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COMPARISON OF AMINOLEVULINIC ACID AND ITS METHYL ESTER MEDIATED PHOTOCYTOTOXICITY ON HUMAN NASOPHARYNGEAL CARCINOMA CELLS

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Nasopharyngeal carcinoma (NPC) is a prevalent cancer in some areas of southern Asia. To explore the potential of photodynamic therapy (PDT) for the treatment of NPC, a small molecule prodrug 5-aminolevulinic acid (ALA) and its methyl ester (MAL) mediated PDT was studied *in vitro*. The results showed that human NPC cells were sensitive to both ALA- and MAL-mediated PDT. However, ALA was more effective than MAL, possiblly due to a higher efficiency of ALA on producing endogenous protoporphyrin (PpIX) in NPC cells. Neither ALA nor MAL caused any significant genotoxicity. The ALA-based PDT might be a useful modality in the treatment of NPC.

Keywords: Aminolevulinic acid; methylaminolevulinate; nasopharyngeal carcinoma; photodynamic therapy.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignant squamous cell carcinoma that has the highest prevalence in Southern China (e.g., Guangdong) followed by some areas of Southeast Asia (e.g., Singapore, Malaysia, Vietnam and Philippines).[1] The common treatment modalities of NPC include radiotherapy and chemotherapy. But the incidences of relapses and side effects associated with those modalities remain high. There is a need to

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develop less invasive and more effective treatment for NPC.[2]

Photodynamic therapy (PDT) is a minimally invasive modality which involves the administration of a photosensitizer followed by illuminating the disease site with visible light of specific wavelength(s). In the presence of oxygen molecules, the activation of photosensitizer inside the cancerous tissue can lead to the generation of cytotoxic species (e.g., singlet oxygen) and consequently destroy the cancer.[3] PDT is a promising modality for treating lesions in the oral cavity, pharynx and larynx.[4,5] Early studies demonstrate that multi-session PDT mediated with the 1st generation photosensitizer hematoporphyrin derivative (HpD) is an effective and safe local treatment modality for NPC.[6–8] However, HpD can cause a prolonged skin photosensitization that makes their application limited.

Aminolevulinic acid (ALA) is a precursor of protoporphyrin IX (PpIX) in the heme biosynthesis pathway. Exogenous administration of ALA or its ester derivatives can induce an intracellular accumulation of PpIX, which is a potent photosensitizer.[3,5] It has been suggested that the use of more lipophilic derivatives of ALA can improve the diffusing properties of ALA and therefore generate higher PpIX yield.[9] Topical application of methylaminolevulinate (MAL) or ALA-methyl ester has been used for various dermatological indications, whereas hexylaminolevulinate (HAL) or ALA-hexyl ester has been approved for fluorescence cystoscopy of bladder cancer detection.[10,11]

The potential of utilizing ALA for the treatment of solid tumor has been proposed in the early 1990's.[12] Several *in vitro* and *in vivo* studies show promising results of ALA-based PDT and fluorescence diagnosis of NPC cells.[13–15] In this study, we compared ALA- and MAL-mediated photocytotoxicity in human NPC cells.

2. Materials and Methods

2.1. *NPC cell lines*

Two human NPC cell lines were used in this study. HK1 cell line,[16] derived from a well-differentiated squamous cell carcinoma (SCC) was provided by the Department of Anatomy, Chinese University of Hong Kong, and CNE2 cell line,[17] derived from a poorly differentiated SCC was purchased from Shanghai Biosis Biotechnology Co. Ltd. Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and antibiotics (50 IU/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL neomycin) at 37°C in a humidified 5% CO_2 incubator.

2.2. *Preparation of ALA and MAL solutions*

δ-Aminolevulinic acid hydrochloride (ALA) and its methyl ester MAL were purchased from Sigma (St. Louis, MO, USA). Stock solutions were prepared in Dulbecco's phosphate buffered saline (PBS) (Gibco) at a concentration of 10 mM. Serum free and phenol red free RPMI medium was used to further dilute the stock solution to the desired working concentration. The working solutions were stored at 4°C and used within a week.

2.3. *Detection of PpIX formation in NPC cells by flowcytometry assay*

Cells (3×10^5 cells/dish) were seeded in 60-mm culture dishes (Corning, Lowell, MA, USA) and incubated at 37°C in a 5% CO_2 humidified incubator for 24 h for attachment. Before the addition of ALA or MAL, the cells were washed twice with PBS, then incubated in phenol red free RPMI medium in the presence of ALA or MAL (0–1 mM) for up to 8 h. Following incubation, the cells were washed twice with PBS and trypsinized with 1 mL 0.25% trypsin-EDTA for 2–5 min. Cells were washed twice with PBS. All procedures were performed in the dark. Cells were resuspended in 0.5 mL PBS and ALA- or MAL-induced PpIX fluorescence was quantified by an EPICS Elite Flow cytometer (Beckman Coulter, Brea, CA, USA). The intracellular PpIX was excited with an argon ion laser (488 nm) at the power level of 200 mW and the fluorescence signal was collected with a 630 longpass filter.[18] 20,000 cells were counted for each sample. Debris was excluded from analysis using forward and side scatter signals. Background fluorescence obtained from cells not exposed to ALA or MAL was subtracted from sample fluorescence. Results were expressed as mean ± S.D. of at least three independent experiments.

