

Chapter 1

UVB and UVA induced formation of photoproducts within cellular DNA

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Abstract

Emphasis is placed in this short survey on recent aspects of the photochemistry of cellular DNA that involve both the effects of UVB and UVA radiations. Direct excitation of the purine and pyrimidine bases of DNA is known to mostly generate dimeric pyrimidine photoproducts in oxygen independent photoreactions. Interestingly, the twelve possible dimeric photoproducts at the four main bipyrimidine sites can now be singled out as dinucleoside monophosphates. This is achieved using a specific and sensitive assay that associates high performance liquid chromatography to tandem mass spectrometry (HPLC-MS/MS) operating in the electrospray ionization (ESI) mode. Thus, it was found that UVB irradiation of human monocyte cells gives rise predominantly to *cis-syn* cyclobutadithymine, thymine-cytosine pyrimidine(6-4) pyrimidone adduct and related cyclobutyl dimer. In contrast the dimeric photoproducts at (di)cytosine sites are generated in very low yields although characteristic tandem mutations of UV-B irradiation are observed at the latter CC sequences. Further, cytosine photohydrate and Dewar valence isomers of the (6-4) photoproducts are at the best minor UV-B photoproducts. Relevant information on UVA-sensitized oxidative damage to cellular DNA was gained from measurements using chromatographic methods and the modified comet assay. Thus, it was shown that 8-oxo-7,8-dihydro-2'-deoxyguanosine is the predominant DNA oxidation product, as mostly, the result of singlet oxygen oxidation. In addition, oxidized pyrimidine bases and DNA strand breaks whose formation involves $\bullet\text{OH}$ radical, are produced in much lower yields. Work is in progress to assess the UVA-induced formation of other markers of oxidative stress. These include on the one hand DNA-protein crosslinks and on the other hand DNA adducts with reactive aldehydes that arise from the breakdown of initially generated lipid peroxides.

1.1 Introduction

Solar UV radiation appears to be the main etiological factor responsible for the induction of skin cancers in human population. It is well established that UVB and UVA radiations act mostly on cellular DNA via direct and photosensitized reactions respectively [for earlier reviews, see 1,2]. Precise assessment of the final products of these photoreactions has been hampered for years by the lack of accurate and quantitative methods of measurement. This particularly concerns the individual determination of dimeric pyrimidine photoproducts including *cis-syn* cyclobutadipyrimidines (CPDs), pyrimidine (6-4) pyrimidone photoadducts (6-4PPs) and related valence Dewar isomers (DewarPPs) for which only limited information was available until recently. However, relevant data on the distribution and repair of the three latter classes of photoproducts within the DNA of plasmids, isolated cells and tissues were gained mostly from serological approaches [3-8]. These include ELISA, RIA and immuno-dot-blot measurements together with immunostaining detection through the availability of monoclonal and polyclonal antibodies [8-12]. We may also mention the recent development of an immunological method aimed at measuring CPDs and 6-4PPs in the DNA of isolated cells in association with the

comet assay [13]. Another suitable method that is receiving major attention is the ligation-mediated polymerase chain reaction (LM-PCR) [14–16]. This allows the mapping within DNA at the nucleotide resolution of dimeric pyrimidine photoproducts and particularly of CPDs, the latter lesions being revealed through the nicking activity of T4 endonuclease V. Thus, it was found that methylation of cytosine residues in 5'-CCG and 5'-TCG sequences leads to a 10-fold increase in the UVB formation of CPDs [17]. Interestingly the latter lesions were found to constitute major p53 mutation hot spots in mouse skin tumors [18]. It was also shown that accumulation of dimeric DNA photoproducts takes place at the same locations of the *p53* gene in both human skin and epidermal cells of Hupki (human p53 knock-in) mice [19]. Another striking information inferred from LC-PCR analysis is the predominant implication of CPDs in a vast majority of UVB-induced mutations in mammalian cells [20]. LM-PCR measurement of CPDs in the DNA of the basal layer of engineered human skin led to the conclusion that upper layers of epidermis protected against the genotoxic effect of UVC but not from those of solar UVB radiation [21]. Evidence was also provided, still using LM-PCR detection of CPDs, that human cells either genetically or functionally compromised for p53 function, are defective in both global and transcription-coupled nucleotide excision repair (TCNER) [22]. Interestingly, cells functionally compromised for retinoblastoma tumor suppressor protein function are only defective in TCNER [21]. Preferential repair of CPDs was found to occur in the promoter and quiescent initiation domain of the *CDC2* gene in both quiescent and proliferating human fibroblasts [23].

