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ORIGINAL ARTICLE

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The cytotoxic and genotoxic potential of 5-aminolevulinic acid on lymphocytes: a comet assay study

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Abstract *Background*: 5-aminolevulinic acid (ALA) and its hexylester (ALA-H) are the drugs currently used in photodynamic therapy (PDT). The side effect, especially the long-term side effect of these drugs is a problem of concern in this field, which has not been clearly understood yet. *Purpose*: The normal lymphocytes and nasopharyngeal carcinoma (NPC) cells were used as the cell models to evaluate the side effects of ALA or ALA-H in the absence of light or under sub-lethal doses of light. *Methods*: The cytotoxic and DNA-damaging effects of ALA or ALA-H on lymphocytes and NPC cells were studied by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the alkaline comet assay. ALA at 0.75 mM concentration and ALA-H at 10-μM concentrations were selected in the studies. This is because under these concentrations, ALA- or ALA-H-mediated PDT can destroy most NPC cells in vitro. The intracellular distributions of the protoporphyrin IX (PpIX), induced by the ALA or ALA-H, were measured by the confocal laser scanning microscope to provide more information for understanding the DNA damage. *Results*: The incubation of 0.75 mM ALA or 10 μM ALA-H alone (without light) did not cause DNA damage as well as the considerable cytotoxic effect on NPC cells. However, after ALA (0.75 mM) incubation and without light irradiation, the serious cytotoxicity and remarkable DNA damage were found in lymphocytes. When the lymphocytes were incubated with ALA-H (10 μM) alone (in the absence of light), no DNA damage could be detected and a slight cytotoxic effect was found. Both ALA and ALA-H induced PpIX in the lymphocytes. The fluorescence images of PpIX intracellular localization demonstrated that the PpIX diffused into the nuclear region in ALA-(0.75 mM)-incubated lymphocytes but not existed in the nucleus of ALA-H(10 μM)- incubated lymphocytes, providing an explanation for the facts that ALA (0.75 mM) induced the DNA damage while ALA-H (10 μM) did not. *Conclusion*: These results suggested that the genotoxic potential of lymphocytes seems high for ALA (0.75 mM) and could be excluded for ALA-H (10 μM).

Keywords Cytotoxicity · Genotoxic potential · 5-aminolevulinic acid · Comet assay · Photodynamic therapy

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Introduction

During the last two decades, photodynamic therapy (PDT) has developed to be an innovative treatment modality for cancers [5], and thousands of cancer patients have been clinically treated with PDT worldwide [2]. The PDT is based on the preferential accumulation of a photosensitizer in a tumor. The subsequent irradiation of visit light activates the tumor-localized photosensitizer and induces the tumor destruction. Differing with the exogenous photosensitizers used in PDT, 5-aminolevulinic acid (ALA) is a precursor to porphyrins in heme synthesis. It was found that ALA can induce the endogenous protoporphyrin IX (PpIX) in tumor cells, and soon the ALA-based PDT was developed to become a new breach of PDT, called ALA-PDT [18]. The ALA-PDT has been carried out in pre-clinical and clinical treatments with promising results, especially for skin cancers [19]. However, the long-term side effects of this investigational modality are still not clearly known [9]. Generally, cancer treatments such as radiotherapy and chemotherapy can cause secondary tumor induction. Whether ALA-PDT has a similar carcinogenic risk is of importance.

The previous results of ALA concerning the DNA damage and genotoxic potential in the literatures are in controversy. The chromosome aberrations were reported after ALA exposure to hepatocytes in the absence of light [8]. The modifications of DNA bases were found after exposure of the isolated DNA to ALA [6]. However, for the cells of normal fibroblast, no detectable DNA damage was found after ALA (1 mM) incubation measured by comet assay [11]. The chromosome aberrations and micronuclei,which are the typical characteristics of mutagenicity, were also not observed in the carcinoma cells of the nasal septum and fibroblasts of the skin when 1 mM ALA hexylester (ALA-H) had been used [27]. The different conclusion in the above reports might be due to the different cell line. The different cells may have different response to ALA. The concentration variation of ALA used in above experiments may be the other reason. After all, the data on this aspect are limited and fragmental, and more works should be done to evaluate the genotoxic potential of ALA and its derivatives.

