Repair of a Dimeric Azetidine Related to Thymine-Cytosine (6-4) Photoproduct by Electron Transfer Photoreduction

Ana B. Fraga-Timiraos, Virginie Lhiaubet-Vallet,* Miguel A. Miranda*

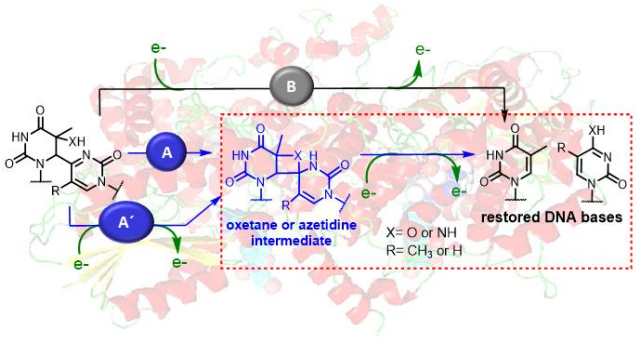
Instituto Universitario Mixto de Tecnología Química (UPV-CSIC)
Universitat Politècnica de València
Consejo Superior de Investigaciones Científicas
Avda de los Naranjos, s/n, 46022 Valencia, Spain
E-mail: lvirgini@itq.upv.es, mmiranda@qim.upv.es

Abstract:

Photolyases are intriguing enzymes that take advantage of sunlight to restore lesions like cyclobutane pyrimidine dimers or (6-4) photoproducts. In this work, the attention is focused on the photoreductive process responsible for splitting of the azetidine ring proposed to occur during (6-4) photoproduct repair at thymine-cytosine sequence. A model compound formed by photocycloaddition between thymine and 6-azauracil has been designed to mimic the elusive azetidine intermediate. The photoinduced electron transfer process has been investigated by means of steady-state and time-resolved fluorescence using photosensitizers with oxidation potentials in the singlet excited state ranging from -3.3 to -2.1 V *vs* SCE. Azetidine ring splitting and recovery of "repaired" bases has been proven by HPLC analysis.

Life would not be feasible on Earth without the efficient DNA repair toolbox that allows safeguarding the integrity of the genome in spite of the continuous exposure to damaging agents. The importance of this machinery has been highlighted by the Nobel Prize in Chemistry 2015 awarded jointly to Lindahl, Modrich and Sancar for mapping the fundamental processes of DNA repair at the molecular level.[1] Among the DNA-damaging agents, UV-radiation is an ubiquitous environmental factor, whose importance is enhanced due to depletion of the ozone layer. The most abundant DNA-lesions formed under direct irradiation are cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (6-4PP).[2] However, the different bipyrimidine combinations are not equivalent for the formation of dimer lesions, as the homodimer resulting from photoaddition between two thymines (TT) is the most efficiently formed CPD, whereas the heterodimer at a thymine-cytosine (TC) sequence predominates in the case of the 6-4PP.[2a] In mammalian cells, these lesions are removed *via* nucleotide excision repair

(NER); nevertheless, in some organisms both CPD and 6-4PP exhibit an additional repair process, which corresponds to DNA photoreactivation catalyzed by photolyases. These enzymes, thoroughly studied by A. Sancar and others, consist of single polypeptide chains with two prosthetic groups.[3] The first one is a light harvesting photoantenna, basically a pterin molecule in the form of methenyltetrahydrofolate (MTHF), while the second one is the catalytic cofactor, a fully reduced deprotonated flavin molecule $FADH^-$. The initial step in the catalytic cycle consists in the absorption of a photon by the MTHF photoantenna; this is followed by energy transfer to the $FADH^-$ cofactor. The photorepair mechanism has been fully resolved for CPDs, where the singlet excited flavin donates an electron to the ground state lesion; subsequent ring splitting of the cyclobutane radical anion restores the native nucleobases.[3] Conversely, the mechanism involved in the repair of (6-4) photoproducts is still under debate.[3b, 4] Until 2008, it was long believed that the (6-4) photolyase operates through intramolecular dark rearrangement of the lesion, to form an oxetane/azetidine intermediate, followed by an electron-induced cycloreversion of the four-membered ring intermediate (Scheme 1, pathway (A)).[3b] However, this paradigm has been challenged, and an alternative mechanism has been proposed on the basis of an *in situ* repair study of the crystallized photolyase containing a single lesion. This new mechanism lacks the oxetane intermediate and consists in a direct photoinduced electron transfer from the singlet excited state of the reduced flavin cofactor to the 6-4PP (Scheme 1, pathway (B)).[4a, 4b] Nevertheless, the possible formation of an oxetane or azetidine-like short-lived species as a result of non-synchronous departure of the protonated XH group and attack of the acylamine moiety has not been ruled out.[3d, 4d] Recently an alternative mechanism has been proposed based on kinetic and theoretical studies involving a two-photon repair process, via photogeneration of the oxetane/azetidine intermediate followed by photoinduced electron transfer (A´).[4c, 4e]



