

Drug–biomolecule interactions in the excited states*

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Abstract: Drug–biomolecule interactions in the excited state are relevant from a photo-biological point of view as they can be correlated with a number of photosensitization disorders such as photocarcinogenicity, photoallergy, phototoxicity, etc. Nonsteroidal anti-inflammatory 2-arylpropionic acids and antibacterial fluoroquinolones have been selected as typical examples of photoactive drugs. Protein photosensitization has revealed photoadduct formation; the major amino acids involved are Tyr, Trp, and His. Generation of specific antibodies has allowed us to identify relevant structures of the drug epitopes. Then, drugs have been submitted to systematic steady-state and time-resolved studies on their photophysical properties, alone and in the presence of biomolecules: proteins, DNA, and their simple building blocks. The results are discussed in the framework of the chemical mechanisms underlying photosensitization by drugs and also in connection with the potential of drug excited states as (chiral) reporters for the binding sites of biomolecules.

Keywords: photobiological; photocarcinogenicity; photoallergy; phototoxicity; arylpropionic acids; fluoroquinolones; photoadducts.

INTRODUCTION

Extensive changes of biomolecules upon irradiation in the presence of drugs are thought to be responsible for the occurrence of photosensitization side-effects. Thus, lipid peroxidation leading to membrane lysis, protein–protein photocrosslinking, or drug–protein photobinding originating photoallergy, as well as DNA damage resulting in photomutagenicity and photogenotoxicity, have been thoroughly studied [1,2]. Obviously, a precise knowledge of the involved active sites and reaction mechanisms would contribute to understanding the photosensitizing potential of new drug candidates. Nevertheless, the complexity of biomolecules renders the study difficult and usually frustrates attempts to establish the detailed course of these processes. So, firstly we have designed and studied synthetic dyads to mimic the interactions between the drug–chromophores and lipids [3], amino acids [4], or nucleosides [5]. Although the use of dyads allowed us to advance in the mechanistic knowledge of photosensitization, these studies are only a first step in the understanding of the photosensitizing mechanisms as they do not totally reflect the complex environment of the biomolecule.

Here, we present the results obtained during the course of our studies on drug–biomolecule interactions, using nonsteroidal anti-inflammatory 2-arylpropionic acids or antibacterial fluoroquinolones

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(Chart 1) as two representative drug families, and proteins, nucleic acids, and their simple building blocks as the target biomolecules.

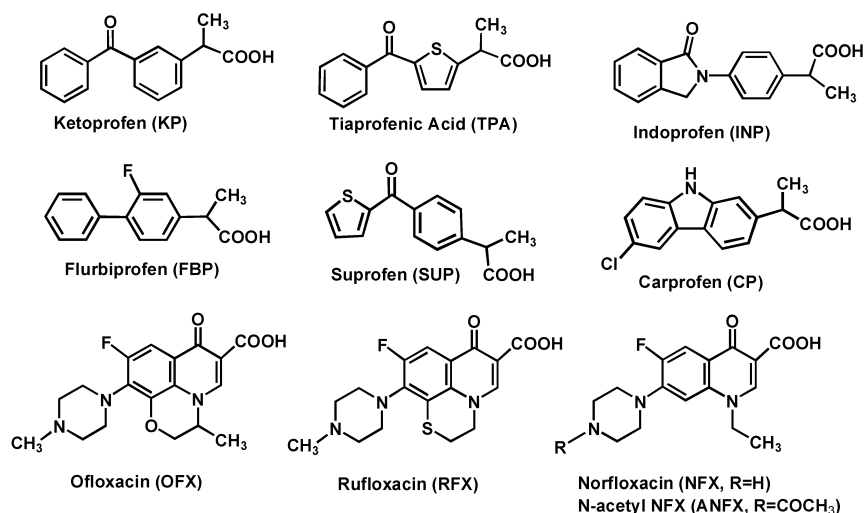


Chart 1 Structures of some photoactive 2-arylpropionic acids and fluoroquinolones.

