Enhancement of aminolevulinic acid based photodynamic therapy by adriamycin

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Abstract

This paper reports on studies that evaluate the interaction between δ-aminolevulinic acid (ALA)-based photodynamic therapy (PDT) and adriamycin (ADM) in an animal model system. Two groups of mice bearing a transplantable mammary adenocarcinoma received ADM i.p. in a single dose of 5 mg (low dose) and 30 mg (high dose) per kg body weight. Sixteen or 40 h after administration of the drug, mice were sacrificed, tumours, livers and hearts were removed and porphyrins, enzyme activities and malondialdehyde content were determined. Tumour explants of ADM-treated mice were incubated with ALA and irradiated with an He-Ne laser. Re-implantation of these in vitro PDT-treated explants into test animals showed that inhibition of tumour growth was significantly enhanced by combined treatment when the low dose of ADM was used. There were no significant changes in porphyrin content, ALA dehydratase and porphobilinogenase activities in the tissues analyzed after ADM treatment as compared with control values. ADM toxicity is thought to be related to semiquinone free radical formation with subsequent generation of reactive oxygen species such as peroxide and hydroxyl radical. These species are considered to initiate lipid peroxidation (LPO) and cause DNA damage. In the case of low-dose treatment with ADM a significant increase in the LPO product, malondialdehyde, was observed after PDT whereas with the high-dose regimen no changes were observed. In the case of explants of (non-irradiated) cardiac tissue malondialdehyde production was also found to be dependent on the dose and time of administration of adriamycin. In our in vivo/in vitro model system we have shown that pre-treatment with ADM increased the cytotoxicity of ALA-PDT at a dosage level of ADM which did not raise LPO levels in heart tissue. The mechanism of this effect has not been clearly elucidated but our data suggest that the observed enhancement of PDT may be attributed in part to the weakening of cellular defence mechanisms by the pre-treatment involving free radical generation by ADM. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

Photodynamic therapy (PDT) shows considerable potential as a treatment modality for malignant tumours. PDT is based on the accumulation in the
target tissue of a photosensitizer. Subsequent illumination with light of an appropriate wavelength provokes a photochemical reaction that results in tissue destruction [14,16,17]. 5-Aminolevulinic acid (ALA) is a heme precursor and its administration results in an increased endogenous production of phototoxic porphyrins, which has been exploited for PDT [20,24,29,44]. ALA-induced porphyrin fluorescence may also assist in the early detection of some malignancies [30]. A combination of in vitro light treatment and in vivo tumour growth allowed us to study the effects of ALA-PDT treatment in the absence of a functioning vascular system and by using this in vivo/in vitro model we have demonstrated significant direct cell killing by the photodynamic action of porphyrins formed endogenously from ALA [20].

PDT in combination with chemotherapy may hold promise for cancer treatment since it may enable a reduction in the doses of the photosensitizer and chemotherapeutic drug required, thus diminishing undesirable side-effects. There are reports on the interaction between PDT, using exogenously administered porphyrins, and cytotoxic drugs used for cancer chemotherapy in vitro [6,12] and in vivo [2,10,33,36,39]. Employing our in vivo/in vitro model [20] we have found a 30% potentiation of PDT by administering the antineoplastic cyclophosphamide 40 h before giving ALA [8].

ADM is an anthracycline antibiotic in current use as an anti-tumour compound. Several mechanisms have been proposed to explain the antitumour properties of ADM which include intercalation into DNA [15], interactions with membranes [23] and bioreductive activation leading to the formation of a semiquinone derivative of the drug, oxygen free radicals and alkylating species [25,46]. Increased photosensitivity in patients undergoing simultaneous PDT and ADM therapy has been noted [36]. Photosensitization mediated by anthracyclines upon excitation in the near-UV [7] and tissue photodamage related to drug exposure [41,42] have been reported.

