]* is also present in the reaction media.

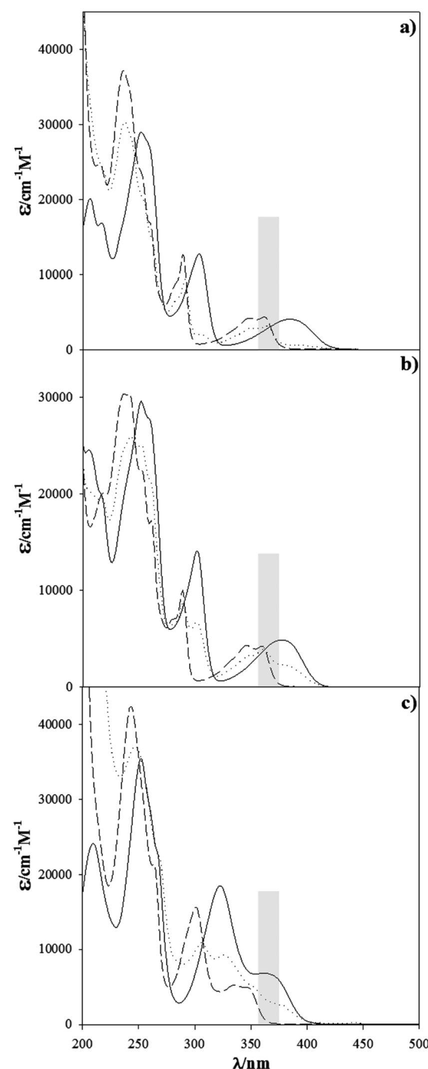

Fig. 1 Absorption spectra of the three β-carboline derivatives investigated recorded under different pH conditions: 4.8 (solid line), 7.4 (dotted line) and 10.0 (dashed line). (**a**) 9-Me–*n*Ho, (**b**) 9-Me–Ho and (**c**) 9-Me–Ha.

It is also important to note that the fluorescence intensity observed upon excitation of non-protonated β-carboline in the

Table 1 Photophysical data of β-carboline derivatives investigated in this work

	pK_a	pH	λ_{em}^{fluo}/nm	Φ_F [a]	τ_F/ns	λ_{em}^{phos}/nm	E_T/kJ mol^{-1}	Φ_Δ [b]
9-Me–*n*Ho	6.3 ± 0.3	4.8	458[c]	0.75 ± 0.05[c]	23.1[c]	482	248	0.10 ± 0.01[c]
		10.0	458 and ~380[c]	0.68 ± 0.05[c]	22.9 and 2.1[c]	426	281	0.08 ± 0.01[c]
9-Me–Ho	6.6 ± 0.2	4.8	444[c]	0.93 ± 0.05[c]	24.5[c]	489	245	0.09 ± 0.01[c]
		10.0	444 and ~374[c]	0.68 ± 0.05[c]	23.8 and 2.5[c]	426	281	0.10 ± 0.01[c]
9-Me–Ha	7.1 ± 0.1	4.8	430	0.43 ± 0.04	9.1	465	257	0.16 ± 0.02
		10.0	430 and ~365	0.35 ± 0.03	9.1 and 0.4	411	291	0.15 ± 0.03

[a] Φ_F values obtained in air-equilibrated aqueous solutions. [b] Φ_Δ values obtained in air-equilibrated D_2O solutions. [c] Data obtained from ref. 38.

