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In vitro studies of the phototoxic potential of the antidepressant drugs amitriptyline and imipramine

Giampietro Viola, Giorgia Miolo, Daniela Vedaldi, Francesco Dall'Acqua *

Department of Pharmaceutical Sciences, University of Padua, and Center for Chemical Investigation of Drugs (associated to the National Institute for the Chemistry of Biological Systems-CNR), via Marzolo 5, 35131 Padua, Italy

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Abstract

Amitriptyline and imipramine, two tricyclic antidepressant drugs, have been studied to evaluate their phototoxic potential using various models. Reactive oxygen species production was investigated. A negligible production of singlet oxygen was observed for both compounds whereas a significant production of superoxide anion was noted for amitriptyline in particular. Moderate red blood cell lysis under UVA light (365 nm) was induced in the presence of the two drugs at a concentration of 50 μM. Cellular phototoxicity was investigated on a murine fibroblast cell line (3T3). The two drugs were phototoxic causing cell death at a concentration of 100 μM and a UVA dose in the range of 3.3–6.6 J/cm^2. Furthermore, the two drugs photosensitized the peroxidation of linoleic acid, as monitored by the formation of dienic hydroperoxides. The presence of BHA and GSH, two free radical scavengers, significantly reduced the lipid oxidation photoinduced by the drugs, suggesting a predominant involvement of radical species. Finally, the involvement of nucleic acids in the phototoxicity mechanism was also investigated using a pBR322 plasmid DNA as a model.

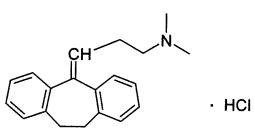
Keywords: Amitriptyline; Imipramine; Phototoxicity; Photohemolysis; Lipid peroxidation; DNA photocleavage

1. Introduction

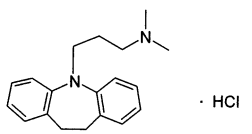
Phototoxic or photoallergic response to systemically or topically administered drugs are frequently reported as adverse drug reactions. Several classes of drugs exhibit this type of side effect, including antibacterials, thiazide diuretics, non-steroidal antiinflammatory drugs, quinolones and tricyclic antidepressant [1,2].

For this last family of drugs various reports exist concerning their phototoxic properties, although they are focalized principally on protriptyline [3–7] which causes skin photosensitization in humans. Moreover, mechanistic studies have addressed these properties regarding the presence of a conjugated C10–C11 double bond in the tricyclic chromophore of protriptyline [5], in terms of photochemical reactivity.

Amitriptyline (3-(10,11)-dihydro-5*H*-dibenzo[a,d]cyclohepten-5-ylidene)-*N*,*N*-dimethyl-1-propanamine) and imipramine (10,11-dihydro-*N*,*N*-dimethyl 5*H*-dibenz[b,f]azepine-5- propanamine) (Fig. 1) are two tricyclic drugs used in the treatment of depression. In contrast with the parent drug protriptyline, they lack



Imipramine



Amitriptyline

Fig. 1. Molecular structures of imipramine and amitriptyline.

* Corresponding author. Tel.: +39-049-8275336; fax: +39-049-8265366.

E-mail address: dacqua@purple.dsfarm.unipd.it (F. Dall'Acqua)

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the double bond and therefore undergo different photoreactions [5].

To date, many anecdotal reports have been made to the GISED (Gruppo Italiano Studi Epidemiologici Dermatologici), suggesting that the two compounds may cause skin photosensitivity reactions.

This paper describes our studies that were aimed at characterizing the phototoxic properties of these two compounds, in general, and reporting a preliminary study of their photodegradation. In particular, observations on the in vitro effects on murine erithrocytes, using photohemolysis as an endpoint, and an evaluation of the cellular phototoxicity on murine fibroblasts are reported. We have also extended our study on the photochemical damage induced by these two drugs on biological molecules, such as lipids and DNA, to better characterize the cellular targets of their phototoxic reactions.

2. Experimental

2.1. Chemicals

Amitriptyline hydrochloride was purchased from Sigma (St. Louis, USA). Imipramine was purchased from RBI (Research Biochemicals International).

N,*N*-Dimethyl-4-nitrosoaniline (RNO) and imidazole were obtained from Merck Schuchardt. Nitroblue tetrazolium (NBT), linoleic acid, 2,6-di-*tert*-butylhydroxyanisole (BHA), glutathione reduced form (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and agarose were obtained from Sigma (St. Louis, USA). pBR322 DNA and HInd III restriction enzyme and ethidium bromide solution were from Pharmacia Biotech AB (Uppsala, Sweden). All other reagents were of analytical grade.

