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ALKALINE LABILIZATION OF DNA PHOTOSENSITIZED BY PROMAZINE DERIVATIVES

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Abstract—Superhelical pBR322 DNA has been photosensitized in the presence of various promazine derivatives. Agarose gel electrophoresis of the photosensitized DNA reveals that true single-strand breaks are induced during irradiation. Alkaline treatment of the photosensitized DNA with a subsequent alkaline agarose gel electrophoresis demonstrates that in addition to true single-strand breaks, these drugs can induce alkali-labile lesions. Although true single-strand breaks are induced randomly into a 5′-[^{32}P]-end labeled pBR322 DNA fragments, the alkaline-labile alterations are located specifically at the level of guanine residues. A strong correlation seems to exist between the visualization of this labilization and the induction of a covalent photoadduct on guanine by the photosensitization mediated by PZ.

Since their development, synthetic phenothiazine tranquilizing drugs have been used in prolonged and high dosage treatment of psychiatric patients [1]. Among these drugs, the members of the promazine family and especially CPZ† have been the most widely used [2]. PZD, however, have been implicated in several phototoxic and photoallergic responses occurring after sunlight exposure [3]. Photodamages to important biomolecules have also been described [4] and it turns out from these studies that nucleic acids are important targets for the photosensitization of living systems by these compounds.

The first demonstration that a DNA base alteration occurs during CPZ mediated photosensitization was done by Kahn and Davies [5]. They showed that CPZ forms a fluorescent photoproduct not dialyzable out the photoreacted DNA solution. The formation of a covalent adduct on DNA was confirmed later [6, 7] and extended to the other members of the promazine family [8]. The photoaddition seems to take place specifically on the guanine residues and be induced more efficiently on single- than on double-strand DNA. Recently, photoaddition products between CPZ and guanosine-5′-monophosphate have been isolated by HPLC and identified by NMR spectroscopy [9]. It appears from this study that in solution, CPZ photoadds by its C2 position to the C8 site of guanosine-5′-monophosphate. The photoaddition products induced on guanine during PZD-mediated photosensitization probably have important consequences in cells because they can block DNA replication of a single-strand template [8] or inhibit DNA transcription into m-RNA by preventing the initiation step [10]. The DNA sugar-phosphate backbone can also be altered by the photosensitization reaction carried out in the presence of these drugs. Indeed, true single-strand breaks have been observed using both neutral agarose gel electrophoresis and supercoiled DNA as substrate of the photoreactions [11]. The breakage reaction has been shown to be promoted by hydroxyl radicals excepted in the case of CPZ, where promazynil or maybe chlorine radicals have been suspected to be the main DNA breaker species [11].

This paper has been mainly focused on the alkaline labilization of DNA photosensitized in the presence of the various PZD. We will show that DNA cleavage occurs efficiently after bringing the photosensitized DNA at high pH values. In addition, the sites of cleavage are completely different from those observed on the photosensitized DNA which has not been treated with alkali. Without alkalinization the cleavage occurs randomly whereas under high pH values the DNA rupture is restricted at the level of guanine residues. These results demonstrate the occurrence of at least two different kind of lesions in the PZD photoreacted DNA, but will also permit an easiest approach of PZD photosensitized cellular DNA by the usual techniques such as the ultracentrifugation in alkaline sucrose gradients.

MATERIALS AND METHODS

Chemicals. PZD were from Specia (Paris, France) excepted TFPZ which was from MS Chemicals (Milan, Italy). They were used as received without any further purification. γ-[^{32}P]-ATP (3000 Ci/mmole) was from Amersham Corp. (U.K.). T_4 polynucleotide kinase was from Pharmacia (Uppsala, Sweden) and calf intestine phosphatase was from Boehringer (Mannheim, F.R.G.).

DNA. pBR322 DNA was amplified in *E. coli*

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† Abbreviations used: PZD, promazine derivatives; PZ, promazine; CPZ, chlorpromazine; MTPZ, methoxypromazine; TFPZ, triflupromazine; ACPZ, acepromazine; HPLC, high performance liquid chromatography.

