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Photosensitizing action of nonsteroidal antiinflammatory drugs on cell membranes and design of protective systems.

G. Condorelli, G. De Guidi, S.Giuffrida and L.L. Costanzo

Dipartimento di Scienze Chimiche, Università di Catania, Viale A.Doria 6, I-95125 Catania, Italy

Abstract

The pathways of photoreactivity of some nonsteroidal antiinflammatory drugs (NSAID), i.e. propionic acid derivatives indicated in a clinical picture as phototoxic and/or photoallergic, are discussed in a general oversimplified scheme. The NSAID photosensitizing activity, estimated from the ability to induce red blood cell lysis, was found to be dependent on the quantum yield of decarboxylation, which in turn determines the superoxide anion formation, on free radical reactivity and on singlet oxygen production efficiency. An investigation into the NSAID photoinduced cell damage in erythrocytes from various mammalian species, erythrocyte ghosts and unilamellar liposomes, evidenced the role played by membrane proteins and phospholipids. To inhibit phototoxic effects, some systems, including β-cyclodextrin and cupric ion and its complexes with bio-functionalized ligands, were considered for their ability in protecting cell membranes from the damage, which is photoinduced by superoxide anion, free radicals and singlet oxygen.

1. INTRODUCTION

The nonsteroidal antiinflammatory acid drugs (NSAID) presently constitute the principal therapeutic agent for controlling the pain and inflammation of rheumatic disease [1]. Several members of this group of drugs are implicated in adverse photosensitivity reactions; the clinical picture, associated with NSAID photosensitivity, suggests phototoxic and/or

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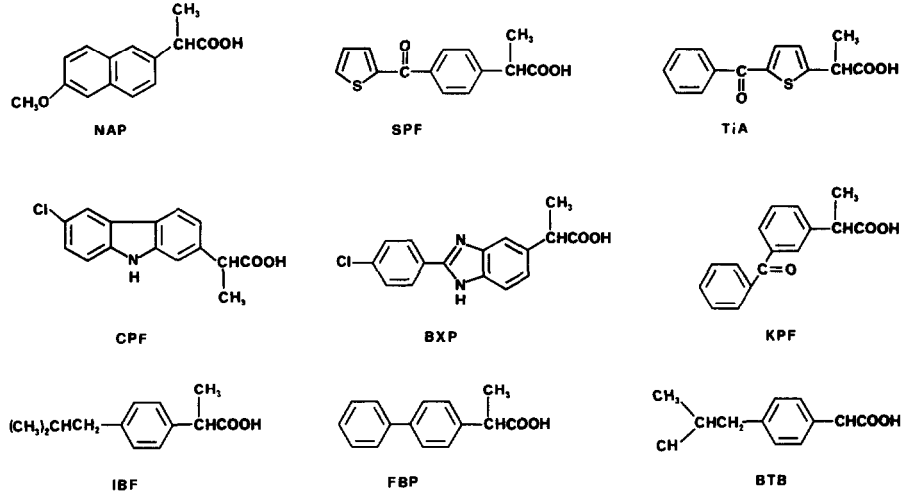
photoallergic reactions [2-11]. NSAID represent a heterogeneous group from a chemical point of view, but many of them are derivatives of propionic acid, substituted with iso- or heterocyclic ring systems at the 2-carbon atom.

In vitro studies of the propionic acid derivatives, such as Naproxen (NAP) [12-14], Suprofen (SPF) [15], Tiaprofenic Acid (TiA) [16-17], Carprofen (CPF) [18], Benoxaprofen (BX) [2,19-23], Ketoprofen (KPF) [24], Ibuprofen (IBF) [25] and Flurbiprofen (FBF) [26] and the butirric acid derivative Butibufen (BTF) [26], have shown that these drugs are the most active in the production of phototoxic effects.



2. MOLECULAR MECHANISM OF PHOTOSENSITIZATION

The NSAID photosensitizing activity has generally been estimated from the ability to induce, under irradiation, lysis of human red blood cells (RBC) in phosphate buffered saline media (pH 7.4); this process is indicative of membrane damage. The photohaemolysis experiments have been carried out in aerobic and anaerobic conditions in the presence of quenchers and scavengers of transient species. The results obtained, associated with the study of direct photochemistry of the drug, show an indication of the molecular mechanism of photosensitization.

