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Sensitivity to ALA-PDT of cell lines with different nitric oxide production and resistance to NO cytotoxicity

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Abstract

In this work, we studied the in vitro interactions between aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) and nitric oxide (NO), as well as the interactions between ALA, porphyrins and some NO donors and precursors. We employed three murine adenocarcinoma cell lines: LM2, which does not produce NO; LM3, which produces NO, and LM3-SNP, a variant of LM3 resistant to NO producing the same amount of NO as the parental. We did not find cross-resistance between NO-induced cytotoxicity and ALA-PDT. In spite of the lower porphyrin synthesis, LM2 cells show the highest sensitivity to ALA-PDT. However, we hypothesised that this is not related to the lack of endogenous NO production, because modulation of NO levels did not modify the response to PDT in any of the cell lines.

Two unexpected results were found: the enhancement of NO production from the donor sodium nitroprusside (SNP) induced by ALA in both cells and medium, and the inhibition by ALA of NO production from arginine. We also found that SNP strongly protected the cells from ALA-PDT by impairing porphyrin biosynthesis as a consequence of an inhibition of the enzyme ALA dehydratase. We were not able to evaluate the action of NO derived from SNP because of the unexpected porphyrin impairment. On the other hand, impairment of NO from Arginine driven by ALA, although not modulating in vitro the ALA-PDT response, by increasing in vivo blood flow, may be contributing to the mechanism of tumour cures.
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Keywords: Photodynamic therapy; 5-Aminolevulinic acid; ALA-PDT; Nitric oxide

1. Introduction

Photodynamic therapy (PDT) is a non-thermal technique for inducing tissue damage with light following administration of a light-activated photosensitising drug, which can be accumulated in malignant or diseased lesions. When the photosensitiser is activated by light, cytotoxic reactive oxygen species and free radicals are generated, thus destroying tumour cells [1].

In recent years, 5-aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) has become one of the most promising fields in PDT research. ALA is the pro-drug of the photosensitiser Protoporphyrin IX (PpIX). After ALA administration, cells generate PpIX through the haem biosynthetic pathway [2,3].

Nitric oxide (NO) is a highly reactive messenger molecule generated from L-arginine in an enzymatic reaction catalysed by nitric oxide synthase (NOS). NO is an important mediator in many biological functions such as tumoricidal action of macrophages, inhibition of platelet aggregation within the microvasculature affecting blood flow, increase of vascular permeability, regulation of neurotransmission and regulation of apoptosis [4,5]. By interacting with reactive oxygen radicals and modulating their activity, NO can either enhance or diminish their biological effects, depending on

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concentration and biological milieu [6]. For example, in some cell types NO can promote apoptosis, whereas in other cells NO inhibits apoptosis [5].

Three isoforms of NOS have been identified: the constitutive neuronal (nNOS or NOS1) and endothelial isoforms (eNOS or NOS3), and the inducible isoform (iNOS or NOS2), all of them are haem enzymes [7].

Most of the published work on NO regulation of PDT toxicity is related to in vivo modulation of tumour blood flow and its influence on PDT response, with a consequent potentiation of the efficacy of Photofrin [8,9]. It has also been demonstrated that the level of endogenous production of NO in tumours appears to be one of the determinants of sensitivity to PDT and that low NO producers exhibit greater sensitivity to PDT with Photofrin [10].

However, some interactions have been found in vitro between PDT and NO. The NO donor spermine-NONOate inhibited necrotic photokilling induced by ALA-PDT in an epithelial breast tumour line [11], and addition of the NO substrate arginine, decreased apoptotic cell death after PDT of lymphoblastoid cells with aluminium phthalocyanine [12].

Some authors have reported increases of NO generation both in vitro and in vivo after photodynamic treatment. An increase in the generation of NO was observed following Phtalocyanine-PDT treatment of the A431 tumour cells in vitro [13]. In vivo, ALA-PDT induced a transient increase in NO production in the site of application [14].

The aims of this work were: (a) to test the ability of ALA to induce PpIX synthesis in three murine adenocarcinoma cell lines: LM2, which does not produce NO; LM3, which produces NO and LM3-SNP which is a SNP-resistant variant of LM3 producing the same amount of NO as the parental [15,16]; (b) to assess the differential response to ALA-PDT treatment of the different cell lines; (c) to elucidate the role of nitric oxide in ALA-PDT-mediated damage, and the interactions between ALA, porphyrins and the NO precursor and donors.