HB101, extracted and purified according to Maniatis *et al.* [12]. pBR 322 DNA was also used as a substrate to generate [^{32}P]-5′-end labeled DNA fragment and then was cleaved by Eco RI and Bam H1 which generated a 375 base pairs DNA. This piece of DNA was purified by 1.5% (w/v) agarose gel electrophoresis and dephosphorylated at 37° during 60 min by calf intestine phosphatase (1 Unit per pmole of 5′-end) in 50 mM Tris–HCl pH 8.0, 0.1 mM EDTA. The reaction was stopped by adding 20 μl of 20 mM Tris–HCl pH 8.0, 1% (w/v) SDS, 200 mM NaCl and 2 mM EDTA and heating at 68° during 15 min. The mixture was extracted with phenol, chloroform and ether and then was ethanol precipitated at −20°. The dephosphorylated DNA fragment was resuspended into 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 M KCl, 25 μg/ml BSA and 80 pmoles of γ-[^{32}P]-ATP. The 5′-end labeling was performed using T_4 polynucleotide kinase (1 Unit per pmole of 5′-end) at 37° for 60 min. After that time, the mixture was extracted as mentioned above and ethanol precipitated. The 5′-end labeled fragment was then restricted by Hae III yielding two DNA pieces of 174 and 78 base pairs in length respectively. The fragments were purified by 8% (w/v) polyacrylamide–7 M urea gel electrophoresis.

DNA photosensitization. DNA–PZD (1 mM) mixtures were done in 10 mM Tris–HCl (pH 7.5) and irradiated during various periods of time using a Xenon vapor lamp (Osram XBO-150, 400 W/m^2) or a high pressure mercury lamp (Osram HBO-500, 2600 W/m^2) equipped with WG305 filters removing light under 295 nm (Schott, F.R.G.). The photosensitized DNA was either pBR322 FI DNA (1 μg/50 μl) or 5′-end labeled pBR322 DNA fragments (10^6 cpm in 50 μl). After irradiation (up to 120 min), the photosensitized DNA was kept either in neutral conditions and directly analyzed or denaturated under alkali (i) by adding NaOH (50 mM final concentration) or (ii) by redissolving the photosensitized DNA in 1 M piperidine and heating at 90° during 30 min. The piperidine was then removed by extensive lyophilisation.

Agarose gel electrophoresis. Photosensitized pBR322 DNA untreated with alkali was analyzed by neutral 1% agarose gel electrophoresis as described by Piette *et al.* [13]. Alkali treated and photosensitized pBR322 DNA was analyzed by alkaline 1% agarose gel electrophoresis (30 mM NaOH, 50 mM NaCl, 1 mM EDTA) as described by Gamper *et al.* [14]. After electrophoresis, the alkaline gels were neutralized, stained by 1 μg/ml ethidium bromide and photographed under u.v. light with a Polaroid camera. Negative films were scanned with a soft laser photodensitometer (Zeineh, F.R.G.).

Polyacrylamide gel electrophoresis. 5′-end labeled pBR322 DNA fragments photosensitized in the presence of PZ either untreated or treated by 1 M piperidine were redissolved in 98% formamide, 0.25% bromophenol-blue, 0.25% xylene cyanol and 0.1 mM EDTA. The samples were denatured by heating for 3 min at 100° and chilling on an ice bath. The samples were then analyzed by 8% or 10% (w/v) polyacrylamide–7 M urea gels (40 cm long, 0.4 mm thickness) run at constant power (60 W). Autoradiography were carried out at −20° for several days with Fuji X-ray Films.

RESULTS

Agarose gel analysis

PBR322 DNA photosensitized by the various PZD has been analyzed by agarose gel electrophoresis under neutral and alkaline conditions (Fig. 1). The electrophoresis performed under neutral conditions reveal that the photoreactions induced true single-

