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Photochemistry and *in vitro* phototoxicity studies of levomepromazine (methotrimeprazine), a phototoxic neuroleptic drug

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The neuroleptic drug levomepromazine (**1**, previously known as methotrimeprazine) is photolabile under UV-A and UV-B light in aerobic conditions. Irradiation of a methanol solution of this drug produces one photoproduct, resulting from the oxidation of **1** to its sulfoxide parent. It is demonstrated that photodegradation occurs via type II mechanism involving irreversible trapping of self-photogenerated singlet molecular oxygen. **1** shows a photohemolytic effect on human erythrocytes and photoinducers lipid peroxidation.

1. Introduction

Levomepromazine (2-methoxy-*N,N*,β-trimethyl-10*H*-phenothiazine-10-propanamine (Scheme, previously known as methotrimeprazine, is a neuroleptic drug with antipsychotic, anxiolytic and sedative effects [1].

The clinical use of this drug has been associated with photosensitivity reactions such as dermatitis, vitiligo, pseudoporphyria and photoallergic reactions [2–5]. The action spectrum of the drug seems to be in the UVA range. Although very useful and almost indispensable, it can produce adverse biological effects such as clinical photosensitization that has been recognized to result from **1**. The interaction of this family of drugs with UV-A (320–400 nm) and UV-B (290–320) radiations, which occur on the skin of patients treated with such drugs, is responsible for a series of photosensitizing effects [6]. Some studies have been published on the clinic photoxicity of levomepromazine, however very few have been carried out to evaluate the phototoxicity potentials of this drug and other phenothiazine derivatives. This phototoxicity could occur through a photodynamic mechanism mediated either by singlet oxygen and hydroxyl radical species, or through an oxygen dependent mechanism, or otherwise directly by the photoproducts or intermediate species as has been suggested for other drugs [7–9]. It appears important that there may be a relationship between photochemical behavior and phototoxicity.

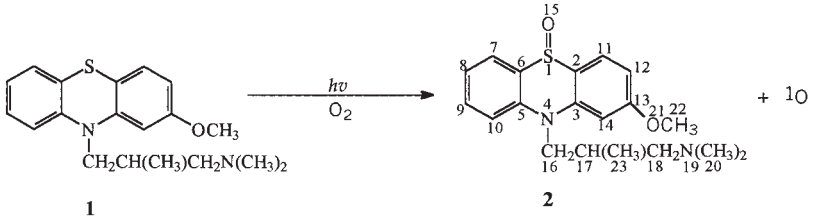
We examined the photolysis of levomepromazine under mild conditions close to those encountered in biological systems, namely, neutral and oxygenated media as well as under argon atmosphere. The irradiation was carried out with UV-B and UV-A light. The main goal was to investigate the photolability of this compound, the role of oxygen in the photoprocesses and in the *in vitro* phototoxicity. The combined approach proved to be useful in understanding the mechanism by which this phenothiazine derivative induces skin photosensitization.

2. Investigations and results

2.1. Photochemistry of levomepromazine (1)

Irradiation with UV-A (290–320) light of a methanol-solution of **1** under oxygen atmosphere affords the photoproduct **2** (yield 80%) and the quantum yield for **1** decomposition was $\Phi = 0.18$ with UV-B and 0.09 with UV-A light.

Scheme



1 **2**

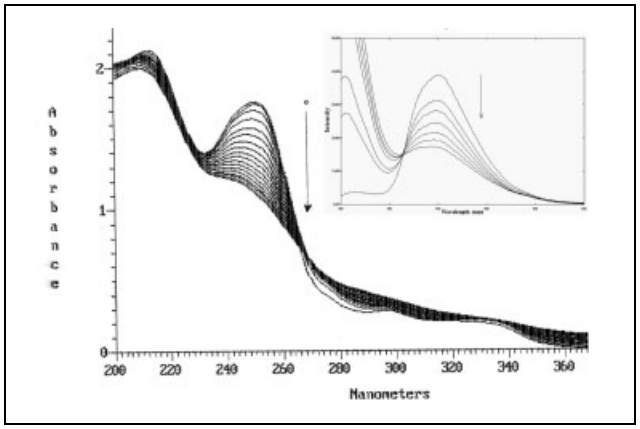


Fig. 1: UV and fluorescence monitoring of the photolysis of levomepromazine by irradiation with UV-A light (laser N_2), irradiation intervals every 5 min