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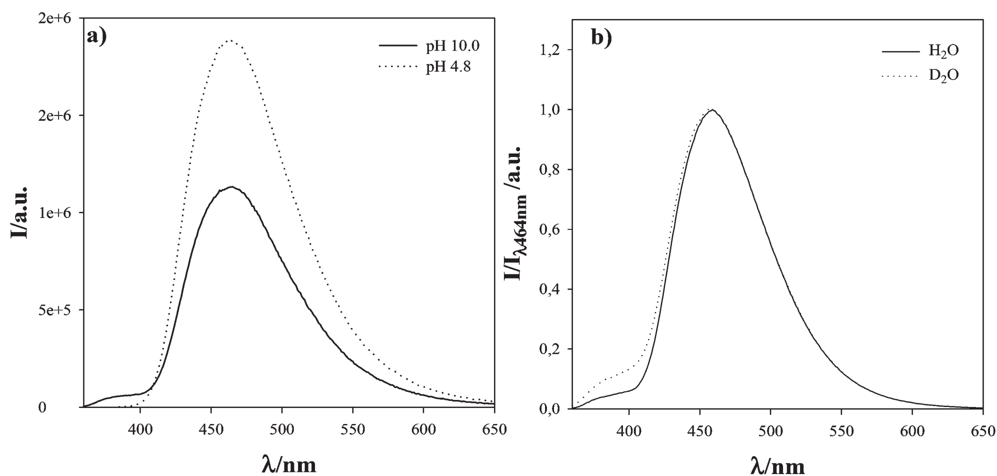


Fig. 2 (**a**) Fluorescence emission spectra of the 9-Me–*n*Ho protonated form (solid line) and the neutral form (dotted line). (**b**) Normalized fluorescence emission spectra of the neutral form of 9-Me–*n*Ho recorded in H_2O (solid line) and D_2O (dotted line) solutions.

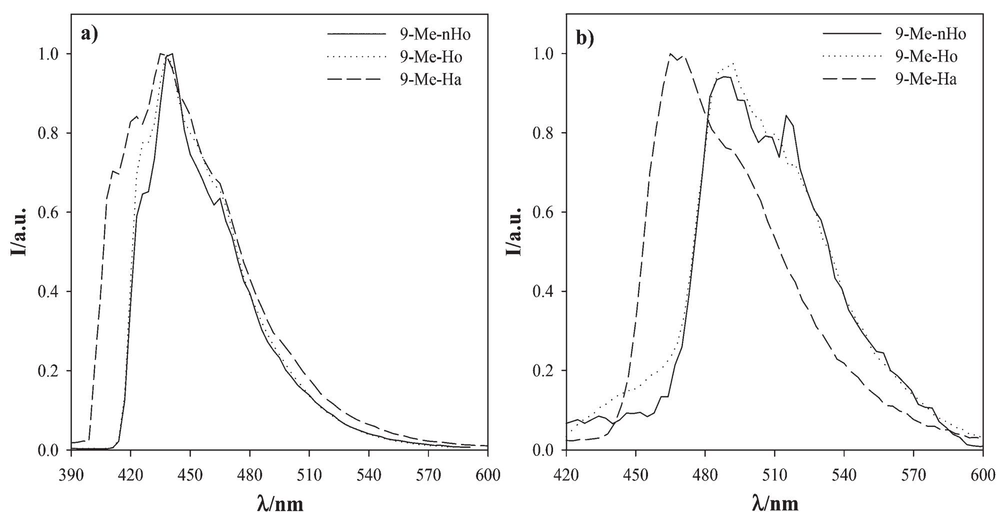


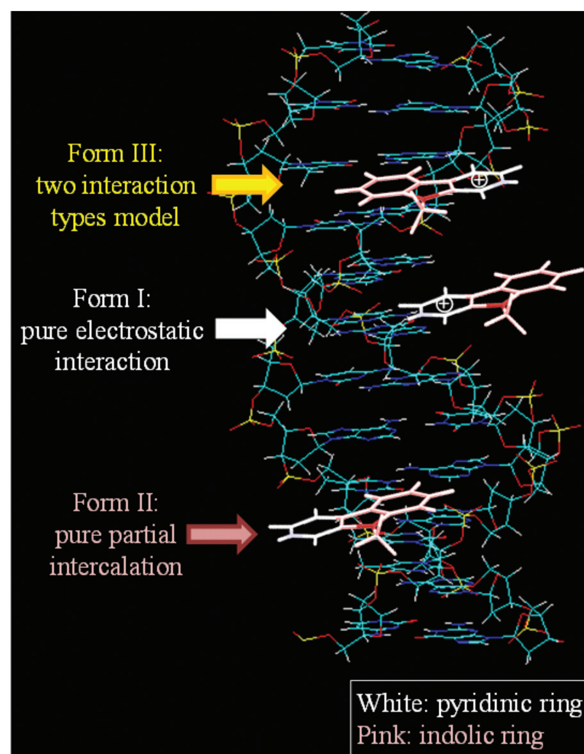
Fig. 3 Phosphorescence emission spectra of β-carboline derivatives recorded at low temperature (77 K). (**a**) Solvent: isopropanol–ethyl ether 1 : 1 (v/v). (**b**) Solvent: isopropanol–ethyl ether 1 : 1 (v/v) with added perchloric acid ($HClO_4$) for protonation of the β-carboline derivatives.