A general scheme for the overall phototoreactivity of these drugs is reported in Fig. 1. Direct action of the light on the propionic acids in buffered saline phosphate (pH 7.4) leads to decarboxylation *via* intermediate radicals with the formation of only one compound, RCH_2CH_3 (I), in anaerobic conditions. This compound is accompanied by the oxydation products

RCH(OOH)CH_3 (II), RCH(OH)CH_3 (III), and RCH(O)CH_3 (IV) under aerated atmosphere. Some of these products show lytic activity towards membranes (Table 1). Intermediate species of oxygen, such as singlet oxygen and superoxide anion, are generated during the photochemical process.

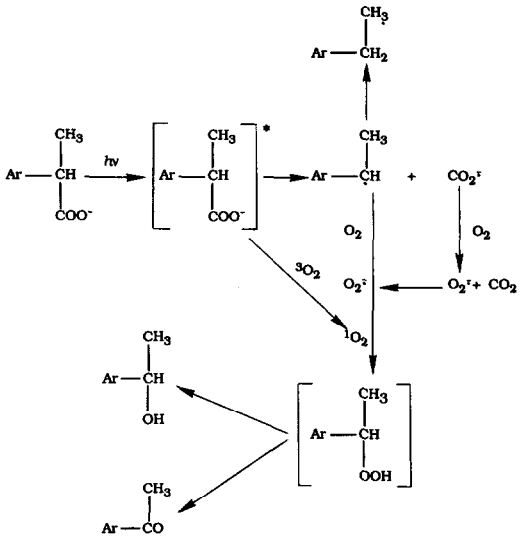


Fig. 1. General scheme of the photoreactivity of propionic acid derivatives.

Table 1
Lytic activity of the photoproducts of propionic acid derivatives.

	I	II	III	IV
NAP				
KPF	+	++	+++	+
CPF				
SPF	++		+	+
IBF			+++	++
BTF				
FBF			++	

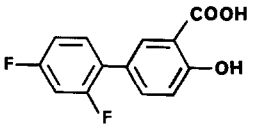
The photoreactivity of each drug, and consequently the associated photosensitization process, are dependent on the quantum yield of decarboxylation, which in turn regulates the formation of $O_2^{\bullet-}$, on the

reactivity of free radicals and on the efficiency of energy transfer to ground oxygen.

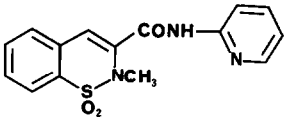
In the case of CPF, the first photochemical process in the presence of cell membrane which functions as a hydrogen donor, is a declorination reaction; then, the declorinated compound follows the general scheme of NSAID photoreactivity.

The butirric acid derivative Butibufen (BTF) can be included in the group of the propionic acids inasmuch as it displays a similar photochemical behaviour.

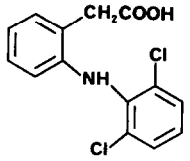
Among the several NSAID with a chemical structure different from the propionic acids, only Piroxicam (PRX), Diclofenac (DCF) and Diflunisal (DFN) were investigated as regard to the adverse light induced biological effects.



DFN



PRX



DCF

Piroxicam represents an *enigma*: a metabolite was found to be phototoxic *in vivo* and *in vitro*, and a second metabolite was mildly phototoxic *in vivo* [27]. However, in a recent review [28] the possibility has been suggested that the photoproducts of the drug are involved in the photosensitivity. Two photoproducts, the N-(2-pyridyl)oxanic acid and the N-methyl saccharin, have been isolated and characterized, but at present their phototoxicity has not been demonstrated [29].

For irradiation with UVA light in aqueous buffer or methanol solution Diclofenac, a drug recently introduced and very often used, leads to a sequential loss of both chlorine substituents and a ring closure to carbazole-1-acetic acid which is the major product. This is a weak phototoxic agent, able to generate singlet oxygen more efficiently than Diclofenac; the photodechlorination process *via* free radicals is considered the more probable initiation step of *in vivo* photosensitivity responses [30].

Irradiation of Diflunisal leads to the production of the compound PhP, which displays high lytic activity towards RBC: in fact PhP (10^{-4} M) induces 50% haemolysis (RBC = 3.3×10^6 cells/ml) within 5 min of incubation. Fig. 2 shows a scheme of the photosensitization mechanism in the presence of or the absence of RBC, from which it can be deduced that DFN induces photosensitized haemolysis with a mechanism involving a concerted action of free radicals, superoxide anion, singlet oxygen and particularly that of a lytic photoproduct [31].

