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Photobiological properties of nabumetone (4-[6-methoxy-2-naphthalenyl]-2-butanone), a novel non-steroidal anti-inflammatory and analgesic agent

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The photolability of nabumetone (NB, **1**, 4-[6-methoxy-2-naphthalenyl]-2-butanone) and its photobiological properties were studied under aerobic and anaerobic conditions using a variety of *in vitro* phototoxicity assays: photohemolysis, photoperoxidation of linoleic acid, and photosensitized degradation of histidine and thymine. The photodegradation rate of NB in methanol and phosphate buffered saline (PBS) was enhanced under oxygenated media. NB was phototoxic *in vitro*. The photohemolysis rate was enhanced by deuterium oxide and inhibited by the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO), butylated hydroxyanisole (BHA), sodium azide (NaN_3) and reduced gluthathione (GSH). The induced photoperoxidation of linoleic acid was inhibited significantly by sodium azide and reduced gluthathione. Histidine and thymine were photodegraded by a photosensitized reaction induced by NB. A mechanism involving singlet oxygen, radicals and photoproducts is suggested for the observed photoxicity.

1. Introduction

Nabumetone (NB, **1**, 4-[6-methoxy-2-naphthalenyl]-2-butanone) is a nonsteroidal anti-inflammatory agent (NSAID) that works as a prodrug. It was developed as a non acidic compound to replace other NSAIDs with high incidence of gastrointestinal side effects [1, 2]. NB is recommended for the treatment of osteoarthritis or rheumatoid arthritis. NB is metabolized by the liver to the active derivative 6-methoxynaphthylacetic acid (6-MNA, **3**) 35% and secondary metabolites 50%; 15% of the drug remain without transformation for at least 24 h [3]. In spite of a non-acidic nature NB has been reported to be a photosensitizing drug (adverse skin reactions reported in 3–9% patients undergoing treatment) and this is not surprising due to the presence of 6-MNA (**2**) as a metabolite, an analog of the phototoxic NSAID, naproxen (**3**) [4–8]. Photophysical and photochemical aspects of NB have been studied recently and two transient intermediates have been identified [9]. The participation of two chromophores in NB are responsible for the photophysical and photochemical behavior.

The present study deals with the photolability of NB in phosphate buffered saline (PBS) pH 7.4 and methanol under aerobic and anaerobic conditions and its *in vitro* phototoxicity using a variety of tests: photohemolysis, photoperoxidation of linoleic acid, and photosensitized degradation of histidine and thymine. The use of additives to see the effects on photohemolysis and photoperoxidation assays allows to establish a type II mechanism for the photosensitizing process.

2. Investigations, results and discussion

NB is photolabile under aerobic and anaerobic conditions in PBS and also in methanolic solutions when irradiated with UV-B, UV-A and visible light. The photolysis under aerobic conditions was followed by monitoring the changes of the absorption bands as shown for a PBS solution of NB (1×10^{-5} M) in Fig. 1. Similar results were observed in methanolic solution (data not shown). The photolability decreased when nitrogen was bubbled through the solutions before irradiation (data not shown). Since the presence of oxygen markedly enhances photodegradation, oxygen should be involved in the degradation mechanism of NB.

Photoinduced hemolysis and lipid peroxidation were observed as model tests for cellular membrane damage while assays with thymine and histidine reveal damage to protein and ADN bases.

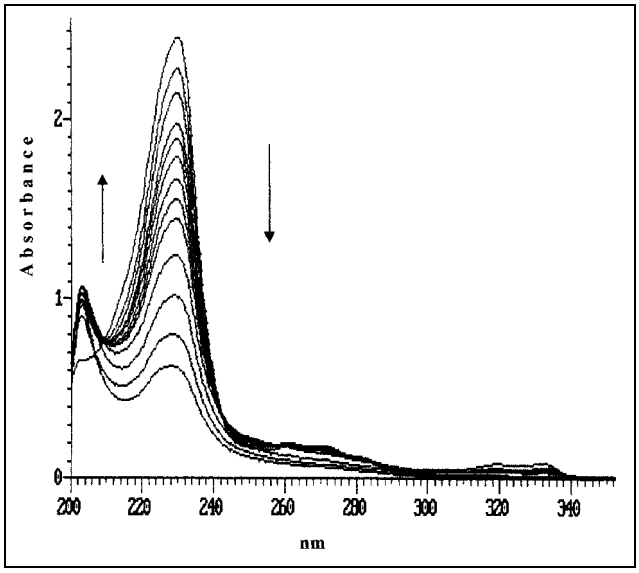

Fig. 1: UV-Vis monitoring of the photolysis of nabumetone (1×10^{-5} M) in PBS under oxygen at regular intervals of 10 min of irradiation