pH 10.0 experiment is slightly lower than that of the respective protonated species in the pH 4.8 experiment, as manifested by a small difference in Φ_F (Table 1). This suggests that only a fraction of $^1[\beta C]^*$ is protonated to yield $^1[\beta CH^+]^*$, which then emits. This is further supported by the spectra recorded in D_2O. Fig. 2b shows, as a representative example, the results obtained for 9-Me–*n*Ho where the isotopic effect is quite clear: the emission intensity of $^1[\beta C]^*$ (~380 nm), relative to the intensity of emission of $^1[\beta CH^+]^*$ (~450 nm), is higher in D_2O than in H_2O. Thus, the steady-state concentration of $[\beta C]^*$ is higher in D_2O than in H_2O. It is noteworthy that, although the latter fact was observed for all the compounds listed in Scheme 1, the effect is stronger in the cases of 9-Me–Ho and 9-Me–*n*Ho than for 9-Me–Ha (results not shown). This could be the consequence of the relatively long fluorescence lifetime of the first two β-carboline derivatives (~20 ns)[38] compared with 9-Me–Ha (9.1 ns).

In addition, phosphorescence emission spectra of both protonated and neutral species of the three compounds investigated were recorded in a solid matrix at low temperature (at 77 K). Briefly, β-carboline derivatives were dissolved in a mixture of 50% isopropanol and 50% ethyl ether (v/v). To record the spectra of the protonated forms, $HClO_4$ was added into the former solutions. As was expected, based on the pH-dependence of the absorption spectra of the β-carbolines, Fig. 3 shows that the phosphorescence spectra of protonated forms are red-shifted in comparison with the spectra of the respective neutral forms. From the onset of the phosphorescence, the energies of the lowest triplet states (E_T) were estimated (see Table 1).

Interaction between 9-methyl-β-carbolines and calf thymus DNA. Spectroscopic studies

The interaction of β-carbolines with ctDNA was investigated under three different pH conditions, pH 4.8, 7.4 and 10.0, using UV-vis absorption and fluorescence spectroscopy: (i) UV-vis spectra of 9-methyl-β-carbolines were recorded in the presence of increasing amounts of ctDNA (results not shown). These results indicate that all the compounds investigated, in their electronic ground states, interact with ctDNA. Although high error margins are associated with the association constant (K_G) determinations, the corresponding K_G values, summarized in Table 2, were estimated by using the Benesi–Hildebrand equation described elsewhere.[28] In addition, the binding of β-carboline to ctDNA has no significant effect on the UV-vis absorption spectra, indicating an important contribution of groove binding interactions between these alkaloids and ctDNA. This fact is in good agreement with the results reported by Taira *et al.*[39] (ii) To further analyze the interaction between 9-methyl-β-carbolines and ctDNA, steady-state fluorescence experiments were performed in the presence of increasing amounts of ctDNA. In the whole pH range investigated (from 4.8 to 10.0) the increase of ctDNA concentration ([ctDNA]) causes a decrease in the fluorescence of the β-carboline derivatives, but no change of the maximum emission wavelengths was observed. These results are in agreement with those reported in the literature for the interaction of 9-Me-*n*Ho and 9-Me–Ho with other types of DNA materials.[27] Briefly, our findings clearly indicate that neither the protonated nor the neutral forms of β-carbolines are fully intercalated into the ctDNA double strand (Scheme 2, pink skeleton). At least, the pyridine ring in the β-carboline moieties is in a polar-protic microenvironment. Note that in the absence of proton transfer between the nucleobase and the excited β-carboline,[28] a full intercalation would certainly provide a non-polar microenvironment to the β-carboline moiety that should be reflected in the emission spectra (*i.e.*, by the increase in the emission band of the neutral form, ~380 nm). Clearly, this is not our case, as is evidenced by the protonation of the pyridine nitrogen in the electronic excited state, even under alkaline conditions, *i.e.*, increasing amounts of ctDNA do not change the maximum of the emission wavelengths of β-carboline derivatives (see the main emission band centered at ~450 nm in ref. 27).