DRUG–PROTEIN INTERACTIONS

Identification of the reactive sites

Protein modifications photosensitized by drugs have been investigated to identify the reactive amino acids and to get more insight into the elucidation of photoallergy mechanisms [6]. In this context, the model protein bovine serum albumin (BSA) has been irradiated in the presence of tiaprofenic acid (TPA) and DTPA (decarboxylated TPA) as photosensitizing agents. The chemical changes occurring under these conditions reveal that tyrosine, histidine, cysteine, and tryptophan constitute the protein key sites for drug-photosensitized protein damage. Mechanistic studies by laser flash photolysis (LFP) show that TPA (and DTPA) triplet state is efficiently quenched by Tyr and Trp (k_q ca. $10^9 \text{ M}^{-1} \times \text{s}^{-1}$). These results together with those obtained in the aerobic and anaerobic photodegradation of isolated amino acids (but also of *p*-cresol and indole as Tyr and Trp models) lead to the conclusion that a mixed Type I/Type II mechanism is involved in the degradation of Trp, while a Type I mechanism predominates in the case of Tyr. On the other hand, His and Cys are well-established targets for Type II photo-oxidation [7].

Moreover, the detection of photocrosslinking corresponding to formation of high-molecular-weight protein aggregates, as well as TPA photobinding to BSA, illustrates other photochemical modifications of protein. This result is highly relevant to understanding the process of photohapten formation, as it represents the primary step in the onset of photoallergy.

Investigation of drug–protein photoadducts

With this background, the structures of drug–protein photoadducts have been determined in order to establish the molecular basis of photoallergy and to understand cross-immunoreactivity between different drugs [8]. The strategy developed to investigate the covalent photobinding of drugs to protein lies in the generation of specific antibodies to drug chemical substructures. In this context, ketoprofen (KP), TPA, and suprofen (SUP) have been chosen for their phototoxic and photoallergic potential. Moreover, an-

other interesting point is the cross-reactivity reported between KP and TPA (but not between TPA and SUP) in photosensitized patients [9].

Thus, a series of antibodies have been prepared against synthetic adducts corresponding to rabbit serum albumin covalently linked with substructures modeling drug epitopes. The set of antibodies obtained is classified according to their specificity as *anti-benzoyl* (Ab A), *anti-phenyl thienyl carbinol* (Ab B), *anti-ethylthenoyl* (Ab C), and *anti-thenoyl* (Ab D). Using this tool, it is found that the vast majority of the TPA photoadducts can be accounted for by means of the Ab A, supporting that the drug binds preferentially via the thiophene ring, leaving the benzene ring more accessible. By contrast, selective recognition of photobound SUP by Ab D evidences coupling via the benzene ring, while KP photoadducts exclusively recognized by Ab A suggest that the unsubstituted benzene ring is again more accessible (Fig. 1). This result is remarkable as, at first sight, the greatest similarities can be found between TPA and SUP both sharing the same benzoylthiophene chromophore; and by contrast, a common drug epitope is identified for TPA–albumin and KP–albumin, but not for SUP–albumin. These results can be paralleled with the reported photocross-reactivity observed between KP and TPA.

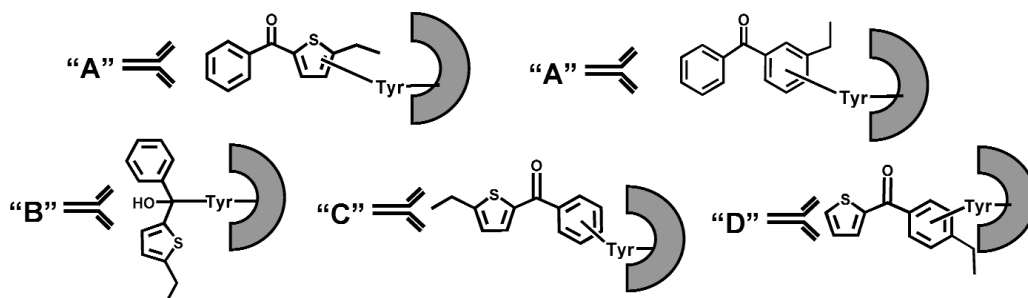


Fig. 1 Possible structures of drug epitopes recognized by specific antibodies.