Cowled et al. [11] and Nahabedian et al. [36] reported that ADM potentiated the photodynamic effects of hematoporphyrin derivative (HPD) in a murine tumour model. However, in contrast to the effects observed in vivo, ADM inhibited the photodynamic destruction of tumour cells in vitro [11].

A major drawback of prolonged clinical use of ADM is its dose-dependent cardiotoxicity, which is attributed to semiquinone free radical formation by microsomal or mitochondrial enzymes [40]. Autoxidation of the semiquinone leads to generation of reactive oxygen species such as superoxide and hydroxyl radical [22]; these species are considered to initiate lipid peroxidation and DNA damage. Although the role of oxygen free radicals in the induction of cardiotoxicity is widely accepted, its involvement in tumour killing is still a matter of debate.

In the studies reported here we analyzed the effects of in vivo ADM on haem biosynthesis and lipid peroxidation and assessed the effectiveness of ALA-induced PDT in tumour tissue from mice previously exposed to ADM, using our in vivo/in vitro model system [20]. The model was devised to maximize control ALA uptake and light exposure of tumour tissue by conducting this PDT procedure in vitro using fragments of equal size.

2. Materials and methods

2.1. Chemicals

ALA was purchased from Sigma (St. Louis, MO, USA) and ADM was a gift from Raffo S.A. Laboratories. All other chemicals were of analytical grade.

2.2. Animals

Male BALB/c mice (12 weeks old) weighing 20–25 g were used. They were provided with food (Purina 3, Molinos Río de la Plata) and water ad libitum. Innocula of 1 mm³ of a mammary adenocarcinoma [45] (M2, Hospital Roffo, Buenos Aires) were implanted subcutaneously under the axilla. Experiments were performed 14 days after implantation. Six mice per group were used.

2.3. Drug administration

Two groups of mice received ADM i.p. in a single dose of 5 mg/kg (low dose) and 30 mg/kg (high dose). Control animals were injected with saline. Sixteen or 40 h after administration of the drug, mice were sacrificed, tumours, livers and hearts were removed and porphyrins, enzyme activities (ALA dehydratase and
porphobilinogenase) and malondialdehyde (MDA) content were determined.

2.4. Tumour growth assessment

The effect of PDT on tumour growth was assessed using the in vivo/in vitro model system previously described [20] (Fig. 1). Briefly, tumour tissue samples cut to a standard size with a biopsy punch and weighing 2 mg obtained from ADM-treated and control animals were incubated for 2 h at 37°C in 5 ml of minimal essential medium supplemented with 1 g/l glucose without serum [46] with or without 0.6 mM ALA. Only non-necrotic, non-haemorrhagic tumour was used. Irradiated explants were exposed for 15 min to light from an He-Ne laser (632.8 nm, Techmet Tube) of 3.5 mW output power, delivering a fluence of 80 mW/cm² and a light dose of 72 J/cm². Appropriate controls of non-irradiated explants incubated with ALA and irradiated and non-irradiated explants incubated without ALA were included.

Immediately after the illumination period, innocula of 1 mm³ were obtained with a biopsy punch from irradiated and non-irradiated tissue and implanted subcutaneously under the right and left flanks, respectively, of recipient mice.

The growth of the re-implanted tumours was measured daily from the 10th day after implantation, when the mass of control tumours was palpable, up to the 25th day, when spontaneous necrosis was a limiting factor. The tumour volume was determined by orthogonal calliper measurement (length × width × thickness × 0.5).

2.5. Enzymatic assays

2.5.1. Homogenates

Tumour and liver tissues were homogenized using an Ultra-Turrax Potter in 0.25 M sucrose (1:5 and 1:10 p/v, respectively), centrifuged at 24 000 × g and the supernatants were employed for measuring enzymatic activities.

2.5.2. ALA dehydratase (ALA-D) assay

ALA-D activity was measured according to Batlle et al. [3] in 50 mM phosphate buffer (pH 6.8) in the presence of 10 mM dithiothreitol (DTT) and 5 mM ALA. Incubations were carried out in aerobiosis at 37°C in the dark for 1 h. The precipitated protein was discarded and the amount of porphobilinogen (PBG) formed was quantified using the Ehrlich reagent [35].