Scheme 2 Model of the interaction between 9-methyl-β-carbolines and double-stranded DNA.

On the other hand, fluorescence data agree with the results obtained from UV-vis spectrophotometric analysis (see above) indicating that these alkaloids are also DNA-groove binders. The acidic nature of the DNA close environment is well known. Thus, such a condition would lead to protonation of the β-carboline moieties that are close to DNA, favoring the electrostatic attraction between β-carbolines and DNA (Scheme 2).

The intercalative mode of binding of 9-substituted-β-carbolines[48,49] and the electrostatic contribution[27,49] has already been proposed. Paul *et al.* have stated that although the interaction mode of harmane (a related β-carboline) with DNA is mainly intercalative, the electrostatic interaction between the cationic dye and DNA should also be taken into account.[49] In addition, when the β-carboline ring interacts with isolated 2′-deoxyadenosine-5′-monophosphate,[28] a quite stable complex is formed, where a clear contribution of π-stacking between the alkaloid and the nucleobase (adenine) and also coulombic interactions are involved. The phosphate group on the nucleotide plays a key role in the relative orientation of the two molecules.

According to our results, a two type interaction model can be proposed that would yield three different types of complexes formed as a consequence of: (i) a pure electrostatic interaction between the protonated pyridinic ring and the phosphate groups of DNA (Form I in Scheme 2), (ii) a partial

Table 2 Summary of β-carbolines/ctDNA interaction parameters: K_G represents the binding constant according to the Benesi–Hildebrand equation and K_S is the equilibrium constant for complex formation (K_S is the K_{SV} values for the static quenching)

	K_G [a]/M in bp^{-1} 10^3		K_S/M in bp^{-1} 10^3		
Compound	pH 4.8	pH 10.0	pH 4.8	pH 7.4	pH 10.0
9-Me–*n*Ho	2.4 ± 0.2	0.6 ± 0.2	6.3 ± 0.1	2.7 ± 0.1	2.6 ± 0.3
9-Me–Ho	2.0 ± 0.3	2.6 ± 0.3	10.5 ± 0.2	6.4 ± 0.7	3.6 ± 0.4
9-Me–Ha	8.3 ± 0.2	0.8 ± 0.5	24.7 ± 0.8	16.8 ± 0.5	10.0 ± 0.5

[a] K_G value could not be obtained at pH 7.4 due to the presence of two different acid–base species of each β-carboline. Therefore, data cannot be analyzed with the Benesi–Hildebrand method.

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intercalation of the neutral species of β-carboline, involving exclusively the indolic ring, see the pink skeleton in Scheme 2 (Form II), and/or (iii) a combination of both types of interaction (Form III).

The decrease of the integrated fluorescence intensity (I_F) as a function of [ctDNA] showed a linear Stern–Volmer behavior (results not shown). The corresponding Stern–Volmer constants (K_S) obtained are listed in Table 2. Taking into account the data analysis of both sets of experiments (UV-vis and steady-state fluorescence), two clear tendencies were observed: (i) for a given β-carboline derivative, the results show a clear pH-dependence on the interaction: the higher the pH the lower the interaction. The correlation between the chemical structure of β-carbolines present at a given pH and the strength of the interaction can be explained in terms of electrostatic interaction: DNA is a negative charged molecule and the three β-carboline derivatives studied have a positive net charge under acidic conditions. (ii) The constant values show the following trend for the interaction with ctDNA: $K^{(9\text{-Me–Ha})} > K^{(9\text{-Me–Ho})} \geq K^{(9\text{-Me–}n\text{Ho})}$.