The above experiments provide very elegant tools to gain important insight into the structure of drug–protein photoadducts. However, they do not allow the full chemical characterization of protein–photosensitizer adducts.

Drug–albumin interaction

Albumin plays a very important role in the photoallergy mechanism, but also in the biodistribution and pharmacological effects of drugs in the body. So, the next step concerning the investigation of biomolecule photosensitization deals with drug–protein interactions in the excited states. As previously reported in the literature [1,10] but also observed during the study of TPA with amino acids, the triplet state seems to be the major excited state involved in the photosensitization of biomolecules. Thus, LFP experiments have been performed with two chiral drugs, carprofen (CP) and flurbiprofen (FBP), associated with human serum albumin (HSA). Moreover, the knowledge of drug–HSA interactions is essential for the understanding of the formation of drug–protein photoadducts, which is responsible for photoallergy.

Carprofen–HSA interactions [11]

The choice of CP is justified by its particular photolysis mechanism. By contrast with other derivatives of 2-arylpropionic acid, the major pathway does not correspond in this case to decarboxylation, but to formation of a photodehalogenated product (PP) [12]. In this way, the chiral center is conserved, allowing the study of stereodifferentiation.

A similar transient absorption spectrum, assigned to the CP triplet state ($\lambda = 450$ nm) is observed for the complexes of (*R*)- and (*S*)-CP with HSA, but remarkably the transient lifetimes show a signifi-

cant stereodifferentiation. The decays at 450 nm are biphasic (with a major long-lived and a minor short-lived component, Table 1). Another striking result is the dramatic lengthening of triplet-state lifetimes when compared with CP in phosphate buffer (τ ca. 0.18 μ s). This observation can be rationalized by the more rigid environment provided by the protein, but also by the suppression of the typical CP self-quenching. The biphasic decays can be correlated with the presence of two binding sites in HSA: the relative contributions of the long- and short-lived components (A_2/A_1) are in excellent agreement with the reported distribution of chiral CP in each binding site (II/I) [13]. Moreover, the presence of the only Trp unit of HSA in site I and the fact that Trp is the most efficient amino acid in the quenching of ^3CP (k_q ca. $6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) are in complete agreement with the shortening of ^3CP lifetime in this site.

Table 1 Photophysical properties of chiral CP in the presence of HSA.

	τ_1 (μ s)	τ_2 (μ s)	A_2/A_1	II/I
(<i>R</i>)-CP	8.9	40	4.8	5
(<i>S</i>)-CP	2.3	24	10.8	10

As observed by steady-state fluorescence, the stereodifferentiating process in the interaction between the chiral CP triplet state and HSA is accompanied by a stereoselective process during protein photosensitization. Thus, the important difference in the emission spectra after UVA-irradiation of (*R*)- and (*S*)-CP in the presence of HSA has been attributed to an enhanced formation of the CP dechlorinated photoproduct (PP) in the case of the (*S*)-CP/HSA complex. This is confirmed by high-performance liquid chromatography (HPLC) experiments where a higher efficiency in the formation of (*S*)-PP is measured. Moreover, another stereoselective process is observed in the photobinding of chiral CP to HSA, as demonstrated by the more intense fluorescence emission of sephadex-filtered (*R*)-CP/HSA photomixture.

Thus, LFP can be used to evaluate drug complexation with proteins via the measurement of their triplet states. Nevertheless, in the case of CP the high reactivity of its triplet state may limit its possibility to be used as a probe.

Flurbiprofen as chiral reporter

For this purpose, the weakly photoreactive drug FBP has been envisaged, and its methyl ester (FBPMe) has been considered as a probe for the binding sites of HSA [14].

As described above for CP, an important lengthening of the triplet lifetimes ($\lambda_{\text{max}} = 360 \text{ nm}$) and a remarkable stereodifferentiation are detected when FBPMe is complexed with HSA (Fig. 2).

