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# Scavengers Protection of Cells Against ALA-based Photodynamic Therapy-induced Damage

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**Abstract.** The exogenously stimulated formation of intracellularly generated protoporphyrin IX, a precursor of haem, is becoming one of the fastest developing areas in the field of photodynamic therapy (PDT). We tested the action of several free radical scavengers, amino acids, antioxidants and sulphur-containing compounds as protectors from photodamage induced by 5-aminolaevulinic acid (ALA)-mediated PDT, employing the LM2 cell line, derived from a mammary murine adenocarcinoma. We exposed the cells to different concentrations of the compounds, 24 h before PDT, during PDT, and 19 h after treatment. We defined the protection grade (PG) as the ratio between cell survival after ALA-PDT treatment in the presence of the protector and cell survival of ALA-PDT treatment alone. We found that L-tryptophan (PG=9.2 at 2 mM), reduced glutathione (GSH) (PG=5.8 at 0.8 mM), *N*-acetyl-L-cysteine (PG=4.86 at 30 mM), melatonin (PG=4.5 at 8 mM) and L-methionine (PG=4.0 at 0.8 mM) are the best protectors from PDT damage, followed by L-cysteine (PG=2.8 at 0.8 mM), mannitol (PG=2.6 at 20 mM) and glycine (PG=2.4 at 40 mM) whereas oxidised glutathione and *S*-adenosyl-L-methionine do not exert any protection. We did not found any photoactive action of the protectors in absence of ALA. These results can be considered to modulate the photodamage induced by ALA-PDT.

**Keywords:** Aminolaevulinic acid; Photodynamic therapy; PDT; Scavengers.

## INTRODUCTION

Photodynamic therapy (PDT) is a cancer treatment modality that, through the administration of an exogenous tumour-localising photosensitiser, mainly porphyrins, followed by an adequate dose of photoactivating light destroys malignant tissues [1]. New perspectives have been opened by the use of the porphyrin precursor 5-aminolaevulinic acid (ALA), from which synthesis of the photosensitiser protoporphyrin IX (PpIX), is accomplished in situ [2,3].

The initial photodynamic effect is the light-catalysed formation of active oxygen species, notably singlet molecular oxygen ($^1O_2$) [4] by a type II reaction and the generation of free radicals and other reactive intermediates such as hydroxyl radical (OH) and the superoxide anion by a type I reaction [5]. The oxidation of cellular constituents by these reactive oxygen species (ROS) damages plasma membranes and subcellular organelles.

Cellular protection mechanisms by way of superoxide dismutase and the scavenger glutathione avoided damage to organelles driven by the phototoxic action of PpIX [6]. When the scavengers trolox or α-tocopherol succinate were present during irradiation, the appearance of apoptotic cells and cytotoxic effects in murine leukaemia cells treated with photosensitisers with lysosomal targets was prevented [7]. Early apoptotic signals generated by PDT were inhibited by the singlet oxygen scavengers L-histidine and α-tocopherol, but not by hydroxyl radical scavengers [8].

Porphyrin accumulation from ALA in epidermal cells was decreased in the presence of sodium azide or mannitol [9]. Mannitol is a well-known hydroxyl radical scavenger, whereas tryptophan, reduced glutathione (GSH) and sodium azide, among others, are antioxidants protecting organisms against singlet oxygen.

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Glutathione has been shown to play a critical role in cellular defence against a variety of injurious agents. Under normal steady-state conditions, most of the glutathione exists in its reduced form. Oxidation of GSH, either non-enzymatically or by the action of glutathione peroxidase, yields glutathione disulphide (GSSG). NADPH-dependent reduction of GSSG by glutathione reductase, as well as its efflux, effectively mantains the intracellular concentration of GSH. Thus, in many cell types, the glutathione oxidation–reduction cycle helps to mantain structural and functional viability in spite of endogenous production of ROS overproduced during acute oxidant injury.

