Experimental studies on the mechanisms of tiaprofenic acid photosensitization

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Abstract

Red blood cell lysis and histidine degradation, photosensitized by tiaprofenic acid (TIA), were investigated. Photohaemolysis was markedly enhanced in oxygenated solutions, but was also intense in the presence of nitrogen. Photohaemolysis was inhibited by butylated hydroxyanisole and reduced glutathione, but was unaffected by sodium azide, superoxide dismutase and mannitol.

The TIA-induced photo-oxidation of histidine was greatly enhanced in the presence of oxygen and almost completely inhibited in solutions bubbled with nitrogen. Sodium azide, butylated hydroxyanisole and reduced glutathione inhibited the photodegradation of histidine. Phototoxicity to histidine was unaffected by mannitol and superoxide dismutase.

The overall results suggest that molecular mechanisms involving free radicals and singlet oxygen are responsible for TIA-photosensitized reactions.

These two *in vitro* models (photohaemolysis and histidine degradation) represent different mechanisms of phototoxicity, but complement one another in the investigation of potential phototoxic substances.

Keywords: Photosensitization, Phototoxicity, Photohaemolysis, Histidine test, Free radicals, Singlet oxygen, Tiaprofenic acid

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most widely used agents in modern medical practice. When first introduced, these drugs were employed primarily in the treatment of inflammatory musculoskeletal disorders, but they are now also prescribed for controlling pain.

NSAIDs are a chemically heterogeneous group of compounds which, nevertheless, share mechanisms of action, therapeutic indications and side effects [1]. Cutaneous eruptions are frequent during treatment with these drugs and range from minor reactions, such as rash or pruritus, to more serious and sometimes fatal side effects, such as vesiculobullous eruptions or toxic epidermal necrolysis.

Benoxaprofen, a propionic acid derivative now withdrawn from the market, is the NSAID with the highest potential for cutaneous toxicity. In some series, cutaneous reactions occurred in 45% of patients and the commonest cutaneous side effect was photosensitivity. In summer, it occurred in about 50% of the patients [2].

Clinical photosensitivity also seems to be the most commonly reported adverse cutaneous reaction to piroxicam [3] and is also frequent with carprofen [4, 5], naproxen [6, 7] and azapropazone [8]. Worldwide, photosensitive reactions have been described with NSAIDs belonging to almost all chemical families [2–4, 8–13], but they are more frequent with the propionic acid derivatives and piroxicam.

The basic mechanism of this photosensitivity is not completely understood, the available data being highly contradictory and controversial. Some clinical, histological and provocative studies with benoxaprofen [14, 15], piroxicam [3, 11, 16], carprofen [17, 18] and sulindac [12] point to phototoxicity. Other papers support photoallergy for piroxicam [19–21], carprofen [4, 22] and suprofen [9].

Results of studies with *in vitro* models of phototoxicity are also very confusing. In almost all *in*

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vitro experiments benoxaprofen and the other propionic acid derivatives sustained their phototoxicity [23–25]. However, azapropazone showed phototoxic potential in photohaemolysis and lymphocyte phototoxicity tests, but was negative in the *Candida albicans* and histidine phototoxicity tests [25].

Piroxicam is almost always negative in *in vitro* phototoxicity tests [19, 25–27]. For this drug there is a large amount of evidence which indicates that photoallergic reactions are induced by previous contact sensitization to thimerosal and thiosalicylic acid, in both man [28, 29] and animal models [30]. It is possible that a photoproduct of piroxicam may bear structural and/or antigenic resemblance to thiosalicylic acid and be responsible for the cross-reaction between these two drugs, a hypothesis first introduced by De La Cuadra [28] and Ljunggren [31], and supported by more recent studies [29, 32].

Tiaprofenic acid (TIA) is a propionic-acid-derived NSAID [33]; a few reports have indicated cutaneous photosensitivity reactions for TIA [13, 34–36]. TIA is phototoxic *in vitro* when a photobasophil-histamine release test is carried out [37] and *in vivo* when the mouse tail technique is used [38]. This paper describes our studies on its phototoxic potential and the mechanisms involved in its phototoxicity using *in vitro* photohaemolysis and histidine tests.