The photolysis of **1**, and its photostability, was followed by monitoring the disappearance of the 250 nm bands at 5 min intervals. The appearance of a new band at 215 nm was also observed. In a same way it was determined by means of the disappearance of the emission band at 450 nm. The isobestic points observed for the photodegradation of **1** suggest that only a single product is likely to have formed. The results are shown for a methanolic solution (1×10^{-3} M) of **1** in Fig. 1.

The photoproduct was isolated (structures **2** see: Scheme) exhibited the following spectroscopic features: Product **2**: ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 7.60$ (m, 1 H, CH-7), 7.58 (m, 1 H, CH-9), 7.42 (d, 1 H, $J_{11,12} = 9$ Hz, CH-11), 7.23 (m, 1 H, CH-8), 7.14 (dd, $J_{12,11} = 9$ Hz, $J_{12,14} =$ 2.3 Hz 1 H, CH-12), 7.06 (m, 1 H, CH-10), 6.24 (d, $J_{14,12} = 2.3$ Hz, 1 H, CH-14), 4.70 (d, $J_{16,17} = 7.3$ Hz 2 H, CH_2-16), 3.80 (s, 3 H, CH_3-22), 2.30 (s, 6 H, CH_3-20) 2.28 (d, $J_{18,17} = 7.4$ Hz, 2 H, CH_2-18), 2.16 (m, 1 H, CH-17), 0.90 (d, $J_{23,17} = 6.0$ Hz, 3 H, CH_3-23). MS: m/e (%) = 344 (M^+, 22), 328 (M^+-16, 78), 299 (M^+-45, 16), 242 (69), 229 (29), 185 (5), 100 (3), 101 (4), 58 (100). IR (KBr): $\nu = 3416$, 2965, 2709, 1710, 1603, 1593, 1465, 1388, 1301, 1270, 1209, 1065, 1029, 978, 763 cm^{-1}.

Degradation of **1** was observed when irradiation was carried out in the presence of rose bengal, using a potassium chromate solution (100 mg/l) as a filter (which allows $\lambda >$ 400 nm) and maintaining all other conditions the same. The photoproduct **2** was detected as the only product of this reaction. Therefore, an interaction or quenching of singlet oxygen with **1** was probable.

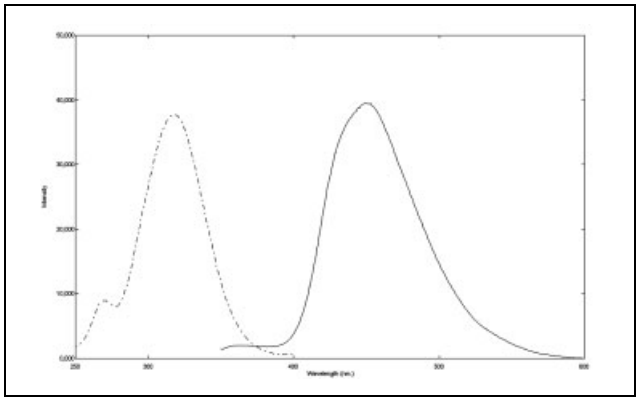


Fig. 2: Excitation and emission spectrum of levomepromazine [1.00×10^{-4} M]; $\lambda_{excitation} = 320$ nm, $\lambda_{emission} = 450$ nm

The excitation and emission fluorescence spectra of **1** in methanol are shown in Fig. 2. This drug show a broad fluorescence band centered around 450 nm and the fluorescence excitation spectrum at 320 nm. The relative quantum yields of fluorescence of **1** at room temperature was $\Phi_F = 0.020$.