2.2. Irradiation procedure

Two HPW 125 Philips lamps, mainly emitting at 365 nm, were used for irradiation experiments. The total energy, detected by a radiometer (Cole-Parmer Instrument Company, Niles, IL), was 0.262 J/cm^2/min.

2.3. Absorption properties and photodegradation experiments

The capacity of the compounds to absorb light in the UV–Vis range was studied in both aqueous and 1:1 water–methanol solutions. The photodegradation spectra on increasing UVA doses was also monitored by a Lambda 12 Perkin–Elmer spectrophotometer.

2.4. HPLC analysis

Amitriptyline and imipramine were diluted at a concentration of 10^{-5} M in double distilled water. Irradiation of the solutions was performed with increasing UVA doses with two HPW 125 Philips lamps, under controlled room temperature (r.t.) (water bath). After irradiation, all samples were filtered (0.22 μm cellulose acetate filter membranes, Lida Manufacturing Corp. Kenosha, WI, USA) before HPLC elution. Analytical HPLC was performed with a reverse phase column C-18 (Waters Spherisorb, S5 ODS2, 4.6 × 250 mm, 5 μm), The mobile phase consisted of a mixture of 60:39.5:0.5 acetonitrile–water–triethylamine, pH 3 (H_3PO_4), flow rate 1.0 ml/min. The eluted species were detected at 250 and 300 nm, using a photodiode array detector (LC-235, Perkin–Elmer).

2.5. Production of reactive oxygen species

Singlet oxygen was determined following the Kraljic and El Moshni procedure [8]. Samples containing the compounds under examination (4.4×10^{-6} M), *p*-nitrosodimethylaniline (4×10^{-5} M) and imidazole (4×10^{-5} M) in phosphate buffer (0.02 M, pH 7.3) were irradiated (365 nm) for different periods of time in quartz cuvettes (1 cm) after oxygen saturation, and their absorbance at 440 nm was then measured.

Superoxide anion was determined following the Pathak and Joshi procedure [9]. Samples containing the compounds under examination (4.4×10^{-6} M) and nitroblue tetrazolium (1.6×10^{-4} M) in phosphate buffer (pH 10) were irradiated (365 nm) for increasing times after oxygen saturation, and their absorbance at 560 nm was measured.

2.6. Cell cultures

Balb/c mouse 3T3 fibroblasts were a kind gift from Dr M. Scarpa (Department of Pediatrics, University of Padova). Cultures were grown in DMEM medium (Dulbecco's modified Eagle medium, Sigma) supplemented with 115 units/ml of penicillin G (Gibco Laboratories), 115 μg/ml streptomycin (Gibco Laboratories) and 10% fetal calf serum (Gibco Laboratories).

Individual wells of 96-well tissue culture microtiter plate (IWAKI, Japan) were inoculated with 100 μl of DMEM containing 5×10^3 mouse 3T3 cells. The plate was incubated at 37°C in a humidified 5% CO_2 incubator for 72 h to form a monolayer of ca. 100% confluence.

The medium was then removed and 100 μl of the drug solution dissolved in ethanol and diluted with Hank's balanced salt solution (HBSS pH 7.2) were added to each well.

The plate was then incubated for 15 min in an atmosphere of 5% CO_2 at 37°C, the control plate was stored in the dark. After irradiation, the solution was

replaced by the medium and the plates were placed in the incubator for 24 h. Cell viability was assayed by the MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide).

2.7. MTT assay

The MTT assay was performed by the Mosmann method [10]. Ten microliters of MTT reagent (5 mg/ml) was added to each well after an additional 24 h incubation (after UVA irradiation).

The plate was then incubated at 37°C for 4 h. Acidic isopropanol (100 μl of 0.08 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the formazan crystals.

After a few minutes at r.t. to ensure that all crystals were dissolved, the plate was read on a microtiter plate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Absorbance was corrected by subtracting the mean value obtained from non-seeded wells. Results were expressed defining the mean absorbance of the HBSS control cultures (unirradiated cells) as 100% viability.

