Enhanced Photo(geno)toxicity of Demethylated Chlorpromazine Metabolites

Fabrizio Palumbo,[1] Guillermo Garcia-Lainez,[2] Daniel Limones-Herrero,[1] M. Dolores Coloma,[3] Javier Escobar,[4] M. Consuelo Jiménez,[1] Miguel A. Miranda*[1] and Inmaculada Andreu*[3]

[1]Instituto de Tecnología Química UPV-CSIC/ Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

[2] Unidad de Cultivos Celulares del IIS La Fe-UPV, Hospital Universitari i Politècnic La Fe, Avenida de Fernando Abril Martorell 106, 46026 Valencia, Spain

[3]Unidad Mixta de Investigación IIS La Fe-UPV, Hospital Universitari i Politècnic La Fe, Avenida de Fernando Abril Martorell 106, 46026 Valencia, Spain

[4]Sabartech S L. Biopolo del IIS La Fe-UPV, Hospital Universitari i Politècnic La Fe, Avenida de Fernando Abril Martorell 106, 46026 Valencia, Spain

Corresponding author:

Email: Inmaculada Andreu – iandreur@qim.upv.es

Miguel A. Miranda – mmiranda@qim.upv.es

Keywords: Excited States, Laser Flash Photolysis, Metabolites, Photosensitized DNA Damage, Phototoxicity

1. INTRODUCTION

Chlorpromazine (CPZ) is an anti-psychotic agent that belongs to the family of phenothiazines. From a clinical standpoint, it is widely used to treat psychotic disorders such as schizophrenia or manic-depression. Unfortunately, CPZ has been often reported as photosensitizing agent, with undesirable side effects such as included phototoxic and photoallergic reactions in humans.[1-8] In addition, CPZ photoproducts like promazine (PMZ) and chlorpromazine sulfoxide (CPZSO) have revealed toxic effects on primary cultures of hepatocytes.[9] More recently, toxic epidermal necrolysis induced by CPZ upon sunlight exposure has been noticed.[10] The phototoxic activity of drug can be related with their genotoxic and mutagenic potential. In this context, CPZ is able to promote DNA photodamage.[6, 11, 12]

From the urine of psychiatric patients, CPZ metabolites have been identified[13] revealing that the metabolic pathways during Phase I led to demethylation, sulphoxidation and hydroxylation.[14, 15] Thus, biotransformation of CPZ results in a variety of derivatives, which have be considered for establishing the pharmacological and toxicological profile.[16]

As a general rule, metabolism converts hydrophobic chemicals into more hydrophilic derivatives that can be easily eliminated through the urine. However, in certain cases, drug-metabolizing enzymes can also produce electrophilic metabolites that react with cellular macromolecules such as DNA, RNA, and proteins, causing cell death and organ toxicity.[17]

Assessment of the phototoxic potential of sunlight-absorbing drugs is highly recommended by Health authorities (FDA; EMEA)[18, 19] but the influence of metabolism is not considered in the appraisal. For that reason, identification of reactive drug

metabolites with phototoxic or adduct forming capability is a major challenge. Hence, the goal of the present work is to investigate the possible photo(geno)toxic potential of drug metabolites, using chlorpromazine as an established reference compound. In this case, the metabolites selected for the study (see Figure 1) are demethylchlopromazine (DMCPZ), didemethylchlopromazine (DDMCPZ) and chlorpromazine sulfoxide (CPZSO). Both DMCPZ and DDMCPZ maintain the CPZ chromophore unaltered, so modulation of the photobiological effects would be associated with the nature of the aminoalkyl side-chain. By contrast, CPZSO displays a modified tricycle aromatic core, which could be responsible for major changes in the phototoxicological properties.

The proposed multidisciplinary approach encompass from photochemical (steady-state and laser flash photolysis) to spectroscopic (Electron Paramagnetic Resonance, EPR) and biological studies (neutral red uptake test, gel electrophoresis, comet assay) in order to obtain mechanistic insight into the involved process.

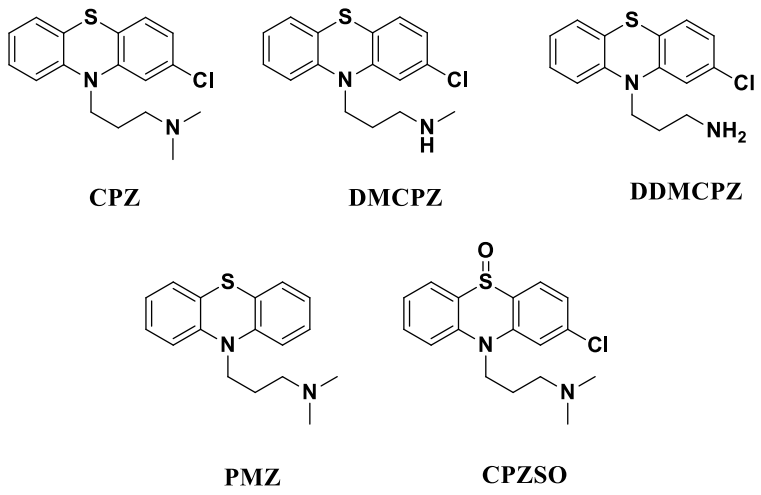


Figure 1. Chemical structures of CPZ, DMCPZ, DDMCPZ, PMZ and CPZSO.

2. MATERIALS AND METHODS

2.1 Chemicals.

All solvents (HPLC grade) and reagents were commercially available and used without additional purification. Chlorpromazine, promazine, *in vitro* toxicology assay kit, neutral red based and DNA repair enzymes *E. coli* formamidopyrimidine DNA glycosylase (Fpg), *E. coli* endonuclease III (Endo III) were purchased from Sigma Aldrich (Madrid, Spain). Supercoiled circular pBR322 DNA, SYBR Safe DNA gel stain and DNA repair enzyme T4 endonuclease V (Endo V) were provided by Roche Diagnostics (Barcelona, Spain), Invitrogen (Madrid, Spain) and Ecogen (Barcelona, Spain), respectively. Phosphate buffered saline solution (PBS, pH 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate amount of deionized water. Reagent kit for single cell electrophoresis assay was supplied by Trevigen (Barcelona, Spain).