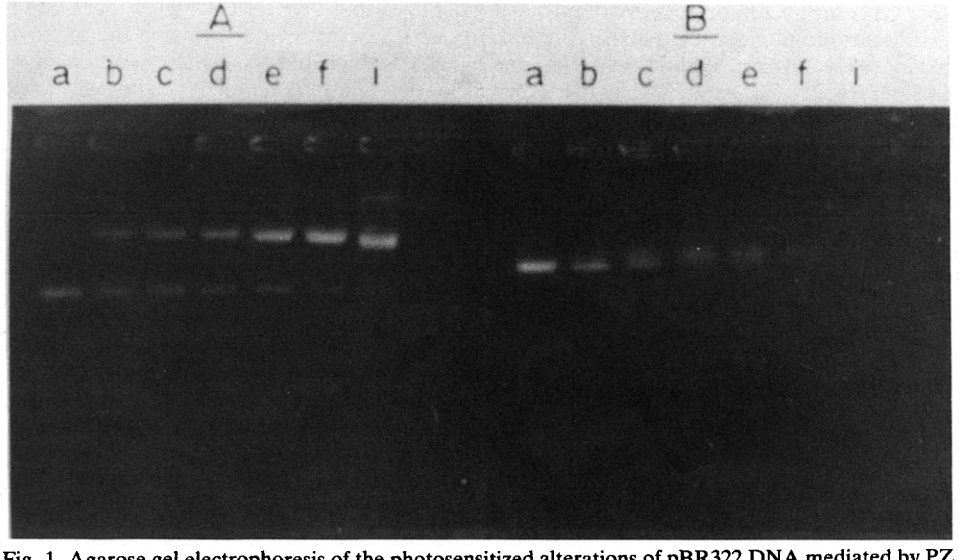

Fig. 1. Agarose gel electrophoresis of the photosensitized alterations of pBR322 DNA mediated by PZ. (A) Electrophoresis performed under neutral conditions showing the photosensitized relaxation of FI DNA into FII DNA. pBR322 DNA is 20 μg/ml, MTPZ (1 mM) and the buffer is 10 mM Tris–HCl (pH 7.5). (B) Electrophoresis performed under alkaline conditions showing the breakage of pBR322 FI DNA. The irradiation conditions are the same as in (A). Irradiation times performed with an OSRAM XBO-150 lamp: 0 min (a), 2.5 min (b), 5 min (c), 7.5 min (d), 10 min (e), 15 min (f) and 30 min (i).

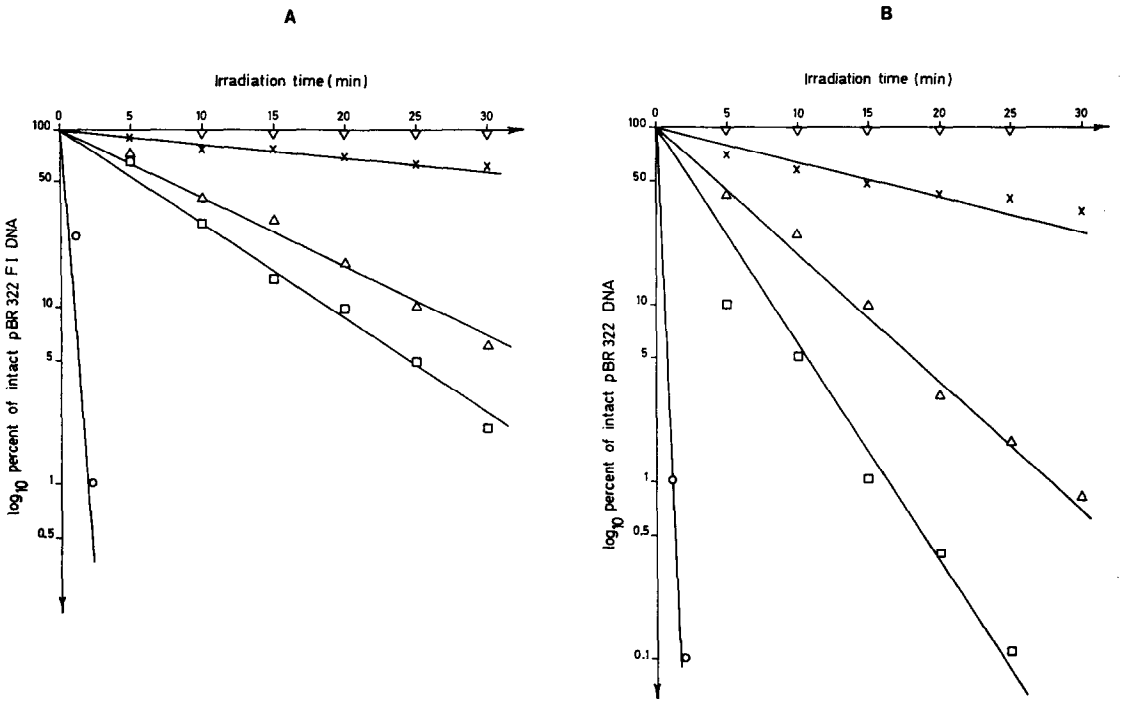

Fig. 2. Nicking activity of the PZD and near-u.v. phototreatment (XBO-150 lamp) on pBR322 FI DNA. The remaining amounts of intact FI DNA are plotted as $\log_{10}$ percent vs the irradiation time (in min): (A) without any alkaline treatment; (B) after alkaline denaturation of the photosensitized DNA. ○, CPZ; □, MTPZ; ×, TFPZ; △, PZ; and ▽, ACPZ.