Photosensitization of cell-free DNA

The indirect DNA damage by photosensitization can be a result of different mechanisms and produces several types of photoproducts. CPDs can be formed by the so-called triplet–triplet energy transfer from the photosensitizers to pyrimidine bases.[40–42] The latter process takes place only if the triplet energy of the photosensitizers is higher than 267 kJ mol^{-1}.[43] In addition, and generally more frequently, oxidized photoproducts are produced. These can be formed following a direct reaction of the excited state of the photosensitizers with the DNA (type I reaction) or by energy and/or electron transfer to molecular oxygen (type II reaction),[44] generating singlet oxygen or superoxide anion as the primary products. These can later (directly or indirectly) oxidize DNA bases. The most frequent oxidized DNA photoproducts are 8-oxo-7,8-dihydroguanine (8-oxoGua), formamidopyrimidines (FapyAde and FapyGua, ring-opened purines), 2,2,4-triamino-(5*H*)oxazolone and apurinic/apyrimidinic sites (AP sites).

Dependence of the DNA damage on the β-carboline derivative concentration. The three 9-methyl-β-carbolines listed in Scheme 1 were investigated as photosensitizers using a supercoiled DNA of bacteriophage PM2. This plasmid was exposed to UVA irradiation (365 ± 20 nm) in the presence of different concentrations of 9-Me–*n*Ho, 9-Me–Ho and 9-Me–Ha. Afterwards, the number of single-strand breaks (SSB) and DNA modifications that are sensitive to the repair glycosylase Fpg, which recognizes 8-oxoGua and some other oxidatively generated purine modifications such as FapyAde and FapyGua, in addition to AP sites,[45] were quantified as described previously.[34,35] Fig. 4 shows that the numbers of DNA modifications recognized by Fpg protein increase nearly linearly with the concentration of the three compounds investigated. SSB were generated in much lower yields than Fpg-sensitive base modifications (<0.07 per 10^4 bp at all the concentrations tested). No significant DNA damage was observed either by

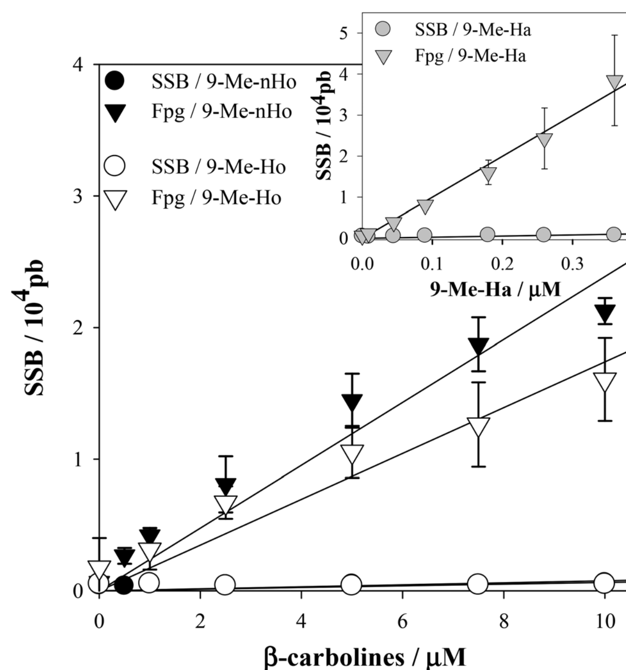

Fig. 4 DNA SSB and modifications sensitive to Fpg protein induced in PM2 DNA by exposure to UVA light (20 min) in the presence of different concentrations of the three 9-methyl-β-carbolines in phosphate buffer (pH 7.4). Inset: 9-Me–Ha. Data are the means of 4 independent experiments (±S.D.).

irradiation of PM2 DNA alone (Fig. 4, data points on the ordinate axis) or by incubation of PM2 DNA in the dark in the presence of each 9-methyl-β-carboline at the highest concentrations (data not shown). This indicates that the damage observed is initiated by the photoexcited β-carboline derivatives.

It is noteworthy that the concentration range needed to induce measurable damage was one order of magnitude lower for 9-Me–Ha than for the other two compounds. This result suggests that 9-Me–Ha is a more efficient photosensitizer than 9-Me–*n*Ho and 9-Me–Ho. This is in good accordance with the fact that 9-Me–Ha has the highest binding constant and triplet quantum yield (Tables 1 and 2).