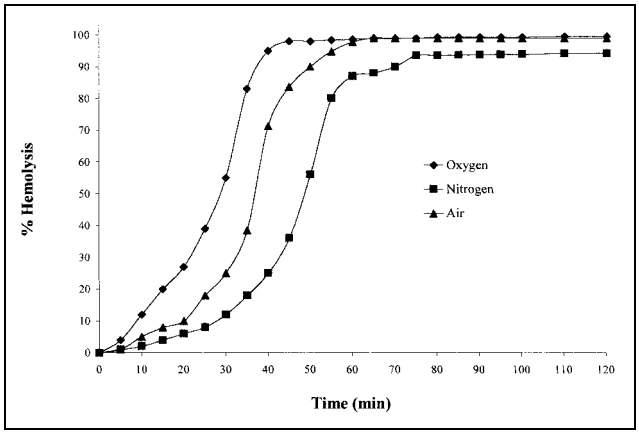

Fig. 2: Photohemolysis of RBCs (3.3×10^6 cells ml^{-1}) sensitized by nabumetone (1×10^{-5} M) under aerated, oxygen and nitrogen conditions

NB sensitized the hemolysis of RBCs when it was irradiated at 1×10^{-5} M in PBS containing a suspension of human erythrocytes. More than 90% of induced hemolysis was observed after one hour irradiation under aerobic conditions. 50% hemolysis was obtained at 37 min of irradiation as is shown in Fig. 2. Dark controls with the same samples revealed less than 0.5% hemolysis. No lysis was observed when cells were irradiated for 1 h in the absence of the drug. NB induced photohemolysis also occurs when the samples were bubbled with nitrogen before adding the RBCs and the presence of oxygen enhanced the lysis. The results are shown in Fig. 2. The photohemolysis studies were repeated in the presence of additives. We found that DABCO, NaN_3, GSH and BHA inhibited the photohemolysis induced by NB.

The results are shown in Table 1. The protection factor obtained for DABCO and NaN_3 showed nearly total inhibition of drug-induced-photohemolysis. The presence of 80% deuterium oxide in PBS solutions had an increased effect on the hemolysis observed as we show a protection factor value below unity for D_2O. Our finding indicated that the protection effect on photohemolysis produced by singlet oxygen quenchers can be attributed to the involvement of singlet oxygen in the process (type II mechanism). Protection factor values greater than unity obtained for GSH and BHA showed delayed photohemolysis. This suggests that free radicals are involved in the process, too. The results are shown in Table 1.

Incubation of preirradiated (1 h and 6 h irradiation) solutions of NB in PBS with red blood cells was able to induce hemolysis in the dark. The results are shown in Fig. 3. This revealed the presence of toxic photoproducts involved in the damage to the cellular membrane. One hour preirradiated samples of NB have a lower concentration of toxic photoproducts than 6 h preirradiated samples as we conclude from the lower lysis observed for 1 h preirradiated samples.

When PBS solutions of linoleic acid were irradiated in the presence of NB significant amounts of dienic hydroperoxides were detected by the appearance of an absorption band at 233 nm. The results are shown in Table 2. The final concentration of hydroperoxides after 120 min irradiation without additives was calculated as 3.7×10^{-5} M on the basis of the extinction coefficient (ε = ca. 31 000).