There is an increasing attention devoted to the assessment of the molecular effects of UVA radiation on DNA in relation with the increased formation of reactive oxygen species (ROS) that for the bulk is mediated by endogenous photosensitizers. This interest is explained, at least, partly by the likely association of UVA with skin cancer risk [24,25] and particularly with skin melanoma incidence whose observation in heavily pigmented hybrids of *Xiphophorus* fish [26] requires, however, further support before to be considered as a suitable model for human. In that respect, UVA does not appear to be a specific mutagen in contrast to UVB that induces a characteristic mutation fingerprint at bipyrimidine sites and more precisely at TC and CC sequences [27]. Thus, the incidence of p53 mutations in UVA-induced skin tumors in hairless mice is very low without any specificity [27]. The relatively high incidence of A:T→T:A point mutations observed on the *LacZ* gene upon exposure of human cells to UVA radiation cannot be correlated with the formation of any known DNA lesions [28,29]. In contrast, the UVA-mediated increase in the frequency of T→G transversions in the *aprt* locus of Chinese hamster ovary cells that was not observed in the nucleotide excision repair-deficient cells may be accounted for by the damaging effects of ROS on DNA [30,31]. It may be pointed out that indirect evidence for the exaltation of the formation of ROS upon UVA irradiation of human skin fibroblasts was provided by the observed induction of heme oxygenase [32] and the release of free iron from ferritin [33]. More direct proofs for the occurrence of oxidation reactions within human and CHO cells upon exposure to UVA radiation was the observed increase in the level of 8-oxo-7,8-dihydroguanine, an ubiquitous biomarker of oxidative

processes, in both nuclear DNA and RNA [34–38]. In addition, relevant information on several classes of oxidative damage induced by UVA and visible radiation to cellular DNA was gained from application of the alkaline elution technique that involves the use of two DNA repair enzymes, namely formamido-pyrimidinone DNA *N*-glycosylase (Fpg) and endonuclease III (endo III). Thus it was shown that the formation of Fpg-sensitive sites, likely to mostly involve 8-oxoGua, are predominant with respect to DNA strand breaks and endo III-sensitive sites (mostly oxidized pyrimidine bases) [39–41].

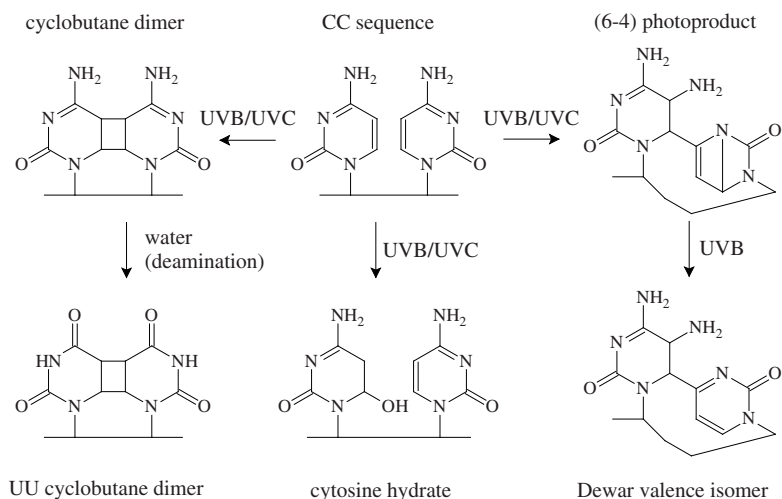
Emphasis is placed in this short survey on recent aspects on the formation of UVB and UVA-mediated damage to cellular DNA that mostly involved dimeric pyrimidine photoproducts and oxidative lesions. The bulk of the measurement of DNA photoproducts was achieved using the recently available HPLC-tandem mass spectrometry technique and the modified comet assay.