Regression analysis of the decay curves for several FBPMe/HSA ratios allows the assignment of $^3\text{FBPMe}$ in the two binding sites of albumin: the major long-lived component corresponds to $^3\text{FBPMe}$ in site I (the high affinity site of 2-arylpropionic acid methyl esters) while the minor short-lived component is assigned to FBPMe in site II. Moreover, the trends clearly show that, for the same FBPMe/HSA ratio, inclusion within HSA is slightly favored for the (*S*)-stereoisomer. As shown in Fig. 2, when the FBPMe/HSA ratio is low, all of the drug is protein-bound with a high selectivity for site I. Progressive increase of the FBPMe/HSA ratio results in the saturation of site I and subsequently of site II, leading to the appearance of increasing amounts of free FBPMe.

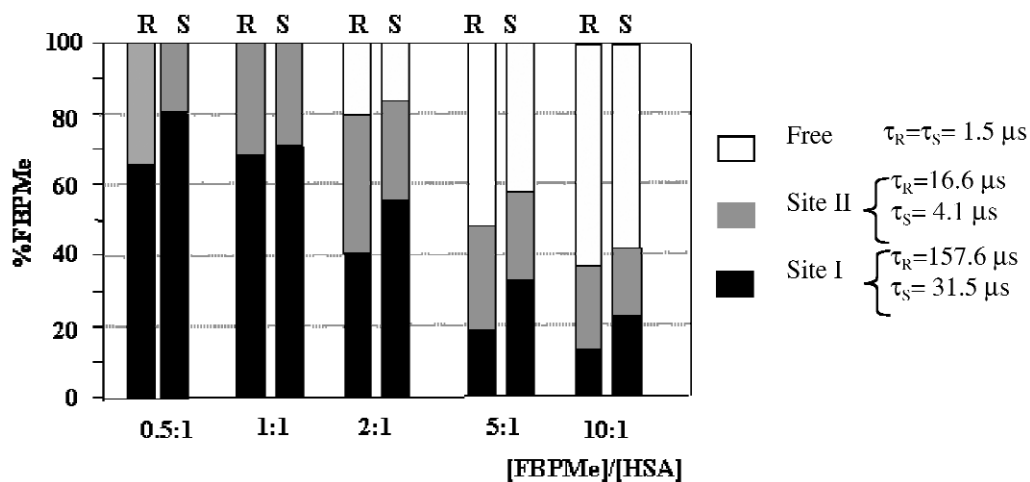


Fig. 2 Percentage of (S)- and (R)-FBPMe location at different [FBPMe]/[HSA] ratios.

DRUG–DNA INTERACTIONS

It is well established that exposure of living organisms to solar radiation may induce lethal mutagenic and carcinogenic effects as a result of photochemical modifications of DNA. At wavelengths shorter than 320 nm, DNA is the absorbing species, while in the UVA the bulk of the photobiological effects is mediated by endogenous and exogenous photosensitizers. Hence, photosensitization has been reported for a number of drugs and especially in the case of nonsteroidal anti-inflammatories [15] and fluoroquinolones [16].

Photosensitization of cellular and isolated DNA by 2-arylpropionic acids

Comet assay

Benoxaprofen, naproxen (NPX), TPA, KP, and SUP, have been reported for their ability to photosensitize formation of single-strand breaks in naked DNA [1]. Nevertheless, the ability of these drugs to induce DNA damage in the more complex environment characteristic of the whole cell, as well as the description of the modified bases and the possible molecular mechanisms involved in base alteration, have been less investigated. By contrast, fluoroquinolones have been shown to produce single- and double-strand breaks, as well as alkali-labile sites, by means of the comet assay [17,18].