2.8. Photohemolysis

Blood was collected from untreated albino mice using heparin as anticoagulant. The blood was washed with PBS (0.01 M phosphate buffer, 0.135 M NaCl, pH 7.4), centrifuged (2500 rpm for 15 min) and the supernatant discarded. The procedure was repeated until the supernatant was colourless. RBC were resuspended in PBS (1:1000) and used within 48 h. For the photohemolysis experiments RBC were incubated with the compounds under study (50 μM) dissolved in 1:1 water–methanol mixture for 15 min at 37°C in the dark. The suspension was then irradiated with increasing doses under gentle shaking in a controlled temperature bath.

Hemolysis was determined by spectrophotometric measurement at 650 nm where intact cells absorb. Control samples: (a) untreated RBC (0% hemolysis); (b) sonicated or diluted 1:1000 with water RBC (100% hemolysis); (c) RBC in the presence of compounds and kept in the dark; (d) RBC irradiated without compounds.

All samples were maintained in the same conditions for the same periods of time, including irradiation times.

2.9. Linoleic acid peroxidation

Linoleic acid was irradiated in the presence of compounds (1×10^{-5} M), as described by Zhou and Moore [11], (pH 7.4, 0.135 M NaCl) containing 0.05% Tween 20, as emulsifying agent, with increasing UVA doses. The peroxidation of linoleic acid was monitored at 233 nm by the absorbance value of the peak corresponding to the conjugated dienic hydroperoxydes formed during irradiation [12].

2.10. PBR322 DNA strand breaks

Each pBR322 DNA sample (100 ng) dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was irradiated with increasing UVA doses in the presence of the compounds under examination (2.5×10^{-4} M). The DNA was precipitated by adding 0.3 M sodium acetate and 2.5 volumes of cold ethanol and kept on ice for 60 min. After centrifugation for 30 min at 12000 rpm at 4°C, the DNA was washed with cold 80% ethanol, centrifuged for 15 min and dried in a Speed-Vac Concentrator (Savant Instruments Inc., NY). After precipitation, the DNA was redissolved in water before loading onto the gel. The samples were loaded on 1% agarose gel ($7 \times 10 \times 1$ cm), after addition of 1 μl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% w/v sucrose) to each sample. The electrophoretic run was carried out in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA) at 25 V for 6 h, using the GNA-100 electrophoretic apparatus (Pharmacia, Uppsala, Sweden). After staining in ethidium bromide solution (1 μg/ml in TAE buffer) for 30 min, the gel was washed with water and the DNA bands were visualized by the Gel Doc 1000 system (BIO-RAD).

As a control, pBR322 DNA was linearized with EcoRI restriction enzyme (9 units/μg of DNA) in a buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiotreitol (high salt buffer) at 37°C for 30 min.

3. Results

3.1. Photodegradation studies

Absorbance spectra of intact drugs are reported in Fig. 2. The decrease in UV absorbance, as a preliminary indication of photodegradation of the compounds in phosphate buffer solution under increasing doses of UVA light, was studied. The extent of UV absorbance decrease is shown in the same figure for both compounds.

The formation of photoproducts after exposure of the two compounds to high UVA doses (up to 33 J/cm^2) was assessed by HPLC analysis. Separation of the photoproducts formed from the intact compound and a calculation of the corresponding area, before and after irradiation, allowed us to establish the amount of photodegraded compound.

Both drugs investigated exhibited very low degradation under UVA exposures. In particular, from the