2. Materials and methods

TIA was gift from Roussell (Portugal). Butylated hydroxyanisole (BHA), reduced glutathione (GSH), sodium azide (AZI), superoxide dismutase (SOD), mannitol (MAN), histidine and sulphanilic acid were obtained from Sigma Chemical Company. Sodium nitrite and sodium carbonate were purchased from Merck. All other chemicals were of reagent grade.

2.1. Photohaemolysis

Blood was collected by venepuncture from normal human volunteers not taking any drugs, using heparin as anticoagulant. Red blood cells (RBCs) were prepared by washing three times with a tenfold volume of physiological saline solution, each time centrifuging the erythrocytes at 3000 *g* for 10 min and carefully removing the supernatant. The RBCs were then diluted in a modified potassium-free Krebs–Henseleit solution (composition (mM): NaCl, 123.30; $CaCl_2$, 2.52; NaH_2PO_4, 1.18; $MgSO_4$, 1.23; $NaHCO_3$, 25.00; glucose, 10.00) containing TIA so that the resulting suspension had a haematocrit of 2.5% and various concentrations of the drug. Drug solutions were bubbled with either oxygen or nitrogen for 20 min prior to the addition of RBCs, or were not bubbled. Finally, the test-tubes were tightly sealed and irradiated, with the respective controls protected from light in the irradiation unit to reproduce temperature effects. Samples of equal haematocrit suspensions without TIA were also irradiated with the respective controls sheltered from light.

Irradiation was performed using a psoralen plus UVA (PUVA) unit (Psoralite, Paul B. Elder Company, OH, USA) equipped with 44 lamps (Voltarc, USA, F72T12-BL-HO) having an emission peak at 365 nm and an irradiance of 16.0 mW cm^{-2} at a distance of 15 cm (as measured with a UVA meter, VLX-365, Vilber Lourmat, France). A merry-go-round irradiation apparatus was used to ensure that all samples received equal radiation. The reaction cells were thin-walled nuclear magnetic resonance (NMR) tubes (ICN Biomedicals, Inc., USA) of 5 mm in diameter with an irradiation surface of 8 cm^2 per millilitre of sample volume.

These studies were repeated in the presence of two free radical scavengers (BHA and GSH), a superoxide radical scavenger (SOD), a hydroxyl radical scavenger (MAN) and a singlet oxygen quencher (AZI). These additives were dissolved in potassium-free Krebs–Henseleit solution before addition to the cells. BHA was dissolved previously in methanol; the same concentration of methanol (1%) was added to the control sample.

After irradiation was complete, the samples were incubated in the dark at room temperature for 30 min. RBC suspensions were then centrifuged for 10 min at 3000*g* to remove the non-lysed RBCs. The haemolysis rate was determined by measuring haemoglobin and potassium concentrations in the supernatant. The haemoglobin was determined by adding 2 ml of Drabkin's solution (KCN, 0.05 g; $K_3Fe(CN)_6$, 0.2 g; KH_2PO_2, 0.14 g; H_2O, 900 ml; pH 7.2 ± 0.1) to 2 ml aliquots in order to convert all types of haemoglobin to cyanmethaemoglobin [25]. The samples were read by spectrophotometry (Baush & Lomb, Spectronic 710, USA) at 541 nm. Potassium concentrations were read by flame photometry (Jencons Scientific Ltd., UK).

2.2. Histidine phototoxicity test [25]

Histidine is photodegradable when irradiated with UVA light according to a degradation curve which was previously determined. It was assumed that this amino acid photodegrades more efficiently in the presence of photosensitizers.

