Laboratorio de Fotoquímica y Fotobiología, Departamento de Química, Universidad Simón Bolívar; Centro de Química, Instituto Venezolano de Investigaciones Científicas; Departamento de Biología Celular Laboratorio de Microbiología y Genética-Laboratorio de Productos Naturales y Biotecnología, Universidad Simón Bolívar, Venezuela

Photosensitizing properties of 6-methoxy-2-naphthylacetic acid, the major metabolite of the phototoxic non-steroidal anti-inflammatory and analgesic drug nabumetone

N. Canudas, D. Zamora, J. E. Villamizar, J. Fuentes, C. Castelli, A. Taddei

Received July 7, 2004, accepted September 28, 2004

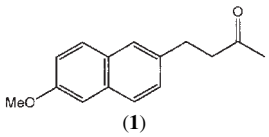
Prof. Dr. Nieves Canudas, Departamento de Química, Universidad Simón Bolívar, Apartado 89000, Caracas 1080-A, Venezuela
ncanudas@usb.ve

Pharmazie 60: 604–608 (2005)

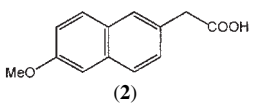
The photobiological properties of 6-methoxy-2-naphthylacetic acid (6-MNAA) were studied using a variety of *in vitro* phototoxicity assays: photohemolysis, photoperoxidation of linoleic acid, photosensitized degradation of histidine and thymine and the *Candida* phototoxicity test. 6-MNAA was phototoxic *in vitro.* 6-MNAA reduced nitro blue tetrazolium (NBT) when irradiated with $\lambda \geq 300$ nm in deoxygenated aqueous buffer solution (pH 7.4). NBT can be reduced by reaction with the excited state of 6-MNAA subject to interference with molecular oxygen. The photohemolysis rate was inhibited by the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO), sodium azide (NaN_3) and reduced glutathione (GSH). Photoperoxidation of linoleic acid and photosensitized degradation of histidine and thymine were significantly inhibited by sodium azide and reduced glutathione. 6-MNAA was phototoxic to *C. albicans*, *C. lipolytica* and *C. tropicalis*. A mechanism involving singlet oxygen, radicals, and electron transfer reactions is suggested for the observed phototoxicity.

1. Introduction

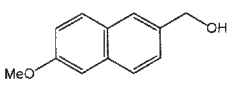
Nabumetone (NB) (**1**), 4-[6-methoxy-2-naphthalenyl]-2-butanone, is a phototoxic non-steroidal anti-inflammatory drug recommended for the treatment of osteoarthritis or rheumatoid arthritis (Carryl 1995; Lister et al. 1993). NB is 35% metabolized by the liver to the active derivative 6-methoxynaphthylacetic acid (6-MNAA) (**2**) (Dahl 1993). Adverse skin reactions have been reported in 3–9% of patients undergoing treatment with NB (Kaidbey and Mitchell 1989; Canudas et al. 2000).



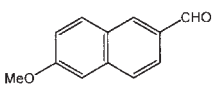
(**1**)



(**2**)



(**3**)



(**4**)

Recently, studies on the intermediates generated from the photoexcitation of NB and its photosensitizing activity using a variety of *in vitro* phototoxicity assays have been published. These studies suggest that NB can induce photosensitizing reactions by a type II photodegradation mechanism (Martínez and Scaiano 1998; Canudas et al. 2000).

6-MNAA is the most important and active metabolite able to reach the skin (Dahl 1993). Its photophysical and photochemical properties have been reported in order to understand the phototoxic effects of NB. Alcohol (**3**) and aldehyde (**4**) have been reported as photodegradation products under aerobic conditions. The photodegradation of 6-MNAA via decarboxylation has been reported to occur by a new mechanism that involves the formation of a non-decarboxylated benzylic radical intermediate that is trapped by O_2, with subsequent loss of CO_2. From these reports it is clear that reactive intermediate species may be formed following the photoexcitation of 6-MNAA to the excited singlet and that these species are capable of initiating the adverse photobiological effects (Bosca et al. 2000).