With this aim, the comet assay has been used to determine whether TPA can cause DNA damage in eukaryotic cells [19]. As shown in Fig. 3, TPA in conjunction with UVA light is able to cause DNA fragmentation. The nature of the damages, assessed by the study of plasmid DNA in the presence of two base excision repair enzymes: formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III, reveals the oxidation of pyrimidine and purine bases, respectively. As evidenced by the distribution of the nucleobase photoproducts, photoreactivity of DTPA (employed instead of TPA for experimental convenience) toward isolated thymidine and 2'-deoxyguanosine involves both free radical and singlet oxygen. Moreover, LFP shows that once again the key entity appears to be the excited triplet state, which is quenched by 2'-deoxyguanosine and to a lesser extent by thymidine.

Sequencing experiments have revealed that other analogs such as SUP, KP, and indoprofen (INP) are acting via mixed Type I/Type II mechanisms [20,21] and that formation of cyclobutane thymine dimers is photosensitized by KP and INP [21,22]. These damages are obtained via a triplet–triplet (T–T) energy transfer from the excited drug to thymine and require that the populated triplet of the photosensitizer be higher in energy than that of the nucleobase. This condition seems to be fulfilled for the

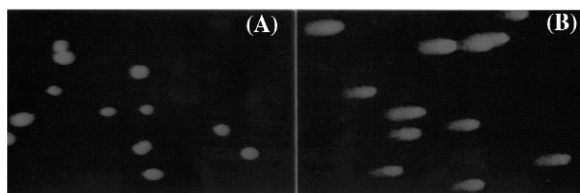


Fig. 3 Comet assay experiment—migration patterns of nuclear DNA from UVA-irradiated cells in the absence (A) and in the presence of TPA (B).

benzophenone-like KP with a very high intersystem crossing quantum yield [1], but not for INP [23]. In the latter case, an interesting hypothesis is that one of the INP photoproducts is in the origin of the photosensitization mechanism. The best candidate is 2-(4-acetylphenyl)isoindolin-1-one (kINP), a *N*-phenylphthalimidine derivative possessing the acetophenone moiety, a chromophore known for its efficiently populated triplet state, whose energy is high enough to photosensitize the formation of thymine cyclobutane dimers [24].

By contrast with INP, kINP triplet state is efficiently formed ($\phi_{ISC} = 0.89$); its energy is ca. 290 kJ mol⁻¹. Photoreactivity toward DNA nucleobases has been firstly addressed by quenching of ³kINP by thymidine, 2'-deoxyguanosine, and DNA. The bimolecular rate constants are ca. 8×10^6 , 1.6×10^9 , and 2×10^7 M⁻¹ s⁻¹, respectively. They are relevant to the ability of ³kINP to photosensitize degradation of these nucleosides. Further experiments have shown that oligonucleotide-photosensitization results not only in the formation of alkali-labile damages on the guanines but also in thymine dimers (T<>T), as revealed by the use of the specific T4 endonuclease V.

Hence, the data obtained by LFP are in agreement with DNA-photosensitization experiments and prove that kINP, and not the parent INP drug, is the key compound in formation of cyclobutane thymine dimers.

DNA-photosensitization by fluoroquinolones

A number of reports have shown that fluoroquinolones may be efficient photosensitizers responsible for undesired cutaneous reactions and are able to operate as photomutagenic and photocarcinogenic agents [25]. In this context, ofloxacin (OFX) and rifloxacin (RFX) photosensitizing properties seem interesting to compare, as despite the close structural similarity (Chart 1), OFX is responsible for a higher degree of DNA oxidation. So, the influence of the sulfur vs. the oxygen atom in position 8 of the ring system has been investigated and correlated with the photosensitizing potential of both drugs [26].