Scheme 1. Schematic representation of proposed scenario for cycloreversion pathway of (6-4) photoproducts. It includes: dark (A) or light mediated (A´) formation of the oxetane/azetidine intermediate prior to the electron transfer process,[3b, 4c] or direct photoreduction the (6-4) photoproduct (B).[4a, 4b]

The mechanistic study of 6-4PP photorepair entails an additional difficulty compared to the CPD case. This relies on the instability of the four membered ring oxetane/azetidine intermediates, which prevents not only their isolation and characterization but also their use as substrates to investigate the electron-induced cycloreversion. Hence, this step has been largely studied using oxetane models;[5] however, until now no analogous report has appeared on model azetidine sytems. Electron transfer cycloreversion of triphenylazetidines has been achieved following a photooxidative pathway,[5-6] whereas the photoreductive approach has only been applied to the ring splitting of structurally unrelated azetidin-2-ones, where the nitrogen atom belongs to a strained β-lactam moiety.[5, 7]

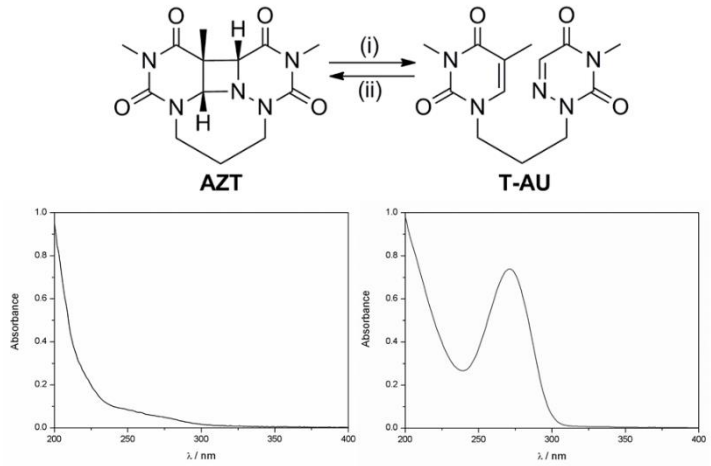


Figure 1. (Top) Model azetidine **AZT** and its cycloreversion product (**T-AU**), (i) Phs + hν (λ_{irr} = 350 nm), (ii) acetone + hν (λ_{irr} > 320 nm). (Bottom) UV-absorption spectra of **AZT** ($1x10^{-5}$ M, left) and **T-AU** ($5x10^{-5}$ M, right) in acetonitrile.

Here, a stable azabipyrimidinic azetidine (**AZT**, Figure 1 top) has been designed as a model for the purported intermediates in the repair of TC dimers by photolyases, in order to address for the first time spectroscopic and photochemical studies of their photoreductive cycloreversion. From a different perspective, AZT is also an aza analog of CPD. This new compound was obtained by the photocycloaddition between the N3-methyl derivatives of thymine and 6-azauracil linked at N1 by a trimethylene bridge (**T-AU**, Figure 1 top). This type of bridge has previously been used in formation[8] and repair[9] studies of model CPD, and it appears to favor the interaction of the lesion with flavin singlet excited state.[9c] The synthesis of **T-AU** was accomplished by reaction of