Table 1: Effect of additives on photohemolysis induced by nabumetone

Time (min)	Without additive	BHA	GSH	NaN_3	DABCO	D_2O*
0	0.0	0.0	0.0	0.0	0.0	0.0
10	5.0	0.1	0.1	0.1	0.1	10.0
20	10.0	2.9	2.5	1.6	1.3	15.0
30	20.0	15.0	17.0	5.0	4.3	30.0
35	38.5	20.0	23.0	9.0	10.0	55.0
40	71.3	26.0	30.0	12.0	14.3	93.0
45	83.6	32.0	36.0	15.0	18.0	94.0
50	90.0	40.0	39.0	17.0	19.0	94.2
55	94.8	45.0	40.0	17.3	19.0	95.0
60	97.8	47.0	42.0	17.3	19.1	97.0
65	99.0	53.0	42.0	17.3	19.0	97.0
70	99.0	54.0	42.3	18.0	19.0	97.4
75	99.3	54.1	42.2	18.0	19.3	97.5
120	–	–	–	19.0	19.0	–
PF**	–	1.6	2.3	>8	> 8	0.8

* D_2O was used to prepare PBS solution

** Protection factor measured from the ratio between the t_{50} obtained with and without additive

In the presence of the additives NaN_3 and GSH, nearly all photoperoxidation reactions induced by NB were inhibited. The results are shown in Fig. 4. The photoperoxidation process induced by NB involves the presence of singlet oxygen and radicals and confirms the results of the observed photosensitized damage to the erythrocyte membrane.

The photolysis of NB in aerobic conditions produced singlet oxygen. This was observed by trapping it with 2,5-dimethylfuran. The reaction between 2,5-dimethylfuran

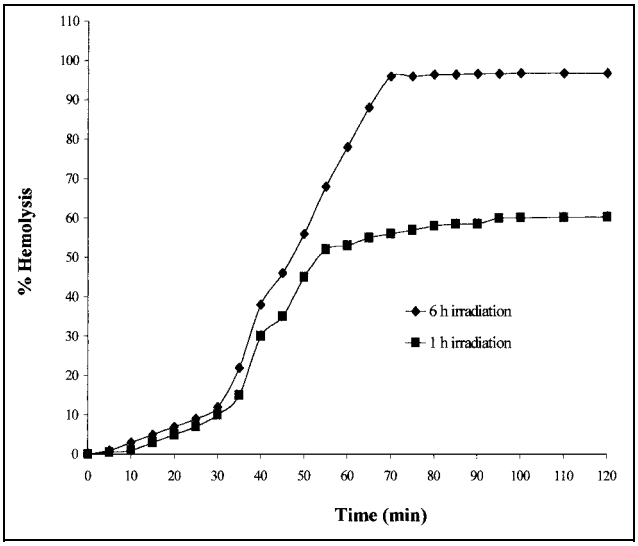

Fig. 3: Hemolysis of RBCs (3.3×10^6 cells ml^{-1}) induced by preirradiated solutions (50 μg ml^{-1}) of nabumetone

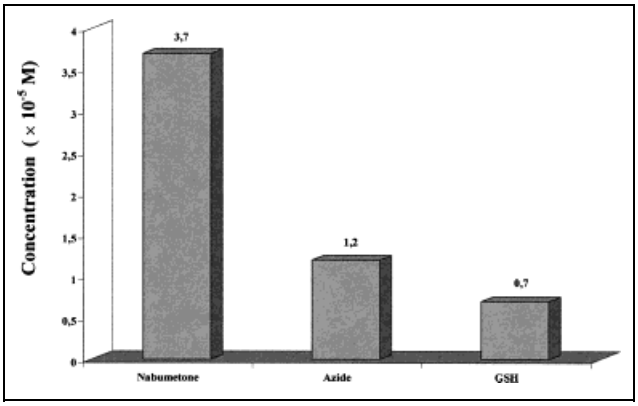

Fig. 4: Comparative concentration of dienic hydroperoxides induced by nabumetone alone and in the presence of additives after 120 min irradiation

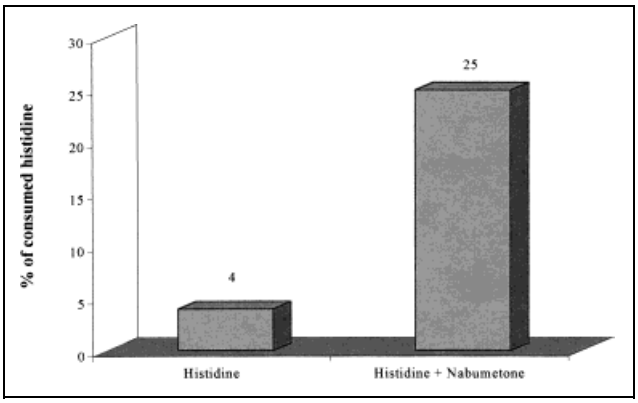

Fig. 5: Percentage of consumed histidine after 1 h irradiation in the presence of nabumetone compared with histidine alone