Solutions of TIA in 10% (v/v) propyleneglycol in sodium phosphate buffer (0.01 M, pH 7.4) were mixed with an equal quantity of L-histidine monochloride solution (0.61 mM) in 0.01 M sodium phosphate buffer (pH 7.4). Samples of this mixture were bubbled with either oxygen or nitrogen for 20 min, or were not bubbled. Finally, the test-tubes were tightly sealed and irradiated with UVA light as in the photohaemolysis study. Samples of the histidine solutions and samples containing the buffered drug solution only (to allow compensation for possible colour changes of the irradiated drug) were also irradiated and the respective controls were sheltered from light. Irradiation was performed using a PUVA unit and NMR tubes as previously described.

The studies involving the samples bubbled with oxygen were repeated in the presence of the same radical scavengers as in the photohaemolysis study. These additives were dissolved in 0.01 M sodium phosphate buffer (pH 7.4) before addition to the histidine–NSAID solution.

When irradiation was complete, the samples were incubated in the dark at room temperature for 30 min. Histidine was determined by a modified Pauly reaction [25]. For this, 200 μl of test solution was made up to 2 ml with phosphate buffer; 200 μl of 1% sulphanilic acid in 0.87 N HCl and 200 μl of 5% sodium nitrite were added and the mixture was left for 10 min; 0.6 ml of 20% sodium carbonate was then added and after a further 2 min, 2 ml of ethyl alcohol. The optical density of the final solution was read at 530 nm in a spectrophotometer (Bausch & Lomb, Spectronic 710, USA) against a reagent blank; the histidine concentration was determined from a standard curve.

2.3. Analysis of data and statistics

In the photohaemolysis test each experiment was repeated at least five times ($n=5$), each time with the blood of a different donor. Also, at least five experiments were performed in the histidine test.

Some results of haemolysis are presented as a percentage of complete haemolysis obtained by hypotonic shock.

Results were expressed as the means $\pm$ standard error of the mean (SEM). Means were analysed for statistical differences using Student's t test. A probability of 0.95 or more was considered significant.

3. Results

3.1. Photohaemolysis

In non-bubbled 0.1 mM TIA solutions, UVA light produced lysis of the RBCs in a dose-dependent manner (Fig. 1). Photohaemolysis induced by TIA was increased in oxygenated solutions, but was also intense in the presence of nitrogen (Fig. 1). TIA did not cause any lysis to erythrocytes kept in the dark and did not interfere with the measurements of both haemoglobin and potassium.

In the dose range tested (0.01–0.1 mM) and with an irradiation of 15 J cm^{-2} UVA, TIA was photohaemolytic using both haemoglobin and potassium measurements (Table 1).