Nasopharyngeal carcinoma (NPC) is one of most prevalent malignancies in southern China [16]. The tests on NPC with ALA-PDT have been carried out recently [1, 15]. In the present study, in addition to the photoinactivation study of NPC cells with ALA-PDT, the dark cytotoxicities including the DNA-damaging potential of ALA or ALA-H on NPC cells were also measured. For the possibility of the secondary tumor induction of drugs, normal cells have the higher risk than tumor cells. Lymphocytes, the typical immune cells circulating in the blood, have a great chance to contact the drugs such as ALA or ALA-H. Thus, the cytotoxic and DNA-damaging effects of ALA or ALA-H on lymphocytes were studied for the first time to estimate the long-term side effect of these drugs.

Materials and methods

Drugs

5-aminolevulinic acid was purchased from Sigma (St. Louis, MO, USA). ALA-H was provided by PhotoCure ASA (Oslo, Norway). Stock solutions were prepared in Dulbecco's PBS (Gibco BRL, Life Technologies, Grand Island, NY, USA) at a concentration of 10 mM for ALA and 1 mM for ALA-H, and stored at 4°C in the dark for using in a few days. Serum-free RPMI medium was used to further dilute the stock solution to the desired concentrations.

Cell lines

The nasopharyngeal carcinoma (NPC/CNE2) cell line was a poorly differentiated squamous cell carcinoma from a 68-year-old Chinese male [28]. The cells were routinely cultured in a RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and antibiotics PSN (50 IU/ml penicillin, 50 (g/ml streptomycin and 100 μg/ml neomycin at 37°C in a humidified 5% CO_2 incubator.

Fresh blood was obtained from the volunteers. The lymphocytes were separated from the blood following the standard Histopaque–lymphocyte isolation procedure [24]. A volume of 3 ml of Histopaque 1077 (Sigma) was added into 3 ml of blood in each centrifuge tube, then these blood-containing tubes were centrifuged at 2,000 rmp for 30 min. The opaque white color layers in these centrifuge tubes were collected, and transferred to the PBS-containing tubes to do centrifuge washing. After three times washing, the lymphocyte pellet in each tube was collected, and transferred to the respective solution contained in the Eppendorf,,. These lymphocytes were srored at 4°C for use within a few days.

Drug incubation, PDT treatment and cytotoxicity assay

The cell viability vide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cytotoxic effects on cells [4]. MTT assay was carried out in 96-well flat bottom plates; 100 μl of NPC cell suspension (3×10^4) was added to each well and incubated for 24 h. Different concentrations of the drug were added to each well and incubated at 37°C in a 5% CO_2 incubator for 4 h. Then, the cells were washed twice with PBS and incubated with the fresh medium. Some groups of cells were kept in the dark for measuring the dark toxicity of the drugs. The other cell groups (six wells each) were irradiated with different light doses from a 400-W quartz–halogen lamp with a heat isolation filter and a 600-nm long-pass filter. The spectral intensity of the light source was basically flat from 600 to 800 nm and the total intensity was measured to be at 14 mW/cm^2 using a power meter [3]. After irradiation, the cells were incubated for another 24 h and then the survival percentage was determined by MTT assay using iEMS Analyzer (Lab-system, Helsinki, Finland). Appropriate controls were included in each experiment. All results were presented as the mean ± SD from three to six independent experiments.

Fluorescence images

After the incubation of ALA (0.75 mM) or ALA-H (10 μM), the cells were washed with PBS for several times and added to the fresh medium for imaging measurements. A laser scanning confocal microscope (Model LSM510, Zeiss, Oberkochen, Germany) was used to acquire the fluorescence images of cellular PpIX. The excitation was 488 nm from the attached Ar^+ laser, and the PMT with a 590-nm long-pass filter in the system was used to capture the fluorescence images. An Achroplan water-immersion objective (63×) was used in the measurements. With the pinhole used

in this confocal system, about 0.4-μm resolution in the *z* direction could be achieved. By using the *z*-scan function, the PpIX intracellular localization in three dimensions was measured.