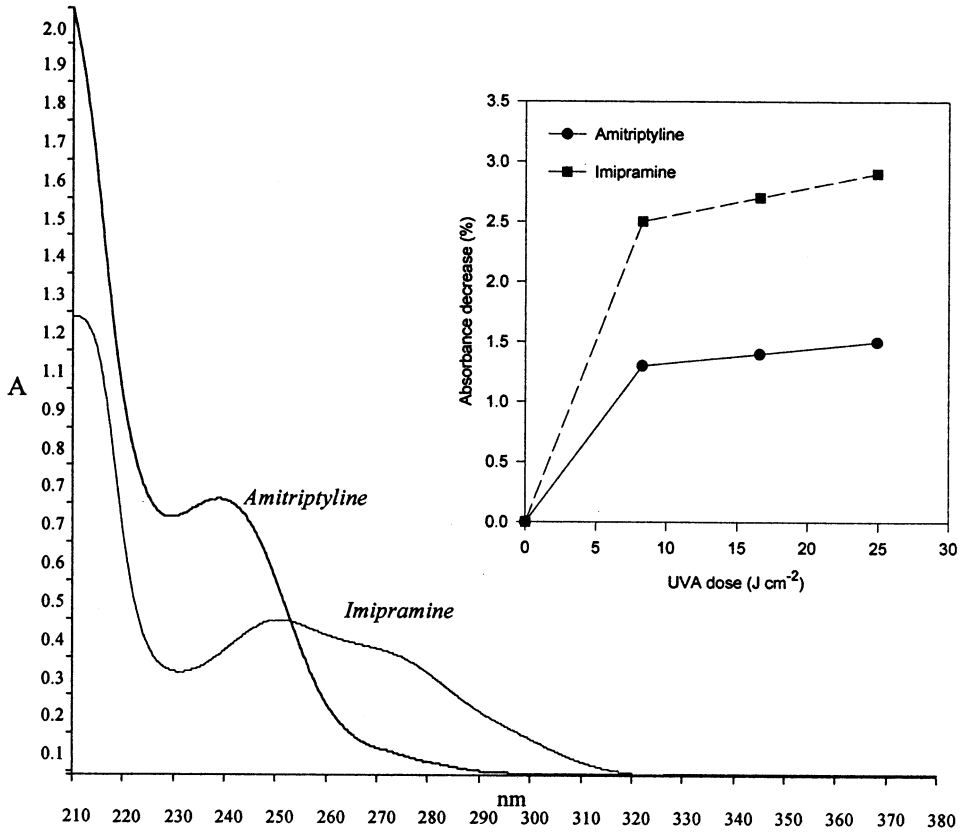

Fig. 2. UV absorbance spectra of imipramine and amitriptyline (50 μM) in phosphate buffer pH 7.2. Inset: percentage of photodegradation of imipramine and amitriptyline drugs evaluated spectrophotometrically by decrease in peaks at 251 and 239 nm, respectively.

HPLC data, it can be seen that imipramine degraded by about 1.2% (98.8 intact) and amitriptyline remained almost intact (99.6%), under UVA light.

Corresponding chromatograms (absorbance at 250 nm), are shown in Fig. 3A (imipramine) and 3B (amitriptyline).

3.2. Production of reactive oxygen species

The production of singlet oxygen ($^1O_2$) by the compounds examined was determined using the method proposed by Kralijc and El Moshni (8), based on bleaching of *N*,*N*-dimethyl-*p*-nitrosoaniline with $^1O_2$ in the presence of imidazole and recording the changes in absorbance at 440 nm.

Equimolar concentrations of proflavine hemisulfate and rose bengal, two well-known singlet oxygen generators, were used as reference compounds.

Fig. 4A shows the kinetics of RNO bleaching after irradiation in the presence of the two drugs. It can be observed that at the highest UVA dose considered we reached a low percentage.

If we consider that, in the same conditions, rose bengal and proflavine produce 36 and 19% of RNO bleaching, respectively, we can conclude that the two drugs are not able to generate singlet oxygen to significant levels.

Superoxide anion ($O_2^{\bullet -}$), was determined following the spectrophotometric method proposed by Pathak and Joshi [9], based on the reduction of nitro blue tetrazolium by superoxide anion and monitoring the increase in absorbance at 560 nm corresponding to the formation of formazan blue.

Fig. 4B shows the increase in absorbance produced by the test compounds after UVA irradiation, exhibiting a pronounced effect of amitriptyline. Instead, a slow increase in absorbance was observed with imipramine.

It seems quite clear from the above data that the photoexcited states of these compounds do not lead to the formation of reactive oxygen species except for amitriptyline that indeed causes a significant production of superoxide anion.

3.3. Red blood cell hemolysis

Red blood cells have been used by a large number of investigators as a model for phototoxicity studies for preliminary indications on potential phototoxic agents. Albino mouse red blood cells (RBC) were used for this experiment since it has been demonstrated that they are

more sensitive than human ones. Moreover, because of their simple cellular structure they can be considered as a model for studying membrane damage [13].