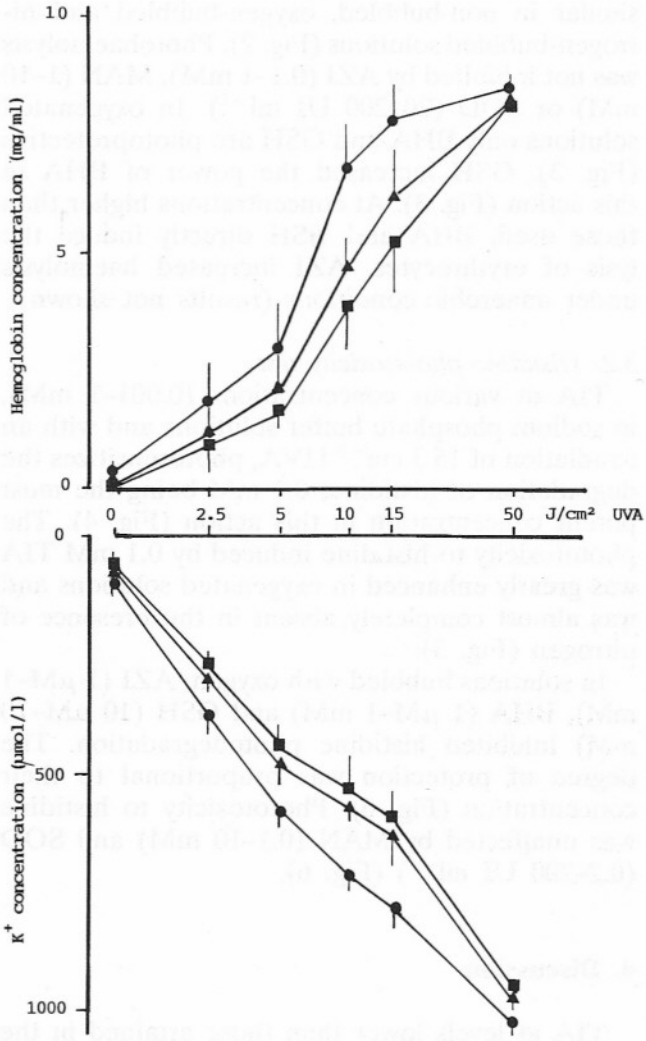

Fig. 1. In the presence of 0.1 mM TIA, UVA significantly produced photohaemolysis in a dose-dependent manner in both haemoglobin and potassium measurements: ▲, air; ●, oxygen; ■, nitrogen. TIA-induced photohaemolysis was increased in oxygenated solutions, but was also intense in the presence of nitrogen. "Air" denotes non-bubbled solutions. Results are expressed as mean $\pm$ SEM ($n=5$).

TABLE 1. Tiaprofenic acid was photohaemolytic using both haemoglobin (Hb) and potassium (K^+) measurements. The results expressed are calculated as the tiaprofenic acid induced photohaemolysis minus the haemolysis in the control sample (without tiaprofenic acid). Mean ± SEM ($n = 5$).

Tiaprofenic acid concentrations	% of total haemolysis (Hb-measurements)	% of total haemolysis (K^+-measurements)
10 μmol l^{-1}	18.6 ± 5.7%	14.4 ± 2.3%
100 μmol l^{-1}	27.5 ± 8.2%	35.1 ± 2.9%

BHA (0.01 mM and 0.1 mM) and GSH (0.1 mM and 1 mM (inhibited the 0.1 mM TIA photoaggression towards the red cell membrane, BHA being the most potent (Fig. 2). These effects were similar in non-bubbled, oxygen-bubbled and nitrogen-bubbled solutions (Fig. 2). Photohaemolysis was not inhibited by AZI (0.1–1 mM), MAN (1–10 mM) or SOD (20–200 UI ml^{-1}). In oxygenated solutions only BHA and GSH are photoprotective (Fig. 3). GSH increased the power of BHA in this action (Fig. 3). At concentrations higher than those used, BHA and GSH directly induce the lysis of erythrocytes. AZI increased haemolysis under anaerobic conditions (results not shown).

3.2. *Histidine phototoxicity test*

TIA at various concentrations (0.001–1 mM), in sodium phosphate buffer solutions and with an irradiation of 15 J cm^{-2} UVA, photosensitizes the degradation of histidine, 0.1 mM being the most potent concentration in this action (Fig. 4). The phototoxicity to histidine induced by 0.1 mM TIA was greatly enhanced in oxygenated solutions and was almost completely absent in the presence of nitrogen (Fig. 5).

In solutions bubbled with oxygen, AZI (1 μM–1 mM), BHA (1 μM–1 mM) and GSH (10 μM–10 mM) inhibited histidine photodegradation. The degree of protection was proportional to their concentration (Fig. 6). Phototoxicity to histidine was unaffected by MAN (0.1–10 mM) and SOD (0.2–200 UI ml^{-1}) (Fig. 6).