Table 2: 233 nm Absorption band increment of dienic hydroperoxides induced by nabumetone with and without additives

Time (min)	Without additive	NaN_3	GSH
0	0	0	0
30	0.22	0.033	0.024
60	0.5	0.079	0.05
90	0.82	0.186	0.123
120	1.15	0.356	0.2

Table 3: Peroxide concentration induced by irradiation of nabumetone, thymine and a mixture of nabumetone + thymine

	Nabumetone	Thymine	Nabumetone + Thymine
Peroxide concentration* ($\times 10^{-7}$ M)	0.30	0.18	1.35

* Concentration after 1 h irradiation

and NB affords 2-methoxy-5-hydroperoxy-2,5-dimethylfuran, 3-hexene-2,5-dione and *cis*- and *trans*-3-oxo-1-butenyl acetate detected by GC-mass spectrometry [10].

The histidine photodegradation assay is a test to observe photosensitized damage to cellular proteins that involves only the attack of singlet oxygen on histidine to yield a photoxidized compound. NB was able to induce photoxidation of histidine as is shown in Fig. 5, representing the amount of histidine consumed by reaction with singlet oxygen after 1 h irradiation in the presence of NB (1×10^{-4} M) [11].

NB sensitized the photodegradation of thymine. This assay is used to predict a possible damage to DNA bases. The reaction between thymine and singlet oxygen generated by NB yields thymine hydroperoxides [12–14]. The concentration of hydroperoxides is shown in Table 3. NB can react with singlet oxygen to yield peroxidic compounds that can be detected in the thymine assay when irradiated solutions of NB and thymine separately are compared with irradiated solutions of thymine and NB together. The data are shown in Table 3. Moreover, NB reacts with singlet oxygen generated by irradiation of a mixture of TPP and NB using a filter, $\lambda > 400$ nm (data not shown). The presence of peroxidic derivatives of NB is important for the ability to oxidize cell membrane components. The presence of peroxidic derivatives was detected as a brown spot when a small quantity of NB photoproduct mixture reacts with an ethanolic solution of KI on a thin layer chromatographic silica gel sheet.

The results obtained suggest that NB is able to generate singlet oxygen and induce photosensitizing reactions on membrane lipids by a type II mechanism. Our results agree with the study by Martinez and Scaiano where a proposal of type II photodegradation mechanism was proposed for NB [9].

The results obtained may be useful to complete photophysical and photochemical studies. They also may be helpful from the medical standpoint to advise patients about the use of these drugs.

3. Experimental

3.1. Chemicals

NB (Relifex®) was provided by Smithkline-Beecham and was extracted by means of a soxhlet extractor with acetone and recrystallized from ethanol (m.p.: 79–80 °C). The purity was 99% as determined by GC-MS (Carlo Erba/Kratos MS25RFA), ^{1}H NMR (Bruker Aspect 3000, 300 MHz) and UV-Vis spectrometry (Milton Roy 3000). Sodium azide (NaN_3), sodium nitrite, 2,5-dimethylfuran (DMF), tetraphenylporphin (TPP), 1,4-diazabicyclo [2.2.2]octane (DABCO), and sulfanilic acid were purchased from Aldrich Chemical Company (Milwaukee, USA), while butylated hydroxyanisole (BHA), reduced glutathione (GSH) and linoleic acid were from Sigma Chemical Company (St. Louis, USA) and histidine, thymine and xylenol orange from Merck (Ohio, USA). Human red blood cells (RBCs) concentrate was supplied by QUIMBIOTEC, C.A (Venezuela). Phosphate-buffered saline pH 7.4 (PBS) was used for *in vitro* assays.