2.4. *Intracellular localization of PpIX induced by ALA and MAL*

Cells (1×10^4 cells/ml) were seeded in 35-mm culture dishes (Corning) and incubated for 24 h for attachment. Washed cells were then incubated with ALA or MAL (0−1 mM). At predetermined time intervals (0.5, 1, 2 or 4 h) cells were washed twice with PBS and a cover slip was placed on top. The cells were examined under a fluorescence microscope equipped with an HBO/100W mercury lamp and 400−440 nm band-pass for excitation. A 510 nm dichroic beam splitter and 590 nm band-pass filter were used for the detection of PpIX fluorescence.[19] The mitochondria were co-stained with Mito-Tracker Green FM (Invitrogen). For comparison, a bright-field image was also captured. All procedures were performed in the dark.

2.5. *Trypan blue assay of PDT-induced photocytotoxicity*

Cells (3×10^4 cells/well) were seeded in 96-well flat-clear-bottomed and black-wall plate (Costar, Corning). After incubating overnight at 37°C in a 5% CO_2 incubator for attachment, the cells were incubated with ALA or MAL (0−1 mM) for 4 h. Following incubation, the sensitized cells were irradiated under various light doses (0−6 J/cm^2) at a power density of 14 mW/cm^2. A 400 W quartz−halogen lamp with heat isolation filter and a 600 nm long-pass filter was used as a PDT light source.[20] Afterward the cells were washed twice with PBS, resuspended in 0.1 mL complete RPMI medium, and further incubated for 24 h. Prior to Trypan blue exclusion assay the cells were washed twice with PBS. 30 μL of 0.1% trypan blue was added to the wells and numbers of cells unstained and stained were counted under a light microscope. All procedures were performed in the dark. Percentage of cell death over the total number of cells (i.e., % of killing) in each field was calculated. Results were expressed as mean ± S.D. of at least three independent experiments.

2.6. *Comet assay of DNA damage*

Cells (7×10^4 cells/mL) were seeded in 35-mm culture dishes (Corning). After incubating at 37°C in a 5% CO_2 incubator overnight for attachment, the cells were incubated with ALA or MAL (0−1 mM) overnight. A total of 0−6 J/cm^2 of irradiation energy was applied to the cell layer at a power density of 14 mW/cm^2. Immediately and 20 h after light treatment, cells were washed three times with PBS and then trypsinized to obtain single cell suspensions for comet assay.[21] Briefly, PDT treated cells were washed twice in cold PBS and resuspended in 85 μl of pre-warmed 1% low gelling temperature agarose (Type VII; Sigma). The cell mixture was transferred to the microscope slide precoated with 85 μL of 1% standard agarose (Sigma) in PBS. The slides were placed in a fridge for 5 min until solidified. Cell lysis was performed by submerging the slides in lysis solution and in the absence of light for 1 h. After electrophoresis, slides were stained with ethidium bromide. Image analysis was performed by measuring the tail moment with the Komet 3.0 Imaging system (Kinetic, Liverpool, UK). 50 cells were scored for each treatment group. Results were expressed as mean ± S.D. of at least three independent experiments and changes in tail moment were tested by one-way ANOVA.

3. Results

3.1. *PpIX formation in NPC cells induced by ALA and MAL*

Intracellular PpIX fluorescence was determined by a flow cytometer at different time points. As expected, the intracellular PpIX formation in NPC cells was mainly influenced by the type and dose of prodrug, and the time of incubation. For HK1 cells, a slightly higher PpIX concentration was obtained for ALA than for MAL at the same dose and incubation time (Fig. 1). Similar trend was seen for CNE2 cells. Figure 2 shows the time course of intracellular PpIX formation in CNE2 cells after incubating with different concentrations of ALA for 2 to 8 h. Compared to HK1 cells, much higher PpIX concentration was obtained in CNE2 cells at the same dose and incubation time. For instance, after incubating with 1 mM of ALA for 4 h the PpIX concentration in CNE2 cells was 2.5 folds higher than that in HK1 cells (2.24 ± 0.793 versus 5.775 ± 2.021).