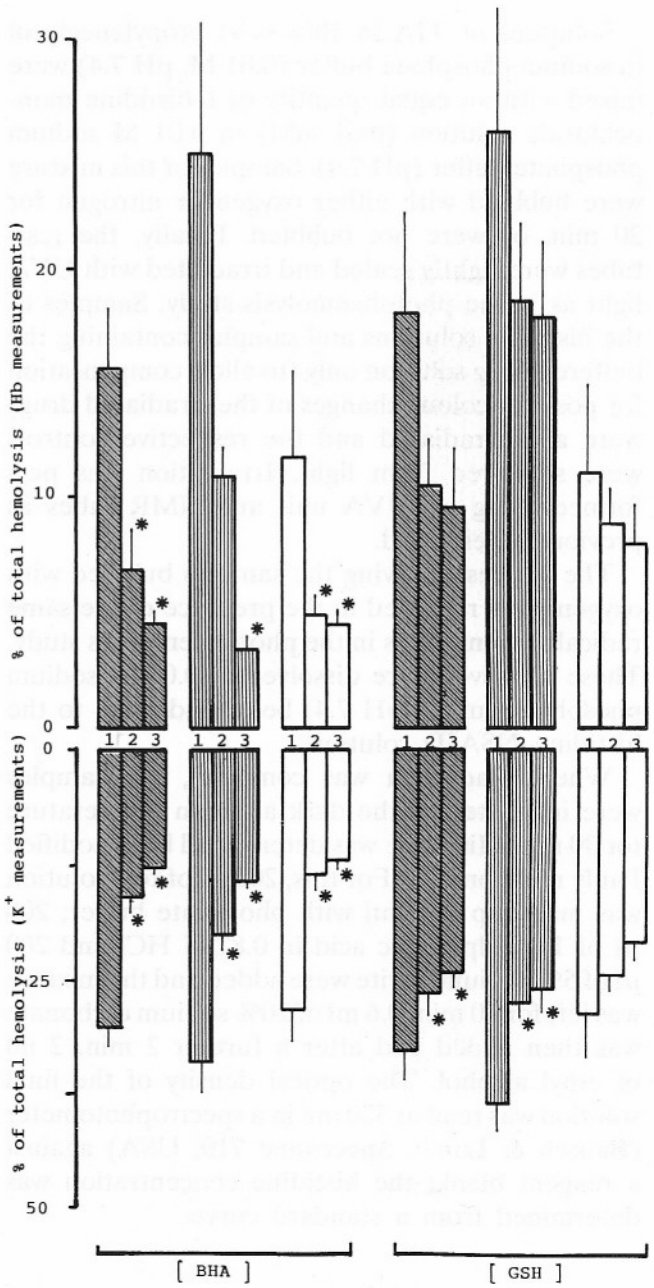

Fig. 2. BHA (0.01 and 0.1 mM) and GSH (0.1 and 1.0 mM), when compared with controls (columns on left), inhibited the 0.1 mM TIA-induced photohaemolysis, BHA being the most potent. These effects were similar in non-bubbled (Air, ▨), oxygen-bubbled (▥) and nitrogen-bubbled (□) solutions. Solutions were irradiated with 15 J cm^{-2} UVA. Results are expressed as the mean ± SEM ($n = 5$). 1, 2 and 3 signify 0, 0.01 and 0.1 mM BHA or 0, 0.1 and 1.0 mM GSH. *$P < 0.05$ (Student's t test).

4. Discussion

TIA at levels lower than those attained in the blood when this drug is used clinically [33], is highly phototoxic in the two *in vitro* phototoxicity models, which confirms the results obtained *in vitro* in a photo-basophil-histamine release test [37] and *in vivo* with the mouse tail technique [38]. These results and the routine photopatch testing in 100 patients [39] confirm its potential for cutaneous photosensitivity [13, 34–36].

The high phototoxic potential of TIA which may be even higher than benoxaprofen [38], contradicts the low incidence of photosensitivity reactions observed with this drug in clinical practice. This may possibly be due to its pharmacokinetic prop-