3.2. Photolysis

The course of the photodegradation of NB (CAS 42924-53-8) and the measure of optical density (OD) for *in vitro* assays was followed by UV-Vis spectrophotometry using Milton Roy 3000 and Perkin Elmer Lambda 2 instruments. NB was irradiated at room temperature (1×10^{-5} M) in PBS and methanol with an Osram HQL 250 W medium pressure Hg lamp in a Pyrex immersion-well photoreactor (Applied photophysics parts no. 3230 and 3307). Carousel modified apparatus was used to make sure that all samples received the same quantity of radiation. The samples (2–3 ml) were irradiated in quartz cuvettes of 1 cm optical path. The photon flux incident on samples was measured by means of a ferric oxalate actinometer and was 1.9×10^{-4} J · s^{-1} · cm^{-2} [15].

3.3. Photohemolysis assays

RBCs where prepared by diluting samples of fresh (no more than five days) packed human erythrocytes in phosphate-buffered saline solution pH 7.4 (PBS) 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 cells · ml^{-1}. For the hemolysis experiments the RBC suspensions with NB 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 interact RBCs [16]. Similar experiments were carried out without irradiation and with a preirradiated solution of NB (1×10^{-5} M). Experiments were performed under aerobic and anaerobic conditions. In the case of anaerobic conditions PBS solution was enriched with N_2 by bubbling it through the solution before the addition of RBCs. The photohemolysis test was repeated with solutions of NB in the presence of (1×10^{-5} M) DABCO and NaN_3 as singlet oxygen quenchers, and BHA and GSH as radical scavengers. Experimental details were similar to previous studies [16, 17]. All the experiments were repeated three times and the average (arithmetic mean) of these experiments is reported.

3.4. Photoperoxidation of linoleic acid in the presence of nabumetone

Duplicate solutions of linoleic acid (1×10^{-3} M) in PBS containing NB (1×10^{-5} M) (Tween 20 was used as surfactant) were irradiated for 2 h keeping the temperature at 34 °C by means of a thermostatic bath. The reaction was monitored by UV spectrometry, following the appearance and subsequent increase of a new absorption maximum at $\lambda = 233$ nm, due to the conjugated dienic hydroperoxides derived from linoleic acid peroxidation [18]. The controls were solutions (1×10^{-3} M) of linoleic acid alone and the drug alone at the same concentrations irradiated under identical conditions, as well as nonirradiated. The test was repeated in the presence of (1×10^{-5} M) NaN_3 and GSH. The concentration of conjugated dienic hydroperoxides was calculated taking into account the molar absorptivities of the hydroperoxides $\varepsilon =$ ca 31 000).

3.5. Photodegradation of thymine

PBS solutions of NB (1×10^{-4} M), thymine (1×10^{-3} M) and mixtures of these solutions were irradiated for 1 h. The controls were the same solutions kept protected from light. The formation of thymine peroxides induced by NB was measured by UV-Vis spectrophotometry according to the procedure outlined by Jiang et al. [14].

3.6. Photosensitized degradation of histidine

Mixtures of PBS solutions of NB (3×10^{-4} M) and L-histidine (3×10^{-4} M) were irradiated for 1 h and incubated for 30 min in the dark. Separate solutions of NB and L-histidine were irradiated under the same conditions. The control samples were the same solutions kept in the dark. The photodegradation of L-histidine induced by NB was measured determining the histidine levels by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents [19]. The absorbance was measured with a spectrophotometer at 530 nm against a blank reagent.

3.7. Singlet oxygen detection

Under the same experimental conditions NB (1×10^{-4} M) was irradiated in the presence of DMF (1×10^{-2} M), a well known singlet oxygen sca-

venger. The photomixtures obtained were analyzed by GC-MS. The irradiation of NB (1×10^{-4} M) was carried out in the presence of TPP using a potassium chromate solution (100 mg/l) as filter allowing $\lambda > 400$ nm and maintaining all other conditions to ascertain whether NB is consumed by reaction with singlet oxygen. The course of the reaction was followed by UV-Vis spectrometry. Photodegradation of NB was not observed under this condition.

Acknowledgements: This research was supported by a grant from Decanato de Investigationes y Desarrollo (DID-Universidad Simón Bolívar) S1-CB-403-PN

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Received July 15, 1999
Accepted September 15, 1999

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