2.2 Synthesis of Metabolites.

Synthesis of DMCPZ was achieved in two-steps following the demethylation procedure described.[20]. In the first step, α-chloroethyl chloroformate (ACE-Cl) was added to an ethylene dichloride solution of CPZ in order to form the intermediate ACE-CPZ, whose methanolysis afforded DMCPZ hydrochloride. As regards, DDMCPZ hydrochloride it was obtained by reduction of the 7-chlorophenothiazinyl nitrile using $LiAlH_4$. Soxhlet extraction was performed in diethyl ether for 3 days, at 40 °C as in the original procedure described for a related compound.[21] Finally, CPZSO was prepared from CPZ by oxidation in aqueous nitrous acid, at room temperature, following the method reported.[22].

All reactions were monitored by analytical TLC with silica gel 60 F254 and revealed with ammonium molybdate reagent. The crudes were purified through silica gel 60 (0.063 – 2 mm). ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ or CD_3OD as solvents on

a Bruker AC-300 at 300 and 75 MHz respectively; NMR chemical shifts are reported in ppm downfield from an internal solvent peak.

DMCPZ. ^{1}H-NMR (300 MHz, CD_3OD) δ 2.19 (m, 2H), 2.64 (s, 3H), 3.10 (m, 2H), 4.06 (t, *J* = 6.3 Hz, 2H), 6.94 – 6.98 (m, 1H), 7.00 – 7.11 (m, 4H), 7.16 – 7.19 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 - 7.28 (m, 1H). ^{13}C-NMR (75 MHz, CD_3OD) δ 24.7, 33.7, 45.2, 48.2, 117.4, 117.6, 123.9, 124.6, 126.0, 127.0, 128.7, 129.0, 129.3, 134.7, 145.6, 147.9.

DDMCPZ. ^{1}H-NMR (300 MHz, CD_3OD) δ 2.13 (dq, *J* = 14.2, 6.3 Hz, 2H), 2.98 – 3.09 (m, 2H), 4.08 (t, *J* = 6.4 Hz, 2H), 6.95 – 7.04 (m, 2H), 7.04 – 7.10 (m, 2H), 7.13 (d, *J* = 8.2 Hz, 1H), 7.18 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.25 (ddd, *J* = 8.2, 7.3, 1.5 Hz, 1H). ^{13}C-NMR (75 MHz, CD_3OD) δ 26.0, 38.6, 45.2, 117.4, 117.6, 123.9, 124.6, 126.1, 127.0, 128.6, 128.9, 129.3, 134.7, 145.7, 148.0.

CPZSO. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.17 (m, 2H), 2.37 (s, 6H) 2.59 (m, 2H), 4.37 (m, 2H), 7.22 – 7.25 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.30 – 7.32 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.53 – 7.55 (d, *J* = 8.1 Hz, 1H), 7.60 – 7.69 (m, 2H), 7.85 – 7.88 (d, *J* = 8.4 Hz, 1H), 7.93 – 7.95 (dd, *J* = 7.8, 1.5 Hz, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 24.3, 45.1, 45.7, 56.1, 116.3, 116.4, 122.2, 122.5, 131.4, 132.6, 133.1, 138.0, 139.1, 139.6.

2.3 Irradiation Equipment

For all *in vitro* photosensitization assays, a photoreactor model LZC-4 (Luzchem, Canada) equipped with 14 lamps for top and side irradiation (λ_{max} = 350 nm, Gaussian distribution) was used as the UVA light source. All irradiations were performed through the lid of the plates and the temperature was controlled by ventilation during the irradiation step.

2.4 *In Vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test.

BALB/c 3T3 fibroblasts cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 4 mM glutamine and 1% penicillin/streptomycin and routinely maintained in exponential growth in 75 cm^2 plastic flasks in a humidified incubator at 37 ºC under 5 % carbon dioxide atmosphere. The 3T3 Neutral Red Uptake Phototoxicity Test was performed as previously described by the OECD guideline 432[23] with minor modifications. Briefly, for each compound two 96-wells plates were seeded (2.5 x 10^4 cells / well). Cells were treated with test compounds at eight concentrations ranging from 0.5 µM to 500 µM and incubated for 1h. Next, one plate was irradiated on ice for 11 min to achieve a dose of UVA equivalent to 5 J/cm^2 (UVA LIGHT), whereas the other plate was kept in a dark box (DARK). The viability of UVA treated control cells was greater than 90% of those kept in the dark indicating the suitability of the UV dose. After irradiation the compound solutions were replaced with DMEM medium, and plates were incubated overnight. Next day neutral red solution (50 µg/mL) was added into each well and incubated for 2 h. Cells were washed with PBS and neutral red was extracted in 100 µL with the desorbs solution (water 49 % (v / v), ethanol 50 % (v / v) and acetic acid 1 % (v / v). The absorbance was measured at 550 nm on a Multiskan Ex microplate reader. For each compound dose-response curves were developed in order to determine the concentration of compound causing a 50% reduction of the neutral red uptake (IC_{50}) in the absence and in the presence of radiation. Finally, the Photo-Irritation-Factor (PIF) was calculated using the following equation: $PIF = \frac{\text{IC50 DARK}}{\text{IC50 UVA LIGHT}}$. According to the OECD Test Guideline[23] a compound is predicted as phototoxic if PIF is > 5, probably phototoxic if PIF>2 and <5, and non phototoxic when PIF<2. CPZ and SDS were used as positive and negative controls, respectively.

2.5 Photosensitized Damage to DNA

The drug (CPZ) or its metabolites (10 μM) in 10 μM PBS at pH 7.4 were added to 200 ng of supercoiled plasmid pBR 322 and mixtures were irradiated as described above. After irradiation, loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol 99% in water) was added to each solution immediately. To reveal specific DNA damages, irradiated mixtures were further incubated with an excess of DNA-repair enzyme (FPG, ENDO V or ENDO III) for 1h at 37 ºC, and loading buffer was then added to each solution. All the samples were loaded on a 0.8% agarose gel containing SYBR® Safe as nucleic acid stain. Electrophoresis was performed in Tris-acetate-EDTA (TAE) buffer (0.004 M Tris-acetate, 1 mM EDTA) at 100V for 1 h. Finally, the DNA bands were detected under irradiation with UV light and visualized using a Gel Logic 200 Imaging System (Kodak). The relative abundance of the supercoiled form (Form I) and the nicked relaxed form (Form II) was quantified by densitometry with the image analyzer software Quantity One (BIO RAD).