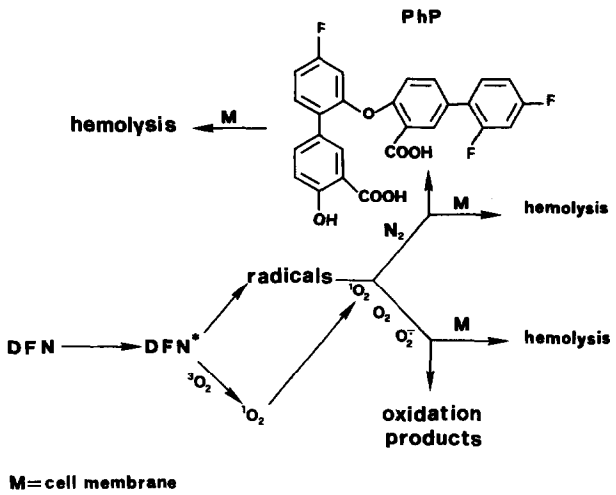


Fig. 2. Mechanism of photosensitization induced by Diflunisal.

3. MEMBRANE ALTERATION

The photosensitizing action of a drug, leading to RBC lysis, causes photomodification in the cell membrane with chemical alterations of its principal constituents: lipids and proteins. The most commonly observed effects are the peroxidation of polyinsaturated fatty acids and cholesterol and the crosslinking of proteins.

The question of the relative importance of these processes in the photoinduced membrane alteration has been subjected to large debate for over 50 years [32-36].

In the investigation into the cell damage photoinduced by NSAID, erythrocytes from various mammalian species, human erythrocyte ghosts and unilamellar liposomes have been used. The mammalian species selected have erythrocytes with a variable composition of the phospholipid classes, particularly in regard to the proportion between phosphatidylcholyne (PC) and sphyngomyelin (Sph) (Table 2) [37-38].

The photoinduced haemolysis rate is influenced by the phospholipid composition: Fig. 3 shows that the semihaemolysis times (t_{50}) increased with the increasing of the Sph content in the RBC membrane and with the decreasing of the PC content. NAP and DFN have been chosen because these drugs have two different mechanisms of photosensitization: the former has a prevalently photodynamic effect, the latter forms a photoproduct with passive permeation activity in the intact cell. In both cases the resistance of

the cell membrane to the photoinduced osmotic shock outlines the importance of the physical state of the membrane; the decreased fluidity in the phospholipid fatty acid chains due to the increase of Sph percentage strongly lowers the haemolysis rate. This finding, together with the lower number of peroxidable double bonds in Sph versus PC, supports the involvement of the phospholipids in the photohaemolytic process [39].

Table 2
Phospholipid composition of erythrocytes from various mammalian species

	Dog	Horse	Cat	Pig	Man	Goat	Cow	Sheep
PC	46.9	42.4	30.5	23.3	28.8	ND	ND	ND
PE	22.4	24.3	22.2	29.7	27.5	27.9	28.8	26.
PS	15.4	18.0	13.2	17.8	13.0	20.8	19.6	15.8
PI	2.2	<0.3	7.4	1.8	1.1	4.6	3.3	3.2
PA	0.5	<0.3	0.8	<0.3	2.2	<0.3	<0.3	<0.3
Sph	10.8	13.5	26.1	26.5	24.5	45.9	46.5	50.8
LPC	1.8	1.7	<0.3	0.9	1.2	ND	ND	ND

PC	Phosphatidylcholine	PA	Phosphatidyl acid
PE	Phosphatidylethanolamine	Sph	Sphingomyelin
PS	Phosphatidylserine	LPC	Lysophosphatidylcholine
PI	Phosphatidylinositole	ND	Not detected

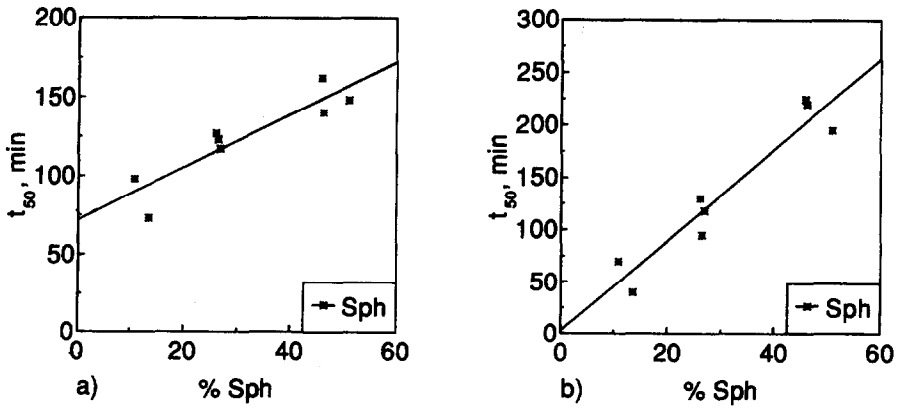


Fig. 3. Photohaemolysis induced by NAP (a) and DFN (b) in various mammalian species.