2.2. Singlet oxygen generation by levomepromazine

1 was capable of producing singlet oxygen when it was irradiated with UV-A and visible light in presence of molecular oxygen. The formation of singlet oxygen by photolysis of **1** was evidenced by trapping it with 2,5-dimethylfuran. Trapping of singlet oxygen induced the formation of hexene-2,5-dione (10%), *cis*- and *trans*-3-oxo-1-butenyl acetate (3 and 16%, respectively), and 2-methoxy-5-hydroperoxide-2,5-dimethylfuran (8%), as determined by GC-MS. This fact was also confirmed by trapping with histidine. A simple and sensitive spectrophotometric method for the detection of $^1O_2$ as produced by different sensitizing dyes in neutral air saturated aqueous solutions (Fig. 3). The reaction between histidine and $^1O_2$ results in the formation of a trans-annular peroxide which can bleach the p-nitrosodimethylaniline (which can be followed at 440 nm). In the absence of histidine, there is no measurable loss of the nitrosoaniline, thus singlet oxygen formed in the system not can cause the bleaching of the last compound. Photooxidation of histidine (which is susceptible to singlet oxygen attack) is produced through levomepromazine acting as singlet oxygen sensitizer (Type II mechanism) [10].

The histidine model should be regarded simply as a test for oxygen dependent photosensitized damage to cellular protein.

The quantum yield of singlet oxygen generation for Rose Bengal is reported as $\varphi(^1O_2) = 0.76$. This parameter can be used as standard to determine a comparative value of $\varphi(^1O_2)$ for **1** levomepromazine [11–12]. We determined a relative value of 0.36 ± 0.01 for this drug.

1 has been shown to photosensitize the reduction of nitro blue tetrazolium (NBT) in PBS solution (pH 7.4, 30 °C). This reaction was more efficient in deoxygenated conditions. This result (Fig. 4) is consistent with the report [24] that in presence of oxygen will suppress the photochemical reduction of NBT to formazan by simple mass action.

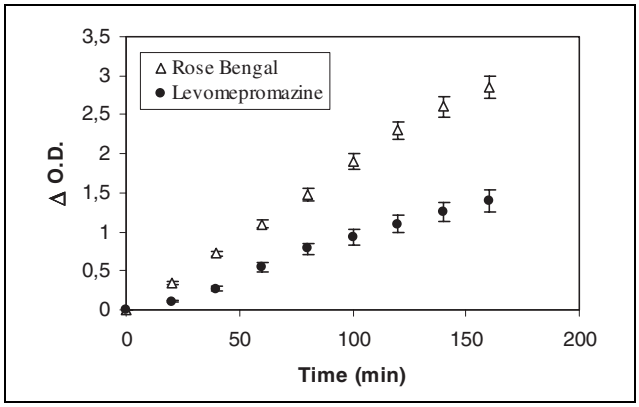


Fig. 3: Influence of the time of irradiation on the bleaching of *p*-nitrosodimethyl-aniline in the histidine-levomepromazine system at 440 nm., Δ O.D. represents the difference in optical density of irradiated and non-irradiated sample. Data are the mean and SEM, (n = 4, $p < 0.05$ vs. control; analysis of variance)

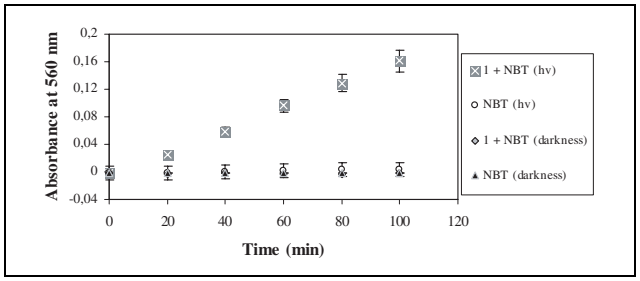


Fig. 4: Photoreduction of NBT (5.1×10^{-4} M) sensitized by levomepromazina (**1**) (5.1×10^{-4} M) in PBS solution. Data are the mean and SEM, (n = 3, $p < 0.05$ vs. control; analysis of variance)

2.3. Phototoxic effects of levomepromazine

1 was able to induce photohemolysis on human erythrocytes (Fig. 5). The *in vitro* experiments of photohemolysis are of obvious significance as a model for the study of diseases involving photoreactive processes. To evaluate the contribution of reactive oxygen species to the photophotosensitized hemolysis, the experiments were performed in argon-purged solutions, as also in presence of singlet oxygen quencher and radical scavengers. A probable mechanism of photohemolysis, based on a radical chain process caused by radical species and singlet oxygen photogenerated from levomepromazine (type I and type II mechanism) [10] is confirmed by the efficient inhibition of the process in the presence of α-tocopherol, Vit. C, GSH and sodium azide which are well-established free radical scavengers and $^1O_2$ quencher. Fig. 5 shows the relative inhibition of the photohemolysis by the addition of specific antioxidants.