Alkaline comet assay (single-cell gel electrophoresis)

Comet assay is a sensitive method for DNA strand-break detection in individual cells, and often used to detect the potential genetic toxicity of the drugs [7]. The cells were incubated with drugs (ALA or ALA-H), then some cell samples were kept in dark to detect the dark effect of drugs and the others were irradiated with a low light dose (sub-lethal level). Twenty-four hours after treatment, the cells were washed three times with PBS and trypsinized to obtain the cell suspensions. The comet assay was carried out as described in the previous work [25]. Briefly, these cell suspensions were centrifuged and the cell pellets were obtained in each tube by removing the liquid supernatants. The cells were then mixed with 85 μl of pre-warmed 1% low-gelling temperature agarose (Type VII, Sigma) in the tubes. Taking one drop of such a mixture to the slide that was pre-coated with 85 μl of 1% standard agarose (Sigma) in PBS, and putting the slides in the refrigerator for 5 min until solidified. To stain the nucleus, the cell membranes were lysed with a cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% Triton X-100), performed by submerging the slides in a staining jar in the dark for 1 h at 4°C. Subsequently, the slides were transferred to an electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA (4°C) for DNA unwinding and expression of alkali-labile sites. Electrophoresis was performed for 30 min at 25 V (0.83 V/cm). The current was maintained at 0.3 A by adjusting the electrophoresis solution level. After electrophoresis, the slides were neutralized in three changes (3×5 min) of 0.4 M Tris with pH 7.5. The slides were dried and stained with 20 μg/ml ethidium bromide for image analysis on the same day. An analysis was performed by measuring the tail moment with the Komet 3.0 Imaging system (Kinetic, Liverpool, UK). Tail moment is an index of DNA damage, which measures the length of the comet tail and the proportion of DNA migrating into the tail [12, 17]. Fifty cells were scored for each treatment group. The results were expressed as mean ± SD of more than three independent experiments, and changes on the tail moment were statistically calculated with *t*-test calculation.

Results

The PpIX formation in NPC cells and lymphocytes

After ALA or ALA-H incubation, the PpIX should be induced in the cells. The intracellular localizations of PpIX in NPC cells and lymphocytes are shown in Figs. 1 and 2, respectively. The PpIX diffusely localized in cytoplasm of NPC cells, no matter the cells have been incubated with ALA or ALA-H. The fluorescence intensities of cellular PpIX in ALA(0.75 mM)-incubated cells were similar to that in ALA-H(10 μM)-incubated cells, demonstrating that the ALA-H possessed better ability to induce the cellular PpIX production than ALA. The pattern of PpIX cytoplasmic localization was also found in ALA-H(10 μM)-incubated lymphocytes, as shown in Fig. 2b. However, in ALA (0.75 mM)-incubated lymphocytes the PpIX not only existed in the cytoplasm but also extended into the nuclear region (Fig. 2a), reflecting that the nuclear membrane of lymphocytes might be damaged during the ALA incubation. Such a special intracellular localization of ALA in the lymphocytes was confirmed by a series *z*-scanning image. The nuclear localization of PpIX is a harmful phenomenon, which could induce the DNA damage subsequently. The PpIX accumulations in two kinds of the cells imply that light irradiation could activate the cellular PpIX and initiate photosensitization resulting in the inactivation of NPC cells and also the damage of normal lymphocytes as the side effect.

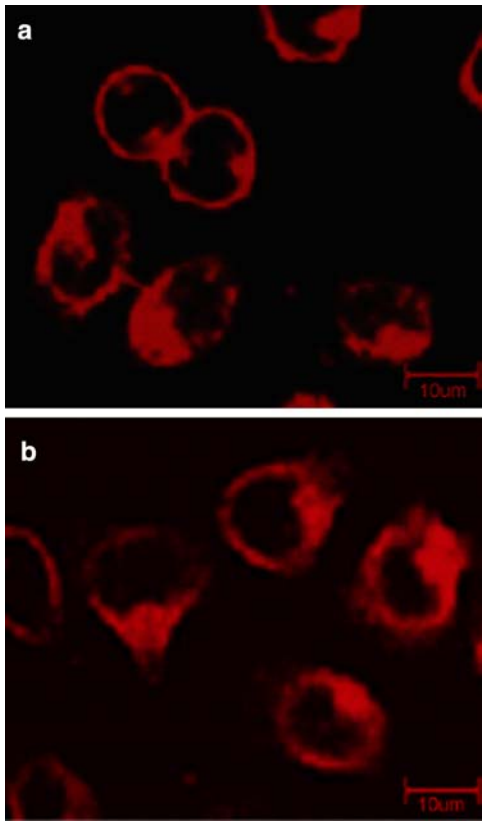

Fig. 1 The intracellular localization of PpIX in NPC cells. Fluo rescen images were acquired by the confocal laser scanning microscope with LP 590 nm filter. Excitation: 488 nm. Cells have been incubated with 0.75 mM ALA or 10 μM ALA-H for 4 h. **a** ALA-loaded cells; **b** ALA-H-loaded cells