Intracellular thiols may play a role in the mechanisms of cancer treatment modalities such as ionising radiation, chemotherapy and hyperthermia [10]. A number of reports have shown that resistance to chemotherapeutic agents may be due to elevated cellular GSH concentrations [11,12]. These studies have indicated that GSH may be involved in protecting cells via detoxification of cytotoxic species, such as scavenging of free radicals and in the repair of oxidative cellular damage by hydrogen donation. It has also been suggested that GSH may influence DNA repair [13] as well as help to mantain membrane integrity via the formation of mixed disulphides [14]. GSH can act against singlet oxygen and superoxide anion radical [15]. Moreover, GSH depletion sensitises cells to photodynamic damage [16].

*S*-adenosyl-L-methionine (SAM) is a precursor of GSH which, unlike GSH itself, can readily cross cell membranes. It has been shown that supplementation of organ preservation solutions with SAM instead of GSH has improved hydroxyl radical and singlet oxygen scavenging, as well as chelation of iron ions [15].

Another sulphur-containing compound, *N*-acetyl-L-cysteine (NAC), also increases GSH levels by providing cysteine and it is known to prevent pathologies elicited by free radicals and ROS [17]. Tao et al. [18] found that NAC significantly overcame the effect on PDT-induced stress-activated protein kinase. It was also found that NAC protected against apoptosis mediated by pyropheophorbide-a-methylester in colon cancer cells [19]. However, NAC failed to attenuate PpIX-induced photosensitivy in erythropoietic protoporphyria [20] and patients treated with Photofrin [21].

In addition, the non-sulphur amino acid glycine, was shown to protect human endothelial cells from $H_2O_2$-induced lethal injury and also to diminish radiation-induced cytotoxicity in bovine endothelial cells [22,23].

The aim of this work was to evaluate the protector action of some free radical scavengers, amino acids, antioxidants and sulphur-containing compounds against the cytotoxic photodynamic action of ALA-PDT.

## MATERIALS AND METHODS

### Chemicals

ALA, L-cysteine, L-trypthophan, L-methionine, glycine, mannitol, melatonin, *S*-adenosylmethionine (SAM), *N*-acetyl-L-cysteine (NAC), reduced glutathione (GSH) and oxidised glutathione (GSSG) were obtained from Sigma Chem Co.

### Cell Line and Cell Culture

Cell line LM2 [24] derived from murine mammary adenocarcinoma M2 (Instituto Roffo, Buenos Aires) was cultured in minimum essential Eagle's medium (MEM), supplemented with 2 mM L-glutamine, 40 μg gentamycin/ml and 5% fetal bovine serum (FBS), and incubated at 37°C in an atmosphere containing 5% $CO_2$.

### ALA-PDT Treatment

Cells ($3.5 \times 10^4$ per well) were incubated in serum-free medium containing 0.2 mM ALA in 24-well plates, and 3 h later, laser irradiations were performed. After 5 min of irradiation, medium was replaced by ALA-free medium+fetal bovine serum, cells were incubated for another 19 h and then tested for viability. Irradiations were performed using a rhodamine dye laser (Model DL30, Oxford Lasers) pumped by a copper vapour laser (CU15A, Oxford Lasers) tuned to 630 nm. The light was focused into a 400 μm-diameter optical fibre coupled to a frontal light distributor (Model FD2, Medlight, Ecublens, Switzerland) to produce a treatment area of uniform intensity. The output power from the fibre was measured with a power meter (Model LM-100XL, Coherent, Auburn, CA) before each

application, and adjusted to the desired light dose (4.6 J/cm$^2$). Non-ALA and non-irradiated controls were also run.

### Drug Exposure

The scavengers, amino acids, antioxidants and sulphur-containing compounds were dissolved in sterile Earle's salts, pH 7.4 and added to the cells 24 h before PDT. They were also present during the 3-h exposure to ALA, during the 5 min of laser irradiation and 19 h after treatment.

### MTT Viability Assay

Phototoxicity/toxicity was documented by the MTT assay [25], a method based on the activity of mitochondrial dehydrogenases, which will be functionally affected by PDT in vitro [26]. At 19 h after treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added to each well at a concentration of 0.5 mg/ml, and plates were incubated at 37°C for 1 h. The resulting formazan crystals were dissolved by the addition of dimethylsulphoxide (DMSO) and absorbance was read at 560 nm.