Quantification of endonuclease-sensitive modifications in PM2 plasmid: DNA damage profiles. The numbers of SSB and various types of repair enzyme-sensitive modifications induced by the photoexcited β-carboline derivatives are shown as DNA damage profiles in Fig. 5. The experiments were performed in phosphate buffer at pH 7.4.

From Fig. 5, it is evident that the three compounds induce predominantly Fpg-sensitive base modifications (oxidized purines), but very few sites oxidized pyrimidines (sensitive to endonuclease III) and SSB. Moreover, a few sites of base loss (AP sites) were identified by endonuclease IV. Also a number of CPDs were detected as indicated by the presence of sites sensitive to T4 endonuclease V, but not to endonuclease IV, *i.e.* the number of sites detected by the combined activities of the two enzymes is higher than the number of sites of base loss detected by endonuclease IV alone.

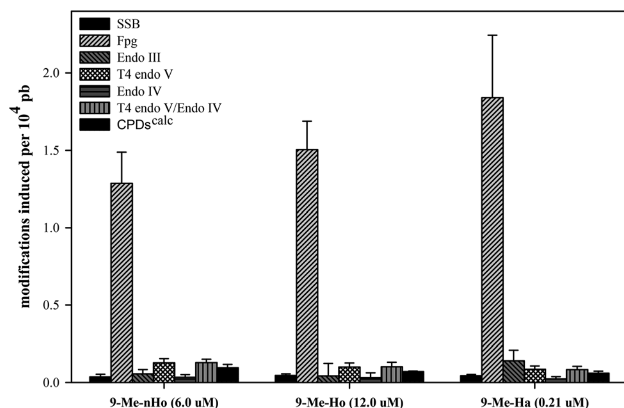

Fig. 5 DNA damage profile showing SSB and several endonuclease sensitive modifications induced in PM2 DNA by β-carboline derivatives exposure to UVA light, in phosphate buffer (pH 7.4). Data are the means of 3 independent experiments (±S.D.).

As already mentioned above, CPDs are generated by direct excitation of the DNA (mostly in the UVB and UVC ranges of the spectrum) but can also be formed by triplet–triplet energy transfer from photosensitizers. At pH 7.4, both protonated and neutral forms of β-carboline derivatives are present in the solution and both can absorb the incident light (Fig. 1). However, only the energy transfer from the triplet state of the neutral form of β-carbolines to the triplet ground state of thymidine nucleobases to generate cyclobutane thymidine dimers (T<>T) is thermodynamically favorable (see Fig. 3 and Table 1). This point is further discussed below.

Role of reactive oxygen species (ROS). Although in a relative small amount, photoexcited β-carboline derivatives are able to produce ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).[38] Hence the contribution of these ROS to the mechanism of DNA damage was investigated. Briefly, Fpg-sensitive sites were measured in the presence of two specific ROS scavengers (SOD and catalase, to evaluate the role of $O_2^{\bullet -}$ and H_2O_2, respectively) and in D_2O buffer (to evaluate the role of $^1O_2$). The results, summarized in Tables 3 and 4, show that the presence of these enzymes and/or the use of buffers in D_2O as solvents have no significant effect on the generation of oxidized purines (Fpg-sensitive modifications), indicating that ROS, albeit present in the reaction mixture, do not play an important role in the mechanisms of DNA photodamage.

Table 3 Effect of SOD and catalase on the generation of Fpg-sensitive modifications photosensitized by β-carboline derivatives at pH 7.4. Data are the means of 3 independent experiments (±S.D.)

	Relative number of Fpg-sensitive modifications (%) in the presence of:		
	SOD	Catalase	SOD + catalase
9-Me–*n*Ho	84 ± 9	91 ± 29	75 ± 12
9-Me–Ha	103 ± 12	90 ± 12	76 ± 5

Interestingly, the replacement of H_2O by D_2O in the solvent resulted in an increase of the yield of CPDs generated by 9-Me–*n*Ho, but not by 9-Me–Ha (Table 4, entry 7). The reason for this isotope effect is analyzed below.