The *in vitro* measurement of the photohemolysis effects of **1** were carried out in the presence of serum proteins (HAS). A similar effect of the photohemolysis was observed but with a 12% minor circa.

It is important to emphasize the phototoxicity effect of photoproduct **2** on erythrocytes. This product is responsible for 50% hemolysis during the 40 min that follow after the irradiation of RBC's in a 1.0×10^{-4} M solution of compound **2**.

When levomepromazine was irradiated in the presence of linoleic acid, significant amounts of dienic hydroperoxides were evidenced by the appearance of the new UV-absorption band at 233 nm. The photoinduced lipid peroxidation by levomepromazine is illustrated in Fig. 6.

When the photohemolysis and lipid peroxidation tests were carried out under argon atmosphere, a decrease of

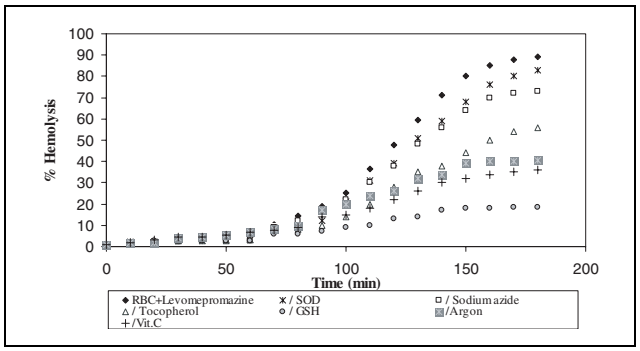


Fig. 5: Photohemolysis of RBC sensitized with levomepromazine in the presence of radical scavengers and singlet oxygen quenchers ([levomepromazine], [SOD], [GSH] and $[NaN_3] = 1.0 \times 10^{-4}$ M, [α-tocopherol] and [Vitamine C] $= 1.0 \times 10^{-5}$ M), RBC $= 3.3 \times 10^6$ cells/ml. Each point represents the mean ±SEM (less than 7%) derived from four observations

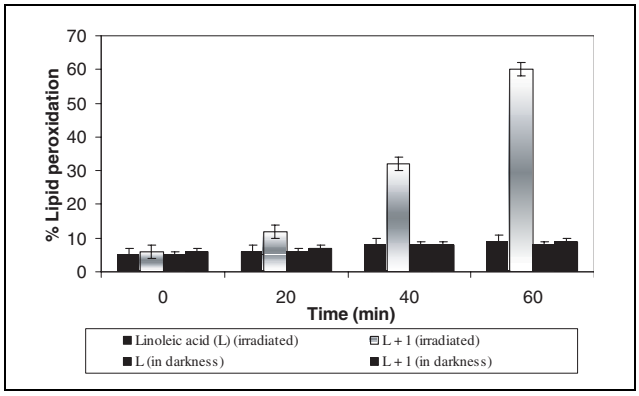


Fig. 6: Photoperoxidation of linoleic acid (L, 10^{-3} M) sensitized by levomepromazine (**1**, 10^{-4} M). Each point represents the mean ±SEM derived from three observations. The SEM was always less than 9%

the photoactivity induced by **1** was observed. The values obtained were approximately 50% and 30% lower for the photohemolysis and for the lipid peroxidation, respectively, than those obtained in aerobic conditions.

The experiments carried out in presence of HSA demonstrated a low catalytic activity of this compound on the photodegradation of **1**. Comparison of this experiment with the photolysis of levomepromazine in the absence of HSA shows no difference between them as far as the photoproducts formed. The yields of the photoproducts were virtually the same in both experiments. The only difference found between the two processes was an acceleration of the photodegradation process of **1**, in the former reaction, followed by the increase of an emission maxima at 433 nm due to the formation of the photoproduct. No photobinding to HSA by levomepromazine was detected.