Photohemolysis data were obtained from experiments with oxygenated suspensions of mouse RBC containing the test compounds at a concentration of 50 μM. It can

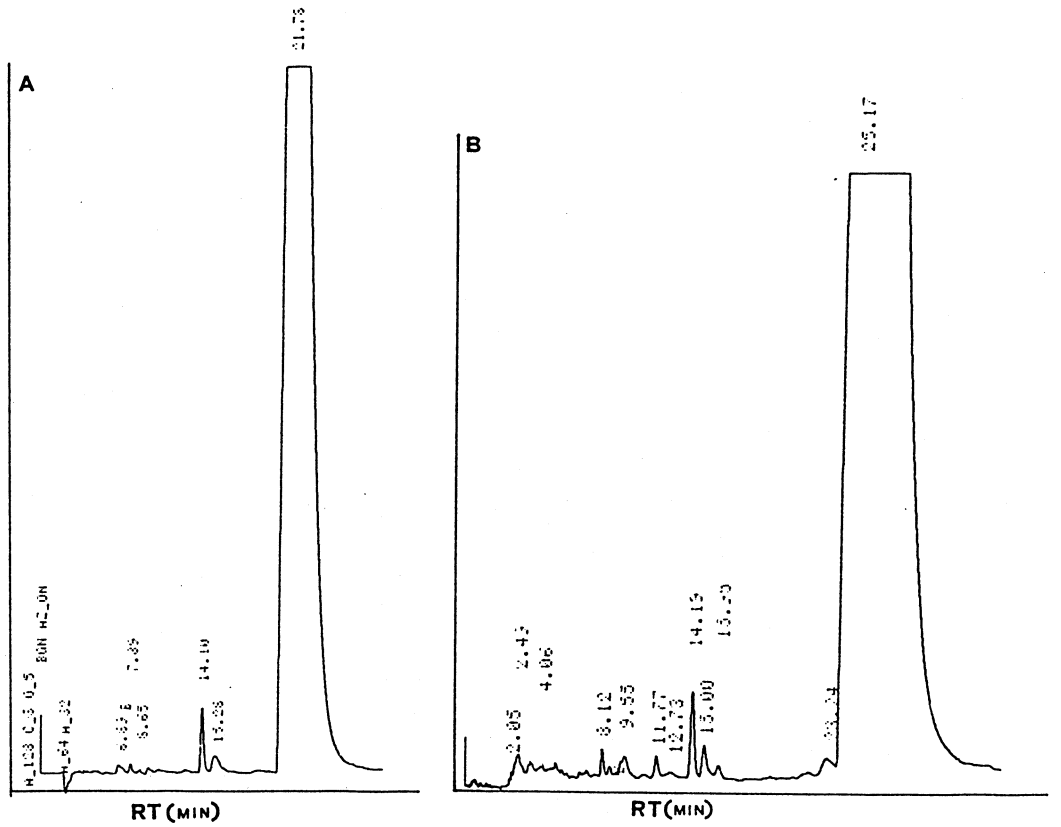

Fig. 3. HPLC chromatograms of irradiated (33 J/cm^2) imipramine (A) and amitryptiline (B). Column: C-18 (Waters Spherisorb, S5 ODS2, 4.6 × 250 mm, 5 μm).; mobile phase: acetonitrile:water: triethylamine, 60:39.5:0.5, pH 3 (H_3PO_4), flow rate 1.0 ml/min; absorbance at 250 nm.