Spin multiplicity of β-carboline's excited states involved in the DNA damage profile. To investigate the nature of the β-carboline's excited states involved in the mechanism(s) of photosensitization, two different strategies were explored: the DNA damage profile generated by 9-Me–*n*Ho was investigated under two different pH conditions (pH 8.0 and pH 6.0) and also in two different solvents, H_2O and D_2O. Taking into account the pK_a value of the investigated molecule, the change in ∼2 pH-units causes a significant difference in the relative concentration of protonated and neutral forms of this β-carboline.

From the results shown in Fig. 6, three major effects are evident: (i) the higher the pH, the lower the yield of Fpg-sensitive site modifications generated and the higher the yield of cyclobutane thymidine dimers (T<>T) produced. (ii) This pH effect is more pronounced when D_2O is used as a solvent. (iii) At a given pH, the amount of CPDs produced in D_2O is larger than in H_2O (see bars for $CPDs^{calc}$).

The results analyzed in connection with our previous findings about the photophysical properties of these alkaloids in

Table 4 The number of DNA modifications sensitive to different enzymes photosensitized by β-carboline derivatives at pH 7.4. Experiments were performed with 9-Me–*n*Ho and 9-Me–Ha. Data are the means of 3 independent experiments (±S.D.)

		Number of DNA modifications (10^4 bp)			
		9-Me–*n*Ho (6.0 μM)		9-Me–Ha (0.21 μM)	
Entry		D_2O	H_2O	D_2O	H_2O
1	SSB	0.08 ± 0.02	0.05 ± 0.02	0.09 ± 0.02	0.05 ± 0.01
2	Fpg	1.9 ± 0.8	1.3 ± 0.3	1.3 ± 0.2	1.8 ± 0.5
3	Endo III	—	0.06 ± 0.03	—	0.14 ± 0.07
4	T4 endo V	0.54 ± 0.09	0.10 ± 0.03	0.07 ± 0.04	0.08 ± 0.01
5	Endo IV	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02
6	T4 endo V + Endo IV	0.55 ± 0.05	0.10 ± 0.02	0.08 ± 0.04	0.08 ± 0.04
7	CPDs^{calc} [a]	0.52	0.09	0.07	0.06

[a] $CPDs^{calc}$ represents the amount of CPDs produced under each experimental condition. These values were calculated by subtracting values of entry 5 from the corresponding values of entry 6 (see the Experimental section).

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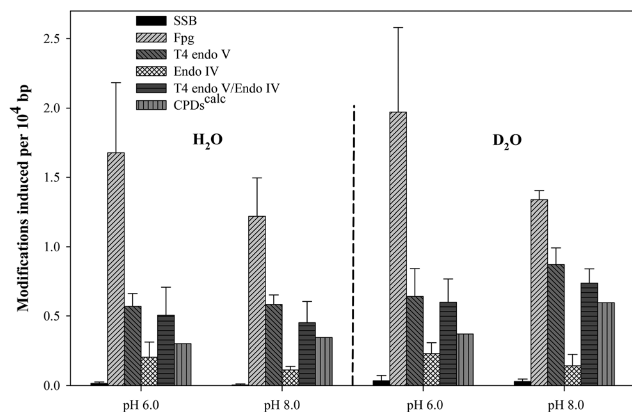


Fig. 6 DNA damage profile showing SSB and several endonuclease-sensitive modifications induced in PM2 DNA by 9-Me–*n*Ho (6.0 μM) exposure to UVA light, in phosphate H_2O and D_2O buffers at different pH (6.0 and 8.0). Data are the means of 3–4 independent experiments (±S.D.).