A confirmation of the importance of the relative percentage of Sph in the membrane is evidenced by a study of liposome resistance to the release of entrapped glucose-6-phosphate (G6P) photoinduced by NAP and DFN: this resistance is directly correlated to the Sph content (Fig. 4).

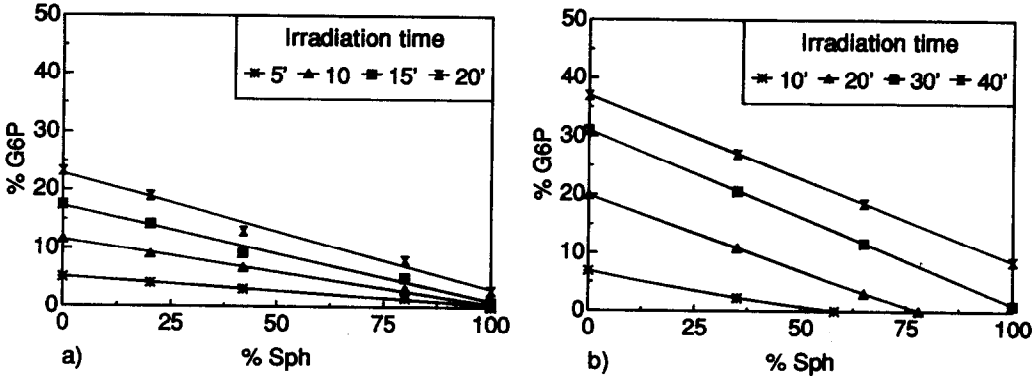


Fig 4. Photosensitization of liposomes at various Sph contents, irradiated in the presence of NAP (a) and DFN (b).

Although the liposomes do not lyse colloid osmotically, the comparison between the course of human RBC lysis and the release of trapped marker from liposomes with lipid composition close to that of the human erythrocytes, can give a useful indication of the involvement of membrane proteins in the photosensitization process (Fig. 5). In aerobic conditions NAP photoinduces both lysis of RBC and the release of marker from liposomes; in anaerobic conditions only photohaemolysis occurs. The absence of marker release can be explained by the lack of proteins in the liposome membrane. Thus, only proteins are involved in the hydrogen abstraction by the decarboxylated radical fragment in anaerobic condition [40].

In the case of DFN, both in aerobic and in anaerobic conditions photohaemolysis and marker release follow the same course, confirming that the same mechanism of passive permeation of the photoproduct PhP in the phospholipid bilayer is operative.

A further study of fatty acyl chain peroxidation and of protein crosslinking, induced by photosensitization with propionic acid derivatives [41], has been carried out with erythrocyte ghosts and PC unilamellar liposomes, with the aim of obtaining a comparation between membranes with and without proteins.

Protein crosslinking was detected as high molecular weight protein by SDS polyacrylammide gel electrophoresis; polyinsaturated phospholipid peroxidation was evaluated from the amount of tiobarbituric acid reactive substances (TBARS). Some data, regarding photosensitization by NAP, are reported in Fig.6.