2. Materials and methods

2.1. Cell line and cell culture

The cell lines LM2 [17] and LM3 [18] were derived from different spontaneous murine mammary adenocarcinomas in BALB/c mice. LM3-SNP, which is resistant to NO cytotoxicity, was derived from LM3 cell line after successive exposures to the NO donor sodium nitroprusside (SNP). The resulting line was 2 times more resistant to NO cytotoxicity than the parental, produced equal NO levels, and when injected in vivo it exhibited lower tumour take and growth rate. In addition, it is less angiogenic than the parental [16]. Cell lines were 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. The LM2 cell line does not produce nitric oxide, whereas LM3 and LM3-SNP produce the same amount [15,16].

2.2. Chemicals

ALA, N^G-nitro-L-arginine methyl ester (L-NAME), SNP and L-arginine were obtained from Sigma Chem Co., USA. DETA NONOate was from Cayman Chemical, USA. The rest of the reagents employed were of analytical grade.

2.3. NO production

The different cell lines were seeded in triplicate in 24-well plates containing 6×10^5 cells/ml and incubated 48 h at 37 °C. Then, medium was replaced by MEM without phenol red plus 5% FBS, and the different treatments followed. After 18 h, NO production was evaluated in cell supernatants by addition of the Griess reagent [1% sulphanilamine in 30% acetic acid with 0.1% *N*-(naphtyl) etylendiamine dihydrochloride in 60% acetic acid] [19], by measuring the absorbance at 550 nm employing an Elisa Reader. NO production is expressed as nmol $NO_2^-/10^6$ cells, using a standard curve of $NaNO_2$ diluted in culture medium. Media without cells incubated under the same conditions were also evaluated and NO production was expressed as nmol $NO_2^-/0.5$ ml medium. The presence of L-NAME, SNP, ALA and arginine in the incubation media did not interfere with the Griess reaction.

2.4. PDT treatment

The cells were seeded in triplicate in 24-well plates containing 7×10^4 cells/ml and incubated 48 h at 37 °C. Afterwards, the cells were incubated in serum-free medium containing 0.6 mM ALA and 3 h later, irradiations were performed. After irradiation, medium was replaced by ALA-free medium + FBS, the cells were incubated for another 19 h to let photodamage occur, and then tested for viability. LD50 was defined as the light dose to kill 50% of cells, employing ALA concentrations leading to plateau porphyrin values.

2.5. Light source

A bank of two fluorescent lamps (Osram L 36W/10) was used. The spectrum of light was between 400 and 700 nm with the highest radiant power at 600 nm. The

plates were irradiated from below, at 20 cm distance from the light source. Fluence rate was measured with a Yellow Springs Kettering model 65 radiometer (Yellow Springs, OH, USA). We used fluences between 0.1 and 1 J/cm^2 and power density was 0.5 mW/cm^2.

2.6. Measurement of porphyrin synthesis after chemical extraction

The cells were seeded in triplicate in 24-well plates containing 7×10^4 cells/ml and incubated 48 h at 37 °C. Afterwards, the cells were exposed 3 h to ALA (0.005–1.2 mM range) in serum-free medium. Porphyrins accumulated within the cells were extracted twice with 5% HCl, leaving the cells for half-an-hour in the presence of the acid. These conditions proved to be the optima for total PpIX extraction. The media were acidified and measured directly in a Shimadzu RF-510 spectrofluorophotometer. The excitation and emission wavelengths producing the highest fluorescence were 406 and 604 nm respectively. PpIX maximum emission in HCl (604 nm) is coincident with Uroporphyrin and Coproporphyrin maxima upon excitation at 406 nm, and thus PpIX (Porphyrin Products, Logan, UT, USA) was used as a reference standard to determine total porphyrins.

2.7. MTT viability assay

Phototoxicity/toxicity was documented by the MTT assay [20]. Following appropriate treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well in 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 dimethyl sulfoxide and absorbance was read at 560 nm.

2.8. Cell number

The number of cells seeded per well and the cell number employed for the calculations of porphyrins per cell were determined by counting viable cells with the Trypan blue exclusion method.

2.9. ALA dehydratase assay in erythrocytes

Bustos et al. [21] method was used. Briefly, human erythrocytes were lysed in Tris–HCl buffer containing Triton X-100. Afterwards, the sample was incubated in 50 mM phosphate buffer, pH 6.8, in the presence of ALA. After 1 h incubation at 37 °C, the reaction was stopped with 5% TCA (final concentration). The formed porphobilinogen was determined in the supernatant after centrifugation, by addition of the Ehrilch reaction, and quantified spectrophotometrically.