In the present paper we deal with a variety of *in vitro* experiments that support the mechanism proposed for the photodegradation of 6-MNAA and show that, like NB, it is an efficient photosensitizer. We studied the *in vitro* phototoxicity of 6-MNAA under aerobic and anaerobic conditions, using *in vitro* tests: photoinduced hemolysis of erythrocytes, induced photoperoxidation of linoleic acid, photosensitized degradation of histidine and thymine, photosensitized reduction of nitro blue tetrazolium (NBT)

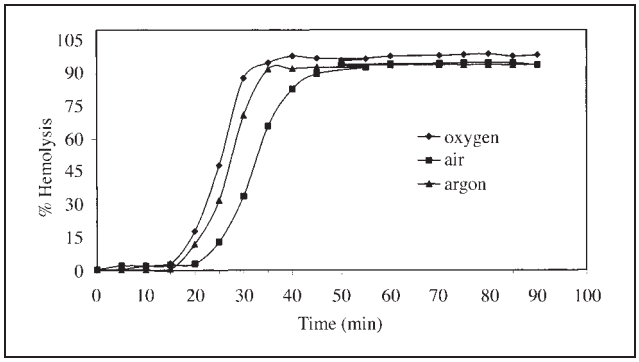


Fig. 1: Photohemolysis of RBCs (3.3×10^6 cells ml^{-1}) sensitized by 6-MNAA (10^{-5} M) under aerated, oxygen and argon enriched atmospheric air

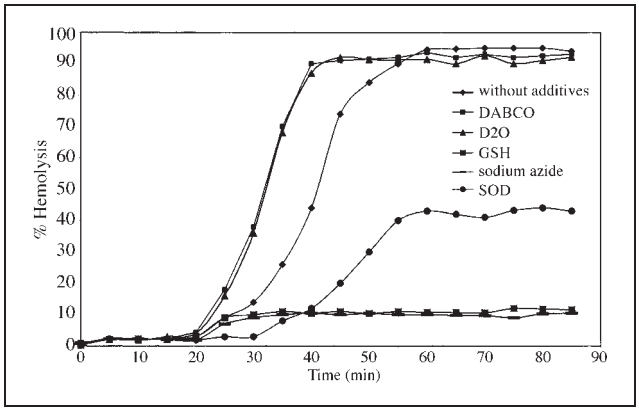


Fig. 2: Photohemolysis of RBCs (3.3×10^6 cells ml^{-1}) sensitized by 6-MNAA (10^{-5} M) under oxygenated conditions in the presence of additives (10^{-5} M)

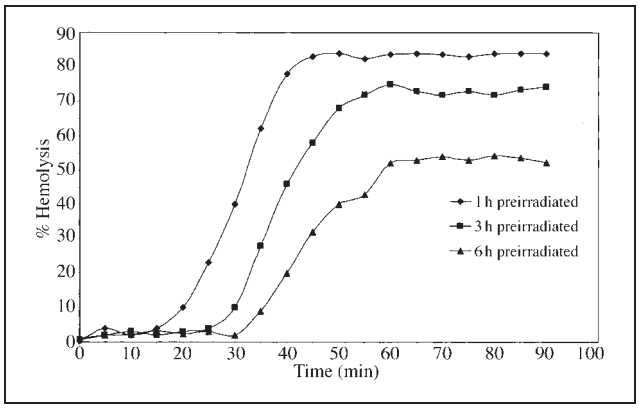


Fig. 3: Photohemolysis of RBCs (3.3×10^6 cells ml^{-1}) sensitized by pre-irradiated solutions of 6-MNAA (10^{-5} M)

and the *Candida* phototoxicity test. The effects of additives on photoinduced hemolysis, photoperoxidation of linoleic acid and *Candida* assays, will help to confirm type II and/or type I mechanisms for the photosensitizing process.