### Cell Number

The number of cells seeded per well was determined by counting viable cells with the Trypan blue exclusion method.

## RESULTS

### Effect of Protectors on Cell Survival induced by ALA-PDT, ALA and Light Exposure

Figures 1–4 show the action of some free radical scavengers, amino acids, antioxidants and sulphur-containing compounds on cell viability under four different experimental conditions: the impact of the protector itself on cell viability, the potential photoactive action of the compound when illuminated, the cytotoxic action of the compound when coincubated with ALA and the effect of the compound in modifying ALA-PDT induced cytotoxicity. ALA concentration and time exposure as well as light conditions were determined in previous work [27].

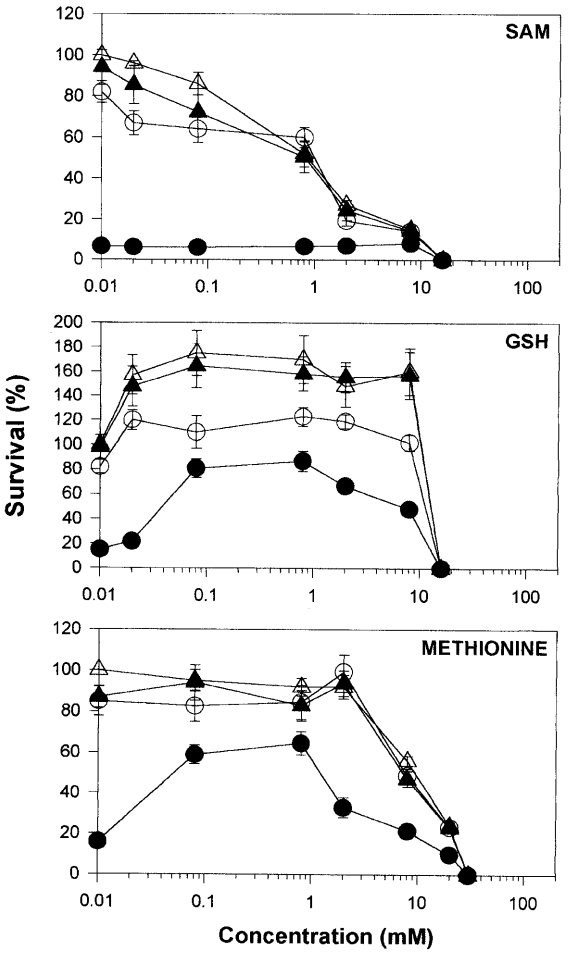


**Fig. 1.** Effects of SAM, GSH and methionine on cell survival induced by PDT, ALA and light exposure. Tryptophan and mannitol were added to the cells from 24 h before PDT to 19 h after treatment, ALA and light exposure period included. PDT-treated cells (●), ALA-treated cells (○), light-irradiated cells (▲) and untreated control cells (△). MTT assay was performed after replacing the medium with free-scavenger medium. The percentage of survival was referred to control cells neither PDT-treated nor exposed to scavengers. Each data point represents the average of three determinations. Error bars show standard deviations.

None of the compounds were cytotoxic by themselves at low concentrations, however, they decreased cell viability by more than 20% at higher and different concentrations for each compound: SAM 0.02 mM, melatonin and cysteine 3 mM, glycine and methionine 4 mM, GSSG 15 mM and NAC, tryptophan, GSH and mannitol 20 mM.

We did not find any photoactive action of the protectors by themselves. When the compounds were coincubated with ALA in the absence of light, in most cases there was a general reversion of the dark toxicity induced by ALA. The exception was GSSG, that enhanced ALA-induced cytotoxicity. As a