3.2. *Intracellular PpIX localization in NPC cells*

The localization of fluorescent PpIX was visualized by a fluorescence microscope. The fluorescence

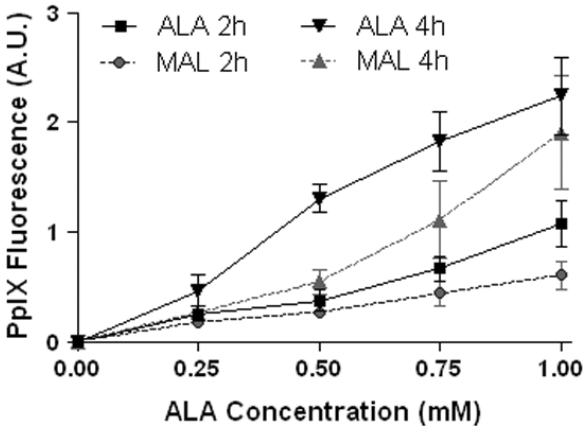


Fig. 1. Intracellular PpIX formation in HK1 cells. Cells were incubated with different concentrations of ALA (solid lines) and MAL (dashed lines) for 2 and 4 h. Results were expressed as mean ± S.D.

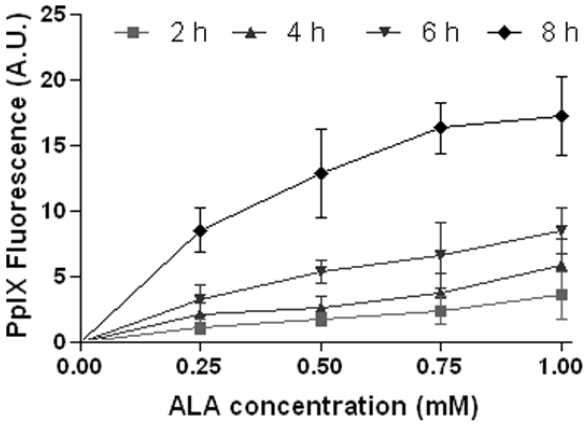
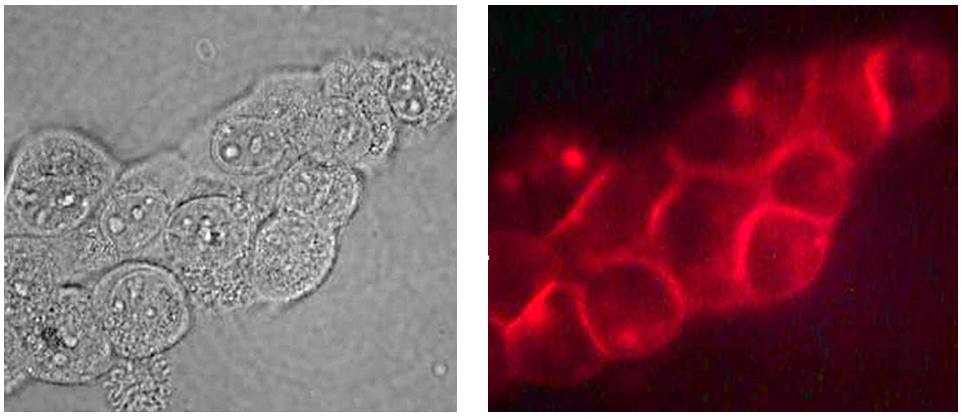


Fig. 2. Time course of intracellular PpIX formation in CNE2 cells. Cells were incubated with different concentrations of ALA for 2 to 8 h. Results were expressed as mean ± S.D.

images revealed that PpIX was endogenously induced in both cell lines after incubating with ALA and MAL. The intensity of PpIX fluorescence increased when prodrug dose and/or incubation time increased. Comparing the fluorescence image with microscopic image of the same cells, it confirmed that the PpIX formation occurred in all cells (Fig. 3). Co-staining with MitoTracker Green clearly demonstrated that PpIX was primarily accumulated in the mitochondria and cytoplasmic membrane. The latter was indicated by the condensed bright red color rim (Fig. 4). As indicated earlier by the flow cytometry assay that a similar level of PpIX fluorescence was obtained between 2 h incubation with 0.5 mM ALA and 4 h incubation with 0.5 mM MAL in HK1 cells, indeed. Figures 3(b) and 3(d) show an identical PpIX localization profile between these two groups.

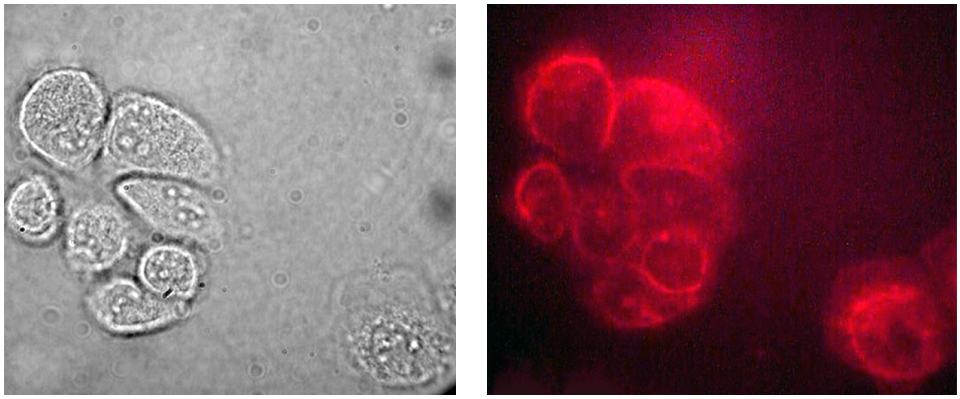
3.3. *Photocytotoxicity of ALA- and MAL-mediated PDT on NPC cells*