an aqueous environment allow the following conclusions: in the whole pH range investigated, Fpg sensitive sites are mostly produced by a direct type I photoreaction from the first electronic excited state of the protonated β-carboline derivatives ($^1[\beta CH^+]^*$). A major contribution of the excited neutral form of β-carboline derivatives ($^1[\beta C]^*$) is unlikely because the yield of Fpg-sensitive modifications is decreased at higher pH and because even at pH 10 the main fraction of $^1[\beta C]^*$ has been demonstrated to undergo rapid protonation, yielding $^1[\beta CH^+]^*$.[47] Because of the relatively long lifetimes of the excited singlet states of these alkaloids and the very low yields of intersystem crossing ($\Phi_T < 0.05$),[46] it appears unlikely that a large number of Fpg-sensitive sites are formed from the excited triplet states ($^3[\beta CH^+]^*$). In contrast, T<>T are most likely produced by triplet–triplet energy transfer from the triplet excited state of the corresponding neutral species ($^3[\beta C]^*$), since only this species and not the protonated one ($^3[\beta CH^+]^*$) has a triplet energy that is high enough to generate CPDs. This origin of the CPDs is supported by the increased yields of CPD formation observed at higher pH and also explains the isotope effect (the increased generation of CPDs observed in D_2O) since the protonation of the excited states, which prevents the energy transfer, is more rapid in H_2O than in D_2O. In the case of 9-Me–Ha, no significant formation of CPDs is observed (Table 4), probably because the absorption coefficient of its neutral form is too low (Fig. 1).

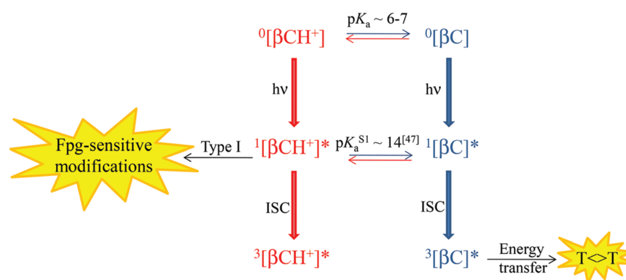
Conclusions

Due to their planar chemical structure, the studied compounds show intercalation abilities. In this regard, the interaction studies indicate that such intercalation should be partial, leaving the pyridinic ring into a protic environment. Moreover, the overall binding constants calculated herein show that the protonated form of each alkaloid (βCH^+) has a higher interaction than the corresponding neutral form (βC). Therefore, coulombic attraction forces between βCH^+ and the negatively charged DNA backbone are of importance.

Under physiological pH conditions, photoexcited 9-methyl-β-carbolines are able to damage cell-free DNA. The analysis with repair enzymes revealed that oxidatively generated purine modifications such as 8-oxo-7,8-dihydroguanine, sites of base loss, oxidized pyrimidines, single-strand breaks (SSB) and CPDs are generated by the three β-carboline derivatives investigated in this work. DNA photodamage is clearly most pronounced when 9-Me–Ha is used as a photosensitizer. The extent of the damage is in agreement with the binding affinity of each β-carboline derivative: the higher the affinity of a given β-carboline to DNA in the ground state, the more β-carboline/DNA complex formation takes place, yielding higher DNA photodamage. Results further indicate that ROS such as $^1O_2$, $O_2^{\bullet -}$ and H_2O_2 are not involved in the main mechanism of damage.

Although under physiological pH both acid–base forms of β-carbolines are present in the solution, our data analysis suggests that Fpg sensitive sites are mostly produced by a direct type-I photoreaction from the first electronic excited state of the protonated β-carboline derivatives ($^1[\beta CH^+]^*$), after a non-fully-intercalative electrostatic binding. On the other hand, cyclobutane thymidine dimers (T<>T) are produced by triplet–triplet energy transfer from the triplet excited state of the neutral species of each β-carboline derivatives ($^3[\beta C]^*$) (see Scheme 3).

Under physiological pH, a mixture of both protonated and neutral β-carbolines species is present. Therefore, basic research on the pH-effect on the β-carbolines' photosensitizing properties certainly provides helpful information to ascertain the contribution of each isolated species and the molecular bases of the mechanisms involved in those biological processes in which these alkaloids take part. In addition, from a biomedical point of view, these studies provide useful knowledge for the design of novel photosensitizers, based on β-carboline's skeleton, with a very distinctive photodynamic behavior according to the microenvironment where they are localized. In this regard, the use of this class of compounds in the design of novel anti-tumor photosensitizers, absorbing in the far UVA (360–400 nm), would represent a very good alternative. In the well-known hypoxic and low pH tumor microenvironment, β-carboline molecules would certainly



Scheme 3 9-Methyl-β-carbolines' electronic excited states involved in the photosensitization pathways of DNA.

show the highest binding affinities, the longest-lived singlet and triplet excited states[50] and, therefore, the highest photosensitizing properties.

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