2.10. Porphobilinogen deaminase assay in erythrocytes

Batlle et al. [22] method was used. Briefly, human erythrocytes were lysed in Tris–HCl buffer containing Triton X-100. Afterwards, the sample was incubated in 0.5 M Tris–HCl buffer, pH 8.2, in the presence of porphobilinogen. After 2 h incubation at 37 °C, the reaction was stopped with TCA. The porphyrins in the supernatant after centrifugation were quantified spectrophotometrically.

2.11. Statistical treatment

The values in the figures are expressed as means ± standard error of the mean, and they are the average of three independent experiments run in triplicate. A paired two-tailed Student's *t*-test was used to determine statistical significance between means. p values <0.05 were considered significant.

3. Results

Fig. 1 shows the dependence of porphyrin synthesis on ALA concentration. We found saturation points at 0.3 mM ALA for the three cell lines. The amount of porphyrins accumulated was markedly lower in the LM2 line. The maximal porphyrin synthesis was around 40 ng/10^5 cells for LM3 and LM3-SNP cell lines and 21 ng/10^5 cells in the LM2 cells.

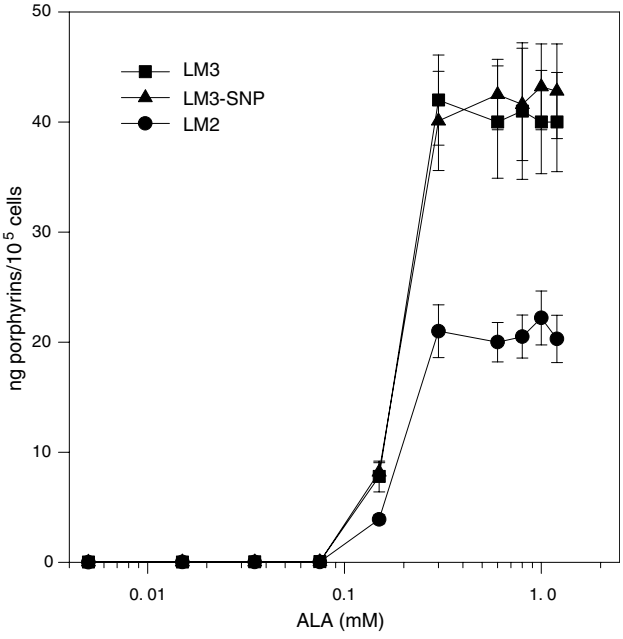

Fig. 1. Porphyrin synthesis from ALA in LM2, LM3 and LM3-SNP cells. 3.5×10^4 cells per well (24-well plates) were incubated for 3 h in the presence of different amounts of ALA. Intracellular porphyrins were determined fluorometrically and normalised per number of cells.

Fig. 2 shows the ALA-PDT induced damage as a function of the increasing light dose. LD50 are: 0.27 J/cm^2 for LM3, 0.10 J/cm^2 for LM3-SNP and 0.047 J/cm^2 for LM2. The most sensitive line to ALA-PDT was LM2, whereas LM3 was the most resistant.

Table 1 describes NO production from the different cell lines. LM3 and LM3-SNP cells produced equal amounts of NO (8.5 nmol $NO_2^-/10^6$), and both were significantly induced by arginine (Arg), the substrate for NOS ($p = 0.01$). LM2 cells showed no detectable levels of NO in either condition. L-NAME, an inhibitor of the constitutive form of NOS significantly inhibited NO production in both LM3 and LM3-SNP cells.

The NO donor SNP, employed under non-toxic concentrations, induced NO release independently on endogenous production.

NO output as a function of the SNP concentration and optimal Arg, L-NAME and SNP concentrations were described previously [15].

We studied the effect of Arg on ALA-PDT. Addition of Arg previously, during, or after PDT treatment, did not modify at all ALA-PDT toxicity in any of the three cell lines. In addition, we did not find increases of intrinsic NO production after ALA-PDT in any of the cell lines (data not shown).