1-bromo-3-chloropropane with N3-methylthymine and subsequent reaction of the resulting 1-(3-chloropropyl)-3-methylthymine with the N3-methyl derivative of 6-azauracil. Then, **AZT** was obtained as the main photoproduct by irradiation of **T-AU** at $\lambda > 320$ nm in the presence of acetone as photosensitizer, to avoid photocycloreversion of the product under direct irradiation.[10] The *cis-syn* configuration of **AZT** was secured by NOE experiments through the interaction of azetidine ring protons with the C5-methyl group of the dihydrothymine moiety (Figure S6). In view of the markedly different absorption properties of **AZT** and **T-AU** (Figure 1 bottom), the thermal stability of **AZT** in acetonitrile was assessed by UV-Vis measurements at 298K, no spectral changes were detected after 48h (Figure S7).

It is assumed that the electron transfer process occurs from the singlet excited state of the reduced flavin ($^1FADH^{-}*$) to the azetidine moiety. Thus, direct mechanistic information should in principle be obtained by monitoring the changes in the intensity and/or kinetics of the cofactor emission in the presence of the azetidine by steady-state and/or time-resolved fluorescence, respectively. However, the very short lifetime of $^1FADH^{-}*$ (in the subnanosecond timescale) does not provide a time-window compatible with diffusion-controlled intermolecular reaction.[11] To overcome this limitation, a series of photosensitizers (Phs) with singlet lifetime in the nanosecond range and oxidation potential close to that of $^1FADH^{-*}$ ($E_{ox}*$ of ca. -2.9 V)[12] was selected (see Table 1).[9a, 9b, 13] In a first stage, steady-state fluorescence experiments were performed. Thus, fluorescence intensity of the selected sensitizers was measured in the absence or in the presence of different concentrations of **AZT** (see Figure 2A for the case of carbazole). Next, time-resolved fluorescence experiments were run in order to conclude about the dynamic character of the quenching process. The singlet excited state lifetime of all photosensitizers was shortened in the presence of **AZT** (see Figure 2B for the case of carbazole). The bimolecular rate constants k_q were determined from the plots representing the reciprocal of the Phs lifetime as a function of **AZT** concentration (Figure 2B, inset). According to the Rehm-Weller equation, the quenching process was more efficient as $E_{ox}*$ became increasingly negative (Table 1 and Figure 3), reaching the diffusion limit near –3.0 V.

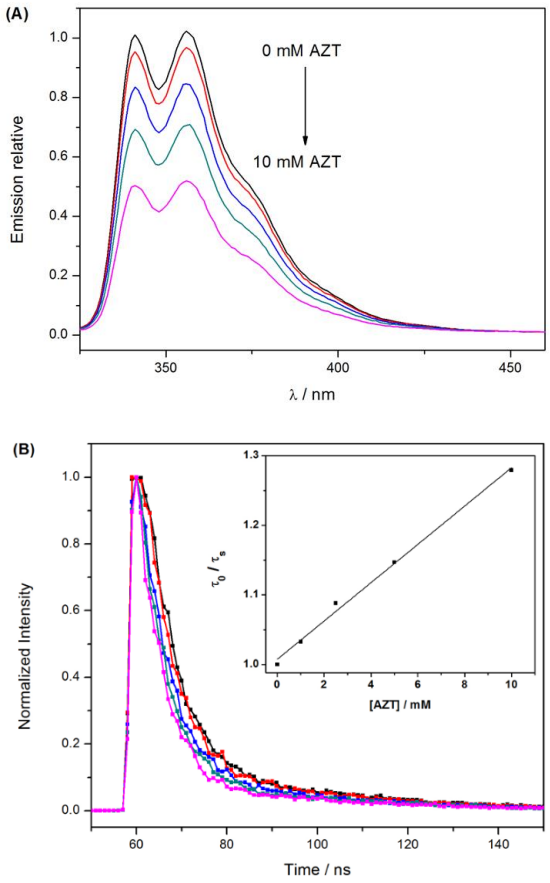


Figure 2. Fluorescence emission spectra (A), and kinetic traces (B) obtained for carbazole in the presence of increasing amounts of **AZT** (from 0 to 10 mM), upon excitation at 310 nm. Inset: corresponding Stern-Volmer plot.