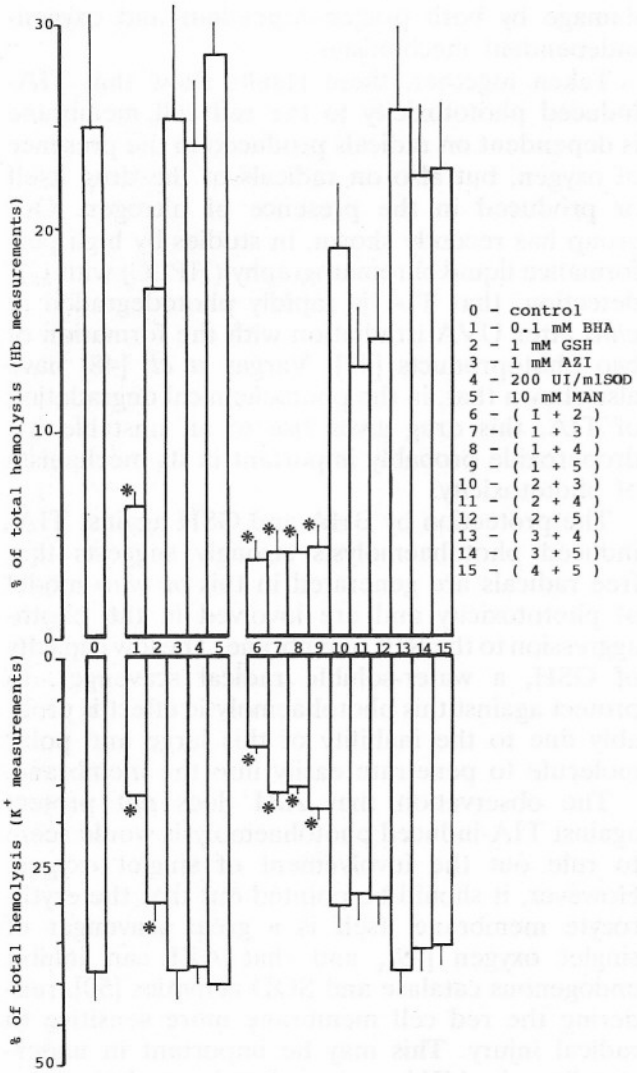

Fig. 3. In oxygenated solutions, only BHA and GSH are photoprotective against TIA-induced photohaemolysis. GSH increased the effect of BHA in this action. Solutions were irradiated with 15 J cm^{-2} UVA. Results are expressed as the mean ± SEM ($n = 5$). *$P < 0.05$ (Student's t test).

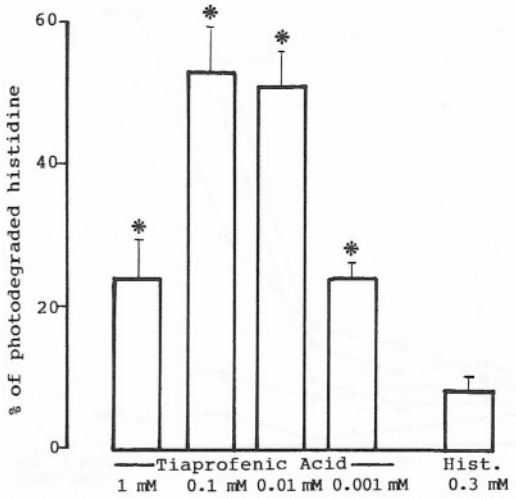

Fig. 4. When the solutions were irradiated by 15 J cm^{-2} UVA, TIA increased the photodegradation of histidine, in a significant manner, compared with the normal photodegradation of histidine (column on right). Results are expressed as the mean ± SEM ($n = 9$). *$P < 0.05$ (Student's t test).

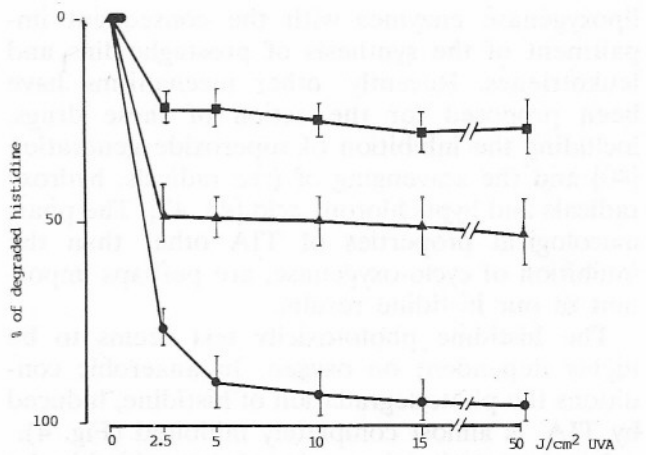

Fig. 5. Influence of oxygen and nitrogen on the photodegradation of histidine induced by 0.1 mM TIA: ■, nitrogen; ▲, air; ●, oxygen. The solutions were irradiated with doses of UVA in the range 0–50 J cm^{-2}. Results are expressed as the mean ± SEM ($n = 5$). *$P < 0.05$ (Student's t test).