3. Discussion

1 undergoes photooxidation to its sulfoxide parent compound (**2**). So, sulfoxidation is identified as the principal degradation pathway for irradiated samples of **1**. Probably the photodegradation of **1** occurs *via* type II mechanism involving irreversible trapping of self-photogenerated singlet molecular oxygen. Similar degradation pathways exist for others neuroleptic drugs as fluphenazine, trifluoperazine, perphenazine and prochlorperazine [6], the photooxidation of organic sulfides [13] and sulfur-centered radicals [14]. It can thus be concluded that sulfoxidation appears to be a common route of degradation for this class of phenothiazines.

Levomepromazine has been shown to photosensitize the reduction of nitro blue tetrazolium (NBT). These results indicate that direct electron transfer occurs from the excited state of **1** to the substrate.

The fact that the photodegradation process was also efficient under oxygen atmosphere and it is essential to produce photoproduct **2** is relevant to understand the mechanism of oxygen-dependent photobiological effects.

The photohemolysis assay, as an *in vitro* phototoxicity test, has evidenced the involvement of radical-mediated cellular membrane damage in the skin photosensitization by levomepromazine. The efficient inhibition of photohemolysis by the well-established radical scavengers GSH, tocopherol and Vit. C and in minor grade by sodium azide (singlet oxygen quencher) determined majority the involvement of a type I mechanism and with minor efficience the type II [15]. Due to that addition of sodium azide did

not exert an appreciable influence on the photohemolysis is in agreement with the minor role played by singlet oxygen.

Lipid photoperoxidation certainly correlates with damage produced in the cell membranes and therefore with the photohemolysis observed. Hydrogen abstraction by the generated intermediate radicals in the levomepromazine photodegradation leads to the linoleic acid peroxidations. The phototoxicity mechanism for levomepromazine probably involves reaction of free radical intermediates and photoproducts with cellular components. The decrease in the photohemolysis and lipid peroxidation tests under argon atmosphere indicates that levomepromazine is capable of photosensitizing red blood cells and lipids throught a process where oxygen plays an important role. Screening for phototoxicity *in vitro* is necessary before introducing drugs into clinical therapy. This may help prevent unwanted drug reactions in humans.

In spite of the cation radical formation of **1** and $O_2^{\bullet -}$ detected by the photosensitized reduction of NBT, the participation of superoxide in the photohemolysis induced by **1** was minor. Only a reduction of 10% of photohemolysis was detected in presence of SOD, a known anion radical quencher. It is can be possible because the fast conversion of $O_2^{\bullet -}$ to other reactive oxygen substances as hidroperoxide or $^{\bullet}OH$ in biological media.

The phototoxicity degree induced by phenothiazines could depend on the balance between the generation of singlet oxygen, oxygen radical and free radical species and the effectiveness of defense systems against toxic radicals. The protective induction by several intracellular signals may represent an adaptive response to cellular stress that might protect the cell against situations as the photosensitization induced by these drugs. On the other hand no protection could be generated by red blood cells in the same situation and the levomepromazine-photoinduced damage can be carrying out.

From a pharmacological point of view it is important that investigations carried out by Dahl and Mao [16, 17] who determined crystal structures of the phenotiazine products suggest that the apparent loss of neuroleptic potency by biotransformation of the drugs to their ring sulfoxides is caused by the introduction of the sulfoxide group itself, and not by concurrent conformational changes in the rest of the molecule. This fact may lead to new investigations to protect this family of drugs from photooxidation and also to find new derivatives with minor photosensibilizing properties.