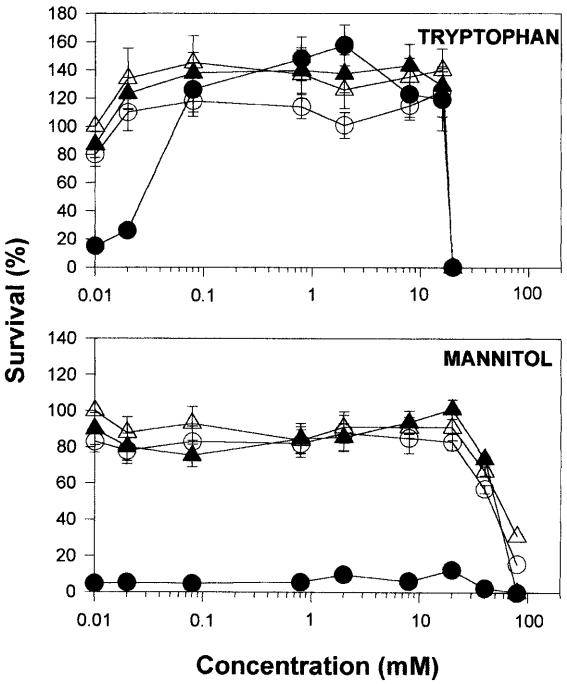


**Fig. 2.** Effects of tryptophan and mannitol on cell survival induced by PDT, ALA and light exposure. SAM, GSH and methionine were added to the cells from 24 h before PDT to 19 h after treatment, ALA and light exposure period included. PDT-treated cells (●), ALA-treated cells (○), light-irradiated cells (▲) and untreated control cells (△). MTT assay was performed after replacing the medium with free-scavenger medium. The percentage of survival was referred to control cells neither PDT-treated nor exposed to scavengers. Each data point represents the average of three determinations. Error bars show standard deviations.

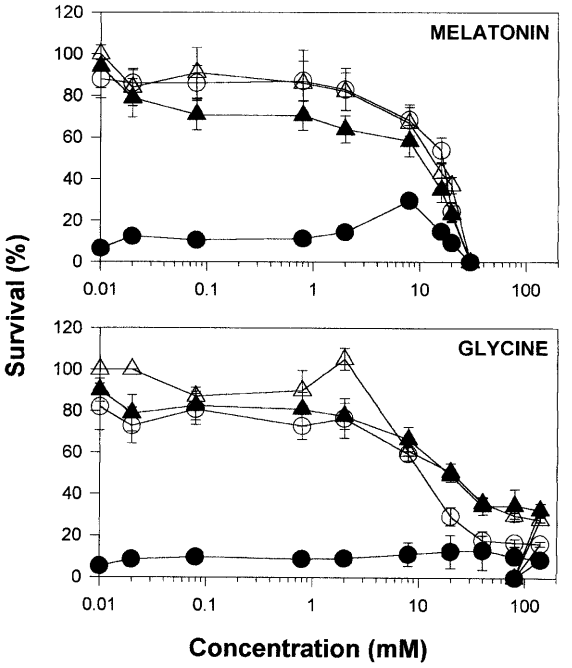


**Fig. 3.** Effects of melatonin and glycine on cell survival induced by PDT, ALA and light exposure. NAC, cysteine and GSSG were added to the cells 24 h before PDT, during ALA and light exposure period and 19 h after treatment. PDT-treated cells (●), ALA-treated cells (○), light-irradiated cells (▲) and untreated control cells (△). MTT assay was performed after replacing the medium with free-scavenger medium. The percentage of survival was referred to control cells neither PDT-treated nor exposed to scavengers. Each data point represents the average of three determinations. Error bars show standard deviations.

general pattern, the concentrations affording protection against ALA-induced cytotoxicity were coincident with the concentrations protecting against PDT. The effect of these protectors on the cytoxicity induced by ALA-PDT compared to the behaviour of PDT-treated cells in their absence, is further examined in Figs 5 and 6.

## Influence of Protectors on Cell Survival after ALA-PDT

To study the effect of the free radicals scavengers, amino acids, antioxidants and sulphur-containing compounds comparatively, we defined the protection grade (PG) as the ratio between cell survival after ALA-PDT treatment in the presence of the different compounds and cell survival without protectors.