Unexpectedly, we found that addition of ALA modifies NO production from Arg in LM3 cells (Fig. 3). After 18 h incubation, 8.2 nmol $NO_2^-/10^6$ cells are formed (Table 1), and further 18 h exposure to Arg increases the production to 19.6 $NO_2^-/10^6$ cells. However, addition of ALA (Arg + ALA) impaired NO cellular production. This feature is also observed in irradiated cells (Arg + ALA + L), showing that light does not interfere in the process. Addition of the ALA dehydratase inhibitor SA did not modify the impairment, suggesting that porphyrin production is not involved in the process. In addition, ALA or ALA-PDT treatment of cells not exposed to Arg modified basal NO production. Media without cells treated under the same conditions did not show any modifications in NO content.

A similar behaviour was observed in LM3-SNP cells but not in LM2 cells, which do not produce NO per se (data not shown).

SNP induced a time-dependent increase in NO production in LM3 cells and in medium without cells (data not shown). Whereas in LM3 cells, 0.001 mM SNP releases 13.1 nmol $NO_2^-/10^6$ cells after 18 h incubation (see Table 1), further 18 h exposure to SNP produces 17.5 $NO_2^-/10^6$ cells. Addition of ALA to the SNP-treated cells (SNP + ALA) induced after 18 h incubation, a significant enhancement of NO production (Fig. 4(a)).

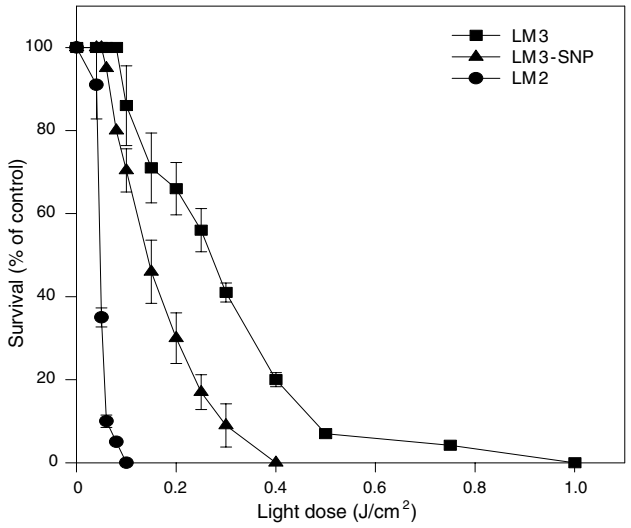

Fig. 2. Cell survival after ALA-PDT with different light doses in LM2, LM3 and LM3-SNP cells. PDT experiments were performed in 6-well plates. Cells were incubated with 0.6 mM in FBS-free medium during 3 h. Immediately after, cells were irradiated with different light doses and incubated in medium containing FBS for further 19 h. MTT assay was performed and cell survival were expressed as percentage of the non-irradiated control incubated in the presence of ALA.

Table 1
NO production from LM2, LM3 and LM3-SNP cells

	Control	Arg	L-NAME	SNP
LM2	ND	ND	ND	5.12 ± 0.25
LM3	8.2 ± 0.45	11.3 ± 1.15	5.7 ± 0.67	13.1 ± 1.17
LM3-SNP	8.5 ± 0.51	11.6 ± 1.10	6.1 ± 0.58	14.3 ± 1.36

Cells were exposed 18 h to 1 mM Arg, 1 mM L-NAME or 0.001 mM SNP, and then NO was measured as explained in Section 2, and results expressed as nmol $NO_2^-/10^6$ cells present at the beginning of the experiment; ND, non-detectable.