2.6 Assessment of Cellular DNA Damage by Comet Assay

For the detection of strand breaks and alkaline labile sites, a single cell gel electrophoresis, also known as comet assay, has been performed as described by Collins A.R *et al*.[24, 25] with slight modifications. First, pre-coated slides were prepared by dipping degrease microscope slides in a hot 1 % normal melting point agarose solution (1w:100v MiliQ water), air dried overnight at room temperature, and then stored at room temperature avoiding high humidity conditions (pre-coated slides can be stored more than two months).

The day of the experiment, fibroblasts (FSK) were trypsinized for 1 min, and after seeding 250.000 cells/ml in PBS per well in three 6-well plates, cells were placed on ice and allowed to stand for 2 h before incubating them with the different chemicals. We have found that trypsin by itself induces DNA damage in the FSK cells, and that it takes 2

hours to recover basal levels. Then, FSK were incubated with 10 μM PBS solutions of CPZ, DCPZ, DDCPZ and positive and negative controls (CCP and CCO) supplied by Trevigen for 1 h. After the incubation time, two 6-well plates were placed in a photoreactor to irradiate the cells with UVA light on ice for 5 min, while 3rd plate of cells were immediately prepared for comet assay without irradiation.

Thus, 30μL of each cell suspension were mixed carefully with 140 μL of 37 ºC 0.8 % low melting point agarose solution (1w:80v PBS-$Ca^{2+/}Mg^{2+}$ free) and drops of 5 μL minigels were loaded on the slides. The coated-slides were placed on an ice-cold tray and twelve minigels were loaded onto each slide, being two replicates per condition. After loading, the minigels were allowed to gelify for 5 min, followed by immersion of the slides in copling Jars containing cold lysis buffer (NaCl 2.5 M, Na_2EDTA 0.1 M, Tris 10 mM, Triton X-100 1% in distilled water and pH 10) and overnight incubation at 4ºC. Next day, the slides were placed in a Trevigen® Comet Assay electrophoresis tank (8 slides per run), covered with 850 mL of cold alkaline electrophoresis buffer (200 mM NaOH, 1mM EDTA in distilled water and pH ≥13) and let during 40 min for DNA unwinding at 4º C. Afterwards, the electrophoresis was run at 21 V (≈ 300 mA) for 20 min at 4º C. When the electrophoresis finished, the slides were neutralized twice in PBS for 5 min and washed once with miliQ water for 1 min; DNA was fixed by slides incubation in 70% ethanol for 5 min followed by other 5 min in 100 % ethanol, and then air-dried.

Comet nucleoids and tails were stained by incubating the slides in a SYBR Gold® ($1:10^4$ TE buffer) bath for 30 min, air dried, and kept in darkness until further visualization. For nucleoids and tails DNA visualization, a distilled water drop and a cover slip were applied onto each slide and placed under an epi-fluorescence microscope with an UV-filter and ≥ 5 pictures were taken for each sample. Finally, visual scoring was applied for DNA damage

quantification for a total of 100 hundred DNA comets.[26] Total comet score (TCS) was calculated by using a classification of 6 DNA damage categories[26] and the following formula: [($N_{class\ 0}$ comets x 0) + ($N_{class\ 1}$ comets x 1) + ($N_{class\ 2}$ comets x 2) + ($N_{class\ 3}$ comets x 3) + [($N_{class\ 4}$ comets x 4) + ($N_{class\ 5}$ comets x 5) + ($N_{class\ 6}$ comets x 6)]/6 and expressing results in 1-100 arbitrary units.

2.7 Steady-State Photolysis.

Ultraviolet absorption spectra were recorded on a Varian Cary 300 scan UV/Vis spectrophotometer. Irradiation of CPZ and its demethylated metabolites was carried out at monochromatic wavelength (330 nm) using a Xenon lamp. The progress of reactions was monitored following changes in emission spectra (λ_{exc} = 330 nm). Steady-state fluorescence experiments were carried out in the wavelength range 350–650 nm using an EasyLife X (Optical Building Blocks) spectrofluorometer, equipped with a monochromator PTI. Measurements were performed in PBS (5×10^{-5} M) at room temperature using 1 cm quartz cells with 3.5 mL capacity under anaerobic conditions.

2.8 Laser Flash Photolysis. Laser flash photolysis (LFP) experiments were done with a pulsed XeCl excimer laser (λ_{exc} = 308 nm, *ca*. 17 ns pulse width, < 100 mJ per pulse) as the excitation source. In general, samples received only one pulse for all kinetic experiments. A pulsed Lo255 Oriel Xenon lamp was used as detecting light source. The observation wavelength was selected with a 77200 Oriel monochromator, and the signal amplified by an Oriel photomultiplier tube (PMT) system made up of a 77348 side-on tube, 70680 housing, and a 70705 power supply. The signal was registered with a TDS-640A Tektronix oscilloscope and subsequently transferred to a personal computer. Concentrations of the samples were fixed by adjusting the absorbance of the solutions at the arbitrary value of 0.3 at the excitation wavelength. All transient spectra were recorded

using 1 cm quartz cells with 3.5 mL capacity at room temperature under aerobic conditions.

2.9 EPR Spin Trapping Measurements. The experiments were done in a flat cell with a Bruker EMX 10/12 EPR spectrometer, using the following parameters: microwave power, 20 mW; modulation amplitude, 1.0 G; and modulation frequency, 100 kHz. As spin-trap 2-methyl-2-nitrosopropane (MNP) was employed. Thus, analysis was performed recording the EPR signal of the MNP adduct of the aryl radical generated by loss of the chlorine atom from CPZ or its metabolites. Anaerobic PBS solutions containing the same concentration (1.4 mM) of MNP and CPZ (or one of its metabolites) were irradiated at 330 nm by means of a Xenon lamp for 3 min.