Maximum protection grades were found at varying concentrations depending on the compound. L-Tryptophan (PG=9.2 at 2 mM), GSH (PG=5.8 at 0.8 mM), NAC (PG= 5.3 at 20 mM), melatonin (PG=4.5 at 8 mM) and L-methionine (PG=4.0 at 0.8 mM) were found to be the best protectors from PDT damage at their optimal concentrations, followed by L-cysteine (PG=2.8 at 0.8 mM), mannitol (PG=2.6 at 20 mM) and glycine (PG=2.4 at 40 mM). Oxidised glutathione and *S*-adenosyl-L-methionine did not exert any protection.

In a parallel set of experiments, cells were exposed to protectors, prior to PDT, during PDT, and after PDT separately, and in a combination of these experimental conditions. We found that the maxima PGs were reached when cells were treated with the protector before and during PDT; its addition after PDT did not improve cell survival (data not depicted).

## DISCUSSION

L-Tryptophan, a well-known and efficient singlet oxygen quencher (Table 1), was the best protector among all the compounds assayed, affording a PG of 9.2 at a concentration as low

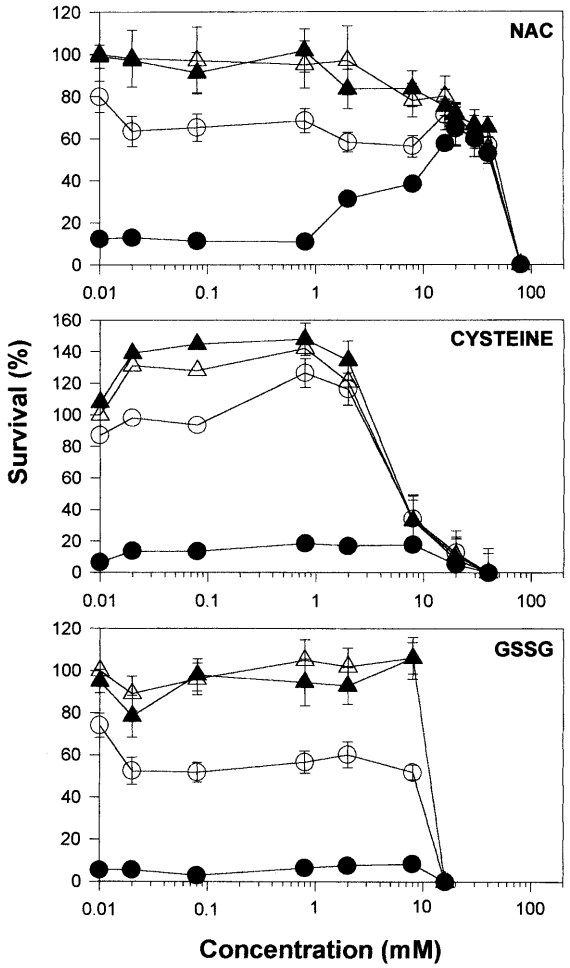


**Fig. 4.** Effects of NAC, cysteine and GSSG on cell survival induced by PDT, ALA and light exposure. Melatonin and glycine were added to the cells 24 h before PDT, during ALA and light exposure period and 19 h after treatment. PDT-treated cells (●), ALA-treated cells (○), light-irradiated cells (▲) and untreated control cells (△). MTT assay was performed after replacing the medium with free-scavenger medium. The percentage of survival was referred to control cells neither PDT-treated nor exposed to scavengers. Each data point represents the average of three determinations. Error bars show standard deviations.