Photosensitization of calf thymus DNA by OFX and RFX results in degradation of the guanine base to give 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), a marker of oxidatively generated damage to DNA, with an efficiency ca. 10 times higher for RFX. However, it is difficult to evaluate the contribution of Type I and Type II pathways of dGuo oxidation in the whole DNA as 8-oxodGuo is obtained by both processes. Nonetheless, one-electron oxidation of guanine gives rise to a more complex pattern of degradation products, which also includes 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 2,2,4-triaminoxazolone. When free dGuo is used instead DNA, two different photoproducts are obtained depending on the involved mechanism: oxazolone and the two diastereoisomeric spiroiminodihydantoin (dSp) for Type I and Type II, respectively. A higher contribution of Type II mechanism (Type II/Type I ratio of 18 and 10 for RFX and OFX, respectively) is observed for the two fluoroquinolones. Moreover, the extent of RFX-photosensitized dGuo is more important than that produced by OFX; this data is in agreement with the relative quantum yields of singlet oxygen production by RFX and OFX (Φ_{Δ} ca. 0.32 vs. 0.13, respectively). Predominance of a Type II mechanism can also be justified in terms of quenching rate constants of the fluoroquinolone triplet state. Indeed, dGuo and molecular oxygen will compete for the quenching of the drug triplet. The fact that the respective

rate constants differ more than one order of magnitude [$k_q(\text{OFX or RFX}/\text{O}_2) = 3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, $k_q(\text{OFX}/\text{dGuo}) = 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and $k_q(\text{RFX}/\text{dGuo}) = 4.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$] justifies the major involvement of a Type II mechanism. On the other hand, the employed reaction medium is important in this case as ^3OFX (but not ^3RFX) is quenched by phosphate anions (k_q ca. $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). In our experiments, the presence of phosphate (10 mM) explains the lower extent of dGuo oxidation by OFX, as singlet oxygen formation will compete with triplet deactivation by phosphate.

TRIPLET-STATE ENERGY OF THYMINE IN DNA [27]

As stated in this work, the triplet state of drugs plays a crucial role in their photosensitizing properties. Among the various mechanisms, T–T energy transfer is of special interest as it is in the origin of thymine dimer formation (the major mutagenic lesion formed upon direct UV-radiation of DNA). Therefore, a key problem is determination of the triplet-state energy of thymine in DNA. Only a limited number of compounds have been found to induce the T–T energy transfer process. As the feasibility of this process is linked to the relative excited-state energies of the donor and the acceptor, the triplet energy of thymine in DNA appears to be a critical parameter. Nonetheless, despite its importance, this value has not yet been definitively established. In DNA, the only approach to address this problem consists in the use of photosensitizers of different triplet energy to produce T \leftrightarrow T lesions. In this way, the upper limit for triplet energy of thymine in DNA has been shifted from 297 kJ mol⁻¹ (methoxyacetophenone) to 290 kJ mol⁻¹ (benzophenone and phthalimidine derivatives) or even lower at 260–280 kJ mol⁻¹ for fluoroquinolones.

In this context, the objective is to find two compounds with triplet-state energy close enough to define a narrow range necessary for measuring the accurate value of thymine triplet in DNA. As fluoroquinolones seem to approach this limit, the rationale is to find two members of this family possessing the same central chromophore but with a peripheral variation. Accordingly, norfloxacin (NFX) and its N(4')-acetylated derivative (ANFX) have been chosen: the former has been taken for convenience as it is the simplest T \leftrightarrow T sensitizing fluoroquinolone derivative, and the latter for the ability of N(4')-acetylation to induce a slight decrease of singlet-state energy [28].

To prove the concept, the DNA-photosensitizing properties of these two compounds are investigated on supercoiled circular DNA. Thus, T \leftrightarrow T formation is revealed by a specific repair enzyme: the T4 endonuclease V. Under the experimental conditions used and without enzymatic treatment, no single-strand breaks are photosensitized by NFX and ANFX. In agreement with previous observations based on HPLC-MS/MS analysis [17], NFX is able to photoinduce T \leftrightarrow T dimers as revealed by the detection of single-strand breaks after subsequent irradiation and enzymatic treatment (Fig. 4). It is noteworthy that the closely related ANFX is inefficient in this assay. Actually, the triplet energy for thymine dimerization in DNA seems to be in the range defined between the NFX and ANFX triplet energies.