3. RESULTS AND DISCUSSION

3.1 Phototoxicity.

Cell viability of the CPZ metabolites and photoproducts in combination with UVA light was assessed by the *in vitro* 3T3 NRU phototoxicity test. Thus, cytotoxicity profiles of BALB/c 3T3 fibroblasts cell treated with CPZ, PMZ, CPZSO, DMCPZ, DDMCPZ were measured in the presence of absence of UVA light, using neutral red as vital dye.

This test is based on the calculation of the PIF that corresponds to the ratio of the IC_{50} for each chemical with and without UVA irradiation. The values obtained are given in Table 1.

Table 1. *In vitro* 3T3NRU phototoxicity assay of CPZ metabolites and photoproducts.

Compound	IC_{50} Dark (µM)	IC_{50} UVA Light (µM)	Photoirritant Factor (PIF)[a]
CPZ	78.6 ± 15.3	2.9 ± 0.6	**27**
PMZ	113.6 ± 18.5	6.6 ± 2,5	**17**
CPZSO	78.5 ± 2.7	25.2 ± 5.0	**3**
DMCPZ	60.9 ± 9.4	1.8 ± 0.6	**35**
DDMCPZ	66.9 ± 5.6	1.8 ± 0.3	**37**
SDS	202 ± 25	244 ± 48	**1**

Data represent Mean±SD from five independent experiments. CPZ and SDS were used as positive and negative control of phototoxicity, respectively. [a]According to the OECD (2004), PIF<2 means "No Phototoxicity", 2<PIF<5 means "Probably Phototoxicity" and PIF>5 means "Phototoxicity".

The IC_{50} were determined from dose-response curves for cell viability of cell treated with chemicals under light or dark conditions. More details are provided in Supplementary Material.

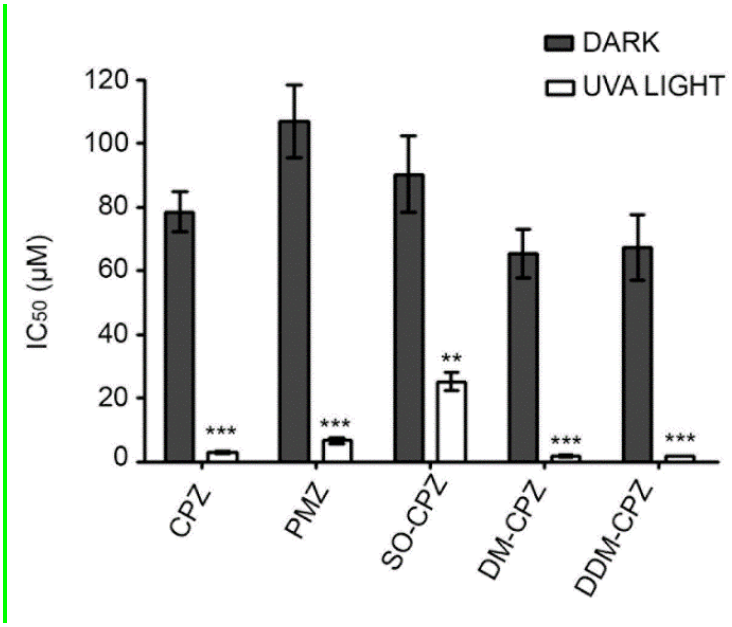


Figure 2. Phototoxicity of CPZ metabolites and photoproducts in the 3T3 NRU assay. 3T3 cells were treated with serial dilutions of CPZ metabolites ranging from 0.5

241 µM to 500 µM for 1h, followed by irradiation or not of a dose of 5 J/cm^2 UVA light. CPZ
242 was used as a positive control of phototoxicity[27] After 24 h cell viability was evaluated
243 by neutral red uptake and IC_{50} values were calculated by non-lineal regression with
244 GraphPad Prism 5.0. Data represent Mean±SD from five independent experiments and
245 asterisks significant differences relative to the IC_{50} Dark by the T-Student test (**$p \geq$
246 0.01; ***$p \geq 0.001$).

247

248 As shown in Table 1 and Figure 2, CPZ was clearly phototoxic as expected, while
249 promazine (dechlorinated photoproduct of CPZ) resulted less toxic upon irradiation with
250 a PIF of *ca*. 17. However, DMCPZ and DDMCPZ resulted be more phototoxic (a 1.6-
251 fold increase of the IC_{50}) than the parent drug. Both CPZ and its demethylated metabolites
252 DMCPZ and DDMCPZ display the same chromophore. Therefore, the different behavior
253 shown in phototoxic assays may be modulated by the alkyl chain for the lipophilicity
254 /hydrophilicity of the different substrates. By contrast, the CPZSO metabolite did not
255 exhibit any phototoxic potential. These results are in agreement with those obtained from
256 the *in vivo* phototoxicity study by means of mouse-tail technique.[28]

257 **3.2 Photosensitized DNA Damage**

258 It is well known that phototoxicity involves damage to biomolecules. In particular
259 photosensitized DNA damage is of interest since it plays an important role in
260 photogenotoxicity and may induce lethal mutagenic and/or carcinogenic effects.[29, 30]
261 Hence, irradiations of CPZ and its metabolites were performed in the presence of
262 supercoiled circular DNA (pBR322) to detect DNA damage. Single strand breaks (ssb)
263 can be observed directly whereas to reveal the nature of base damage the use of DNA-
264 repair enzymes is needed.