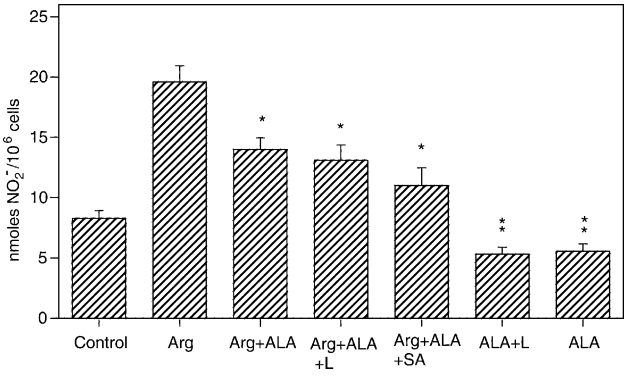

Fig. 3. NO production in LM3 cells after ALA and arginine exposure. LM3 cells were exposed 18 h to 1 mM Arginine and then received the following treatments: Arg: exposed to 1 mM Arg for another 18 h. Arg + ALA: exposed to 1 mM Arg and 0.6 mM ALA for 18 h. Arg + ALA + L: exposed to 1 mM Arg and 0.6 mM ALA for 3 h, irradiated (0.27 J/cm^2) and left in the presence of ALA and Arg for further 15 h. Arg + ALA + SA: exposed to 1 mM Arg, 0.5 mM SA and 0.6 mM ALA for 18 h. ALA: exposed to 0.6 mM ALA for 18 h. ALA + L: exposed 3 h to 0.6 mM ALA, irradiated (0.27 J/cm^2) and further exposed 15 h to ALA. NO quantification was performed at the end of the treatment, and it was expressed as nmol $NO_2^-/10^6$ cells present at the beginning of the experiment. Controls, basal NO values; *$p < 0.05$ (compared to the Arg condition); **$p < 0.05$ (compared to the control).

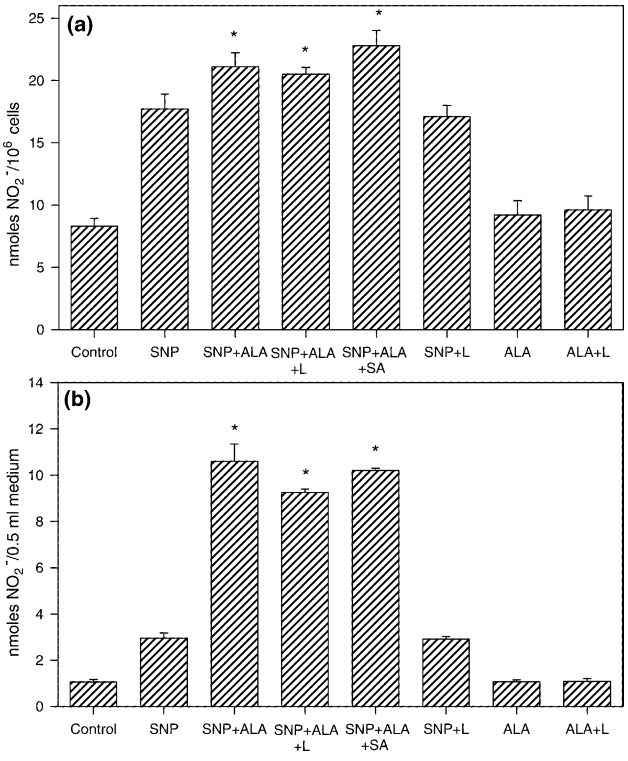

Fig. 4. NO production in LM3 cells and media after SNP and ALA exposure. (a) in the cells; (b) in the media. LM3 cells or serum-containing media without cells were incubated with 0.001 mM SNP for 18 h and then: SNP: exposed to 0.001 mM SNP for another 18 h. SNP + ALA: exposed to 0.001 mM SNP and 0.6 mM ALA for 18 h. SNP + ALA + L: exposed to 0.001 mM SNP and 0.6 mM ALA for 3 h, irradiated (0.27 J/cm^2) and left in the presence of SNP and ALA for another 15 h. SNP + ALA + SA: exposed to 0.001 mM SNP, 0.5 mM SA and 0.6 mM ALA for 18 h. SNP + L: exposed to 0.001 mM SNP, irradiated (0.27 J/cm^2) and exposed to SNP for further 18 h. ALA: exposed 18 h to 0.6 mM ALA. ALA + L: exposed 3 h to 0.6 mM ALA, irradiated (0.27 J/cm^2) and further exposed 15 h to ALA. NO quantification was performed at the end of the treatments. NO production is expressed as nmol $NO_2^-/10^6$ cells present at the beginning of the experiment or nmol $NO_2^-/0.5$ ml medium. Cell control: basal cell NO production. Medium control: basal medium NO production; *$p < 0.05$ (compared to the SNP condition).