Photocytotoxicity of PpIX was determined by the Trypan blue exclusion assay at different prodrug and light doses. ALA/MAL alone or light alone had no cytotoxic effect. As expected, the photocytotoxicity induced by the combination of PpIX formation and light irradiation was mainly influenced by the type and dose of prodrug, the length of incubation, light dose and cell type. The percentage of photodynamic killing increased when prodrug dose and/or light dose increased. As shown in Fig. 5, a complete killing of HK1 cells could be



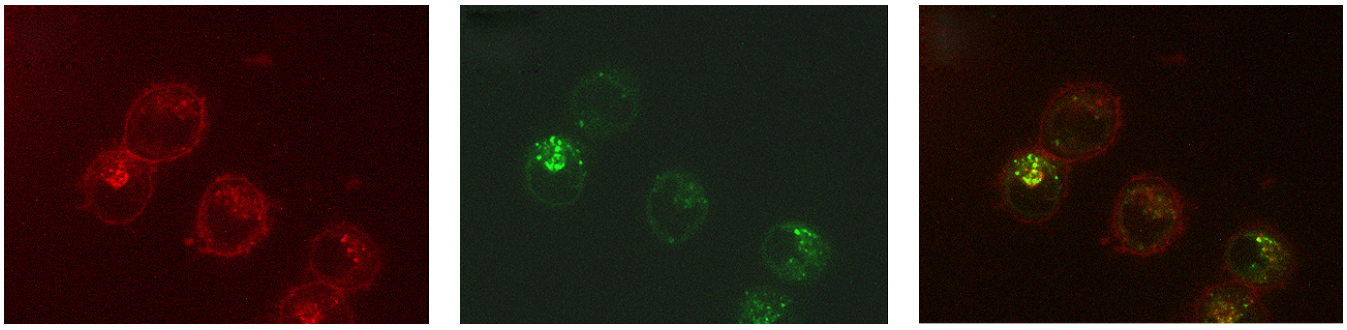
(a) (b)

Fig. 3. Microscopic and fluorescence images of HK1 cells. Top panel: cells were incubated with ALA (0.5 mM) for 2 h; bottom panel: cells were incubated with MAL (0.5 mM) for 4 h.



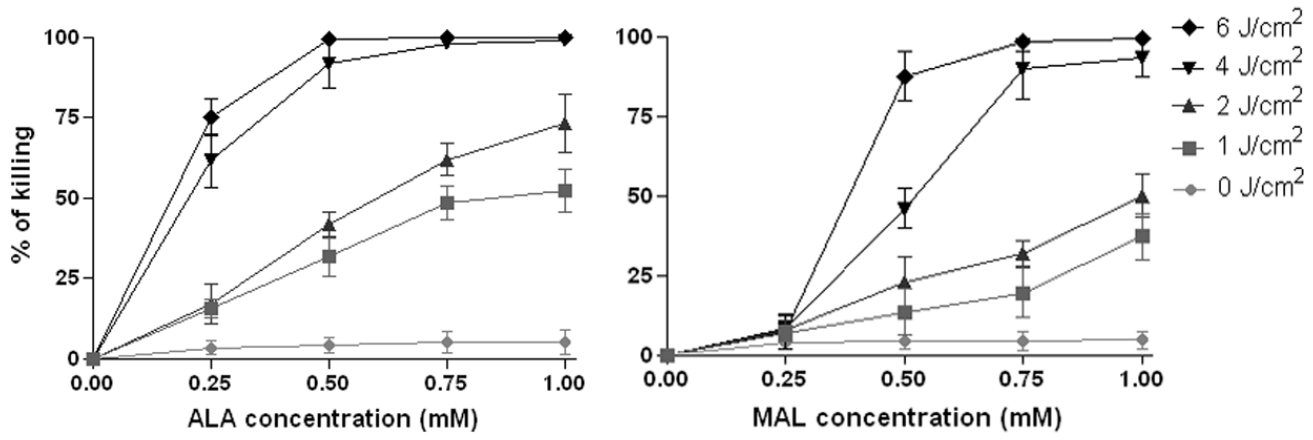
(c) (d)

Fig. 3. (*Continued*)



(a) (b) (c)

Fig. 4. MitoTracker Green stain of CNE2 cells. (a) Cells were incubated with ALA (0.5 mM) for 4 h; (b) MitoTracker Green staining; and (c) overlay image.