2. Investigations, results and discussion

Erythrocytes are a convenient model system for studying damage to cellular membranes. Here, photoinduced membrane damage by 6-MNAA was investigated in intact human red blood cells (RBCs). Irradiation of a suspension of RBCs in buffered aqueous solutions (phosphate 0.05 M, pH 7.4) (PBS) in the presence of 6-MNAA (1.0×10^{-5} M) led to hemolysis of erythrocytes both aerobically (air and O_2 enriched atmospheres) and anaerobically (Ar enriched air atmosphere). 50% of photoinduced hemolysis was obtained with 34 min of irradiation under an air atmosphere, while only 24 min were required to observe the same degree of hemolysis under an O_2 enriched atmosphere. 6-MNAA induced photohemolysis also occurred when the samples were bubbled with argon before adding the RBCs and 50% hemolysis was obtained after 27 min irradiation. Hemolysis was enhanced under O_2 or Ar enriched atmosphere as is shown in Fig. 1. Since the presence of oxygen markedly enhances induced photohemolysis our results are in agreement with the previous study of the involvement of oxygen in the photodegradation of 6-MNAA (Bosca et al. 2000). In all the samples more than 95% of photoinduced hemolysis was observed after 55 min irradiation. Dark controls revealed less than 1% hemolysis. No lysis was observed when cells were irradiated for 50 min in the absence of the drug. The mechanism of photodynamic reactions in photoinduced membrane damage can be probed with reagents which reveal whether singlet oxygen, radicals or superoxide are involved. Thus, the photohemolysis studies were repeated in the presence of additives. We found that sodium azide and gluthathione (GSH) showed nearly total inhibition of drug-induced photohemolysis. The presence of 1,4-diazabicyclo[2.2.2]-octane (DABCO) increased the observed photoinduced hemolysis. The presence of 75% deuterium oxide in the PBS solutions slightly increased hemolysis. The results are shown in Fig. 2. The protection factor obtained for sodium azide and GSH showed nearly total inhibition of drug-induced photohemolysis (data not shown). A probable mechanism of photoinduced hemolysis caused by radical species (type I mechanism) photogenerated from 6-MNAA is strongly suggested as GSH (radical trapping) showed total inhibition of hemolysis. The fact that the addition of sodium azide (a singlet oxygen quencher) exerts nearly total inhibition of photoinduced hemolysis suggests a type II mechanism (Quintero and Miranda 2000). Incubation of pre-irradiated (1 h, 3 h and 6 h) solutions of 6-MNAA in PBS with red blood cells in the dark failed to induce hemolysis (data not shown). Irradiation of the same pre-irradiated solutions of 6-MNAA at a concentration of 10^{-5} M in PBS with red blood cells sensitized the hemolysis which was low for solutions that have been pre-irradiated for long times (Fig. 3). A sample of 6-MNAA pre-irradiated for one hour had a lower concentration of photoproducts than a sample pre-irradiated for 6 h. This indicated the absence of toxic and phototoxic photoproducts of NB involved in the damage to the cellular membrane. Standard tests for assessing the intermediacy of superoxide were used, comparing the results of photoinduced hemolysis experiments performed in the presence and in the absence of superoxide dismutase (SOD). In the presence of SOD, erythrocytes were protected under aerobic conditions and showed nearly 60% inhibition of hemolysis (Fig. 2). The effect of SOD involves the ability of NB (3×10^{-5} M) to act as an electron transferring photosensitizer, which was tested using NBT as an electron acceptor in aerated phosphate buffer solution at pH 7.4 (Beauchamp and Fridovich 1971). The photoreduction of NBT was observed as a function of irradiation time by monitoring the increase in absorbance at 560 nm due to the appearance of the formazan product (Moore et al. 1998; Auclair et al. 1978). Control experiments showed that no reduction occurred when NBT solutions were irradiated in the absence of sensitizer or when the sensitizer

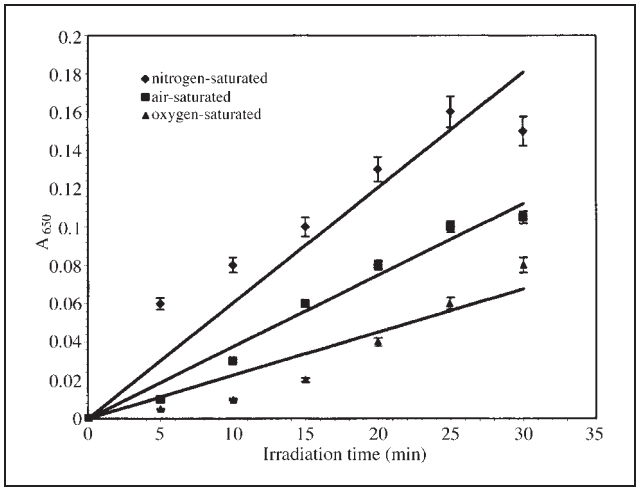


Fig. 4: Photoreduction of NBT (5.0×10^{-5} M) sensitized by 6-MNAA (3×10^{-5} M) in aqueous buffer solution, pH 7.4. Experiments in triplicate, the arithmetic mean is reported

and NBT solutions were maintained in the dark. The reduction occurred in an air-saturated solution, but at a lower rate when an O_2-saturated solution was employed under identical conditions. The reaction occurred rapidly in a N_2-saturated solution (Fig. 4). Thus it appears that reduction of NBT photosensitized by 6-MNAA is probably a reaction of the excited state of 6-MNAA with NBT subject to interference with molecular oxygen.