erties: the peak plasma concentrations are attained 40 min to 2 h after oral ingestion, the half-life of elimination is about 1.5–2.5 h and the volume of distribution ranges from 4% to 10% of body weight with very low tissue levels [33]. These characteristics decrease the probability of being exposed to solar light with adequate skin concentrations of TIA for cutaneous photoreactions.

In these photohaemolysis studies, we determined not only, as is usual, the haemoglobin, but also the potassium which leaks through the membrane and reflects the erythrocyte damage. Potassium measurements in the supernatant gave us more reproducible results than the determination of haemoglobin.

TIA sensitizes the photodegradation of histidine in an unusual dose–effect manner. The most potent concentration in this action was 0.1 mM, and the concentrations of 1 mM and 0.001 mM were of similar potency. We assume that TIA, on the one hand, sensitizes the photodegradation of histidine and, on the other, by its complex anti-inflammatory activity, protects histidine from photodegradation. This protection is evident at high concentrations and decreases at low concentrations of the drug.

The therapeutic activity of TIA and other NSAIDs appears to depend, to a large extent, on the inhibition of cyclo-oxygenase and, sometimes,

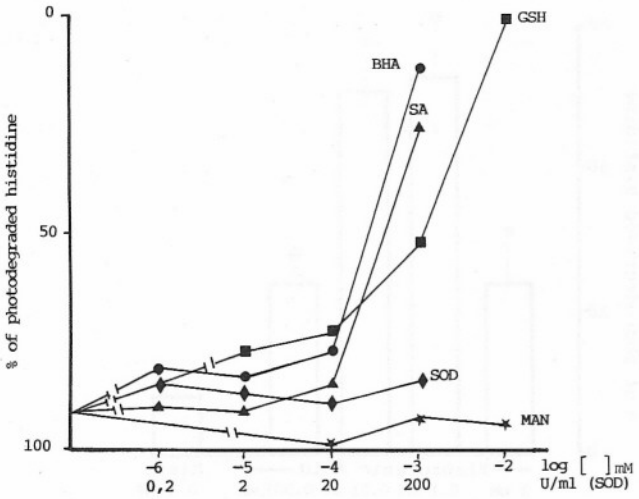

Fig. 6. In oxygenated solutions, AZI (SA), BHA and GSH inhibited the 0.1 mM TIA-induced histidine photodegradation. Solutions were irradiated with 15 J cm^{-2} UVA. Results are expressed as the mean ($n=5$); SEM was always less than 10%.

lipoxygenase enzymes with the consequent impairment of the synthesis of prostaglandins and leukotrienes. Recently, other mechanisms have been proposed for the action of these drugs, including the inhibition of superoxide generation [40] and the scavenging of free radicals, hydroxy radicals and hypochlorous acid [41, 42]. The pharmacological properties of TIA other than the inhibition of cyclo-oxygenase, are perhaps important in our histidine results.

The histidine phototoxicity test seems to be highly dependent on oxygen. In anaerobic conditions the photodegradation of histidine, induced by TIA, is almost completely inhibited (Fig. 4).

In contrast photohaemolysis is intensified in the presence of oxygen, but is also observed in nitrogenated solutions (Fig. 1). There is some controversy about the oxygen dependence of the photohaemolysis induced by other NSAIDs. Webster *et al.* [43] have demonstrated *in vitro* that in the presence of UV radiation benoxaprofen produces a dose-dependent lysis of sheep erythrocytes, which does not appear to be dependent on the presence of oxygen. Photohaemolysis induced by naproxen [44] and ketoprofen [45] also occurs under anaerobic conditions, but the presence of oxygen markedly enhances the lysis. In contrast, with diflunisal, photohaemolysis occurs under anaerobic conditions at an increased rate [46]. In the present study, we found that TIA, like other propionic-acid-derived NSAIDs, induces photohaemolysis in oxygenated and nitrogenated solutions, but is more evident in the presence of oxygen. These observations suggest that TIA can cause membrane damage by both oxygen-dependent and oxygen-independent mechanisms.