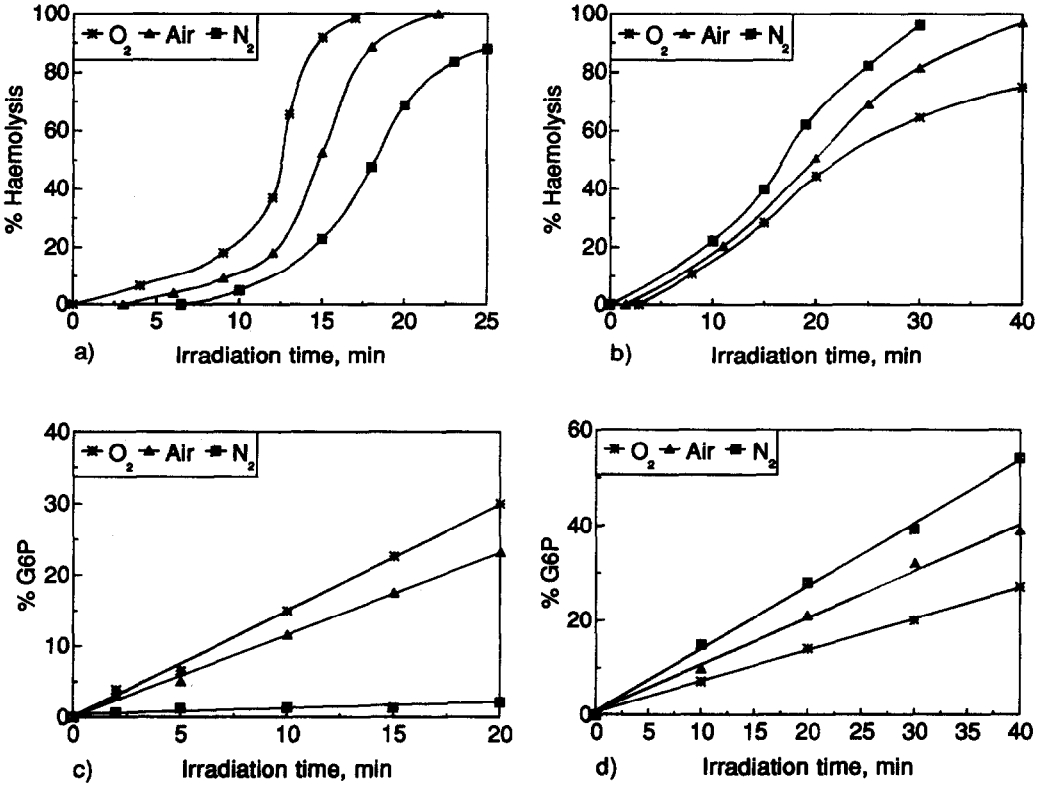


Fig. 5. Comparison between the lysis of human RBC and the release of trapped marker from liposomes sensitized by NAP (a,c) and DFN (b,d).

Photoinduced protein crosslinking, generated from a loss of spectrin and Band 3 protein, was partially oxygen dependent. Singlet oxygen is involved in the process of crosslinking, as well as in phospholipid peroxidation. Superoxide anion can provoke phospholipid peroxidation in the presence of iron traces *via* the Haber-Weiss reaction. This can occur in erythrocyte ghosts with hemoglobin residue, but in iron free liposomes no oxidation occurs by superoxide anion

On the basis of the overall results a general scheme can be proposed in order to relate the photochemistry of the drug, the membrane alteration and the photohaemolytic process (Fig. 7).

4. PHOTOPROTECTION SYSTEMS

Since NSAID photoinduce damage to the cell membrane, it has been considered useful to find some systems potentially able to reduce such damage.

β-Cyclodextrin, which forms inclusion complexes with NSAID, reduces the photoinduced haemolysis [42,43]. The protective action was found only in a restricted range of concentrations. At concentrations above this range, β-cyclodextrin damages the membrane.

Knowledge of the mechanisms of photosensitization (Fig. 7) suggests a possible design of other photoprotective systems, which permit the scavenging of the single intermediate active species. Among these systems, some copper complexes with biofunctionalized ligands, providing SOD-like activity, were considered [44]. These complexes reduce the photohaemolysis induced by KTP, a drug which undergoes photodegradation with $O_2^{\bullet-}$ formation; their protective action is related to their SOD-like activity, as shown by the comparison between the catalytic constants of the complexes and the semihaemolysis times (Tab. 3).