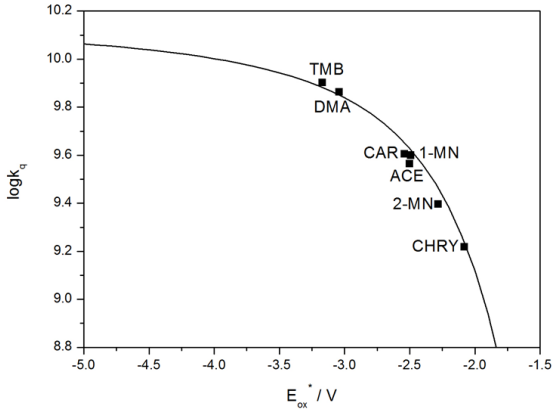


Figure 3. Rate constants (k_q in M^{-1} s^{-1}) versus singlet excited state oxidation potentials (E_{ox}* in V vs SCE) for quenching of the Phs fluorescence by **AZT**.

By contrast, no clear correlation was obtained between k_q and the Phs singlet excited state energy (Figure S8) ruling out a singlet-

singlet energy transfer process as responsible for the deactivation of ^{1}Phs*. Altogether, these data point to an electron transfer mechanism between ^{1}Phs* and **AZT**. It is noticeable that E_{ox}* for the $FADH^-$ photolyase cofactor is of ca. -2.9V vs SCE;[9b] this value, when included in Figure 3, corresponds to an expected rate constant for the electron transfer process with **AZT** of ca. 6 x 10^9 M^{-1} s^{-1}.

Table 1. Oxidation potential in the singlet excited state (E_{ox}*) of the selected Phs, and bimolecular rate constant (k_q) for the quenching of the Phs by **AZT** determined by time-resolved fluorescence

Phs	E_{ox}* (V *vs* SCE)	τ (ns)	k_q (x10^9 M^{-1} s^{-1})[a]
N,N,N´,N´-tetramethyl-1,4-phenylenediamine (TMPD)	-3.3[b]	1.5	N.D. [c]
N,N,N´,N´-tetramethylbenzidine (TMB)	-3.2[b]	6.0	8.0±0.7
N,N,-dimethylaniline (DMA)	-3.0[b]	2.8	7.3±1.0
$FADH^-$	-2.9[d]		
Carbazole (CAR)	-2.5[e]	7.4	4.0±0.2
Acenaphthene (ACE)	-2.5[b]	10.6	3.7±0.2
1-methoxynaphthalene (1-MN)	-2.5[f]	6.2	4.0±0.3
2-methoxynaphthalene (2-MN)	-2.3[b]	6.6	2.5±0.2
Chrysene (CHRY)	-2.1[b]	11.9	1.7±0.2

[a] The experiments were performed twice and the errors correspond to average deviations. [b] From ref [9a]. [c] Not determined because of the temporal resolution of the setup. [d] From ref [12]. [e] From ref [13]. [f] From ref [9b].

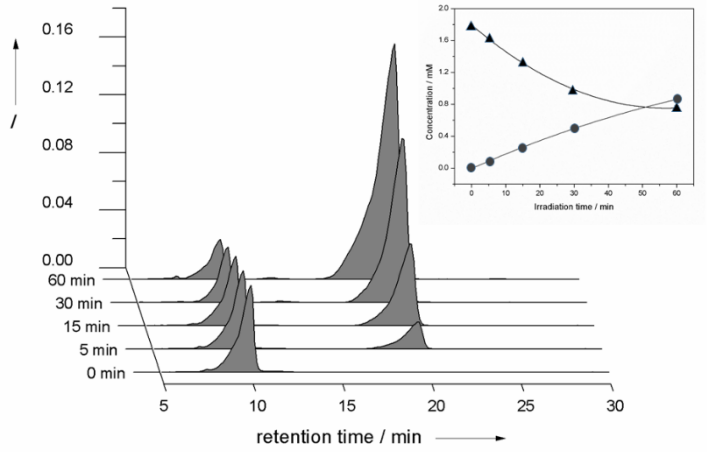


Figure 4. HPLC chromatograms obtained after 0, 5, 15, 30 and 60 min of irradiation of a mixture of **AZT** (2 mM) and **TMPD** (4 mM) in acetonitrile with 350 nm light. Assay conditions: C18 reverse-phase column, 20% acetonitrile and 80% water as eluent, detection at 270 nm. Inset: Variation of the concentration of **AZT** (▲) and repaired **T-AU** (●) with the irradiation time.