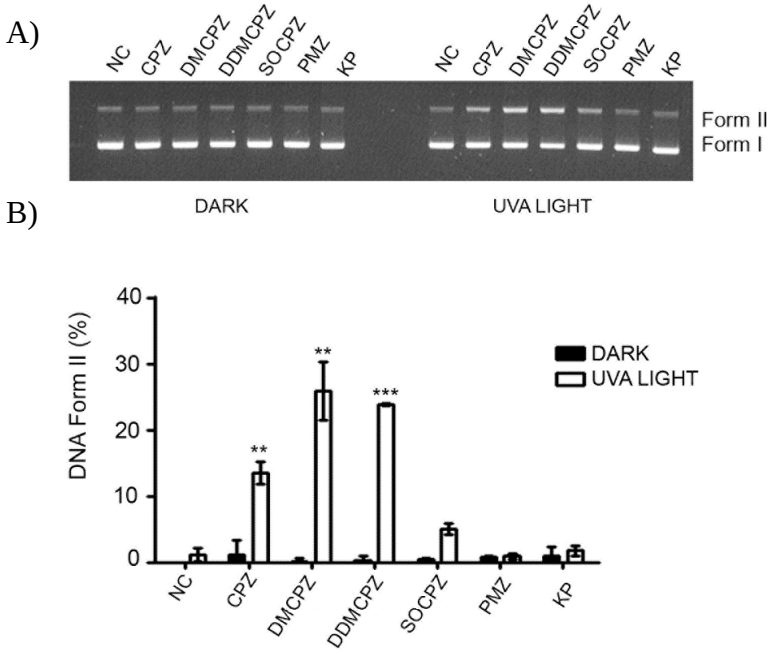


Figure 3. Photogenotoxicity of CPZ and its metabolites. (A) Agarose gel electrophoresis of supercoiled DNA (pBR322, 200 ng/µL) alone or in the presence of CPZ and its metabolites (10 µM) in the dark or upon irradiation (5 min) using a multilamp photoreactor λ_{max} = 355 nm. NC: Negative Control, KP: ketoprofen used as standard. (B) Formation of DNA Form II quantified by densitometry. Data represent Mean±SD from three independent experiments and asterisks significant differences relative to the formation of DNA Form II in Dark conditions by the T-Student test (**p ≥ 0.01; ***p ≥ 0.001). The initial value of Form II was subtracted from all samples.

Agarose gel electrophoresis (Figure 3 A) revealed a conversion of native supercoiled form I into circular form II, indicating ssb formation. Interestingly, quantification by densitometry of form II showed that DMCPZ and DDMCPZ display higher photogenotoxic potential than the parent drug (Figure 3 B). The DMCPZ and DDMCPZ metabolites resulted to be more hydrophilic and reactive than the parent drug, demonstrating that CPZ metabolism modulates the potential to photosensitize DNA damage.

Different DNA–repair enzymes Endo III, Fpg and Endo V were used in order to reveal oxidized pyrimidines, oxidized purines and cyclobutane thymine dimers, respectively. In all cases, ssb formation was accentuated for DMCPZ and DDMCPZ metabolites (data reported in Supplementary Material).

3.3 Photogenotoxicity detected by Comet Assay

Comet assay under alkaline conditions was performed to reveal the combination of single-strand breaks, double-strand breaks and alkali-labile sites in the cellular DNA. Thus, human fibroblasts (FSK) were embedded in agarose on a slide and incubated for 1 h with CPZ, DMCPZ and DDMCPZ. After 5 min of UVA exposure and immediate lysis, alkaline electrophoresis was performed. During electrophoresis, the damaged and fragmented DNA migrates away from the nucleus. Upon staining with SYBR Gold the fluorescence of the nuclei was examined and the total comet score (TCS) was calculated by using a classification of 6 DNA damage categories.[26]

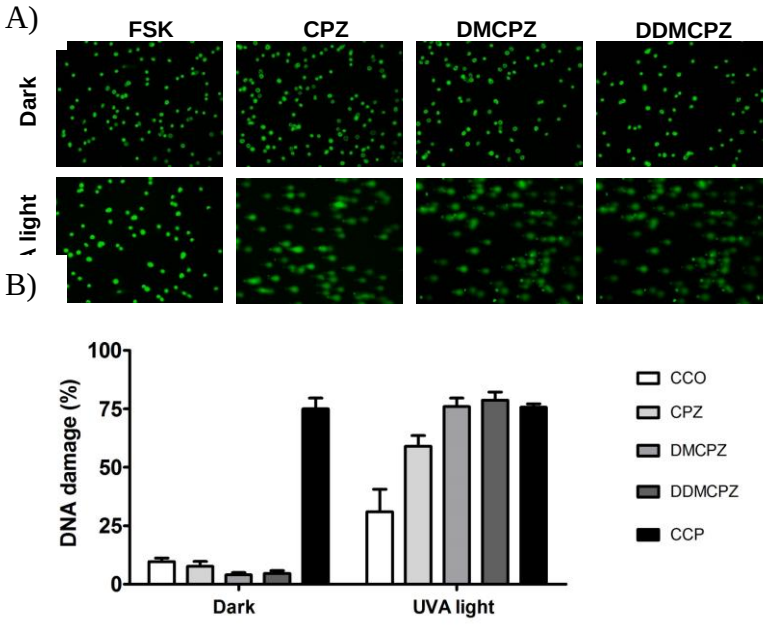


Figure 4. (A) Top: Unexposed FSK alone or treated with 10 μM of CPZ, DMCPZ and DDMCPZ. Bottom: Untreated or incubated with 10 μM of CPZ and its metabolites cells upon UVA irradiation. (B) Percentage of DNA damage obtained for chemicals tested calculated by means of 6 DNA damage categories. CCO and CCP were used as positive and negative control, respectively.

As shown in Figure 4, comet assays confirmed the damage induced by CPZ and its metabolites to cellular DNA, as fragmented DNA moves faster through agarose gel, forming a tail. These results are in agreement with CPZ metabolites displaying a higher photogenotoxic potential than the parent drug.