(a) (b)

Fig. 5. Photocytotoxicity of ALA- and MAL-mediated PDT on HK1 cells. Cells were incubated with different concentrations of ALA (a) or MAL (b) for 4 h. Results were expressed as mean ± S.D.

achieved by ALA at a dose level of 0.75 mM and a light dose of 4 J/cm^2, whereas, a complete killing of HK1 cells was achieved by MAL at a dose level of 1 mM and a light dose of 6 J/cm^2. This suggested that ALA was more effective than MAL, possibly due to a higher yield of PpIX induced by ALA. For CNE2 cells, it was expected that a complete killing could be achieved by ALA at a drug dose level greater than 1 mM and a light dose level greater than 4 J/cm^2 (Fig. 6). This indicated that CNE2 cells were less sensitive than HK1 cells.

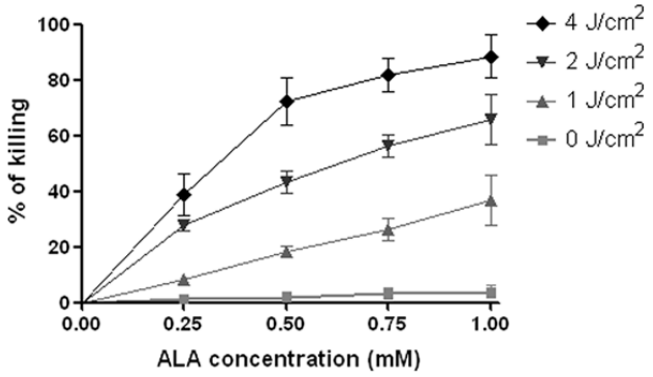


Fig. 6. Photocytotoxicity of ALA-mediated PDT on CNE2 cells. Cells were incubated with different concentrations of ALA for 4 h. Results were expressed as mean ± S.D.

3.4. *Genotoxicity of ALA- and MAL-mediated PDT on NPC cells*

Potential genotoxicity of ALA- and MAL-mediated PDT on NPC cells was examined using the comet assay. The results from the comet assay showed that the mean total tail DNA ranged from 2% to 4% for HK1 cells (Fig. 7). Similar results were seen for CNE2 cells (3% to 5% mean tail DNA). There was no significant DNA damage immediately and 20 h