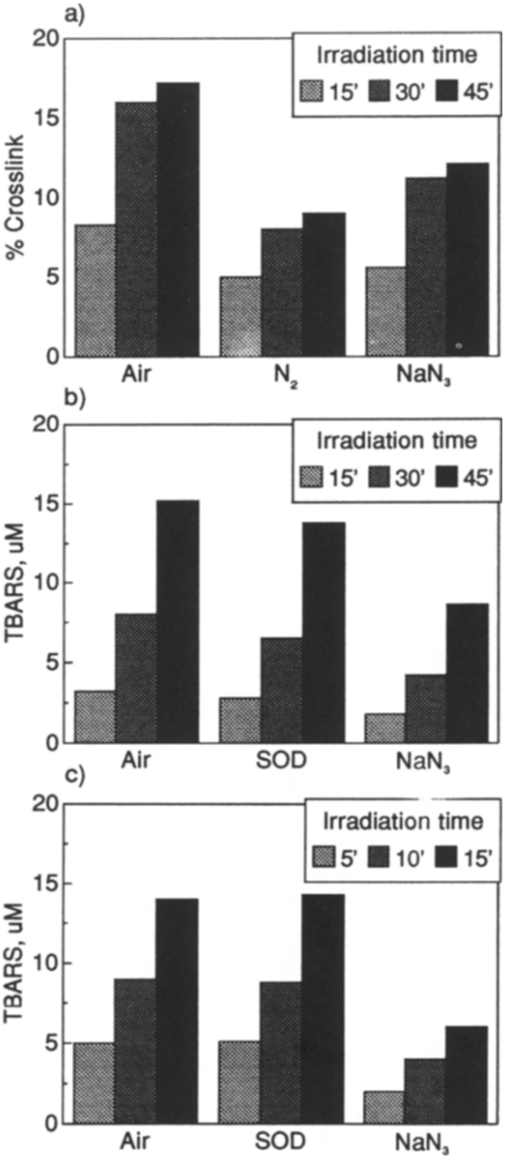


Fig 6. Protein crosslinking (a), erythrocyte peroxidation of ghosts (b) and liposomes (c) photoinduced by NAP.

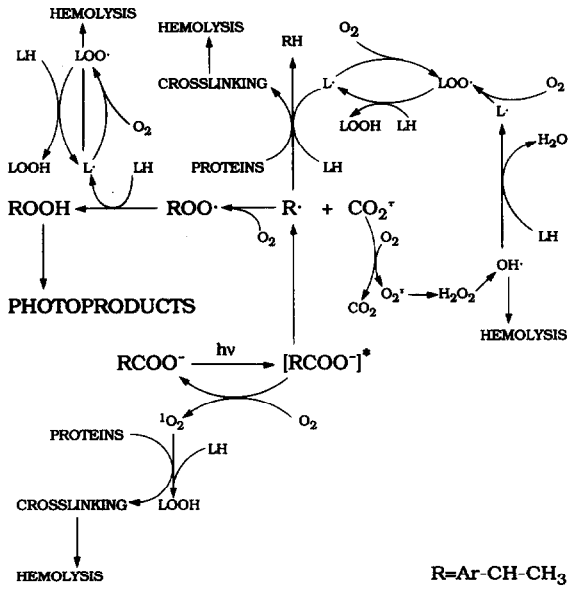


Fig. 7. General scheme of photosensitization induced by propionic acid derivatives

Table 3
Catalytic constants for the dismutation of superoxide anion by copper complexes and semihaemolysis times in KPF photosensitization in the presence of these complexes.

Copper (II) complexes	Kc x 10^{-7} / M^{-1} s^{-1}	t_{50} / min
= =		50
[Cu(HPO_4)]	4.72	73
[Cu(Leu-Leu)H_{-1}]	6	225
[Cu(Leu-D-Leu)H_{-1}]	9.1	280
[Cu(CD Hm)]$^{2+}$	10	325
[Cu(c-His-His)]$^{2+}$	45	350

Besides this, Cu^{2+} inhibits lipid photoperoxidation at concentrations higher than those needed for SOD-like activity (> 10^{-7} M) [45].

This behaviour was evidenced in phosphatidilcholine liposomes, where the Haber-Weiss reaction cannot be operative; thus peroxidation occurs *via* a radical pathway and Cu^{2+} may act as a free radical scavenger. The mechanism is not clear. However all the results are consistent with a mechanism in which Cu^{2+} is reduced to Cu^{+} by superoxide anion and then it is reoxidized to Cu^{2+} by the radicals present ($L^{\bullet}$, $LOO^{\bullet}$, $ArOO^{\bullet}$).

Among the ligands reported in Tab. 3, *c*-His-His in a concentration higher than 10^{-4} M was found to be able to quench the singlet oxygen and so give photoprotection. Thus a system containing Cu^{2+} and *c*-His-His in the most suitable concentrations is potentially able to protect cell membrane from the damage photoinduced by superoxide anion, free radicals and singlet oxygen.

5. PERSPECTIVES

The problem "sunlight + drug + biomolecules → adverse biological effects", needs thorough research on:

in vitro

a) the photophysical and photochemical properties of the drugs,

b) the photodamage induced in cellular components.

in vivo

a) the metabolism of the drug,

b) the amount of the drug which reaches the site of exposure or specific sites within the cell,

c) the repair processes by which the organism may respond to photochemically induced damage.

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