strand breaks in pBR322 DNA. This conclusion can be raised from the observations that photoexcited PZD are able to promote the conversion of the superhelical form into the relaxed one. The various PZD exhibit variations in the efficiencies to induce the superhelical relaxation. As observed by Decuyper *et al.* [11] CPZ turns out to be highly efficient whereas PZ and MTPZ appear equivalent but less efficient than CPZ. TFPZ exhibits a low efficiency and ACPZ seems to be unable to photosensitize pBR322 relaxation (Fig. 2A). Irradiation without PZD is without any effect. Alkaline denaturation of the photosensitized pBR322 DNA does not modify the relative efficiencies of the various PZD tested: CPZ remains the most active and ACPZ is still without any effect (Fig. 2B). However, the detected kinetic rates are always higher than those recorded under neutral conditions. The factors by which the kinetic rates are increased are: 1.85 for PZ, 2.5 for MTPZ and 2.25 for TFPZ. In the case of CPZ, it turns out that the alkaline treatment increases only the alteration rates by a factor of 1.25. No DNA alteration has been observed by alkaline denaturation of pBR322 DNA photosensitized by ACPZ. In order to characterize better the alkali-labile lesions induced during the photosensitization reactions by PZD and to know whether they are introduced sequence specifically into DNA, we used 5′-[^{32}P]-end labeled DNA fragments of define sequence as substrates of the photosensitization and analyzed the products by high resolution polyacrylamide gels.

Polyacrylamide gel analysis

True single-strand breaks induced in 5′-end labeled pBR322 DNA fragments during photosensitization mediated by PZ can be observed by running the products of the photoreaction on polyacrylamide gel electrophoresis. Under these electrophoretic conditions, DNA fragments are separated from each other according to their length. Figure 3 shows such an analysis carried out with a 174 base pairs DNA fragment photosensitized in the presence of PZ. Without irradiation, the DNA fragment appears on the gel as a single band of 174 nucleotides in length. Increasing the irradiation time up to 120 min, the band intensity corresponding to the intact fragment decreases and concomitantly numerous bands show up on the gel. They correspond to polynucleotides differing from each other only by one nucleotide. Their lengths range between 173 and very short DNA fragment even smaller than 10 base pairs. In addition, their intensities are similar, allowing to conclude that the photosensitization reaction mediated by PZ cleaves DNA with the same frequency at the level of each base. The true single-strand breaks are introduced regardless of the nucleotide sequence of the DNA.

A different band pattern appears on the autoradiograms when the DNA fragments are photosensitized by PZ and treated with hot piperidine before loading on the polyacrylamide-urea gels (Fig. 4). Weak banding occurs at the level of each base excepted for those situated at the levels of guanine