1.2 Distribution of UVB radiation-induced dimeric pyrimidine photoproducts within cellular DNA

The advent of the HPLC in the mid 70's together with the availability of new stationary phases including octadecylsilyl silica gel (ODS) packing material has provided a strong impetus to the development of sensitive and highly resolutive analytical method aimed at monitoring the formation of tiny amounts of lesions within cellular DNA. Interestingly, it was shown as early applications that the *cis-syn* isomer of cyclobutadithymine (*c,s* Thy <> Thy) was efficiently separated from the DNA hydrolysis products [42,43]. It should be reminded that the only one available sensitive detection approach at that time was the radioactive measurement of the content of HPLC fractions; however, this was not achieved on line due to the lack of suitable detector. One of the main advantages provided by the HPLC separation on the ODS columns is that thymine is eluted less rapidly than the targeted *c,s* Thy <> Thy, avoiding any contamination of the fractions containing the latter minor photoproduct due to the trailing of [³H]-thymine [42,43]. However, the measurement of *cis-syn* cyclobutane dimer involving cytosine and [³H]-thymine that is released as Ura <> Thy was difficult due to a co-eluting radioactive contaminant, the likely 5-hydroxy-5-methylhydantoin that arises from self-radiolysis process [44]. Interestingly, the assay despite some limitations has received several relevant applications including the assessment of repair kinetic of *c,s* Thy <> Thy in UVC-irradiated of normal and xeroderma pigmentosum fibroblast cells [43]. Subsequently, a suitable HPLC method that does not require radioactive pre-labeling of DNA has become available for monitoring the formation of fluorescent pyrimidine (6-4) pyrimidone photoproducts (6-4PPs).[45] This has required the use of HF-pyridine at room temperature as a mild hydrolytic reagent to obtain a quantitative release of relatively unstable 6-4PPs as nucleobase derivatives. The detection of the latter photoproducts that exhibit a fluorescence emission spectra with maxima around 380 nm upon excitation in the UVB range was achieved at the output of the HPLC column using a fluorescence detector. The distribution of the 6-4PPs including either two thymine or one thymine and a cytosine was assessed in

UVC irradiated DNA. However, the relatively low sensitivity of the steady-state fluorescence detection technique has prevented the application of the assay for monitoring the formation of 6-4PPs within nuclear DNA of UVC or UVB irradiated cells.

Interestingly, most of the limitations that have been encountered for measuring the dimeric pyrimidine photoproducts (*vide supra*) were overcome using the accurate HPLC-MS/MS method operating in the electrospray ionization (ESI) mode. This method that recently became available has already found interesting applications for the measurement of DNA damage including several oxidized pyrimidine and purine nucleosides and nucleobases [46,47]. The overall strategy involves enzymatic digestion of DNA [48,49] from UVB irradiated cells by a cocktail of several enzymes including 3'- and 5'-exonucleases after the quantitative conversion of Cyt <> Cyt and 5'-end cytosine 6-4PPs into the corresponding uracil derivatives through deamination. Therefore, in one HPLC analytical run it is possible to accurately measure at the output of the chromatographic column the twelve possible bipyrimidine adducts at TT, TC, CT and CC sequences both in isolated and cellular DNA upon exposure to low doses of UVC and UVB radiations [50,51]. Interestingly, the tandem mass spectrometric measurement which is achieved in the sensitive multiple reaction monitoring (MRM) mode provides also a specific way of distinguishing CPD from 6-4PP for a given bipyrimidine site due to the occurrence of a different fragmentation pattern [52]. Similar trends in the photoproduct distribution are observed in isolated and cellular DNA upon either UVC or UVB irradiation. As a first remark it may be noted that the formation of the Dewar valence isomers of 6-4-PPs is barely detectable upon exposure of cellular DNA to biologically relevant doses of UVB radiation. Under the latter conditions, only the Dewar isomer at CC sites (Figure 1) was found to be produced, however, in a very low yield. The three main UVB-induced dimeric



photoproducts appear to be generated in the following decreasing order of importance: *c,s*Thy <> Thy > 6-4PP at TC sequence > Thy <> Cyt (Table 1). In contrast, the CT sites and to a lower extent, the CC sequences exhibit a low photoreactivity as inferred from the low yield formation of both CPDs and 6-4-PPs. It should be reminded that the CC photoproducts although generated with a low efficiency exhibit a high mutagenic potential that leads to the observation of the characteristic UV-induced tandem mutation CC→TT. However, further work is required to definitively establish the nature of the highly mutagenic UVB-induced photolesion(s). As a final remark, it may be stressed that the comparison of the HPLC-MS/MS measurements and LM-PCR analysis of dimeric pyrimidine photoproducts in the DNA of UVC irradiated cells [53] shows that application of the latter method leads to a strong underestimation of the yield of 6-4PPs at TT and TC sites. This is likely due to the low efficiency of the piperidine-mediated conversion of the 6-4PPs into strand cleavage at the 3'-side since only the related valence Dewar isomers appear to be strongly alkali-labile.