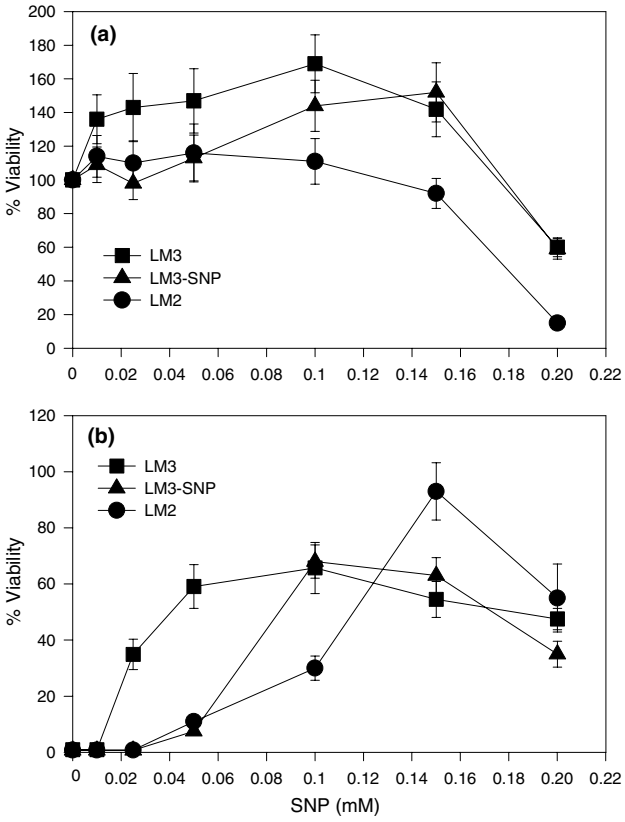

Fig. 5. SNP on cell proliferation and protection from ALA-PDT toxicity. Cells were preincubated 18 h with different SNP concentrations in serum-containing medium. (a) Afterwards, medium was replaced for serum-free medium and further incubated for 21 h. MTT was performed and percentage of viability was calculated from the control without SNP. (b) Medium was replaced for serum-free medium containing 0.6 mM ALA and after 3 h, cells were irradiated with a light dose producing 99% cell death. MTT was performed after 18 h incubation. Percentage of viability was calculated from −PDT + SNP conditions (graph a).

In serum-containing medium, addition of ALA induced an even higher, 3.5-fold increase, of NO release (Fig. 4b).

Such increases in NO production, in both cells and media, are independent on light treatment (SNP + ALA + L) and SA action, showing that neither photodynamic effects nor porphyrins interfere in the process. ALA alone or irradiated did not modify basal NO content, and SNP was not affected by light treatment. Similar patterns were observed in LM3-SNP and LM2 cell lines (data not shown).

Treatment of LM3, LM2 and LM3-SNP cells with Arg or L-NAME and exposed to ALA-PDT did not modify PDT response (data not shown). However, SNP at a certain concentration range, protected from ALA-PDT damage (Fig. 5). Low SNP concentrations (0.005–0.15 mM) induced around 40% increase on proliferation of both LM3 and LM3-SNP cells, whereas in LM2 cells only a slight non-significant increase was observed (Fig. 5(a)). Higher SNP concentrations were cytotoxic for the three cell lines.

Upon ALA-PDT photosensitisation of these cells previously exposed to SNP (Fig. 5(b)), we observed a concentration dependent protection from ALA-PDT cytotoxicity, and the protection degree slightly depended on the cell line. We confirmed the results with the colony formation assay (data not shown), discarding the hypothesis of some SNP interference with the MTT assay.

We also found that the higher the light dose, the higher the protection (data not shown), and this protection was exerted even at SNP concentrations toxic for non-PDT treated cells (0.2 mM).

To investigate SNP protection of ALA-PDT damage, we studied SNP effect on porphyrin synthesis (Fig. 6).

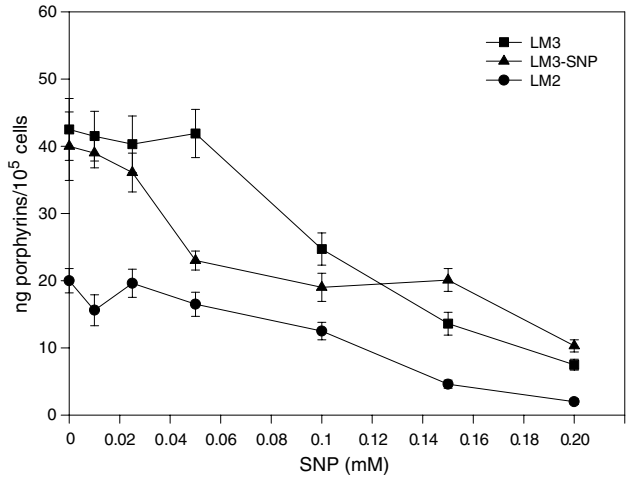

Fig. 6. Porphyrin synthesis in cells exposed to SNP Cells were preincubated 18 h with different SNP concentrations in serum-containing medium. Afterwards, medium was replaced for serum-free medium containing 0.6 mM ALA and after 3 h porphyrins were extracted with HCl. Porphyrins were normalised by the number of cells after SNP induction of proliferation.