2.5.3. Porphobilinogenase (PBGase) assay

PBGase (porphobilinogen deaminase and isomerase complex) was determined according to Batlle et al. [4] in 50 mM Tris–HCl buffer (pH 8) with 0.6 mM NaCl, 0.12 mM MgCl₂ and 40 mM PBG. Incubations were carried out at 37°C aerobically in the dark for 2 h. Trichloroacetic acid was added (final concentration 5% v/v), the precipitated protein was discarded by centrifugation and porphyrins were estimated spectrophotometrically.

2.5.4. Enzyme units

One enzyme unit was defined as the amount of
enzyme that catalyzes the formation of 1 nmol of product per hour.

2.5.5. Proteins
Proteins were estimated by the method of Lowry et al. [32].

2.5.6. Porphyrins
Basal levels of porphyrins in tissue (tumour, liver and heart) and incubation media were determined spectrophotometrically after extraction with ethylacetate/acetic acid (4:1 v/v) [19].

2.5.7. Lipid peroxidation
Lipid peroxidation in tumour and heart homogenates was measured by MDA content through the reaction with thiobarbituric acid according to Niehaus and Samuelson [37].

2.6. Statistics
Results are presented as the mean and standard deviation of three duplicate experiments and comparisons between groups were made with the Student’s t-test. Values of $P < 0.05$ are considered significant.

3. Results

3.1. Effects of ADM on porphyrin biosynthesis and basal LPO
In order to detect possible changes in porphyrin in biosynthesis or basal LPO, we determined these parameters in control and ADM-treated mice.

Treatment with either the low or high dose of ADM did not significantly alter the porphyrin content of tumour, liver or heart tissue (data not shown) which remained at normal levels (tumour 0.22 ± 0.12, liver 0.43 ± 0.20 and heart 0.81 ± 0.34 μg/g wet weight tissue).

ALA dehydratase and PBGase are enzymes involved in the conversion of ALA to porphyrins. There was no significant alteration in the level of PBGase activity in liver (0.33 ± 0.05 units/mg protein) or tumour tissue (0.25 ± 0.04 units/mg protein). Conversely, ADM treatment resulted in a fall of 57% in hepatic ALA-D activity 40 h after injection with a low dose of ADM (Table 1) and a fall of 45% after 16 h with a high dose. Tumour ALA-D activity was not modified by ADM treatment (13.60 ± 3.12 units/mg protein).

Basal levels of LPO were not affected either in tumour (0.57 ± 0.32 nmol MDA/mg protein) or in heart (1.27 ± 0.55 nmol MDA/mg protein) by ADM treatment, although basal LPO was relatively higher in heart tissue.

3.2. ALA-stimulated porphyrin synthesis in tumour explants from mice treated with ADM
Tumour explants of 50 mg from mice treated with ADM were incubated with or without 0.6 mM ALA and porphyrin content was measured in the explants and the incubation medium (Fig. 2). The levels of endogenous porphyrins were very low in the explants incubated in ALA-free medium.

There were no modifications of porphyrin content of explants incubated with ALA 16 and 40 h after i.p. injection with ADM, indicating that the drug does not affect porphyrin biosynthesis from ALA.

The incubation media showed similar profiles to those found in the explants, indicating that the amount of porphyrins released to the medium is proportional to the synthesis rate in the tumour explant, although a slight effect on the retention of porphyrins was observed 40 h after ADM administration.

3.3. Effect of ADM on lipid peroxidation of tumour and heart explants incubated with ALA
Mice treated with a low or high dose of ADM were sacrificed after 16 or 40 h and tumour and heart tissue was homogenized and the level of lipid peroxidation was measured by MDA content through the reaction with thiobarbituric acid according to the method of Niehaus and Samuelson [37].