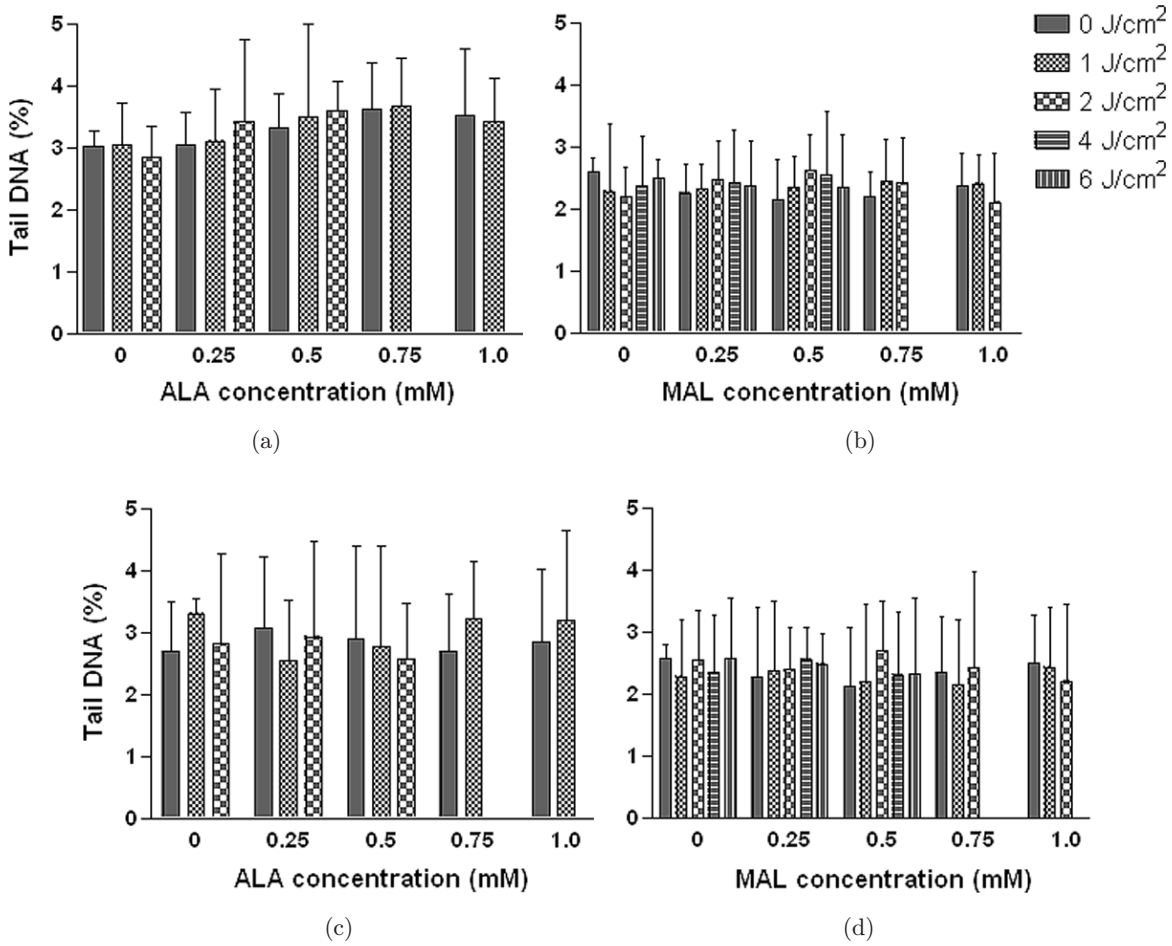


Fig. 7. Tail moment of HK1 cells after PDT. Cells were incubated with ALA (a) and (c) or MAL (b) and (d) overnight. Top panel: immediately after PDT; bottom panel: 20 h after PDT. Results were expressed as mean ± S.D.

after PDT in controls and treated groups received LD_{25} to LD_{75} PDT dose (one-way ANOVA, $p > 0.05$). These results suggested that ALA- and MAL-mediated PDT had no genotoxicity on NPC cells.

4. Discussion

The interest in potential applications of the small molecule prodrug ALA and its ester derivatives continue to grow. In this study, we explored the feasibility of ALA-based prodrugs for the PDT treatment of NPC. The photocytotoxicity of ALA and its methyl ester MAL on human NPC cells was examined *in vitro*. As expected, when the prodrug incubation concentration was increased, the fluorescence intensity of PpIX in both cell lines was increased accordingly (see Figs. 1 and 2), implying that the PpIX formation was elevated as the concentration of ALA and MAL increased. The PpIX accumulation was also proportional to the incubation time for both prodrugs (see Fig. 2). Taken together, we found a incubation concentration- and time-dependent in PpIX accumulation in both human NPC cell lines. This is consistent with previous report on human NPC and other cancer cell lines.[13,22]

Interestingly, for the same prodrug concentration and incubation time, the PpIX formation was obviously higher in ALA treated cells than in MAL treated cells (see Figs. 1 and 2). These results reflected why the phototoxicity of ALA-PDT was higher than that of MAL-PDT at the same dose level (see Figs. 5 and 6). This suggests that MAL is not so favorable in penetrating the NPC cells in *in vitro* condition and therefore the PpIX production was consequently lower. This phenomenon might be cell-type dependent. Gaullier *et al.* reported that compared to ALA, in order to produce the same among PpIX, 5−10 times higher concentration of MAL was required for WiDr cells (adenocarcinoma colon cells).[23] Unlike MAL, the hexyl ester ALA (HAL) is more readily taken up by NPC cells and exhibits a strong photocytotoxicity.[19] Nevertheless, ALA could be considered as a candidate for PDT treatment of NPCs. Based on the fact that PpIX formation in CNE2 cells was similar to that in HK1 cells, ALA-mediated PDT might be applied not only in well-differentiated NPC cells but also in poor-differentiated NPC cells. An *in vitro* study suggests that the status of Epstein-Barr virus (EBV) infection might affect the sensitivity of NPC cells to hematoporphyrin-mediated PDT.[25] The effect of EBV infection on ALA-based PDT should be explored in future study.

According to the heme synthesis pathway, PpIX is produced initially in the mitochondria and can diffuse into the cytoplasm. In this study, we demonstrated that PpIX could diffuse into the cytoplasmic compartments of HK1 cells (see Fig. 3). However, the mitochondria was still the main binding target of PpIX (see Fig. 4). This unique subcellular distribution profile determines that ALA-mediated PDT can induce apoptosis shortly after light irradiation and activate p38 MAPK and JNK signalling pathway.[24,26,27]

Although the administration of photosensitizer and lesion site-directed light irradiation are safe and the topical or systemic administration of prodrug does not cause a prolonged general cutaneous photosensitization, unpredicted complication in the normal and cancerous tissue that unavoidably accumulate photosensitizers might incur due to exposure to light.[3] In order to explore the genetic safety of utilizing ALA-based prodrug, possible DNA damaging effect was investigated with or without light irradiation. In general, under the drug and light dose of LD_{25} PpIX is primarily formed in the mitochondria and the PDT dose is strong enough to initiate the mitochondria-mediated apoptosis together with DNA fragmentation.[28,29] Under higher doses (e.g., $>LD_{25}$), the Comet assay of fragmented DNA might give a false-positive genotoxic response.[30] Hence, in order to avoid a false alarm the genotoxicity study was carried out under the LD_{25}. Results suggested that there was no DNA strand break detected for dark controls and PDT groups. Though ALA-mediated PDT is a valuable treatment and clinically used widely, the genotoxic potential is of great concern and now was proven to be safe. However, the potential genotoxicity of ALA ester derivatives in lymphocytes is still inconclusive and needed to be further studied.[31]