Lipid photoperoxidation can be related to damage produced in the cell membranes and can be responsible for the induced photohemolysis results observed for 6-MNAA. When aerated PBS solutions of linoleic acid were irradiated in the presence of NB (2.5×10^{-6} M), significant amounts of dienic hydroperoxides were detected by absorption at 233 nm (Miranda et al. 1992) (Fig. 5). The final concentration of hydroperoxides after 120 min of irradiation without additives was calculated as 1.32×10^{-5} M on the basis of the extinction coefficient (ε = ca. 31000). In the presence of the additives GSH and DABCO, nearly 80% of the photoperoxidation reactions induced by 6-MNAA were inhibited. In the presence of sodium azide (an efficient singlet oxygen quencher) the photoperoxidation was inhibited by 50%. These results suggest that the phototoxicity mechanism of 6-MNAA probably involves the reaction of singlet oxygen and radicals, respectively, with cellular membrane components, as they are important in the photosensitized peroxidation of linoleic acid under aerobic conditions as well as in the observed induced photohemolysis. The histidine photodegradation assay is a test to ob-

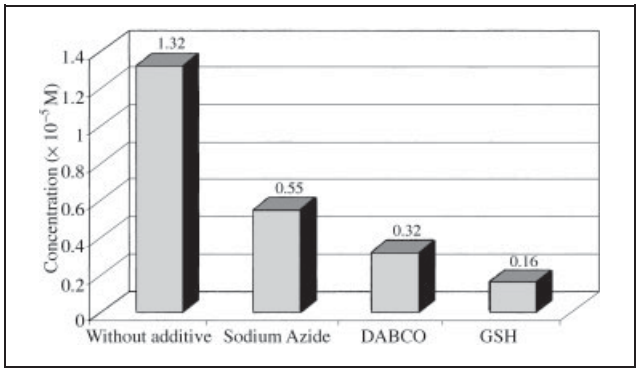


Fig. 5: Comparative concentrations of dienic hydroperoxides induced by 6-MNAA with and without additives after 120 min of irradiation

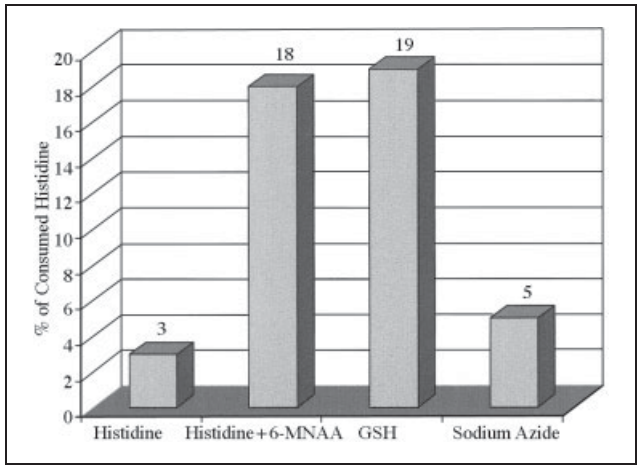


Fig. 6: Percentage of L-histidine consumed after 40 min of irradiation alone, in the presence of 6-MNAA and in the presence of additives

serve photosensitized damage of cellular proteins that involves the attack of singlet oxygen on histidine to yield an oxidized addition compound. 6-MNAA (2×10^{-4} M in PBS) photoinduced oxidation of histidine by reaction with singlet oxygen after 40 min of irradiation (Fig. 6). The presence of GSH, as a radical scavenger, did not protect against the photoinduced oxidation of histidine, while complete protection was observed in the presence of sodium azide (Lowell 1993).

The possible damage to DNA photoinduced by 6-MNAA was investigated by two *in vitro* assays (Gupta 1973; Jiang et al. 1990; Jiang et al. 1992). The thymine photodegradation assay is used to predict possible damage to DNA bases. Singlet oxygen generated during the photodegradation of 6-MNAA reacts with thymine yielding thymine hydroperoxides. The formation of peroxides by the reaction of thymine with singlet oxygen, can be observed in the thymine assay when solutions of 6-MNAA were irradiated separately. The concentration of hydroperoxides is shown in the Table. The formation of thymine hydroperoxides during irradiation of thymine and a solution of 6-MNAA together was completely inhibited by the presence of GSH. Marked (60%) thymine hydroperoxide inhibition was observed in the presence of sodium azide. Oxidative species other than singlet oxygen can be involved in hydroperoxide formation. The *Candida albicans* phototoxicity test can also be used for quantitative estimation of reactions involving DNA damage (Daniels 1965; Kavli and Volden 1984). This test can show injury to cell constituents like the nucleus. When exposed to irradiation in the presence of test drugs, dose-dependent inhibition of yeast growth around the discs was seen with NB and 6-MNAA. The use of 8-MOP as a positive control was included (Knudsen 1985). PBS/30% ethanol and piroxicam (PRX) never inhibited growth, either on irradiated or non-irradiated plates. When exposed to radiation ($\lambda > 300$ nm) for 15 min, dose-dependent inhibition was seen with 6-MNAA and 8-MOP in *C. albicans, C. tropicalis and C. lipolytica*. 6-MNAA did