Upon pre-incubation with SNP, we found a decrease in porphyrin synthesis per cell in all the cell lines. Even in LM2 cells, where SNP effect on proliferation was almost negligible, porphyrin impairment was also observed, showing that the phenomenon is not related to cell density.

We also investigated if the effectiveness of PDT with exogenous PpIX was also affected by SNP exposure (data not shown), and we found that the NO-donor did not modify the PDT response, thus reinforcing the hypothesis that SNP exerted its action through impairment of porphyrin synthesis.

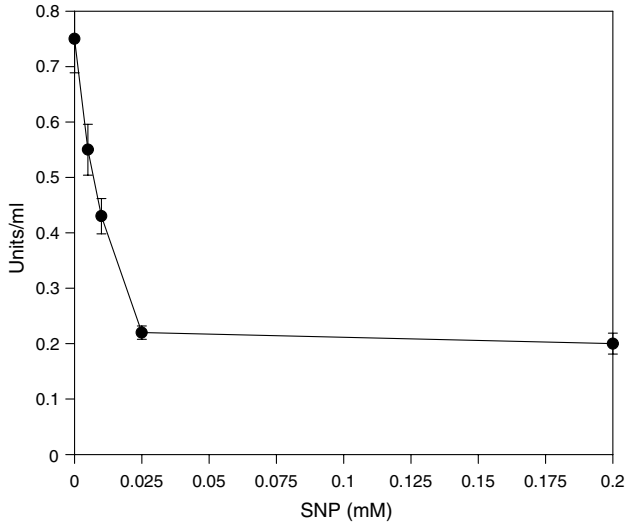

Fig. 7. ALA dehydratase activity in the presence of SNP in erythrocytes. Human erythrocytes were exposed 3 h to different SNP concentrations. Afterwards, ALA dehydratase assay was performed.

In addition, we tested if SNP was capable of inhibiting ALA dehydratase and/or Porphobilinogen deaminase, the enzymes catalysing the two steps of conversion from ALA to Uroporphyrinogen III, the first porphyrin in the haem pathway. We employed human erythrocytes as a high activity enzyme source. We found that ALA dehydratase (Fig. 7) was dramatically inhibited even at low SNP concentrations. On the contrary, Porphobilinogen deaminase, was not inhibited by SNP in the same concentration range (normal value: 70 ± 10 U/ml) (data not shown).

In addition, we tested the effect of another NO donor, Deta NONOate, and we found that, employed under conditions of equal NO release, it did not protect from ALA-PDT toxicity or inhibit porphyrin synthesis from ALA either, showing that this feature was not related to NO production (data not shown).

4. Discussion

ALA enhances NO production from SNP in cells and in medium, although more markedly in the former. The mechanism by which SNP releases NO is unknown, but a leading theory is that it occurs spontaneously, on exposure to vascular tissue and after reaction with various reducing agents. The proposed mechanism of NO production from SNP is that an electron is transferred to the NO^+ ligand from another molecule as the SNP anion is reduced. Oxidising agents such as iodine inhibit NO release from SNP; KCN also blocks the release of CN^- and formation of the intermediates necessary for NO release [23], while under hypoxic conditions, NO release from SNP is enhanced [24].

According to our results, the oxidising agents such as ALA and/or free radicals induced by ALA do not block but enhance SNP release. ALA may behave as a pro-oxidant in vitro, through the generation of reactive oxygen species upon iron-catalyzed oxidation to 4,5-dioxovaleric acid [25].

In the presence of ALA, NO production from SNP increases by 2.4 nmol in cell-containing medium, but the increase in cell-free medium is much more marked (7.65 nmol more than the SNP control). This feature is not related to porphyrin production in cells, as the SA-treated cells show equivalent NO increase. It is likely that cell-conditioned medium provides a different redox microenvironment due to progressive loss of vitamins and aminoacids, a slightly different pH, or other possible alterations which do not favour ALA-driven SNP release.