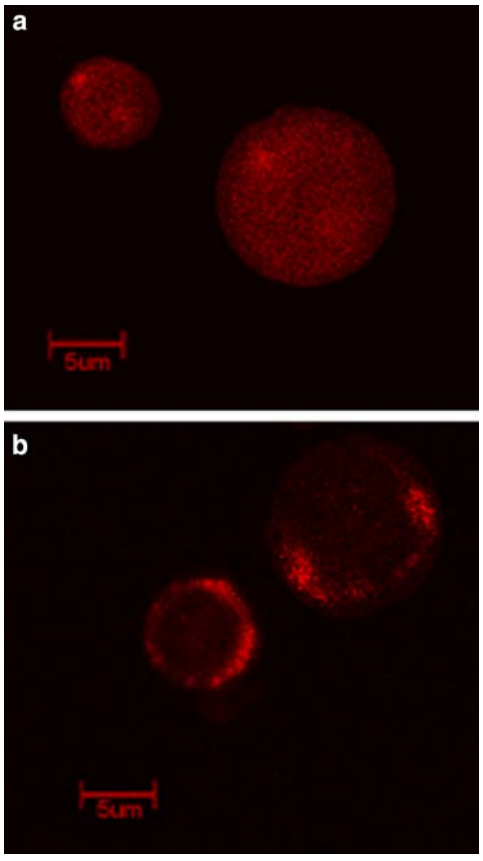

Fig. 2 The intracellular localization of PpIX in the lymphocytes. Fluorescence images were acquired by the confocal laser scanning microscope with LP 590 nm filter. Excitation: 488 nm. Cells have been incubated with 0.75 mM ALA or 10 μM ALA-H for 4 h. **a** ALA-loaded cells; **b** ALA-H-loaded cells

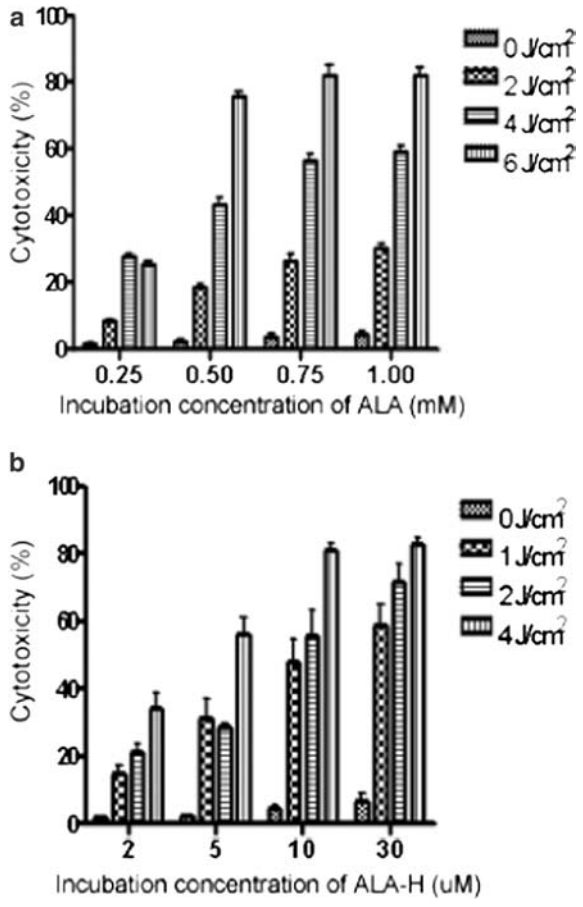

Fig. 3 Photocytotoxicity of ALA or ALA-H on NPC cells. the cells were incubated with different ALA or ALA-H concentrations for 4 h, and then irradiated with various light doses. The death rate was measured by MTT assay. *Error bars* (SD) were from three to six independent experiments. **a** ALA; **b** ALA-H

The photocytotoxicity of NPC cells and lymphocytes

Figure 3 shows the PDT effects of ALA or ALA-H on NPC cells. The inactivation of cells is dependent both on the drug-incubation concentration and the irradiation dose. When the incubation concentration of ALA was around 0.75 mM and the light dose reached 6 J/cm^2, the 80% death rate (LD_{80}) of cells could be achieved. While in the case of ALA-H-PDT, 10 μM incubation concentration and 4 J/cm^2 light dose were adequate for the LD_{80}. These results demonstrate that the ALA- and ALA-H-mediated PDT have the potential to become the new modality for NPC treatment, especially the ALA-H which is much more effective than ALA.