Table: Peroxide concentration (1×10^{-5} M) induced by 1 h irradiation of 6-MNAA, thymine and a mixture of 6-MNAA + thymine with and without additives

	Without additive	Sodium azide	GSH
6-MNAA	0.24	0.28	0.98
Thymine	0.03	0	0.02
6-MNAA + Thymine	0.82	0.33	0

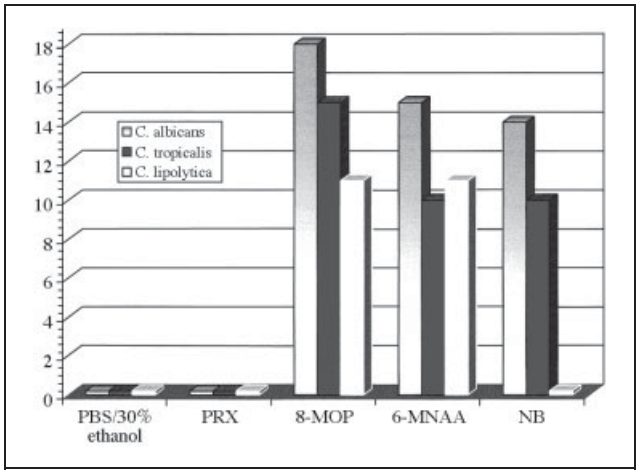


Fig. 7: Dose response relations for *C. albicans*, *C. tropicalis* and *C. lipolytica* tested with PBS/30% ethanol solutions of NB (10 mg/ml), 6-MNAA (10 mg/ml), 8-MOP (5 mg/ml), PRX (5 mg/ml) and PBS/30% ethanol alone after 15 min of irradiation

not inhibit growth of *C. lipolytica* on irradiated or non-irradiated plates; nevertheless, *C. albicans* and *C. tropicalis* were sensitive to NB with 15 min of irradiation (Fig. 7). When exposed to radiation for 1 h, dose dependent inhibition was seen with NB, 6-MNAA and 8-MOP on the three *Candida* sp. samples. The diameter of inhibition on *C. albicans* on the discs after 1 h irradiation was twice the diameter observed at 15 min of irradiation for 6-MNAA and 8-MOP. *C. tropicalis* and *C. lipolytica* were less sensitive than *C. albicans* to all the compounds tested at 1 h irradiation time (Fig. 8). All the compounds showed only a little toxic effect in non-irradiated plates but the phototoxic effect was greater in irradiated dishes (data not shown). Doubling the irradiation dose from 1 h to 2 h did not increase the phototoxic potency of the compounds tested. Only *C. tropicalis* was killed by 2 h of irradiation ($\lambda > 300$ nm). We note that the best results were observed for a 1 h irradiation time, in which the inhibition diameters of yeast growth reached their maximum value. 6-MNAA was more potent against the growth of all three *Candida sp.* than NB at 1 h irradiation time and the values obtained were comparable with those of 8-MOP under

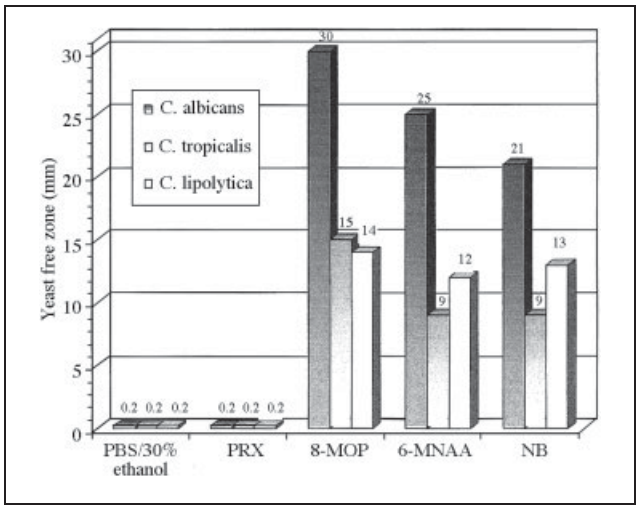


Fig. 8: Dose response relations for *C. albicans*, *C. tropicalis* and C. *lipolytica* tested with PBS/30% ethanol solutions of NB (10 mg/ml), 6-MNAA (10 mg/ml), 8-MOP (5 mg/ml), PRX (5 mg/ml) and PBS/30% ethanol alone after 1 h of irradiation