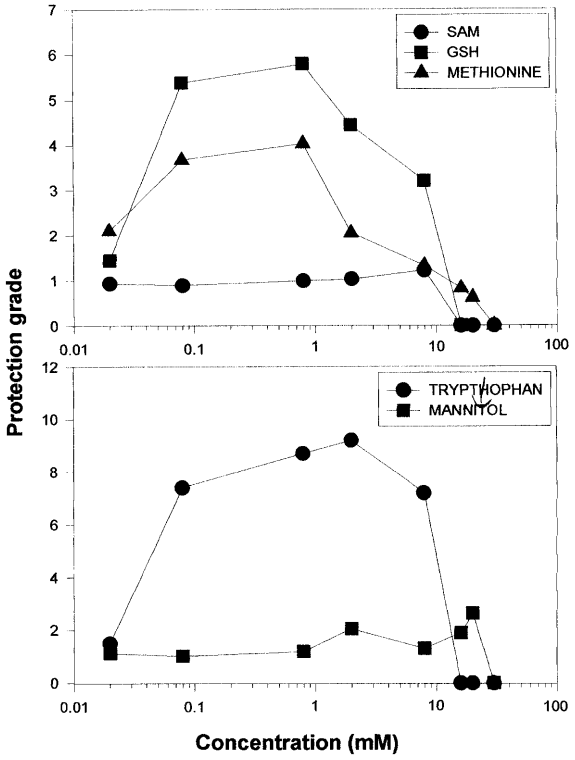


**Fig. 5.** Influence of protectors on cell survival after ALA-PDT. SAM, GSH, methionine, tryptophan and mannitol were added to the cells 24 h before PDT, during ALA and light exposure period and 19 h after treatment. MTT assay was performed after replacing the medium with free-scavenger medium. PG is the ratio between cell survival after ALA-PDT treatment in the presence of the different compounds and cell survival without protectors.

as 2 mM. On the other hand, the hydroxyl radical scavenger mannitol afforded little protection (PG=2.6 at 20 mM). Although lack of specificity of free radical quenching should be considered, these findings may underline the predominant role of $^1O_2$ in ALA-PDT cell killing.

Regarding the sulphur-containing compounds, GSH was highly protective. The essential amino acid methionine can be converted into SAM, which through a series of transulphuration reactions yields cysteine and from cysteine comes GSH. The aminothiol NAC can scavenge free radicals through binding of the reduced sulphydryl group, either by thiol metabolism or by enhancing GSH metabolism. NAC itself can act as a singlet oxygen quencher. In vivo, NAC forms cysteine, cystine, methionine, glutathione and mixed sulphides [28].

In this study GSH (PG=5.8 at 0.8 mM), NAC (PG=5.3 at 20 mM) and methionine (PG=4.0 at 0.8 mM), when used at their maximal protection concentrations, afforded higher protection against ALA-PDT damage than L-cysteine (PG=2.8 at 0.8 mM), which afforded only a slight protection. However it should be noted that much higher NAC concentration is needed to induce the same effect as that produced by GSH. Although for most of the compounds maxima protection grades were reached at relatively high concentrations, it has to be taken into account that concentrations above 1 mM are not pharmacologically relevant.

Unexpectedly, SAM did not show any protection against ALA-PDT, perhaps because at the concentrations tested, higher than 0.02 mM, it appears to have some toxic effect. As

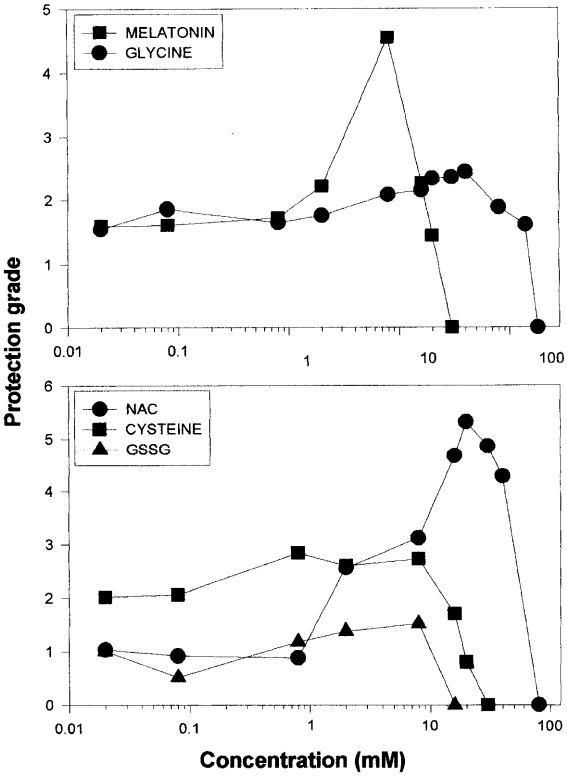


**Fig. 6.** Influence of protectors on cell survival after ALA-PDT. Glycine, melatonin, NAC, cysteine and GSSG were added to the cells 24 h before PDT, during ALA and light exposure period and 19 h after treatment. MTT assay was performed after replacing the medium with free-scavenger medium. PG is the ratio between cell survival after ALA-PDT treatment in the presence of the different compounds and cell survival without protectors.

expected, the oxidised form of glutathione, GSSG, neither protected nor enhanced PDT-induced photoxicity.