Apparently, another very important advantage of ALA-based PDT and photodiagnosis is that the drug incubation time (i.e., the PpIX formation time) is very short (a few hours) in contrast to the long incubation time of other porphyrin-based sensitizer (>24 h). Short sensitizing time is more beneficial in clinical practice. ALA and its ester derivatives have been widely applied orally, systemically, or topically with successful results in

different cancers.[32] Therefore, it is feasible to develop ALA-based PDT protocols by taking advantage of the availability of existing 630 nm light sources for treating NPC.[4,8]

In summary, available data in this study demonstrated that the role of ALA-based PDT in both NPC cell lines was promising as a treatment modality. ALA and MAL could endogenously produce PpIX. ALA favorably generated more PpIX than MAL under similar drug concentration and sensitization time in both NPC cell lines. ALA exhibited a more potent effect than MAL in photoinactivation of NPC cells *in vitro* without causing genotoxicity.

Acknowledgments

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References

1. A. T. Chan, "Nasopharyngeal carcinoma," *Ann. Oncol.* **Suppl 7**(vii), 308−312 (2010).
2. J. Guigay, "Advances in nasopharyngeal carcinoma," *Curr. Opin. Oncol.* **20**(3), 264−269 (2008).
3. Z. Huang, "A review of progress in clinical photodynamic therapy," *Technol. Cancer Res. Treat.* **4**(3), 283−293 (2005).
4. M. A. Wildeman, H. J. Nyst, B. Karakullukcu, B. I. Tan, "Photodynamic therapy in the therapy for recurrent/persistent nasopharyngeal cancer," *Head Neck Oncol.* **1**(40), 1−6 (2009).
5. P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piett, B. C. Wilson, J. Golab, "Photodynamic therapy of cancer: an update," *CA Cancer J. Clin.* **61**(4), 250−281 (2011).
6. Z. Q. Sun, G. Y. Luo, "Photodynamic therapy for recurrent nasopharyngeal carcinoma after radiotherapy (An analysis of 191 cases)," *Chin. J. Laser Med. Surg.* **5**, 134−136 (1996) (in Chinese).
7. M. C. Tong, C. A. van Hasselt, J. K. Woo, "Preliminary results of photodynamic therapy for recurrent nasopharyngeal carcinoma," *Eur. Arch. Otorhinolaryngol.* **253**(3), 189−192 (1996).
8. L. Li, R. Luo, W. Liao, M. Zhang, Y. Luo, J. Miao, "Clinical study of Photofrin photodynamic therapy for the treatment of relapse nasopharyngeal carcinoma," *Photodiag. Photodyn. Ther.* **3**(4), 266−271 (2006).
9. J. Bedwell, A. J. MacRobert, D. Phillips, S. G. Bown, "Fluorescence distribution and photodynamic effect of ALA-induced PP IX in the DMH rat colonic tumour model," *Br. J. Cancer* **65**(6), 818−824 (1992).
10. C. S. Betz, J. P. Lai, W. Xiang, P. Janda, P. Heinrich, H. Stepp, R. Baumgartner and A. Leunig, "*In vitro* photodynamic therapy of nasopharyngeal carcinoma using 5-aminolevulinic acid," *Photochem. Photobiol. Sci.* **1**(5), 315−319 (2002).
11. V. Manivasager, P. W. Heng, J. Hao, W. Zheng, K. C. Soo, M. Olivo, "Macro-microscopic fluorescence imaging of human NPC xenografts in a murine model using topical vs intravenous administration of 5-aminolevulinic acid," *Int. J. Oncol.* **21**(5), 1003−1007 (2002).
12. V. Manivasager, K. K. Yee, P. W. Heng, K. C. Soo, M. Olivo, "A study comparing endogenous protoporphyrin IX induced by 5-ALA and ALA-methyl ester with exogenous PpIX and PpIX dimethyl ester in photodynamic diagnosis of human nasopharyngeal carcinoma xenografts," *Int. J. Oncol.* **29**(4), 997−1002 (2002).
13. N. Fotinos, M. A. Campo, F. Popowycz, R. Gurny, N. Lange, "5-Aminolevulinic acid derivatives in photomedicine: Characteristics, application and perspectives," *Photochem. Photobiol.* **82**(4), 994−1015 (2006).
14. S. B. Brown, "The role of light in the treatment of non-melanoma skin cancer using methyl aminolevulinate," *J. Dermatolog. Treat.* **14**(Suppl. 3), 11−14 (2003).
15. J. A. Witjes, J. Douglass, "The role of hexaminolevulinate fluorescence cystoscopy in bladder cancer," *Nat. Clin. Pract. Urol.* **4**(10), 542−549 (2007).
16. D. P. Huang, J. H. Ho, Y. F. Poon, E. C. Chew, D. Saw, M. Lui, C. L. Li, L. S. Mak, S. H. Lai, W. H. Lau, "Establishment of a cell line (NPC/HK1) from a differentiated squamous carcinoma of the nasopharynx," *Int. J. Cancer* **26**(2), 127−132 (1980).
17. Z. Sizhong, G. Xiukung, Z. Yi, "Cytogenetic studies on an epithelial cell line derived from poorly differentiated nasopharyngeal carcinoma," *Int. J. Cancer* **31**(5), 587−590 (1983).
18. E. A. Hryhorenko, K. Rittenhouse-Diakun, N. S. Harvey, J. Morgan, C. C. Stewart and A. R. Oseroff, "Characterization of endogenous protoporphyrin IX induced by delta-aminolevulinic acid in resting and activated peripheral blood lymphocytes by four-color flow cytometry," *Photochem. Photobiol.* **67**(5), 565−572 (1998).
19. R. W. K. Wu, E. S. M. Chu, C. M. N. Yow, J. Y. Chen, "Photodynamic effects on nasopharyngeal carcinoma (NPC) cells with 5-aminolevulinic acid or