similar conditions. The use of UVB in our experiments shows that it is feasible to carry out the *Candida* phototoxicity test for drugs that have important absorption at $\lambda > 300$ nm. The use of 8-MOP under these conditions results in a good approach to the positive reference.

The results obtained suggest that 6-MNAA is able to induce damage to biological components. Photosensitizing peroxidation of membrane lipids, proteins and possible damage to DNA bases by 6-MNAA can be explained by type I and type II mechanisms. Our results agree with photochemical and photophysical studies on 6-MNAA where both mechanisms were proposed (Bosca et al. 2000).

The results obtained may be useful to understand the photosensitizing properties of NB, as 6-MNAA is its major metabolite.

3. Experimental

3.1. Chemicals

Nabumetone (Relifex®) was provided by SmithKline Beecham and was extracted and characterized according to the procedure already outlined (Canudas et al. 2000). 6-Methoxy-2-naphthylacetic acid was synthesized from 6′-methoxy-2′-acetonaphthone (Aldrich) according to the procedure reported previously (Bosca et al. 2000). The purities of NB and 6-MNAA were 99% (GC-MS, Fisons HRGC 8000), ^{1}H NMR (Jeol Eclipse 400 MHz), TLC (general purpose silica gel on polyester) and UV-Vis spectrophometry (Varian Cary 50). Reduced glutathione (GSH), linoleic acid, nitro blue tetrazolium (NBT), bovine superoxide dismutase (SOD) and piroxicam (PRX) were purchased from Sigma. 8-Methoxypsoralen (8-MOP), NaN_3, sulfanilic acid and 1,4-diazabicyclo[2.2.2]octane (DABCO) were purchased from Aldrich Chemical Company (Milwaukee USA). All analytical and HPLC grade solvents, thymine, histidine and xylenol orange were obtained from Merck (Darmstadt, Germany). Human red blood cell (RBC) concentrate was supplied by QUIMBIOTEC, C.A. (Venezuela). Phosphate Buffered Saline pH 7.4 (PBS) was used for *in vitro* assays.

3.2. Irradiation conditions

In all the photosensitization experiments, irradiation was performed using an OSRAM HQL 450 W medium pressure Hg lamp located inside a Pyrex immersion-well photoreactor (Applied Photophysics parts no. 3230 and 3307) for $\lambda \geq 300$ nm irradiation. A merry-go-round modified apparatus was used to make sure that all samples received the same quantity of radiation. For the photoinduced hemolysis, lipid photoperoxidation, histidine and thymine tests, the samples (2–3 ml) were irradiated at a distance of 10 cm in quartz cuvettes of 1 cm optical path and monitored by UV-Vis measurements of optical density (OD) using a Cary 50 VARIAN instrument. The photon flux incident on samples was 1.9×10^{-4} J · s^{-1} · cm^{-2} as measured previously (Canudas 2000). The temperature inside the samples during irradiation was between 25 and 28 °C.

3.3. Candida growth inhibition

The yeasts tested were: *C. albicans*, *C. lipolytica* and *C. tropicalis* provided by the "Lorenzo De Montemayor" Fungi Collection at Simón Bolívar University. For the test, a modification of the methods described by Daniels (1965) and Knudsen (1985) was used. In order to avoid toxic response due to the solvent, the drugs were dissolved in a solution of PBS (pH 7.6) and 30% v/v ethanol, using concentrations according to the dose required to observe normal growth. NB and 6-MNAA were tested at a concentration of 10 mg/mL and 8-MOP and PRX at a concentration of 5 mg/mL, respectively. The three *Candida* species from a 24 h growth on Sabouraud-dextrose agar were dissolved in saline solution (0.83% p/v NaCl) to achieve a concentration of approximately 3×10^8 cells/mL (McFarland Standard Nº 1) and 100 µL of each of these solutions were spread on plates of Sabouraud-dextrose agar (4 plates for each species). Filter papers (Whatman Nº 1, 5 mm diameter) were placed on top of the spread plates at a distance of 2 cm from each other. 5 µl of each of the tested compounds and the solvent alone was added to each filter, and the filters were put in the refrigerator for 3 h to permit diffusion of the drugs. The prepared plates were covered with a Pyrex filter ($\lambda > 300$ nm) and irradiated for 15 min, 1 h and 2 h at a distance of 10 cm from a medium pressure mercury lamp (Osram HQL 450 W). The photon flux incident on samples was 1.8×10^{-4} J · s^{-1} · cm^{-2} as measured previously (Canudas 2000). Dark controls were performed to exclude toxic effects of the drugs themselves. After irradiation, the plates were incubated in the dark at 30 °C for 48 h. The results were observed as growth inhibition around the disc, and the diameters were measured. The procedure was carried out 3 times with each sample, and the mean values were calculated.