Since the concentrations of 0.75 mM ALA and 10 μM ALA-H can sufficiently perform the PDT to destroy the NPC cells; these incubation concentrations were selected to measure the damaging effects on normal lymphocytes. Figure 4 demonstrates the cytotoxicity of ALA or ALA-H on lymphocytes in the absence of light or with the low light doses. The lymphocytes are very sensitive to ALA- and ALA-H-mediated PDT. When the incubation concentration approached 0.5 mM for ALA or 10 μM for ALA-H and the light dose was over 2 J/cm^2 for the ALA-incubated cells or 1 J/cm^2 for ALA-H-incubated cells, most lymphocytes were destroyed. Under such overall PDT dose (incubation concentration of drug×irradiation dose), the damage on NPC cells was obviously lower. The dark toxicity of ALA to lymphocytes is serious. More than 50% cells were dead after incubation by 0.75 mM ALA alone (without light). The dark effect of ALA-H at selected incubation concentrations (10 μM or lower) is slight on lymphocytes, only a small portion of the cells (less than 20 percent) were hurt. Therefore, the lymphocytes are very fragile to stand against the PDT and even the ALA alone.

The DNA damage on lymphocytes and NPC cells

The DNA-damaging effects of the drugs on the cells under the conditions of low light doses and in the absence of light were investigated by the comet assay. The DNA tail moments represent the extent of DNA damage in this assay. The DNA tail moments remained small

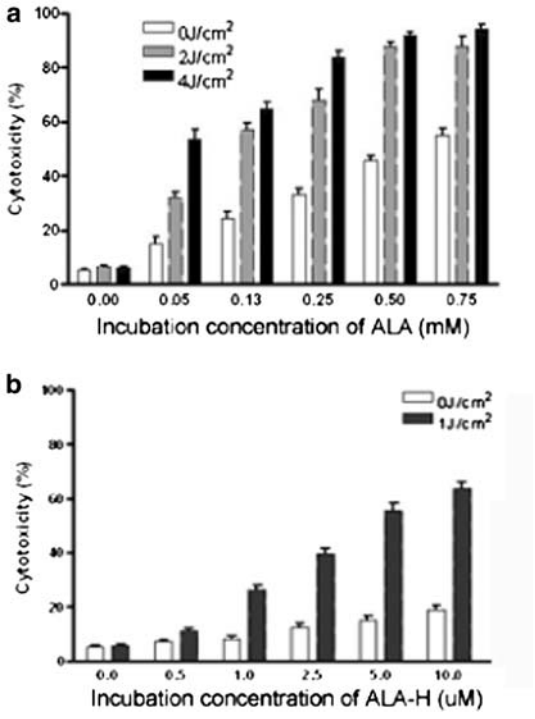

Fig. 4 Photocytotoxicity of ALA or ALA-H on lymphocytes. The cells were incubated with different concentrations of ALA (0.75 mM or lower) or ALA-H (10 μM or lower) for 4 h, and then irradiated with various light doses. The death rate was measured by MTT assay. *Error bars* (SD) were from three to six independent experiments. **a** ALA; **b** ALA-H