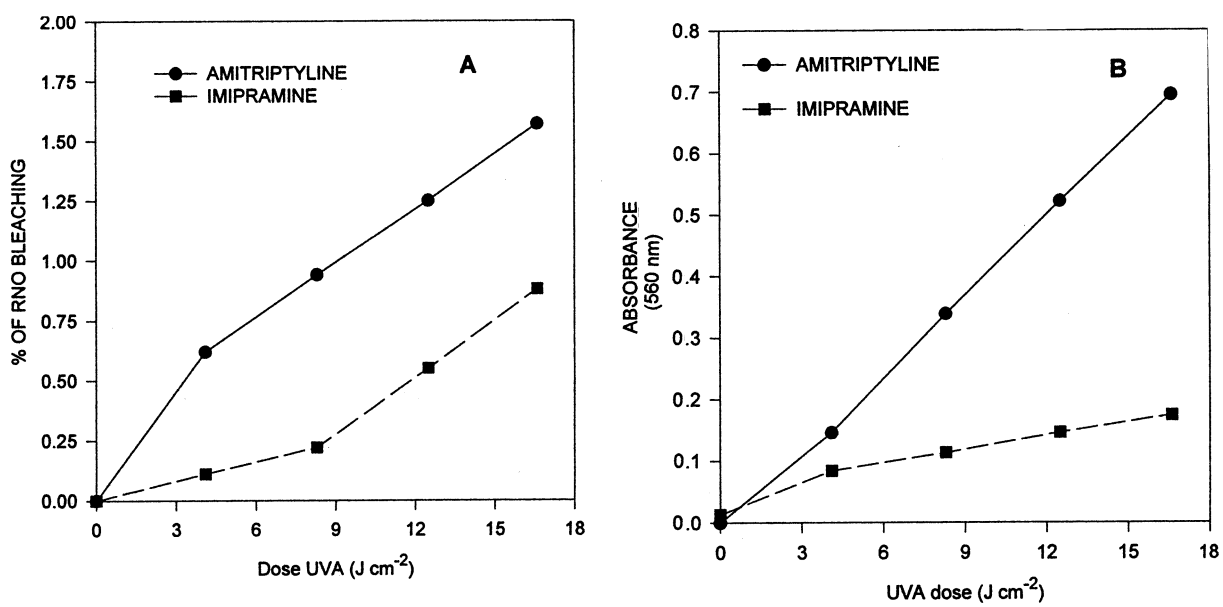

Fig. 4. Percentage of RNO bleaching at 440 nm (A) and increase in absorbance of nitroblue tetrazolium at 560 nm (B), induced by imipramine and amitriptyline.

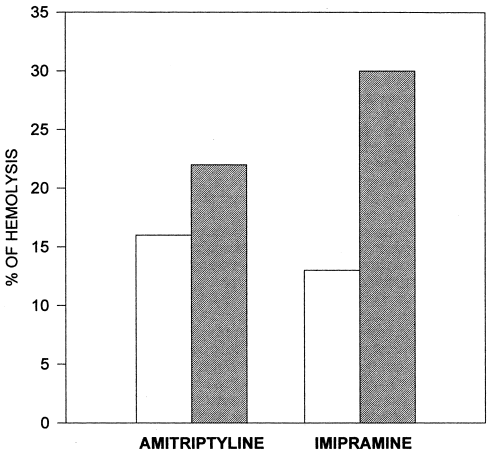

Fig. 5. Percentage of hemolysis of mouse red blood cells induced by imipramine (50 μM) and amitriptyline (50 μM) in the dark (white bars) and after UVA irradiation at the dose of 16.6 J/cm^2 (shaded bars).

be seen in Fig. 5 that the extent of photohemolysis induced by imipramine is not relevant; in fact, the percentage of hemolysis reached after a UVA dose of 16.6 J/cm^2 is about 30% (the contribution of UVA alone is about 20%). For amitriptyline there is no difference between the hemolysis induced by the drug in the dark and after irradiation. In this case a significant dark hemolysis is observed. This effect is related to the lipophilic properties of the two molecules, in particular to the ability of these drugs to accumulate inside the biological membrane modifying its permeability.

3.4. Cellular phototoxicity

The phototoxicity of amitriptyline and imipramine was investigated on a cultured cell line of murine fibroblasts (Balb/c 3T3) considered as an in vitro model for skin irritation and photosensitization.

Table 1 shows the extent of cell survival expressed as a percentage of cell viability after UVA irradiation. The number of viable cells was determined by the MTT test 24 h after irradiation. The two antidepressant drugs were tested at two concentrations, 50 and 100 μM. Cell viability was slightly reduced in the presence of imipramine incubated in the dark, in the same conditions used under UVA light, and at both concentrations tested. The values obtained at 100 and 50 μM were 86.8 ± 1.6 and 90.6 ± 6.6, respectively. In contrast, no dark toxicity was observed for amitriptyline.

After irradiation, neither compound at a concentration of 50 μM had a phototoxic effect. A significant effect was seen at a concentration of 100 μM for both drugs and for all doses of UVA light used.

3.5. Linoleic acid peroxidation

To determine whether lipid peroxidation could play a role in the photosensitivity caused by the compounds examined, their photosensitizing effects on a simple model of lipid peroxidation was investigated. The ability of the two drugs to photoinduce lipid peroxidation was investigated using linoleic acid, a polyunsatured fatty acid, measuring the progressive increase in absorbance at 233 nm corresponding to the formation of dienic hydroperoxides [12].

Fig. 6 shows the extent of peroxidation as a function of UVA dose for amitriptyline and imipramine, at a concentration of 50 μM.