3.4. Hemolysis assays

Red Blood Cells (RBCs) were prepared by diluting samples of out-of-date (no more than 20 days from the date stated) packed human erythrocytes in PBS at pH 7.4 until the resultant suspension had an OD of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponds to 3.3×10^6 cell $\times$ ml^{-1}. For the hemolysis experiments the RBC suspensions with 6-MNAA 1×10^{-5} M were irradiated and the percentage hemolysis was determined by measuring the decrease in OD at 650 nm, since the OD is linearly proportional to the number of intact RBCs (Giuffrida et al. 1995; Kagan et al. 1992). Similar experiments were carried out without irradiation and with pre-irradiated solutions of 6-MNAA (1×10^{-5} M). Experiments were performed under aerobic and anaerobic conditions. In the case of anaerobic conditions the PBS solution was enriched with Ar by bubbling prior to the addition of RBCs. The photohemolysis test was repeated with solutions of 6-MNAA in the presence of DABCO (1×10^{-5} M) and NaN_3 as $^1O_2$ quenchers and GSH and SOD as radical scavengers. Experimental details were similar to previous work (Canudas et al. 2000). The experiments were repeated three times and the average (arithmetic mean) is reported.

3.5. Lipid peroxidation assays

Duplicate solutions of linoleic acid (1×10^{-3} M) in PBS containing 6-MNNA (1×10^{-5} M) (Tween 20 was used as a surface active agent) were irradiated for two hours at 34 °C. The reaction was monitored by UV-Vis, observing the appearance and subsequent increase of an absorption at 233 nm, due to the conjugated dienic hydroperoxides derived from linoleic acid peroxidation (Miranda et al. 1992). The controls used were solutions (1×10^{-3} M) of linoleic acid and of the drug which were irradiated under identical conditions, as well as not irradiated. The test was repeated in the presence of (1×10^{-5} M) NaN_3 and DABCO and GSH. The concentration of conjugated dienic hydroperoxides was calculated taking into account their molar absorptivities (ε = ca. 31000).

3.6. Photoreduction of NBT sensitized by 6-MNAA

Buffered aqueous solutions (PBS, pH 7.4, flushed with gas: oxygen or Ar as required, for 10 min) with 50 μM of 6-MNAA containing NBT (5.5×10^{-5} M) were placed in quartz vessels of 1 cm path length. The vessels were stoppered and irradiated at a distance of 10 cm from the light source. Photoreduction of NBT was followed as a function of the irradiation time by determining the increase in absorbance at 560 nm (Moore et al. 1998).

3.7. Photosensitized degradation of histidine

Mixtures of PBS solutions of 6-MNAA (1) (5×10^{-4} M) and L-histidine (3×10^{-4} M) were placed in quartz vessels of 1 cm path length, irradiated for 1 h and incubated for 30 min in the dark at room temperature. The same solutions were irradiated in the presence of GSH and NaN_3 (1×10^{-5} M). Separate solutions of 6-MNAA and L-histidine alone were irradiated analogously. Identical solutions maintained in the dark were used as controls. The photodegradation of L-histidine induced by 6-MNAA was measured by determining the histidine levels in the irradiated samples by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents (Figuereido et al. 1993). The absorbance was read at 530 nm.

3.8. Photodegradation of thymine

PBS solutions of 6-MNAA (3×10^{-4} M), thymine (1×10^{-3} M) and mixtures of these solutions were irradiated for 1 h with and without GSH and NaN_3 (1×10^{-5} M). Controls were kept protected from light. The formation of thymine peroxides was measured by UV-Vis as reported (Jiang et al. 1992).