Mice were treated with a single dose of 5 or 30 mg/kg of ADM and were sacrificed after 16 and 40 h. Control animals were injected with saline. ALA-D activity measured in hepatic tissue is expressed in units/mg protein. Control levels were 37.34 ± 3.22 units/mg protein.
explants of 50 mg were incubated for 2 h in the presence of ALA. Tumour explants were then irradiated, but heart explants were not. Because both PDT and ADM antitumour action is thought to be mediated through reactive oxygen species, we investigated the effect of the anthracycline on tumour LPO induced by the photodynamic treatment. Fig. 3a shows that there was a 300% increase in MDA content in the tumour explants incubated for 2 h with ALA and irradiated when they derived from mice treated with a low dose of ADM 16 or 40 h beforehand. The higher dose did not induce any significant alterations in tumour LPO.

It is well-known that ADM provokes cardiotoxicity and therefore we considered it of interest to assay the LPO status of ALA-incubated (non-irradiated) heart tissue (Fig. 3b). A significant increase in MDA content was observed in cardiac tissue derived from animals treated with the higher dose of ADM 40 h prior to the assay but not after 16 h or in the case of the lower dose ADM treatment.

3.4. Effect of ADM on the efficacy of ALA-based PDT treatment

We compared the size of tumours after re-implan-
tation into recipient mice of tissue fragments which
derived from saline-treated control animals and mice
 treated according to the scheme in. Tumour volumes measured at 20
days after re-implantation are shown. (Open box) explants incubated
without ALA and non-irradiated (ALA−/light−); (shaded
box) explants incubated without ALA and irradiated (ALA−/light+); (XX) explants incubated with 0.6 mM ALA and non-irra-
diated (ALA+/light−); (hatched box) explants incubated with 0.6
mM ALA and irradiated (ALA+/light+). The histogram shows the
mean values ± SD.

Fig. 4. The effect of adriamycin pre-treatment on ALA-PDT. Mice
were treated with a single dose of 5 mg/kg of ADM. At 16 or 40 h
after dosing the animals were sacrificed and tumour explants were
treated according to the scheme in. Tumour volumes measured at 20
days after re-implantation are shown. (Open box) explants incubated
without ALA and non-irradiated (ALA−/light−); (shaded
box) explants incubated without ALA and irradiated (ALA−/light+); (XX) explants incubated with 0.6 mM ALA and non-irra-
diated (ALA+light−); (hatched box) explants incubated with 0.6
mM ALA and irradiated (ALA+/light+). The histogram shows the
mean values ± SD.

4. Discussion
We have found that whilst ADM administration
does not change haem biosynthesis or basal MDA
levels, explants of tumour from ADM-pre-treated ani-
mals incubated with ALA and irradiated exhibit raised
levels of LPO. The PDT-induced rise in MDA content
was only observed at low doses of ADM pre-treat-
ment. At the higher dose of ADM this response was
lower or absent. The observed increase in tumour LPO
caused by pre-treatment with the low dose of ADM
may be explained by the relative stabilization [6] of
hydroperoxides and other reactive oxygen species
(ROS) generated during the PDT process. Adminis-
tration of anthracyclines has been shown to provoke
decreases in the activity of glutathione peroxidase
[43], superoxide dismutase and catalase [31] and to
deplete the cell of glutathione by formation of thiol
adducts with the drug [47]. Consequently, impairment
of the antioxidant levels and antioxidant enzyme
activities produced by ADM may make cells more
susceptible to photodynamic damage.

The high dosage ADM pre-treatment increased
LPO products in non-irradiated heart but this effect
was absent in the low dosage regimen. This is an
interesting observation and suggests that low doses
of ADM may be capable of enhancing photo-oxida-
tive damage to tumour tissue without significantly
increasing the risk of cardiopathy.

ALA-D, a well-known sulphydryl enzyme [21],
appears to be sensitive to ADM treatment, particularly
in liver tissue. This effect is possibly due to oxidation
of sulphhydryl groups at the active site of the enzyme
by interaction with ADM-generated free radicals. In
a previous study [9] this enzyme activity was shown to
be impaired after cyclophosphamide chemotherapy.