4. Experimental

4.1. Chemicals

Levomepromazine (**1**) (CAS 60-99-1) was extracted from the commercial medicament Sinogan® (Rhône-Poulenc Rorer laboratory, France) with a soxhlet extractor using methanol as the solvent, purified by TLC and recrystallized from the same solvent. The purity was 99% as determined by mass spectroscopy, ^{1}H NMR and by comparison with an authentic pure commercial sample. Reduced glutathione (GSH), vitamin C, α-tocopherol, human serum albumin (HSA) and superoxide dismutase (SOD) were purchased from Sigma (St. Louis, MO, USA) while sodium azide (NaN_3) and 2,5-dimethylfuran (2,5-DMF) from Aldrich (Steinheim, Germany). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

4.2. Photolysis

Solutions of levomepromazine in PBS as well as in MeOH (1.00×10^{-4} M) were irradiated under aerobic conditions at room temperature, with a nitrogen laser with spectral output 337 nm (GL-3300 Photon Technology International, New Jersey, USA) and peak power at 5 Hz of 2.4 MW and 1.45 mJ of energy per pulse; as well with an Osram HQL 250 W medium pressure Hg lamp located inside a Pyrex immersion-well photoreactor (Applied Photophysics parts No. 3230 + 3307) for UV-A irradiation (output spectral 320–400 nm) with a maximum at 350 nm and a total irradiance of 17 mW/cm^2 as measured with a UVX Digital Radiometer (Melles Griot, USA). The experiments were also carried out with a Rayonet photochemical chamber reactor (model RPR-100, Southern New England Ultraviolet Company-USA) equipped with 16 phosphorus lamps with a emission maximum in UV-A between 320–400 nm and UV-B 290–320 nm (23 mW/cm^2 of irradiance). The distance between the light source to the test aliquots was 10 cm. The temperatures detected in the cuvette during a standard 1 h irradiation were no higher than 27 °C. The course of the reaction was followed by UV-Vis absorption spectrophotometry using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA) and by emission spectrophotometry with a Shimadzu RF 1501.

The photostability of **1** was determined in alcoholic solution at 30 °C upon illumination of 1.0×10^{-3} M solutions with UV-A and also with a N_2-Laser light previously described. The concentration of the **1** samples was monitored spectrophotometrically at different irradiation times, and the photostability was expressed as the percent residual absorbance referred to the absorbance measured before irradiation.

Preparative irradiations (1.6×10^{-2} M) were monitored by liquid chromatography (HPLC, Waters Delta Prep 4000) equipped with an analytic and preparative Porasil 125A column using a MeOH/CH_2Cl_2 gradient as mobile phase at a flow rate of 0.8 ml · min^{-1}, with monitoring at 250 nm. When irradiation was completed, the PBS solution was extracted with CH_2Cl_2 and the organic phase evaporated at reduced pressure (14 Torr) at room temperature. The residue was purified by preparative HPLC. The isolated products were analyzed by ^{1}H NMR spectroscopy (Bruker Aspect 3000, 300 MHz), FT IR (Nicolet DX V 5.07) and GC-MS (Carlo Erba/Kratos MS25RFA).

In the determination of quantum yields the photolysis was allowed to proceed to less than 10% product formation to minimize light absorption by the photoproducts and further side reactions. The photon flux incident on 3 ml of solution in quartz cuvettes of 1 cm optical path was measured by means of a ferric oxalate actinometer and was of the order of 10^{15}–10^{16} quanta · s^{-1}.

The relative quantum yields of fluorescence at room temperature were determined by comparing the corrected fluorescence intensity of the levomepromazine in ethanol with that of rhodamine B (at a concentration of 1.00×10^{-6} M in ethanol, fluorescence quantum yield $\varphi_f = 0.69$) or with that of quinine bisulfate in 0.05 M H_2SO_4 (fluorescence quantum yield $\varphi_f = 0.55$) [18]. The fluorescence spectra were registered with a Shimadzu RF 1501 spectrofluorophotometer.

1 was also irradiated in the presence of rose bengal with an Osram HQL 250 W medium pressure Hg lamp using a potassium chromate solution (100 mg/l) as a filter allowing only $\lambda > 400$ nm and maintaining all other conditions the same. In this way, the reaction of singlet oxygen with **1** was carried out and studied.

4.3. Singlet oxygen generation

Irradiations of **1** were carried out under the same experimental conditions, in the presence of 2,5-dimethylfuran (2,5-DMF, 5.00 mmol) which is normally used as a trap for singlet oxygen ($^1O_2$) [19]. This process was followed by GC and by MS as in the previous experiment. Rose bengal, a well known $^1O_2$ sensitizer, was used as a standard for comparison with **1** as far as $^1O_2$ formation, under identical conditions of photolysis.