Acknowledgements: This research was supported by grants from FONACIT (S1-2001000688) and Decanato de Investigaciones y Desarrollo (DID-Universidad Simón Bolívar). The authors wish to thank the Fungi Collection "Lorenzo De Montemayor" at Simón Bolívar University for supplying the yeasts used in this study and José Ignacio Juncosa for technical assistance.

References

Auclair C, Torres M, Hakim J (1978) Superoxide anion involvement in NBT catalysed by NADPH-Cytochrome P-450 reductase. FEBS Lett 89: 26–28.

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287.

Bosca F, Canudas N, Marin ML, Miranda MA (2000) A photophysical and photochemical study of 6-methoxy-2-naphthylacetic acid. The major metabolite of the phototoxic nonsteroidal anti-inflammatory drug nabumetone. Photochem Photobiol 71: 173–177.

Canudas N, Moulinier J, Zamora D, Sanchez A (2000) Photobiological properties of nabumetone (4-(6-methoxy-2-naphthalenyl)-2-butanone). Pharmazie 55: 282–285.

Carryl OR (1995) Comparative effects of nabumetone, naproxen, piroxicam and diclofenac on rat gastric irritancy following acute exposure to OTC non-steroidal anti-inflammatory agents and other gastric irritants. Scan J Rheumatol 24: 336–338.

Dahl S (1993) Nabumetone: a "non acidic" non-steroidal anti-inflammatory drug. Ann Pharmacother 24: 457–459.

Daniels F (1965) A simple microbiological method for demonstrating phototoxic compounds. J Invest Dermatol 44: 259–267.

Figuereido A, Fontes C, Gonzalo M, Poiares A, Teixeira I (1993) Experimental studies on the mechanism of tiaprofenic acid photosensitization. J Photochem Photobiol B: Biol 18: 161–168.

Giuffrida S, De Guidi G, Sortino S, Chillemi R, Constanzo L, Condorelli G (1995) Molecular mechanism of drug photosensitization, part. 5. photohemolysis sensitized by suprofen. J Photochem Photobiol B: Biol 23: 125–133.

Gupta B (1973) Microdetermination techniques for hydrogen peroxide in irradiated solutions. Microchemical J 38: 363–367.

Jiang Z, Hunt JV, Wolff S (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide. Anal Biochem 202: 384–386.

Jiang Z, Woollard A, Wolff S (1990) Hydrogen peroxide production during experimental protein glycation. FEBS Lett 268: 69–72.

Kaidbey KH, Mitchell FN (1989) Photosensitizing potential of certain nonsteroidal anti-inflammatory agents. Arch Dermatol 125: 783–786.

Kagan J, Wang T, Kagan I, Tuveson R, Wang G, Lam J (1992) Photosensitization by 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol: Damage to erythrocyte membranes. Photochem Photobiol 55: 63–73.

Kavli G, Volden G (1984) The *Candida* test for phototoxicity. Photodermatology 1: 204–207.

Kirby TW, Fridovich L (1982) A picomolar spectrophotometric assay for superoxide radical. Anal Biochem 127: 435–440.

Knudsen EA (1985) The *Candida* phototoxicity test. The sensitivity of different strains and species of *Candida*, standardization attempts and analysis of the dose response curves for 5- and 8-methoxypsoralen. Photodermatol 2: 80–85.

Lister BJ, Poland M, DeLapp RE (1993) Efficacy of nabumetone versus diclofenac, naproxen, ibuprofen and piroxicam in osteoarthritis and rheumatoid arthritis. Am J Med 95: 25–95.

Ljunggren B (1985) Propionic acid-derived non-steroidal anti-inflammatory drugs are phototoxic *in vitro*. Photodermatol 2: 3–9.

Lowell W (1993) A scheme for in vitro screening of substances for photoallergenic potential. Toxic. *In vitro* 7: 95–102.

Martinez LJ, Scaiano JC (1998) Characterization of the transient intermediates generated from the photoexcitation of nabumetone: comparison with naproxen. Photochem Photobiol 68: 646–651.

Miranda MA (1992) in: Castell JV (ed.) Alternative in vitro models to animal experimentation in Pharmaco-Toxicology, Farmaindustria, Barcelona, pp. 239–240.

Moore DE, Ghaebremeskel KA, Chen BC, Wong ELL (1998) Electron transfer processes in the reactivity of nonsteroidal anti-inflammatory drugs in the ground and excited states. Photochem Photobiol 68: 685–689.

Quintero B, Miranda MA (2000) Mechanism of photosensitization induced by drugs. Ars Pharm 41: 27–46.