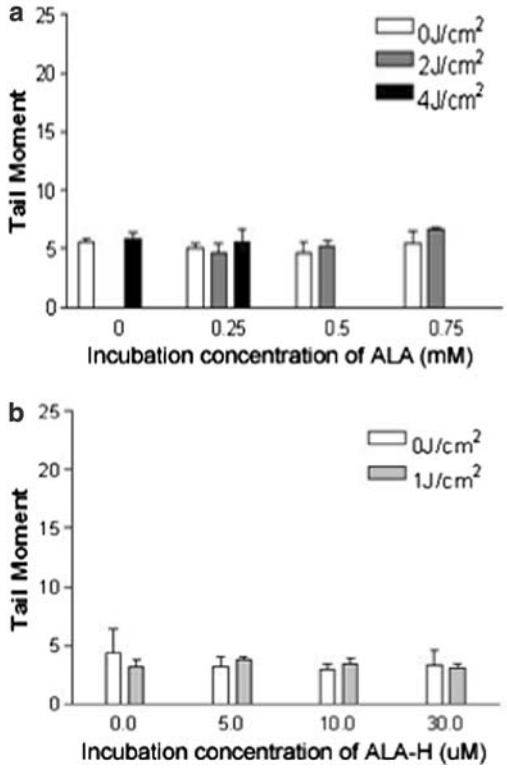

Fig. 5 The DNA-damaging measurements of ALA or ALA-H on NPC cells. The cells have been incubated with various ALA or ALA-H concentrations for 4 h and then irradiated with different light doses or in the absence of light. The expression of DNA damage in tail moments was measured by the comet assay. **a** ALA; **b** ALA-H

(about 5%) in all of the conditions in ALA- or ALA-H-treated NPC cells (Fig. 5), and no significant increment of the tail moment ($P > 0.05$) was found as compared to the control cells. Here, the cell-death rates were lower than 25% under selected overall PDT doses.

Figure 6 shows the results of comet assay in ALA- or ALA-H-treated lymphocytes. In ALA-PDT- or ALA-H-PDT-treated cells, the DNA damage was very obvious with the significant increase of tail moment ($P < 0.01$) compared to the control. However, as shown in Fig. 4, the cell-death rates were already as high as 90% for ALA group and 60% for ALA-H group at such overall PDT doses. The ALA-PDT is well known to induce the apoptosis in sensitized cells resulting in the nuclear condensation and fragmentation [13]. Thus, the increment on tail moment is probably due to the expression of these apoptotic cells. The DNA damage caused by the drug alone (without light) is the problem of great concern. The ALA-H (10 μM) alone did not cause any increment on the tail moment as compared to the control cells ($P > 0.05$), indicating that no DNA damage occurred in ALA-H-incubated cells. While the tail moment increase ($P < 0.01$) was found in ALA-(0.75 mM)-incubated cells in the absence of light, reflecting ALA alone also caused a considerable DNA damage in the lymphocytes.

Discussion

The dark cytotoxic and genotoxic effects of ALA and its derivatives have been considered as a crucial problem in the field of ALA-PDT [9]. In previous reports, the DNA damage was found in hepatocytes but not in normal fibroblasts when the cells have been incubated with ALA in the absence of light [8, 17]. These results imply that the cell resistance to drugs is dependent on the cell line. To investigate the side effects of ALA type drugs, especially the genotoxic effect, the selection of the cell model is certainly important. Lymphocytes are the blood cells circulating around the whole body. They have more chances than other non-blood normal cells to encounter the drug molecules that are delivered to the tumor. Thus, the lymphocyte is a suitable model to study the cytotoxic and genotoxic effects of ALA and its derivatives. The other parameter of the dark cytotoxic study is the selection of drug concentration. When the concentration used is too high, any drug can cause the toxicity in the treated cells. For most in vitro works, the concentrations of ALA around millimoles and that of ALA-H with several micromoles were commonly used [10, 14, 22, 23]. In the present work, 0.75 mM of ALA and 10 μM of