An evident increase in the absorbance at 233 nm can be seen as a function of UVA dose, showing similar kinetics for both drugs. In particular they reach a plateau at about 10 J/cm^2 of UVA light, with a final hydroperoxide concentration (between 10^{-4} and 10^{-5} M) calculated on the basis of a known extinction coefficient [14], suggesting that the capability of the two drugs to photoinduce lipid oxidation has reached a steady state. This behavior is similar to that observed in photodegradation studies for which the curves displayed almost the same shape.

Moreover, to investigate the molecular mechanisms involved in drug-induced photoperoxidation of lipids, the extent of linoleic acid peroxidation was determined in the presence of BHA (2,6-di-*tert*-butylhydroxyanisole) and GSH (reduced glutathione) two well-known scavengers of free radical species. On the basis of the results presented above regarding the negligible production of singlet oxygen by the drugs, we did not use singlet oxygen quenchers in these experiments.

Fig. 7 shows that the addition of BHA at a concentration of 1 μM significantly inhibits lipid peroxidation.

Table 1
Percentage of cell viability of Balb/c 3T3 fibroblast cells after exposure to the indicated doses of UVA, in the presence of amitriptyline and imipramine [a]

UVA dose (J/cm^2)	UVA controls	Amitriptyline		Imipramine	
		50 μM	100 μM	50 μM	100 μM
3.3	105.2 ± 3.3	101.4 ± 14.5	72.7 ± 8.5.	104.7 ± 12.9	69.8 ± 8.4
6.6	98.8 ± 3.1	108.2 ± 4.1	68.2 ± 8.2	97.3 ± 11.5	54.7 ± 9.2

[a] The cytotoxicity was determined by the MTT test after 24 h from irradiation. Data expressed as mean ± SEM for at least five independent experiments performed in quadruplicate.

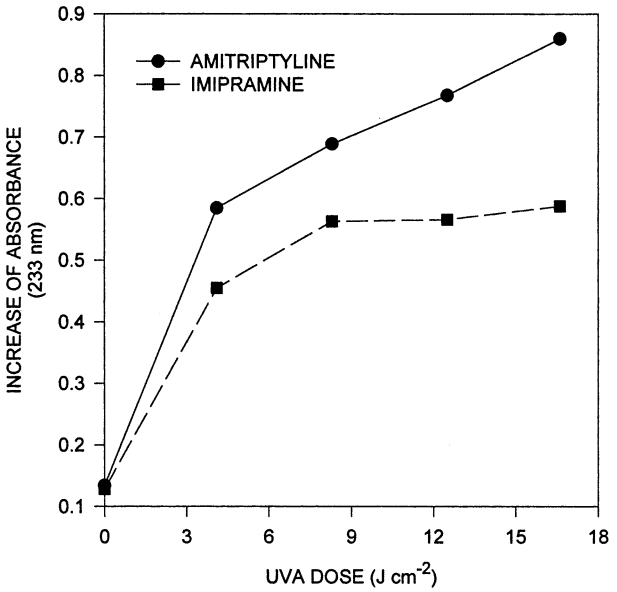

Fig. 6. Photoperoxidation of linoleic acid by amitriptyline (50 μM) and imipramine (50 μM) as a function of UVA dose. The formation of dienic hydroperoxides was followed by the increase in absorbance at 233 nm.

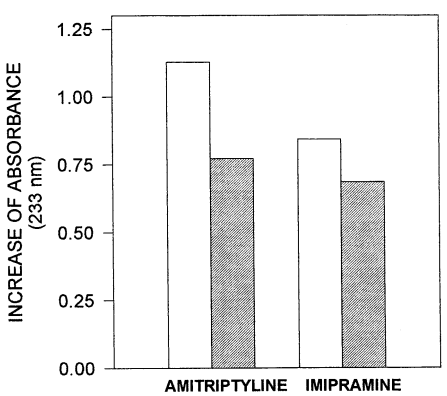

Fig. 7. Effect of BHA (2,6-di-*tert*-butylhydroxyanisole) on drug-induced photoperoxidations. White bars: level of peroxides sensitized by the drugs at a concentration of 50 μM and at UVA dose of 16.6 J/cm^2. Shaded bars: plus BHA at a concentration of 1 μM.

GSH, at a concentration of 100 μM, also exhibits substantial antioxidant effects, although to a lesser extent than BHA (data not shown), suggesting that free radical species are involved.

3.6. DNA strand breaks

Another important cellular target of photoreaction mediated by drugs is DNA. DNA alterations induced by phototoxic drugs have been investigated for several drugs [15–17].