1.3 UVC and UVB-induced formation of monomeric base photoproducts

It was recently confirmed that UVB is able to generate 8-oxoGua in DNA of mouse keratinocytes [54], mouse epidermis [55] and Chinese hamster ovary cells [56]. However, as shown in a comparative study on the UVB-induced-formation of several classes of photodamage to cellular DNA, the contribution of 8-oxoGua is rather low. Thus, the yield of 8-oxoGua measured as the corresponding 2'-deoxyribonucleoside by HPLC-electrochemical detection in the DNA of UVB irradiated CHO cells was 2.1 lesions per 10⁶ bases and kJ.m⁻² [56]. This is about two orders of magnitude lower than the level of CPDs that was assessed by immunodetection. Further work is required to better delineate the mechanisms of 8-oxoGua formation that may involve a Fenton type chemistry, hole migration from initially photo-ionized pyrimidine and adenine bases or singlet oxygen oxidation. In that respect, we may anticipate a notable contribution of •OH radical or related reactive oxygen through the Fenton reaction since UVB irradiation of cellular DNA was found to lead to similar yields of strand breaks and Fpg-sensitive sites when detected using the alkaline elution technique [39,40].

Table 1. Distribution of bipyrimidine photoproducts* within DNA of human THP1 monocytes upon exposure to UVB radiation expressed in number of lesions per kJ.m² and 10⁴ bases (dose range 0–2.6 kJ.m²)

	TT	TC	CT	CC
CPD	3.147±0.07	1.286±0.047	0.577±0.51	0.279±0.067
6-4PP	0.245±0.007	1.4000±0.034	<0.01	0.062±0.028
DewarPP	<0.01	<0.01	<0.01	<0.03

*From Douki and Cadet [51].

Table 2. Oxidative damage to DNA of human THP1 monocytes upon exposure to UVA and ionizing radiations expressed in number of lesions per either kJ.m^{-2} (dose range 0–2.6 kJ.m^{-2}) or per Gy (dose range 0–40 Gy)^e

Lesions (per 10^9 bases)	Control	UVA radiation (per kJ.m^{-2})	Gamma rays (per Gy)
8-OxodGuo ^a		11	0.8
FapyGua ^b		27	not determined
DNA strand breaks ^c	265	130	0.9
Fpg-sensitive sites ^d	190	18	1.9
Endo III-sensitive sites ^d	195	53	0.3

^aHPLC-ECD.

^bHPLC/GC-MS.

^cComet assay (single strand breaks, double strand breaks and alkali-labile sites).

^dModified comet assay.

^eFrom Pouget et al. [62].

Another putative UVB DNA photodamage that has received a lot of attention in early model studies is 6-hydroxy-5,6-dihydrocytosine the so-called “cytosine photohydrate” that arises from hydration of singlet excited state cytosine [for a comprehensive review, see 1]. A relevant piece of information on the formation of cytosine photohydrate in both isolated and cellular DNA was recently gained from the application of a suitable HPLC-MS/MS assay [57]. This allows the measurement of 2'-deoxycytidine photohydrates as the 6*R* and 6*S* diastereomers of 6-hydroxy-5,6-dihydro-2'-deoxyuridine upon DNA enzymatic hydrolysis and quantitative deamination. Thus, it was found that UVC-induced formation of cytosine hydrate in isolated DNA is a minor photochemical event with a yield of formation which is about 2 orders of magnitude lower than that of CPDs. The formation ratio CPDs/cytosine hydrate was found to be even lower by a factor of 10 in cellular DNA as the likely result of lower accessibility of water molecules for hydration of the cytosine moieties in compacted cellular DNA. These data that, at the best, suggest a minor contribution of cytosine hydrate to the overall biological effect of far-UV radiation are in agreement with a previous estimation of endonuclease III-sensitive sites that were to be 2 orders of magnitude lower than the level of CPDs [58].

1.4 Damage induced by UVA radiation to cellular DNA

Several lines of evidence that underline the major role played by endogenous photosensitizers in promoting oxidative reactions to cellular DNA upon activation by UVA radiation are now available [for a recent review, see 59] are available. Another indirect support for the UVA-induced generation of reactive oxygen species is provided by the observation of the enhancement of the cytotoxic and DNA damaging effects of this component of solar radiation upon addition of L-arginine to human keratinocyte HaCaT cell cultures [60]. A reasonable explanation involves the implication of peroxyxynitrite as a damaging species as