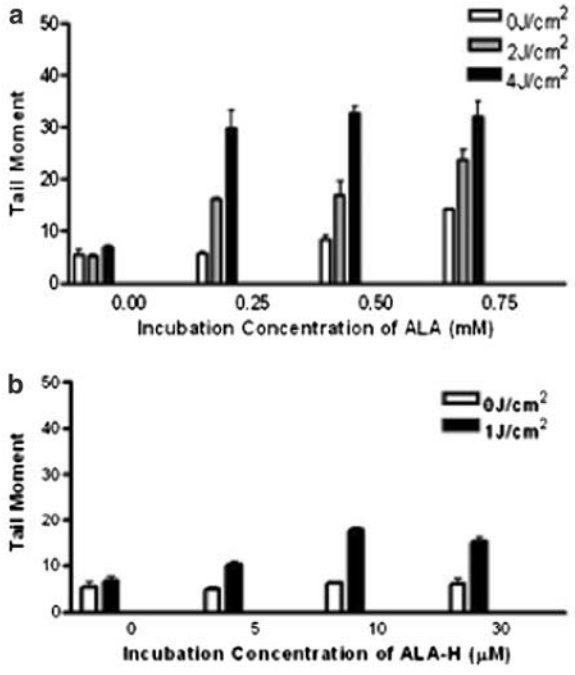

Fig. 6 The DNA-damaging measurements of ALA or ALA-H on lymphocytes. The cells have been incubated with various ALA or ALA-H concentrations for 4 h and then irradiated with different light doses or in the absence of light. The expression of DNA damage in tail moments was measured by the comet assay. **a** ALA; **b** ALA-H

ALA-H were selected because ALA or ALA-H with these concentrations can mediate the destruction of most NPC cells by PDT. Here, we found for the first time, that 0.75 mM ALA alone caused the both cytotoxicity and DNA damage in lymphocytes while 10 μM ALA-H did not induce detectable DNA damage and gave rise to a slight cytotoxic effect in the lymphocytes.

The alkaline comet assay is particularly sensitive to single-strand breaks and alkali-labile sites of DNA, and thus becomes a reliable method to detect the genotoxic potential in the cells [20]. Based on our results, the genotoxic potential of ALA-H on lymphocytes can be excluded. Under the concentrations commonly used (10 μM or lower), ALA-H is safe. Whereas ALA, should be very carefully used in the clinic. A considerable portion of lymphocytes is harmed when ALA with the concentration around millimoles exists in the system. More serious response is that lymphocytic DNA damage would be induced resulting in a genotoxic potential of lymphocytes. In fact, the ALA-H only has an additional hexylester on the one end of the molecule compared to ALA. However, the hexylester makes the ALA-H to become more lipophilic and thus increases the ability of PpIX cellular production as well as the efficiency of ALA-H-PDT. Due to the high PDT efficiency, ALA-H with low concentrations could achieve the inactivation of NPC cells, and did not cause DNA damage in the lymphocytes. Regarding the low PDT efficacy of ALA, high concentration is needed to fulfill the cancer therapy, but which would induce a serious side effect such as the DNA damage of lymphocytes. According to the side effects of ALA on normal lymphocytes, the ALA delivery way becomes very important provided the ALA-PDT would be applied in vivo. Among the several ways of ALA delivery, the intravenous administration is of high risk, because the lymphocytes in the blood vessel encounter the ALA in a very early stage after ALA transport into the vessel and they stay together until the elimination of ALA in about 2 days [19]. The topical application of ALA seems to be a better way for drug delivery, in which the chance of mixing of the lymphocytes with ALA is dramatically reduced leading to a much lower risk of the genotoxic potential.

The concentration of drug seems to be a key factor in inducing the genotoxic potential in lymphocytes. When high concentration of ALA (mmol) is used in the in vitro study, a large number of ALA molecules enter the cells and approach or even enter the nucleus because their small size is associated with high penetration ability. It was reported that ALA itself could undergo oxidation to yield the reactive oxygen species (ROS) in the absence of light [6]. The ROS certainly can damage the DNA when they are produced in the nucleus, and also break the nuclear membrane to allow the PpIX to diffuse into the nuclear region, which may explain the nuclear localization of PpIX in Fig. 2a. The PpIX in the nucleus might produce singlet oxygen, thus accelerating the DNA damage [9]. When the concentration of the drug is low such as in the ALA-H (10 μM) case, the cellular amount of ALA-H molecules should accordingly be low. In such a situation, the nuclear membranes remain intact and PpIX cannot enter the nucleus as shown in Fig. 2b.

To explore the new modality of NPC therapy, the PDT treatment on the NPC patients was carried out clinically with the first generation photosensitizer of hematoporphyrin derivative (HPD) and promising results were obtained [21]. To search for better PDT drugs for NPC inactivation, our group has investigated several different photosensitizers [25, 26]. Summarizing, among the drugs we have tested, namely, HPD, mTHPC, MC540, ALA and ALA-H, the best one seems to be ALA-H which has the potential for PDT treatment of NPC and is worth investigating further in vivo. ALA-H has high PDT efficacy on NPC inactivation, and has no risk of the genotoxic potential on normal lymphocytes.

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