Inside cells, it seems that ALA is inhibiting Arg conversion to NO through the NOS. PpIX has been shown to inhibit the three NOS isoforms [26]. However, from experiments with SA where no porphyrins are formed [27], we can discard the hypothesis of a mechanism

involving the porphyrins. Therefore, ALA may either react with Arg thus consuming NO substrate, or inhibit NOS activity or enhance arginase activity.

In rodents, inhibition of NO production has been found to reduce vessel diameter and blood flood in tumours, modulating vessel permeability and inducing tumour hypoxia [28,29], factors that modulate PDT response [9,10]. Therefore, impairment of NO endogenous levels by ALA, may lead in vivo to increased blood flow and oxygen availability, and consequently ALA-PDT efficacy in tumour cures may be partly due to this unexpected interaction.

A poor oxidant itself, NO reacts extremely rapidly with superoxide (O_{2^-}) to give the radical peroxinitrite ($ONOO^-$), a nitrating agent and strong oxidant [30]. On the other hand, if NO is more persistent than O_{2^-} in a biological setting, it may exhibit antioxidant properties [31]. In addition, NO can also react with the free radicals, thereby blocking the lipid chain propagation reaction [32], ligate to pro-oxidant iron complexes [31] or inactivate apoptosis-inducing caspases via *S*-nitrosilation [33]. Under our PDT regimen, neither NO nor NO derived compounds should be acting as cytoprotectors against photodamage or as enhancers of free radicals mediated reactions.

Some investigations have found that certain NO donors prevented photodamage. Addition of arginine or NO donors decreased apoptotic cell death after photosensitisation of cells with aluminium phthalocyanine [12]. The NO donor spermine–NONOate inhibited lipoperoxidation and necrotic photokilling induced by ALA-PDT in an epithelial breast tumour line [11] by impairment of post-photooxidation chain reactions. However, in our cell lines, neither the NO donors SNP nor DETA NONOate inhibited ALA-PDT cell damage by a NO-mediated action. We found that SNP strongly protected the cells from ALA-PDT by impairing porphyrin biosynthesis as a consequence of an inhibition of ALA dehyratase. The fact that Deta-NONOate did not either protect of ALA-PDT toxicity or inhibit porphyrin synthesis from ALA, shows that this effect is not related to NO production.

Moreover, the lack of effect of SNP on the efficacy of PDT with exogenous PpIX reinforces the hypothesis that abrogation of porphyrin synthesis is the only factor involved in ALA-PDT photoprotection. The effects of SNP on ALA dehydratase activity are not surprising due to the sulphydrilic nature of this enzyme, but such effects have not been previously reported. As a consequence of ALA dehydratase inhibition, it is likely that the haem pool decreases with the SNP treatment, thus impairing the synthesis of NOS and consequently inhibiting endogenous NO synthesis. Since SNP is clinically used for the immediate control of very high blood pressure, congestive heart failure and control bleeding during surgery, the characterisation of such inhibition will be the subject of future studies.

LM3-SNP and LM3 cells produced equal amount of ALA-induced porphyrins and exhibited similar NO production. However, the LM3-SNP cells were more sensitive to ALA-PDT and more resistant to NO-induced cytotoxicity. Not only was no cross-resistance between NO and PDT found but, on the contrary, in this case, resistance to NO implies PDT sensitivity and vice versa.

Unexpectedly, LM2 cells show the highest sensitivity to ALA-PDT, in spite of the lower porphyrin synthesis. We considered the possible relevance of the lack of endogenous NO production. However, neither increasing NO levels of LM2 cells by Deta NONOate exposure nor modulating of NO levels of LM3 cells with arginine or NAME, modified the response to PDT photodamage. However, we do not disregard the possibility of NO modulation of PDT that cannot be mimicked by exogenous intervention.

To sum up, we were not able to evaluate the action of NO derived from SNP because of its unexpected porphyrin impairment. On the other hand, we found an interaction between ALA and Arginine, with a consequent decrease of NO production. However, this modulation does not modify the in vitro ALA-PDT response, but we can predict that in vivo interactions of ALA with endogenous arginine may contribute to the mechanisms of tumour cures by blood flow modulation.

5. Abbreviations

ALA	5-aminolevulinic acid
Arg	arginine
L-NAME	N^G-nitro-L-arginine methyl ester
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
NO	nitric oxide
NOS	nitric oxide synthase
PDT	photodynamic therapy
PpIX	Protoporphyrin IX
SNP	sodium nitroprusside
SA	succinyl acetone

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