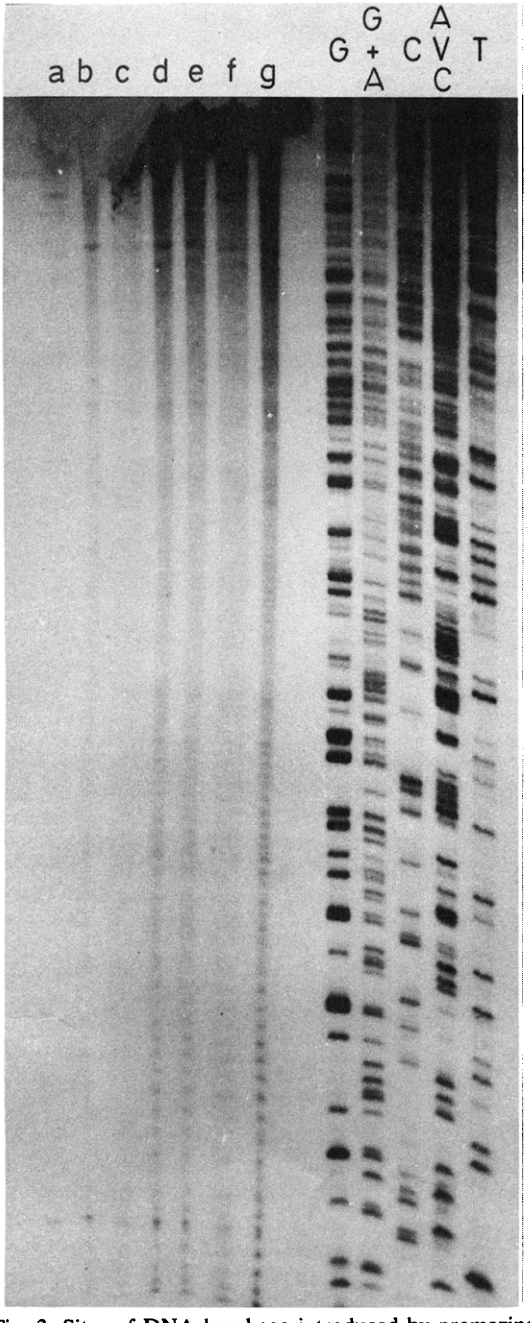

Fig. 3. Sites of DNA breakage introduced by promazine photosensitization. The EcoRI-HaeIII 5′-end labeled pBR322 fragment has been irradiated in the presence of 1 mM PZ (HBO-500, Osram, 2600 W/m^2). After various irradiation times, samples were removed and denaturated at neutral pH in the presence of 98% formamide. The various aliquots were analyzed by 8% polyacrylamide-urea gels. Irradiation times are: (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min, (e) 60 min, (g) 90 min, and (f) 120 min. These samples were loaded in parallel with a Maxam–Gilbert procedure [18].

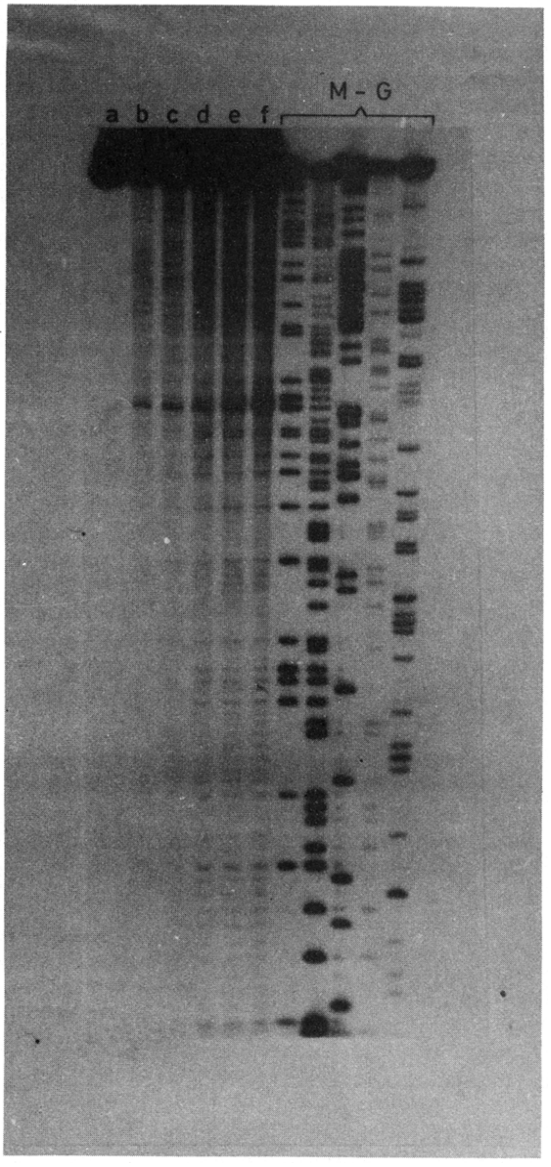

Fig. 4. 10% polyacrylamide–7 M urea gel analysis of the Hae III-Bam HI 5′-end labeled pBR322 fragment irradiated in the presence of 1 mM PZ (XBO150, Osram, 400 W/m^2). After various irradiation times, samples were removed, ethanol precipitated and treated with hot piperidine (90°) for 30 min. The piperidine was removed by lyophilization and the samples were loaded on the gel. Irradiation times are: (a) 0 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, and (f) 30 min. G, G + A, T, A > C and C represent the various channels of the DNA sequenced by the Maxam–Gilbert procedure.

residues which are between two- and five-fold more intense than the others (Fig. 4). In this case, the alkaline treatment performed prior to the electrophoresis allows to detect DNA alterations which are alkali-sensitive in addition to the previously described true single-strand breaks. Regarding the data presented above on the randomness of the true single-strand breaks, we can conclude that the alkali-labile sites are specifically located at the level of the guanine residues. No strand breakage is detectable by an alkaline treatment of the non-photosensitized DNA fragments.