We found an additive effect on PDT-induced cell

Photodynamic treatment (ALA+/light+) of ADM-treated explants
further inhibited tumour growth. On the basis of comparative
tumour size there was a 30% enhancement in the
photodynamic killing of tumour cells by previous
ADM.

When the higher dose of 30 mg/kg of ADM was
employed, there were no differences in the growth of
tumour tissues compared to the non-treated controls
under the four conditions analyzed (data not shown).
killing when ADM was administered in low doses 16 or 40 h before PDT, which correlates with the rise in tumoral LPO. When the time intervals between ADM administration and ALA incubation were less than 16 h, we found neither enhancement of the antitumoral effect nor increased MDA levels. Nahabedian et al. [36], employing the same low dose of ADM, found a potentiation in the antitumour action of HPD-based PDT in the murine tumour EMT-6 but not with 10 mg/kg nor in the RIF-1 tumour line. Cowled et al. [11] have observed that 0.5–4.0 mg ADM/kg administered with HPD at the time of irradiation potentiates the photodynamic effect, doubling the time for recurrence of murine tumours, whereas ADM administered after PDT was not as effective. It was reported that ADM increases the uptake of HPD in vivo [11] and mitomycin C, another antineoplastic drug, increases the uptake of Photofrin [33].

Another explanation for the enhancement of the ALA-based PDT by ADM could be related to possible changes in receptors or carrier proteins of molecules such as ALA and/or porphyrins. ADM interactions with membranes [23,49] may modify the permeases involved in ALA uptake and, by analogy, since ADM localizes in mitochondria [23], it may interact with the mitochondrial benzodiazepine receptor, which is postulated to be involved in the transport of porphyrins [48]. As yet, ALA transport systems have not been characterized in animal cells [51]. However, our results do not support the hypothesis that ADM might facilitate ALA uptake and/or inhibit ALA or porphyrin release from tumour cells.

The relatively little tumoricidal effect of ALA incubation alone could be attributed to oxidative damage promoted by this compound, as proposed by Hermes-Lima et al. [27], whilst the slight response to light treatment may be due to photosensitization of basal porphyrins. Inhibitory effects on cell viability after He-Ne laser irradiation were also noticed by Cantacuzino and Ionescu-Mihaiesti [6].

Anthracyclines have been shown to bind covalently to duplex DNA when irradiated with UV or visible light [13]. In agreement with in vivo results of Cohen et al. [10] we have found that ADM may have photosensitizing properties with 630 nm light, although Andreoni et al. [1] failed to demonstrate this effect with 488 nm light in an in vitro assay. Since ADM action is potentiated by heat [50] it is possible that the mild hyperthermia induced by laser irradiation [5] could partially account for the effect of ADM.

Whilst the main subcellular targets of PDT are cell membranes and organelles, most antitumour drugs appear to act through DNA damage. However, it is not so clear that ADM and PDT act by different mechanisms. Photodynamic effects at the level of DNA have been reported [18,28] and as has been recently found for ADM [38], an apoptotic response of the tumour cells to PDT has also been postulated [26]. In addition, ADM interactions with membranes [22] and mitochondria [34] through free radical generation is proposed as one of its tumoricidal effects. The lack of additive effect at 30 mg/kg ADM may also be due to competition for the same sensitive site within the tumour.

If chemotherapy is contemplated in patients undergoing PDT, then any beneficial interaction between the therapeutic modalities should be understood to permit optimum timing and choice of agents. In this study we found that pre-treatment with low doses of ADM adds to the antitumour effects of ALA-based photodynamic therapy and this correlates with an increase in PDT-induced LPO. Although further studies will be necessary to elucidate the mechanism of this enhancement, this is the first report on the possible therapeutic benefit of a combination of ALA-based PDT and ADM.

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