Strand breaks are readily observed by following the conversion of supercoiled pBR322 (SC) to its nicked form (OC) and linear form (L) by agarose gel electrophoresis.

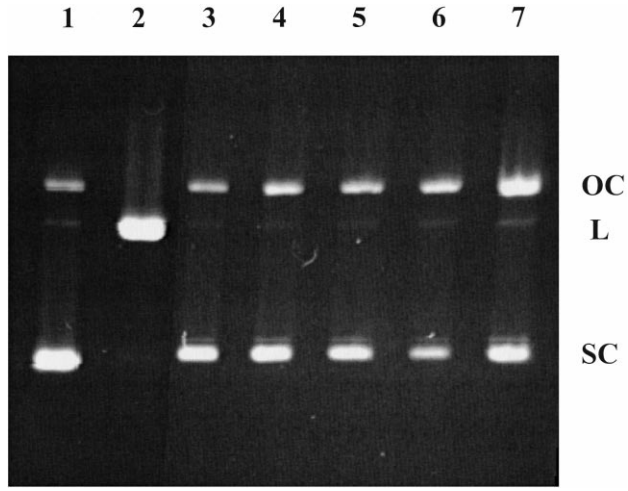

Fig. 8. Photocleavage of plasmid pBR322 by imipramine and amitriptyline. Plasmid pBR322 was irradiated (16.6 J/cm^2) in the presence of the two drugs at a concentration of 2.5×10^{-5} M. Lane 1: untreated pBR322; lane 2: pBR322 linearized with EcoRI; lanes 3 and 5: pBR322 in the presence of imipramine and amitriptyline, respectively, incubated in the dark; lanes 4 and 6: irradiated pBR3223 in the presence of imipramine and amitriptyline, respectively; lane 7: irradiated pBR322 without any photosensitizer. OC, Open circular; L, Linear; SC, supercoiled DNA.

Fig. 8 depicts the photocleavage of supercoiled pBR322 plasmid DNA by the two drugs investigated. For the sake of comparison, the EcoRI linearized plasmid is shown in lane 2.

It can be seen that pBR322 DNA is slightly modified by the two drugs, both in the dark (lanes 3: imipramine and 5: amitriptyline) and after irradiation (lanes 4: imipramine and 6: amitriptyline) to the same extent; only single strand breaks can be detected. Furthermore, irradiation alone produces the same slight modification to DNA (lane 7). Therefore, the two drugs, in combination with UVA light, do not induce marked changes in DNA conformation.

4. Conclusions

The study of the photosensitizing properties of amitriptyline and imipramine have evidenced that the two drugs are phototoxic in vitro as demonstrated by their capacity to photosensitize red blood cell hemolysis, cell killing of fibroblast and lipid peroxidation.

The observed photohemolysis, induced by imipramine and amitriptyline, may reflect peroxidation of membrane lipids, suggesting that biological membranes may be involved. Moreover, the MTT assay carried out on fibroblast measures mitochondrial respiratory chain enzyme activity of living cells, so it is possible that phototoxicity induced by these drugs is targeted against this organelle.

Lipid peroxidation induced by the test compounds certainly correlates with damage produced to the cell

membrane and therefore skin phototoxicity. A possible mechanism of lipid peroxidation induced by the compounds examined appears to involve free radicals, as confirmed by efficient inhibition of the process in the presence of BHA and GSH, well-defined free radical quenchers, and also by the remarkable nitro blue tetrazolium reduction observed.

We have also considered DNA damage as a consequence of phototoxic reactions mediated by the two drugs. For the drugs examined there was a slight production of single strand breaks, the extent of which was observed before and after UVA irradiation for both drugs.

This may be very useful when considered with the results obtained since they provide, to the best of our knowledge, a first report of the phototoxic properties of amitriptyline and imipramine in vitro on biological systems and photochemical tests. These results correlate well with the reported in vivo photosensitizing potential of the drugs. Indeed, although these drugs induce photosensitizing reactions in humans, these adverse effects are far less common than those observed for other classes of compound (i.e. fluoroquinolones and phenothiazines). This may explain why amitriptyline and imipramine exhibit some phototoxicity, which is not as high as other drugs, on the substrates studied here.

Furthermore, the approach utilized here appears to be a reasonable screening method for an evaluation of the phototoxic potential of new substances and for achieving an insight into the mechanisms involved in phototoxic and/or photosensitized effects.

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