3.4 Photophysical Studies

3.4.1 Absorption Spectra

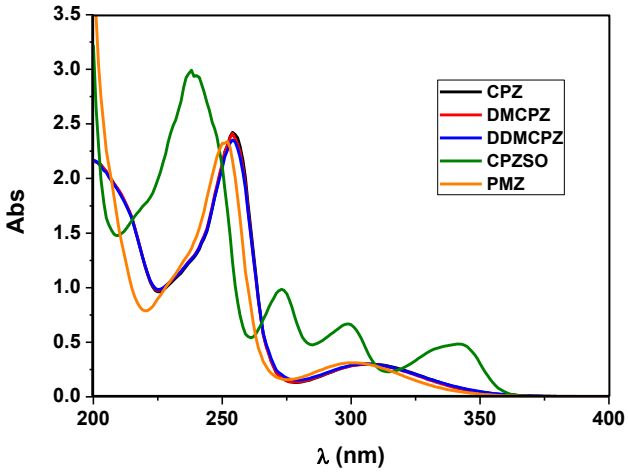


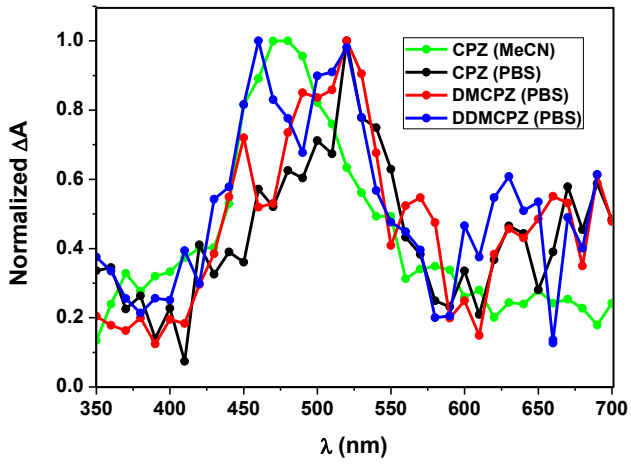
Figura 5. UV absorption spectra of CPZ, DMCPZ, DDMCPZ, CPZSO and PMZ in PBS at 5×10^{-5} M.

The absorption spectra of CPZ, DMCPZ, DDMCPZ and PMZ showed the typical UV-Vis spectral features of the phenathiazine chromophore, while CPZSO displayed bands that absorb more in the UVA region (Figure 5).

3. 4.2 Laser Flash Photolysis Studies

The transient absorption spectra of CPZ (λ_{exc} = 308 nm, air, flow cell) were recorded in MeCN (as model of lipophilic environment) and in aqueous medium (PBS). The normalized spectra are shown in Figure 6. The absorption band in MeCN exhibited a maximum at λ = 470 nm, attributed to the triplet excited state, in agreement with literature data.[31, 32] However, the maximum in PBS appeared at λ = 520 nm, and was due to the CPZ radical cation.[33-37] In aqueous medium, DMCPZ and DDMCPZ, exhibited both contributions from the triplet excited state and the radical cation. The decay traces monitored at λ = 470 nm (PBS, air) showed that the triplet lifetimes values of the three compounds were in the submicrosecond domain and increased with the methylated degree (Figure 6B).

A)



B)

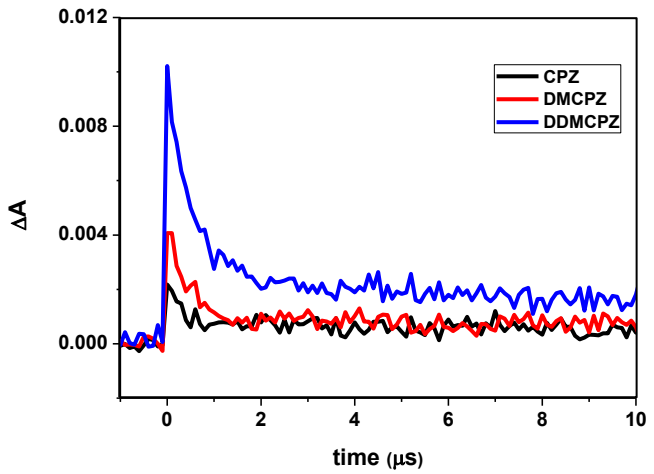
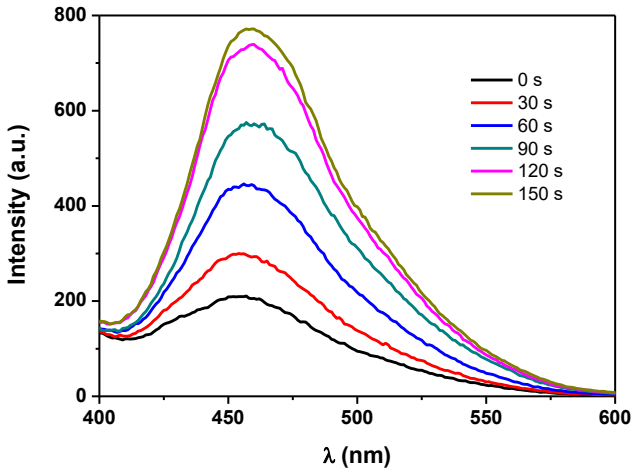


Figure 6. Laser flash photolysis (λ_{exc} = 308 nm, air) of CPZ and its metabolites. (**A**): Normalized spectra of CPZ in MeCN (green), CPZ in PBS (black), DMCPZ in PBS (red) and DDMCPZ in PBS (blue), recorded 0.2 μs after the laser pulse. (**B**): Normalized decay traces monitored at λ = 470 nm in PBS/air.

3.4.3 Steady-State Photolysis

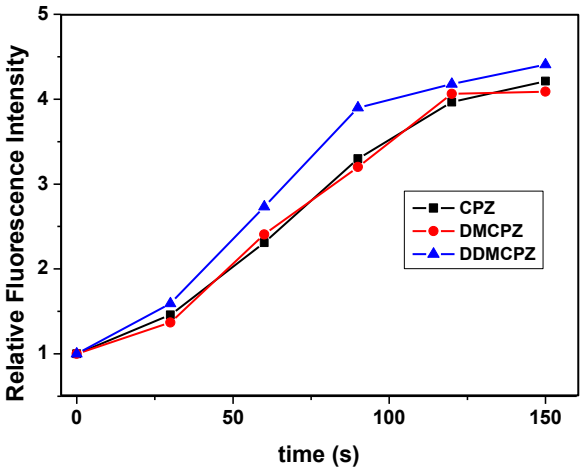
The course of the photoreaction of CPZ (λ_{irr} = 330 nm, PBS) was followed by monitoring the changes in its fluorescence spectra. The initial emission of CPZ (λ_{exc} = 330 nm/PBS) consisted of a band centered at 460 nm with quantum yield lower than 0.005. The fluorescence bands of the photomixtures were located in the same region, but their intensity increased with irradiation times (Figure 7). This is in agreement with the occurrence of a photodehalogenation reaction, leading to the corresponding promazines, whose fluorescence quantum yields are much higher. A comparison of the fluorescence intensity changes at 500 nm with irradiation times showed a similar trend for CPZ, DMCPZ and DDMCPZ (Figure 7B).