Finally, steady-state photolysis was run to ensure that the fluorescence quenching resulted in the expected ring splitting reaction. For this purpose, *N*,*N*,*N´*,*N´*-tetramethyl-1,4-phenylenediamine (**TMPD)** was selected as photosensitizer on the basis of the above results. Thus, an acetonitrile solution of **AZT** in the presence of **TMPD** was irradiated monochromatically at 350 nm to make sure that the light is selectively absorbed by **TMPD**, avoiding this way the possibility of direct photolysis (Figure S9).[10] The sample was purged with nitrogen, irradiated for different times, and analyzed by HPLC to determine the amounts of obtained photoproduct and remaining **AZT** (Figure 4). Only one photoproduct was observed (Φ=0.3), which was assigned to compound **T-AU** by comparison with an authentic sample. This demonstrates that the electron transfer process leads to a clean cycloreversion of the azetidine ring.

Further experimental evidence in support of the electron transfer mechanism was obtained by UV-Vis spectrophotometry. As shown in Figure S19, irradiation of **TMPD** in the presence of **AZT** resulted in the formation of a species absorbing between 500

and 650 nm, which is coincident in shape and position with the visible band of **TMPD** radical cation.[14] However, it is important to mention that the observed species does not correspond to the "in cage" $\mathbf{TMPD}^{+\bullet}$ responsible for **AZT** cycloreversion but to the longer-lived free radical cation escaped from the solvent cage, which is present at very low concentration (ca. 5×10^{-6}M).

In summary, the present study has clearly demonstrated for the first time that photoinduced injection of one electron into a dimeric azetidine derived from thymine leads to a clean cycloreversion and therefore to "repair" of the nucleobases. This is relevant to understand the role of (6-4) photolyase and supports the feasibility of the mechanistic pathway involving reductive splitting of an azetidine intermediate.

Experimental Section

Fluorescence Quenching. The absorbance of the sensitizer for the fluorescence experiments was kept 0.15 at the excitation wavelength (λ_{exc} = 310 nm). On the other hand, a stock solution of azetidine **AZT** (0.15 M) was prepared, so it was only necessary to add microliter volumes to the sample cell to obtain appropriate concentration of the quencher.

The rate constants (k_q) for the reaction were obtained from the Stern-Volmer plots[15] following equation (1):

$$1/\tau = 1/\tau_0 + k_q \times [AZT] \qquad (1)$$

where τ_0 is the lifetime of the photosensitizer in the absence of **AZT** and τ is the lifetime after addition of a quencher concentration [AZT].

Steady State Photolysis. Acetonitrile solutions (3 mL) of **TMPD** (2 mM) and azetidine **AZT** (1 mM) were irradiated at room temperature with a Microbeam system (model L-201) including a Xe lamp (150 W) equipped with a monochromator (model 101). The excitation wavelength was fixed at 350 nm.

HPLC Analysis. The irradiated solutions were analyzed by analytical HPLC using reversed phase column (MEDITERRANEA SEA 18, 5 μ Teknokroma, 25 x 0.46 cm), which was used with a 20/80 CH_3CN/H_2O v/v mobile phase and a flow of 1 mL min^{-1} over 30 minutes. Products were detected by a UV detector set at 270 nm and assigned by comparison with an authentic sample of compound **T-AU**. Areas of peaks detected during the analysis were correlated to calibration curves derived from authentic samples **T-AU** and **AZT** allowing the determination of their concentration as a function of irradiation time. Quantum yields were determined by using a calibrated photodiode to measure the photon flux of the lamp source at 350 nm.

Acknowledgements

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Keywords: 6-azauracil • cycloreversion • DNA damage • fluorescence • photosensitization

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