the result of the reaction of L-arginine-stimulated formation of NO^\bullet with photogenerated $\text{O}_2^{\bullet-}$. As a more direct proof of the occurrence of oxidation reactions, it was recently shown that UVA irradiation of cellular DNA gives rise to 8-oxodGuo as assessed by HPLC-ECD measurements [56,61]. Insights into the mechanism of 8-oxodGuo formation were gained from a study that has involved the measurement of three main classes of UVA-induced oxidative damage to DNA in human monocytes using a modified comet assay [62]. These included frank DNA strand breaks together with alkali-labile lesions on the one hand and additional nicks provided by incubation with Fpg and endo III enzymes respectively on the other hand. Interestingly it was found in agreement with previous measurements achieved using the alkaline elution technique that the level of Fpg-sensitive sites was much higher than that of either strand breaks or lesions recognized by endo III [39,40]. Interestingly, the distribution pattern of the oxidative lesions is different from that induced by exposure to gamma rays (Table 2). Under the latter conditions where $\bullet\text{OH}$ radical is the predominant reactive species, the yield of endo- and Fpg-sensitive sites is similar, each of them being about three times lower than that of DNA strand breaks. It should be added that recent investigations using a suitable derivatized naphthalene endoperoxide as a chemical source of singlet oxygen ($^1\text{O}_2$) [63] have shown that the latter ROS reacts in a highly specific way with the guanine moiety of both isolated and cellular DNA to produce exclusively 8-oxoGua [64,65]. It was found that $^1\text{O}_2$ is not able to act as a one-electron oxidant as inferred from the lack of formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) which is also generated by the reaction of $\bullet\text{OH}$ radical with guanine. It should be added that attempts to detect FapyGua in the DNA of UVA-irradiated cells were unsuccessful [62], suggesting either the lack or the low involvement of $\bullet\text{OH}$ radical and/or one-electron oxidation process in the formation of 8-oxoGua. In fact the predominance of the latter compound over DNA strand breaks and endoIII-sensitive sites, mostly oxidized pyrimidine bases, may be rationalized in terms of predominant participation of $^1\text{O}_2$ (85%) together with a low contribution of $\bullet\text{OH}$ radical (15%). It is expected that the qualitative and quantitative formation of the different classes of oxidatively generated damage to DNA is likely to vary with the nature of the cells since they are expected to contain different types of photosensitizers. This may explain the absence of detection of 8-oxoGua in the DNA of UVA-irradiated human epidermoid carcinoma cells [66].

It clearly appears that 8-oxoGua, the main oxidative lesion identified so far in the DNA of UVA-irradiated cells is at the best generated in very low amounts. In fact exposure of monocyte cells to a dose of UVA radiation up to $50 \text{ kJ}\cdot\text{m}^{-2}$ is required to double the level of steady-state level of 8-oxoGua [62] that arises mostly from oxidative metabolism in non-irradiated cells. This strongly suggests that the contribution of 8-oxoGua to the overall biological deleterious effects of UVA radiation is expected to be very low. In that respect, the role of CPDs that have been shown to be formed in much higher yield than 8-oxoGua (ratio 19) has to be further investigated. Interestingly, it was recently found that Thy \leftrightarrow Thy is the predominant UVA-induced dimeric pyrimidine photoproduct at the exclusion of other CPDs and 6-4PPs [67,68], suggesting the occurrence of an energy transfer process for its formation.

1.5 Conclusions

Major progress has been recently made in the assessment of the main UVB and UVA-induced damage to cellular DNA through accurate HPLC and biochemical measurements. Thus, the complete pattern of the dimeric pyrimidine photoproducts is now available for the main bipyrimidine sequences. It is confirmed that there is a strong primary sequence effect on the formation of both CPDs and 6-4PPs. However, information is still lacking on the UVB-induction of 5-methylcytosine (5-MeCyt) containing dimeric pyrimidine photoproducts in both isolated and cellular DNA. Indirect measurement has suggested that the presence of a 5-MeCyt residue in a bipyrimidine sequence would prevent the formation of related 6-4PP photoproducts in cellular DNA [69]. However, this contrasts with the fact that both CPDs and 6-4PPs were found to be efficiently produced in 5-MeCyt containing dinucleoside monophosphates [70]. Further applications of the HPLC-MS/MS assay are expected for assessing the kinetic of repair of individual bi-pyrimidine photoproducts in various cell lines as already shown for the thymine dimeric lesions within the DNA of *Arabidopsis thaliana* [71]. A major analytical development is expected for the latter assay with the association of either micro-HPLC or capillary electrophoresis to the tandem mass spectrometry detection that may result in a significant increase in sensitivity. Other types of oxidative DNA damage have to be considered as potential UVA-induced lesions. Relevant candidates are represented by adducts that arise from the reaction of aminobases with aldehydes such as malonaldehyde and 4-hydroxy-2-nonenal, breakdown products of lipid peroxides for which a suitable HPLC-MS/MS is available [72]. A second class of photosensitized DNA photodamage for which there is still a paucity of structural and mechanistic information deals with DNA-protein cross-links.

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