A)



345

B)



346

347 **Figure 7**. Monitorization of irradiations by fluorescence. Conditions: λ_{irr} and λ_{exc} = 330 nm, 5 ×
348 10^{-5}, PBS, air; (**A**) Spectra obtained after irradiation of CPZ from 0 to 150 s; (**B**) Fluorescence
349 intensity values at λ = 500 nm for CPZ (black), DMCPZ (red) and DDMCPZ (blue) at different
350 irradiation times.

351 **Electron Paramagnetic Resonance**

352 To confirm the presence of intermediates of radical nature, an EPR study was performed.
353 As a matter of fact, the spin adducts obtained from 2-methyl-2-nitrosopropane (MNP)
354 and the aryl radicals of CPZ, DMCPZ and DDMCPZ were observed in all cases (Figure
355 8). This is in agreement with the photodehalogenation reaction, initiated by homolytic
356 cleavage of the C-Cl bond.

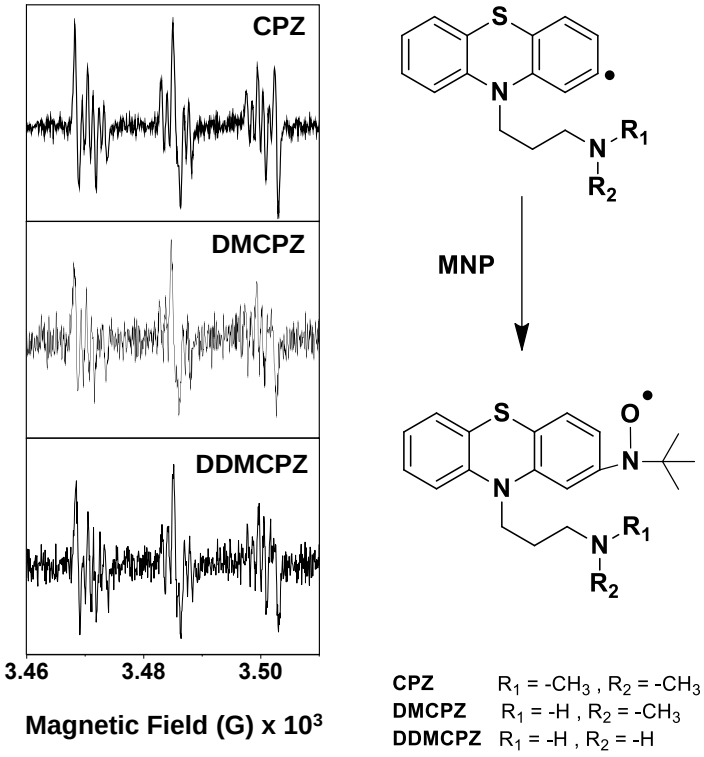


Figure 8. EPR spectra of MNP adduct of aryl radical from CPZ, DMCPZ and DDMCPZ in H_2O (λ_{exc} = 330 nm, N_2). The concentrations of drug/metabolite and MNP were 1.4 mM in all cases.

Acknowledgements

Miguel Servet Contract for I. A. and FPU fellowship for F.P. is gratefully acknowledged.

Funding

This work was supported by the Carlos III Institute of Health (Grants: RD12/0013/0009 and CP11/00154) and the Generalitat Valenciana (Prometeo Program).

REFERENCES

(1) T B Fitzpatrick, M A Pathak, I A Magnus, a., and Curwen, W. L. (1963) Abnormal Reactions of Man to Light. *Annual Review of Medicine 14*, 195-214.