its hexyl ester," *Cancer Lett.* **242**(1), 112−119 (2006).
20. C. M. N. Yow, J. Y. Chen, N. K. Mam, N. H. Cheung, A. W. Leung, "Cellular uptake, subcellular, localization and photodamaging effect of temoporfin (mTHPC) in nasopharyngeal carcinoma cells: comparison with hematoporphyrin derivative (HPD)," *Cancer Lett.* **157**(2), 123−131 (2000).
21. P. L. Olive, J. P. Banath, "Induction and rejoining of radiation-induced DNA single-strand breaks: "tail moment" as a function of position in the cell cycle," *Mutation Res.* **294**(3), 275−283 (1993).
22. S. L. Gibson, J. J. Havens, T. H. Foster, R. Hilf, "Time-dependent intracellular accumulation of delta-aminolevulinic acid, induction of porphyrin synthesis and subsequent phototoxicity," *Photochem. Photobiol.* **65**(3), 416−421 (1997).
23. J. M. Gaullier, K. Berg, Q. Peng, H. Anholt, P. K. Selbo, L. W. Ma, J. Moan, "Use of 5-aminolevulinic acid esters to improve photodynamic therapy on cells in culture," *Cancer Res.* **57**(8), 1481−1486 (1997).
24. K. P. Uberriegler, E. Banieghbal, B. Krammer, "Subcellular damage kinetics within co-cultivated WI38 and VA13-transformed WI38 human fibroblasts following 5-aminolevulinic acid-induced protoporphyrin IX formation," *Photochem. Photobiol.* **62**(6), 1052−1057 (1995).
25. B. Li, Z. Chen, L. Liu, Z. Huang, Z. Huang, S. Xie, "Differences in sensitivity to HMME-mediated photodynamic therapy between EBV+ C666-1 and EBV- CNE2 cells," *Photodiagnosis Photodyn. Ther.* **7**(3), 204−209 (2010).
26. C. M. Yow, C. K. Wong, Z. Huang, R. J. Ho, "Study of the efficacy and mechanism of ALA-mediated photodynamic therapy on human hepatocellular carcinoma cell," *Liver Int.* **27**(2), 201−208 (2007).
27. R. W. Wu, C. M. Yow, C. K. Wong, Y. H. Lam, "Photodynamic therapy (PDT) — Initiation of apoptosis via activation of stress-activated p38 MAPK and JNK signal pathway in H460 cell lines," *Photodiagnosis Photodyn. Ther.* **8**(3), 254−263 (2011).
28. J. M. Gaullier, M. Gèze, R. Santus, T. Sa e Melo, J. C. Mazière, M. Bazin, P. Morlière, L. Dubertret, "Subcellular localization of and photosensitization by protoporphyrin IXhuman keratinocytes and fibroblasts cultivated with 5-aminolevulinic acid," *Photochem. Photobiol.* **62**(1), 114−122 (1995).
29. B. C. Wilson, M. Olivo, G. Singh, "Subcellular localization of Photofrin, aminolevulinic acid, photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy," *Photochem. Photobiol.* **65**(1), 166−176 (1997).
30. L. Henderson, A. Wolfreys, J. Fedyk, C. Bourner, S. Windebank, "The ability of the Comet assay to discriminate between genotoxins, cytotoxins," *Mutagenesis* **13**(1), 89−94 (1998).
31. E. S. Chu, R. W. Wu, C. M. Yow, T. K. Wong, J. Y. Chen, "The cytotoxic, genotoxic potential of 5-aminolevulinic acid on lymphocytes: A comet assay study," *Cancer Chemother. Pharmacol.* **58**(3), 408−414 (2006).
32. R. Musiol, M. Serda, J. Polanski, "Prodrugs in photodynamic anticancer therapy," *Curr. Pharm. Des.* **17**(32), 3548−3559 (2011).