Taken together, these results show that TIA-induced phototoxicity to the red cell membrane is dependent on radicals produced in the presence of oxygen, but also on radicals of the drug itself or produced in the presence of nitrogen. Our group has recently shown, in studies by high performance liquid chromatography (HPLC) with UV detection, that TIA is rapidly photodegraded *in vitro* after UVA irradiation with the formation of two photoproducts [47]. Vargas *et al.* [48] have also shown that, in the photochemical degradation of TIA, this drug gives rise to an unstable hydroperoxide probably important in its mechanism of phototoxicity.

The protection by BHA and GSH against TIA-induced photohaemolysis strongly suggests that free radicals are generated in this *in vitro* model of phototoxicity and are involved in the photoaggression to the RBC membrane. The low capacity of GSH, a water-soluble radical scavenger, to protect against this photohaemolytic effect is probably due to the inability of this large and polar molecule to penetrate easily into the membrane.

The observation that AZI does not protect against TIA-induced photohaemolysis would seem to rule out the involvement of singlet oxygen. However, it should be pointed out that the erythrocyte membrane itself is a great scavenger of singlet oxygen [49], and that AZI can inhibit endogenous catalase and SOD activities [50], rendering the red cell membrane more sensitive to radical injury. This may be important in understanding why AZI increases photohaemolysis under anaerobic conditions. AZI is very insoluble in organic solvents and should remain mostly in the aqueous phase and not in the erythrocyte membranes. The lifetime of singlet oxygen is much longer in organic than in aqueous media, and so the circumstances are very unfavourable for the inorganic reagent to quench much of the singlet oxygen and to prevent it from damaging the erythrocyte [51].

Similarly, the lack of efficacy of SOD does not mean that superoxide is not generated in this "biological" model of phototoxicity, because the efficacy of SOD depends on the erythrocyte levels of catalase, glucose-6-phosphate dehydrogenase and glutathione peroxidase which suppress SOD-generated hydrogen peroxide. For this reason the enrichment of RBCs with SOD can sometimes have a pro-haemolytic effect [52].

The ability of BHA, GSH and AZI to provide dose-dependent protection against NSAID-induced photodegradation of histidine suggests that

free radicals and singlet oxygen are generated in this *in vitro* phototoxicity model and are involved in the photosensitized oxidation of this amino acid. The ineffectiveness of SOD does not necessarily mean that superoxide is not important in this model of phototoxicity, because SOD-generated hydrogen peroxide can also oxidize histidine. In this "chemical" model it would be almost impossible to generate hydroxyl radical and so the lack of efficacy of MAN was expected.

In vitro models of phototoxicity have essentially two functions. Firstly, they may provide a screening method for the phototoxic potential of new substances; secondly, they may achieve some insight into the mechanisms involved in photosensitization and hypothetically in skin photoreactions.

The RBC is a system devoid of organelles. Therefore photosensitized haemolysis has been attributed to membrane damage which results in disturbed cation permeability leading to colloid osmotic swelling and lysis. However, the erythrocyte membrane has its own means of protection. Phototoxicity in this "biological" model results from the interaction between the means of aggression and defence in the RBC membrane.

The histidine test is a "chemical" model of phototoxicity which is highly dependent on oxygen and has no intrinsic protection for histidine. Histidine is a ubiquitous amino acid in biological systems and a major site of damage during radical attack on proteins [53]. The results of this test are hence of consequence when studying medical phototoxicity.

These two *in vitro* models test different mechanisms of phototoxicity, but complement one another in the investigation of potential phototoxic substances and mechanisms of phototoxicity.

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