DISCUSSION

We can draw two major conclusions from the data presented above: firstly, alkaline conditions can labilize the DNA photosensitized by PZD specifically at the levels of the guanine residues. Secondly, the photosensitization reactions promote also the formation of true single-strand breaks which take place randomly into the sugar-phosphate backbone. The detection of true single-strand-breaks during DNA photosensitization mediated by PZD (except ACPZ), confirmed those reported by Decuyper *et al.* [11]. However, TFPZ turns out to be less efficient toward pBR322 than ØX174 RF DNA whereas this derivative has been found to relax rather efficiently this viral DNA. The origin of this difference is unknown but cannot be due to the quality of the drug itself because the same commercial product has been used in both studies. True single-strand breaks are introduced randomly into pBR322 fragments, thus without any specificity regarding the DNA primary sequence. The absence of sequence specificity has also been observed in the case of other kind of photosensitizers such as for example copper (II)-camptothecin [15] and tetracycline derivatives [16]. Hydroxyl radicals have been shown to be generated during the near-u.v. irradiation of the PZD–DNA complexes [11] and these radicals are known to react easily with the DNA sugar moiety leading to a hydrogen abstraction, to the deoxyribose ring opening and finally to the sugar-phosphate backbone rupture [17]. Since this radicalar species reacts equivalently with the various deoxyribose units, it is very easy to understand why no DNA sequence specificity has been recorded during the PZD-mediated DNA breakage.

On the other hand, the photosensitization reactions carried out in the presence of PZD lead to the introduction of damages into DNA which under alkaline conditions are transformed into a DNA chain rupture. All the PZD except ACPZ give rise to this behaviour. [^{32}P]-5′-end labeled DNA pieces of known sequence have been used in this work for modification by the photosensitized action of PZD. Piperidine treatment of the modified DNA induced strand breaks and the comparison of the length of the cleavage products with those produced by the Maxam–Gilbert procedure [18] on the same sequence of DNA reveals that the modifications by the treatment create the alkali-labile sites at the position of guanine.

It has been shown for several years that photodynamic treatment of DNA with dyes such as methylene blue [19], proflavine [20], rose Bengal [21] and porphyrins [22] result in the specific destruction of guanine. Singlet oxygen has been identified as one of the reactive oxygen species responsible for the generation of guanine oxidation which could be alkali-sensitive [23]. In the case of PZD, these drugs do not produce singlet oxygen in aqueous medium [24, 25] and then it is not possible to implicate this activated molecule in the alkaline sensitivity of guanine residues observed after the photosensitization reactions mediated by these compounds. On the other hand, evidences that CPZ and other PZD excepted ACPZ form covalent adducts has appeared in the literature [6–8]. Using DNA sequencing techniques, Merville *et al.* [8] demonstrate that the photoaddition occurs specifically on guanine and is introduced more efficiently when the DNA is under a single-strand form. In the work presented above, it is clearly shown that the alkaline treatment labilizes guanine; then it is very likely that the photoaddition products previously recorded in single-strand DNA are also introduced in double-strand DNA and are situated at the level of guanine. The detailed structure of such adducts are still unknown but Ciulla *et al.* [9] have recently isolated photoaddition products of CPZ on guanosine-5′-monophosphate generated in solution. Using NMR spectroscopy, these authors have postulated that the covalent addition occurs between the C_2 position of CPZ and the C_8 of the imidazole ring of guanosine-5′-monophosphate. However, it is still difficult to speculate about the events which lead to the alkaline disruption of these photoadducts in DNA and to the DNA chain opening.

Although neither the principal target nor the damage responsible for the phototoxic action of PZD have been identified, photolesions in membranes [26] and to nucleic acids [27] remain probably the main sites of the PZD induced photoeffects. Further researches are then necessary to determine if guanine specific modifications occurs in cell, since it is not obvious to extrapolate the data presented above to living tissues.

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