(2) Satanove, A., and McIntosh, J. S. (1967) PHototoxic reactions induced by high doses of chlorpromazine and thioridazine. *JAMA 200*, 209-212.
(3) Epstein, J. H., Brunsting, L. A., Petersen, M. C., and Schwarz, B. E. (1957) A Study of Photosensitivity Occurring with Chlorpromazine Therapy11Presented at the Seventeenth Annual Meeting of The Society for Investigative Dermatology, Inc., Chicago, Illinois, June 10, 1956. *Journal of Investigative Dermatology 28*, 329-338.
(4) Johnson, B. E. (1974) Cellular mechanisms of chlorpromazine photosensitivity. *Proceedings of the Royal Society of Medicine 67*, 871-873.
(5) Ljunggren, B., and Moller, H. (1977) Phenothiazine phototoxicity: an experimental study on chlorpromazine and related tricyclic drugs. *Acta dermato-venereologica 57*, 325-329.
(6) Kochevar, I. E., Chung, F.-L., and Jeffrey, A. M. (1984) Photoaddition of chlorpromazine to DNA. *Chemico-Biological Interactions 51*, 273-284.
(7) Motten, A. G., Buettner, G. R., and Chignell, C. F. (1985) SPECTROSCOPIC STUDIES OF CUTANEOUS PHOTOSENSITIZING AGENTS–VIII. A SPIN-TRAPPING STUDY OF LIGHT INDUCED FREE RADICALS FROM CHLORPROMAZINE and PROMAZINE. *Photochemistry and Photobiology 42*, 9-15.
(8) Beijersbergen van Henegouwen, G. M. J. (1997) *Medicinal Photochemistry (phototoxic and phototherapeutic aspect of drugs)*.
(9) Castell, J. V., José Gómez-Lechón, M. A., Miranda, M. A., and Morera, I. M. (1987) Toxic effects of the photoproducts of chlorpromazine on cultured hepatocytes. *Hepatology 7*, 349-354.
(10) Huang, H.-T., Chang, C.-L., and Tzeng, D.-S. (2010) Toxic epidermal necrolysis after sun-exposure probably due to lamotrigine and chlorpromazine. *Asian Journal of Psychiatry 3*, 240-242.
(11) De Mol, N. J., and Busker, R. W. (1984) Irreversible binding of the chlorpromazine radical cation and of photoactivated chlorpromazine to biological macromolecules. *Chemico-Biological Interactions 52*, 79-92.
(12) Viola, G., Latterini, L., Vedaldi, D., Aloisi, G. G., Dall'Acqua, F., Gabellini, N., Elisei, F., and Barbafina, A. (2003) Photosensitization of DNA Strand Breaks by Three Phenothiazine Derivatives. *Chemical Research in Toxicology 16*, 644-651.
(13) Beckett, A. H., Beaven, M. A., and Robinson, A. E. (1963) Metabolism of chlorpromazine in humans. *Biochemical Pharmacology 12*, 779-794.
(14) Hartmann, F., Gruenke, L. D., Craig, J. C., and Bissell, D. M. (1983) Chlorpromazine metabolism in extracts of liver and small intestine from guinea pig and from man. *Drug Metabolism and Disposition 11*, 244-248.
(15) Chetty, M., Moodley, S. V., and Miller, R. (1994) Important Metabolites to Measure in Pharmacodynamic Studies of Chlorpromazine. *Therapeutic Drug Monitoring 16*, 30-36.
(16) Wójcikowski, J., Boksa, J., and Daniel, W. A. (2010) Main contribution of the cytochrome P450 isoenzyme 1A2 (CYP1A2) to N-demethylation and 5-sulfoxidation of the phenothiazine neuroleptic chlorpromazine in human liver—A comparison with other phenothiazines. *Biochemical Pharmacology 80*, 1252-1259.
(17) Pearson, P. G., and Wienkers, L. C. (2009) *Handbook of Drug Metabolism, 2nd Ed.*
(18) EMEA. (2002) *Note for guidance on photosafety testing*.
(19) FDA, C. (2003) *Guidance for industry photosafety testing*.
(20) Kitamura, K., Fujitani, K., Takahashi, K., Tanaka, Y., Hirako, S., Kotani, C., Hashimoto, T., and Takegami, S. (2000) Synthesis of [N-13CH3] drugs (chlorpromazine, triflupromazine and promazine). *Journal of Labelled Compounds and Radiopharmaceuticals 43*, 865-872.
(21) Zhou, Z., Franz, A. W., Bay, S., Sarkar, B., Seifert, A., Yang, P., Wagener, A., Ernst, S., Pagels, M., Müller, T. J. J., and Thiel, W. R. (2010) Redox Active Mesoporous Hybrid Materials by In situ Syntheses with Urea-linked Triethoxysilylated Phenothiazines. *Chemistry – An Asian Journal 5*, 2001-2015.

(22) Owens, M. L., Juenge, E. C., and Poklis, A. (1989) Convenient oxidation of phenothiazine salts to their sulfoxides with aqueous nitrous acid. *Journal of Pharmaceutical Sciences 78*, 334-336.
(23) OECD. (2004) *OECD Guidelines for the Testing of Chemicals Test No. 432: In Vitro 3T3 NRU Phototoxicity Test (Original Guideline, adopted 13th April 2004)*.
(24) Azqueta, A., and Collins, A. R. (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Archives of Toxicology 87*, 949-968.
(25) Collins, A. R., El Yamani, N., Lorenzo, Y., Shaposhnikov, S., Brunborg, G., and Azqueta, A. (2014) Controlling variation in the comet assay. *Frontiers in Genetics 5*.
(26) Møller, P. (2006) Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. *Mutation Research/Reviews in Mutation Research 612*, 84-104.
(27) Nathalie, D., Yannick, G., Caroline, B., Sandrine, D., Claude, F., Corinne, C., and Pierre-Jacques, F. (2006) Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay. *Toxicology in Vitro 20*, 480-489.
(28) Ljunggren, B., and Möller, H. (1977) Phenothiazine Phototoxicity: an Experimental Study on Chlorpromazine and its Metabolites. *Journal of Investigative Dermatology 68*, 313-317.
(29) Loveday, K. S. (1996) Interrelationship of Photocarcinogenicity, Photomutagenicity and Phototoxicity. *Photochemistry and Photobiology 63*, 369-372.
(30) Klecak, G., Urbach, F., and Urwyler, H. (1997) Fluoroquinolone antibacterials enhance UVA-induced skin tumors. *Journal of Photochemistry and Photobiology B: Biology 37*, 174-181.
(31) García, C., Oyola, R., Piñero, L. E., Arce, R., Silva, J., and Sánchez, V. (2005) Substitution and Solvent Effects on the Photophysical Properties of Several Series of 10-Alkylated Phenothiazine Derivatives. *The Journal of Physical Chemistry A 109*, 3360-3371.
(32) Navaratnam, S., Parsons, B. J., Phillips, G. O., and Davies, A. K. (1978) Laser flash photolysis study of the photoionisation of chlorpromazine and promazine in solution. *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases 74*, 1811-1819.
(33) Joshi, R., Ghanty, T. K., and Mukherjee, T. (2008) Reactions and structural investigation of chlorpromazine radical cation. *Journal of Molecular Structure 888*, 401-408.
(34) Rosenthal, I., Ben-Hur, E., Prager, A., and Riklis, E. (1978) PHOTOCHEMICAL REACTIONS OF CHLORPROMAZINE; CHEMICAL AND BIOCHEMICAL IMPLICATIONS. *Photochemistry and Photobiology 28*, 591-594.
(35) Piette, J., Decuyper, J., Merville-Louis, M.-P., and Van De Vorst, A. (1986) Molecular, Cellular and Medical Aspects of PhotosensitizationBiomolecular photoalterations mediated by phenothiazine derivatives. *Biochimie 68*, 835-842.
(36) Nath, S., and Sapre, A. V. (2001) Photoinduced electron transfer from chloropromazine and promethazine to chloroalkanes accompanied by cleavage of C–Cl bond. *Chemical Physics Letters 344*, 138-146.
(37) Garcia, C., Smith, G. A., McGimpsey, W. G., Kochevar, I. E., and Redmond, R. W. (1995) Mechanism and Solvent Dependence for Photoionization of Promazine and Chlorpromazine. *Journal of the American Chemical Society 117*, 10871-10878.