Indirectly, photosensitized degradation of histidine [20] was measure in the presence of 0.25, 0.50, 1.0 and 1.5×10^{-5} M solution of levomepromazine. These solutions were mixed with a equal quantity of L-histidine solution at 0.60 to 0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of this mixture were irradiated with a Osram HQL 250 W medium pressure Hg lamp through a filter Pyrex (radiation dose 4.5 J/cm^2) at time intervals from 60 to 180 min. and the respective controls were maintained protected from light. Histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The optic density was read on a spectrophotometer at 530 nm against a blank reagent, a modified Pauly reaction and by bleaching of *p*-nitrosodimethylaniline [21, 22].

4.4. Electron transfer mechanism detection by reduction of NBT

Under the same condition of the photololysis of **1** (5.1×10^{-4} M), the photoreduction of nitro blue tetrazolium ([NBT] = 5.1×10^{-4} M) was followed in presence of NBT, in presence and absence of oxygen, as a function of the irradiation time by determining the increase in absorbance at 560 nm due to the formation of diformazan product [23, 24].

4.5. Photoinduced hemolysis of RBC by levomepromazine

For the photohemolysis experiments, a red blood cells (RBC) suspension from three different samples of freshly obtained human erythrocytes was prepared by washing them four times with a tenfold volume of a phos-

phate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl), centrifuging each time the cells at $2500 \times g$ for 15 min and carefully removing the supernatant. Finally, the RBC were diluted in PBS containing 1 so that the resultant suspension had an optical density (OD) of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponded to 3.3×10^6 cells $\cdot$ ml^{-1}, which was read on a Milton-Roy 3000 spectrophotometer. Parallel experiments were carried out in a desareated (Argon) solution of PBS. The hemolysis rate and the hemolysis percentage were determined by measuring the decreasing OD at 650 nm, since the optical density is proportional to the number of intact RBC [25]. **1** was dissolved in the RBC solution and irradiated continuously at concentrations of 1.0 to 4.0×10^{-4} M under aerobic conditions in a Rayonet photochemical reactor equipped with 16 phosphor lamps with an emission maximum at 300 nm or alternatively with a nitrogen laser (output 337 nm), or with an Osram HQL 250 Watt medium pressure Hg lamp in a Pyrex immersion-well photoreactor, for periods ranging from 10 to 200 min in order to study the photohemolysis effect. Similar experiments were carried out without irradiation and with a preirradiated solution of **1**.
The photohemolysis test was repeated in the presence of serum proteins, reduced gluthatione (GSH, 1.0×10^{-4} M) and ascorbic acid (1.0×10^{-5} M) as radical scavengers, α-tocopherol (1.0×10^{-5} M) and sodium azide (NaN_3, 1.0×10^{-4} M) as singlet oxygen quenchers, superoxide dismutase (SOD, 1.0×10^{-5} M) and also under inert atmosphere (argon).

4.6. Photosensitized peroxidation of linoleic acid

Linoleic acid 1.0×10^{-3} M in PBS was irradiated in the presence of compound **1** and also in a pre-irradiated solution of **1** (1.0×10^{-5} M); the formation of dienic hydroperoxides was monitored by UV-spectrophotometry, through the appearance and progressive increase of a new band at $\lambda = 233$ nm [26, 27]. This test was also carried out under argon atmosphere.

4.7. Photodegradation in presence of human serum albumin

The photoreaction of levomepromazine (**1**) in presence of human serum albumin (HSA) was monitored using a Shimadzu RF 1501 spectrofluorometer at $\lambda_{exc} = 320$ nm and $\lambda_{emis} = 450$ nm. The sample in 1 cm^2 Suprasil quartz cells was irradiated as indicated before. The concentration of **1** was 1×10^{-4} M and that of HSA 2×10^{-8} M. Control experiments were carried out without HSA as well as in the dark.

4.8. Statistical treatment of results

At least three independent experiments were performed except where indicated. The results are expressed as a mean ±S.E.M. derived from 3–4 observations. The level of significance accepted was $p = 0.05$.

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