Cell viability of both non-treated controls and ALA-PDT-treated cells increased 60% in the presence of GSH, showing an unspecific cell metabolism activation. On the other hand, tryptophan increases 40% non-treated cells viability but a much higher increase (140%) is observed in PDT treated cells, demonstrating in addition, a specific protection against photodamage.

The pineal hormone melatonin is a potent hydroxyl radical scavenger [29]. Additionally, melatonin has been reported to neutralise hydrogen peroxide, singlet oxygen, peroxynitrite anion and nitric oxide, and it also stimulates several antioxidative enzymes [30]. In our study we proved that this hormone is an excellent protector (PG=4.5 at 8 mM), at nearly the same level as the the sulphur-containing compounds NAC and methionine.

In addition to singlet oxygen, which is the primary cytotoxic agent in PDT, other interconvertible reactive oxygen species, in particular OH [31] are also generated. Moreover, $^1O_2$, which is a powerful active oxygen form but not a free radical, may induce the formation of oxygen free radicals [32,33]. These two facts explain the ability of the radical scavengers to protect from ALA-PDT-induced damage.

The protection exerted by the non-sulphur amino acid tryptophan is probably due to quenching of $^1O_2$ to photooxidation [31] and the slight action of glycine may be ascribed to direct oxidation of the molecule [21]. In agreement with our results, Henderson and Miller [31] found that tryptophan largely abolished PDT cell damage induced by Photofrin in CHO

**Table 1.** Action of some free radical scavengers, amino acids, antioxidants and sulphur-containing compounds agains ALA-PDT damage

| | Mode of action | Protection from ALA-PDT damage |
|---|---|---|
| SAM | Precursor of GSH. Can Cross cell membranes | No protection |
| NAC | Scavenge by thiol metabolism or enhancing GSH metabolism. $^1O_2$ quencher | Protective |
| GSH | Scavenger of free radicals. Hydrogen donation | Protective |
| Cysteine | Precursor of GSH | Slight protection |
| GSSG | Product of GSH oxidation. No protective action | No protection |
| Methionine | Precursor of GSH | Protective |
| Tryptophan | Mainly $^1O_2$ quencher | Best protection |
| Mannitol | Mainly OH scavenger | Slight protection |
| Glycine | Oxidation helps to maintain cellular redox state | Slight protection |
| Melatonin | Mainly OH but also scavenger of many free radicals | Protective |

SAM, *S*-adenosylmethionine; NAC, *N*-acetylcysteine; GSH, reduced glutathione; GSSG, oxidised glutathione.

cells. Glycine was found to protect against ionising radiation [21], but there were no reports on protection against PDT.

Maximum PGs were found when the cells were protected before and during PDT, whereas protection after PDT did not improve cell survival. In contrast to our results, addition of the radical scavengers trolox or α-tocopherol during the 60 min after irradiation of photosensitised murine leukaemia cells with tin etiopurpurin, afforded partial protection from apoptosis and phototoxicity [7]. However, the lysosomal localisation of the photosensitiser may induce long-persisting photoproducts, which may not occur with PpIX formed from ALA, which localises mainly in mitochondria.

In conclusion, our results concerning cell survival following PDT in the presence of various agents affecting cytotoxic molecular species show that L-tryptophan, an efficient singlet oxygen quencher is the best protector from PDT damage, followed by the sulphur-containing compounds GSH, NAC and methionine, and the hormone melatonin. The presence of the protectors before and during laser illumination is crucial for protection. These findings are useful in the design of a phototherapeutic strategy imparting better protection for normal tissue against the undesirable effects of PDT.

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