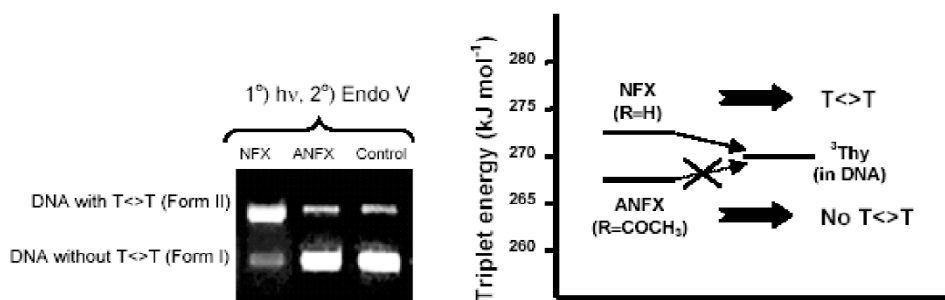


Fig. 4 Energetic diagram and agarose gel showing T \leftrightarrow T formation.

Hence, LFP has been performed to characterize the triplet states of both photosensitizers in buffered aqueous solution (pH = 7.4). A transient T–T absorption with λ_{max} around 600 nm is detected in both cases. Determination of intersystem crossing quantum yield (Φ_{ISC} , ca. 0.52 and 0.40 for NFX and ANFX, respectively) rules out the possibility that the failure of ANFX in T \leftrightarrow T formation is due to an inefficient population of its triplet state.

Triplet-state energies of both photosensitizers are determined by energy transfer quenching of the T–T signal by several potential acceptors: FBP, 4-biphenylcarboxylic acid (BPC), and NPX, whose corresponding triplet energies are 271, 265, and 259 kJ mol⁻¹. The obtained quenching rate constants summarized in Table 2 show the different behavior of ANFX and NFX toward the quenchers. It is generally accepted that the rate constant for energy transfer is nearly diffusion-controlled when the triplet state of the donor is at least 8 kJ mol⁻¹ above that of the acceptor. So, the energy of NFX and ANFX triplet state can be reasonably estimated at 273 and 268 kJ mol⁻¹, respectively. From this assumption, a “functional” value of 270 kJ mol⁻¹ should be given for thymine triplet state in DNA.

Table 2 Quenching rate constants (k_q , M⁻¹ s⁻¹) of NFX and ANFX triplet state.

Quencher	NFX	ANFX
FBP	0.09×10^9	$<0.005 \times 10^9$
BPC	1.5×10^9	0.9×10^9
NPX	2.2×10^9	2.1×10^9

In conclusion, the important information is the energy level required for a given compound to be a possible photosensitizer of thymine cyclobutane dimer formation in DNA. The estimated 270 kJ mol⁻¹ value constitutes a plausible threshold and provides the basis for an alert rule: any chemical with a triplet energy higher than 270 kJ mol⁻¹ should be considered as regards its capability to induce formation of T \leftrightarrow T.

CONCLUSION

In this review, photosensitization is addressed by different techniques. First, study of photosensitization of the whole protein shows that Tyr, Trp, and His are the key amino acids involved in these processes. Moreover, it is established that specific antibodies directed to drug epitopes are elegant tools to investigate the structure of drug–protein photoadducts, the key intermediates of photoallergy. On the other hand, drug triplet excited states are shown to be the key chemical entities responsible for the photosensitizing processes. In this context, drug–HSA interactions in the excited triplet state are studied for CP and FBPMc, and it is established that they can be used as chiral reporters for the two binding sites of the protein. Moreover, a remarkable stereodifferentiation is found for the triplet-state lifetimes within the protein microenvironment.

Then, DNA-photosensitization, investigated at cellular level (comet assay experiment), or with naked DNA and isolated nucleosides, reveals that both Type I and Type II mechanisms are involved in the oxidative processes. Once again, the crucial role of the photosensitizer triplet state is evidenced, particularly for the formation of thymine dimers by a T–T energy transfer. In this context, the critical value of triplet-state energy of thymine in DNA is estimated at 270 kJ mol⁻¹.

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