Enhancement of 5-aminolevulinic acid-induced photodynamic therapy by a bioadhesive polymer

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Background/purpose: Using a topical formulation of 5-aminolevulinic acid (ALA), a mediated photodynamic therapy (PDT) was developed to treat oral precancerous and cancerous lesions. Several clinical results demonstrated that this special ALA preparation containing Carbopol 971P (CP971P), a bioadhesive polymer, resulted in a satisfactory PDT effect. It is believed that this PDT efficacy may be due to the increased bioadhesion of the preparation at the treatment site. We investigated whether there was a beneficial effect of CP971P on ALA PDT in vitro.

Materials and methods: CP971P was co-incubated with ALA in a cell culture system. The effect of CP971P was evaluated by monitoring the uptake of ALA, the fluorescence intensity of protoporphyrin IX, and the cell survival rate.

Results: The polymer was found to enhance the fluorescence intensity of protoporphyrin IX and the subsequent PDT effect when concurrently dosed with ALA.

Conclusion: Our results showed that a formulation of ALA containing CP971P could provide a better ALA PDT effect in cancer treatment.

Introduction

Photodynamic therapy (PDT) is a new treatment modality in which a combination of a photosensitizer and visible light is programmed to result in the destruction of selected cells. In 1987, Malik and Lugaci1 first reported the use of endogenous protoporphyrins as clinical photosensitizing agents. Later in 1990, Divaris et al.2 proposed a new approach to PDT, which involves the photosensitizer prodrug, 5-aminolevulinic acid (ALA). ALA is a metabolic prodrug of protoporphyrin IX (PpIX), a photosensitizing agent, in the heme biosynthesis pathway. Endogenous synthesis of ALA is regulated by the synthesis of heme via feedback control; following the exogenous administration of ALA, the feedback mechanism is bypassed with subsequent overproduction and accumulation of porphyrin precursors, the predominant one of which is PpIX.3 The use of PpIX generated through the heme biosynthetic pathway after administration of ALA has many applications in PDT against various cancers.4,5 A simplified
Excipient effect on photodynamic therapy

schematic expression of ALA PDT is shown in Fig. 1. The formation of one PpIX molecule requires eight ALA molecules for synthesis. Selective PpIX accumulation may be related to several factors, such as increased uptake of ALA, a low concentration of iron, a low activity of ferrochelatase, and a high activity of porphobilinogen deaminase in tumor cells. The passage of ALA through the cell membrane is restricted by the rate of uptake by neoplastic cells and its poor diffusion through tissues and cellular membranes. In order to increase its transport across cellular membranes, rather high concentrations of ALA generally must to be applied over long periods of time. Except for the Levulan Kerastick Topical Solution and a sol-gel preparation, the reasons for including any single vehicle material in the formulation of most ALA formulations published in the literature were not explained. The Levulan Kerastick Topical Solution and the sol-gel preparation showed comparatively better ALA PDT results in clinical treatments, and the reason may have been due to a rational formulation design that ensured the performance of ALA and the formation the photosensitizer, PpIX, and a subsequent PDT effect upon irradiation. The Levulan Kerastick Topical Solution is a two-component system: one component containing 1.5mL of solution vehicle, and the other component containing 354mg of ALA HCl as a dry solid. The reason for the two-component system is mainly due to stability concerns with ALA. ALA is not stable in solution and easily undergoes dimerization, so it is better to keep it in a dry powder form during storage. Since the Levulan Kerastick Topical Solution is mainly for skin application, the solution vehicle they include in the preparation is basically for enhancing penetration of ALA through the skin. The target of the sol-gel preparation is the oral mucosa, and a combination of a mucoadhesive polymer and thermal-responsive polymer is included to enhance the retention time of ALA at the application site in order to increase drug uptake at the target site. Recently, the ALA sol-gel formulation was reported to exert a satisfactory result for skin wart treatment. In that report, the authors reportedly achieved a complete response 3 weeks after ALA PDT, and no recurrence was observed for up to 2 years in 94% of patients. However, the reason this clinical result using the ALA sol-gel was better than those using ALA cream is not clear.

Recently, a new field of “polymer therapeutics” has garnered lots of attention for pharmaceutical formulators, and mucoadhesive polymers seem to have the potential to enhance the performance of active ingredients. It would be interesting to study whether the mucoadhesive polymer, Carbopol 971P (CP971P), in the ALA sol-gel preparation holds the answer as to why the sol-gel formulation showed a better PDT effect than the traditionally favored cream preparation. Carbopol polymers (including CP971P) are generally regarded as safe polymers composed of acrylic acid crosslinked with polyalkenyl ethers or divinyl glycol. Carbopol polymers are commonly used in oral suspensions, tablets, and topical formulations, and their mucoadhesive properties provide intimate contact between the dosing formulation and the mucosal surface, resulting in prolonged residence time at the site of absorption. Since the clinical results demonstrated that the ALA sol-gel preparation can produce a satisfactory PDT effect on oral cancers and precancerous lesions, it would be interesting to know whether such a formulation can benefit other mucous routes, such as the lung. In this study, the effect of CP971P on a PpIX formation and the subsequent PDT effect after incubation with ALA in cell culture systems were studied.

Materials and methods

Cell culture conditions and ALA incubation

Two cell lines were tested for the effect of CP971P on ALA PDT. KB cells (ATCC CCL-17; American Type Culture Collection, Manassas, VA, USA), originally obtained from an epidermal carcinoma of the mouth, were cultured in Eagle’s minimum essential medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA). CL1-0 cells, established from a 64-year-old man with a poorly differentiated lung adenocarcinoma, were kindly provided by the National Health Research Institutes (Miaoli, Taiwan). A stock culture of CL1-0 cells was maintained in RPMI 1640 (Sigma) with 10% FBS. Cell cultures were maintained at 37°C in a
humidified atmosphere of 95% air and 5% CO₂. The cells seeded in culture plates or dishes were incubated with 1mM ALA (with or without CP971P, diluted in serum-free medium and neutralized to pH 7.2 with NaOH immediately before use) for 3 hours. CP971P was kindly provided by Lubrizol (Wickliffe, OH, USA), and a stock solution (1% wt/vol) was prepared by slowly adding the polymer powder into water with stirring for 3 hours or longer. Various concentrations of CP971P were obtained by taking aliquots of the stock solution and diluting them with water.

Measurement of ALA-induced PpIX fluorescence by flow cytometry

Cells were seeded in triplicate in six-well plates containing 2 × 10⁵ cells per well and incubated for 24 hours at 37°C. Afterwards, cells were incubated in phenol red-free, serum-free medium containing 1mM ALA for 3 hours. Following ALA incubation, cells were trypsinized, removed from the culture dishes, and resuspended in fresh culture medium supplemented with serum. Cellular fluorescence was quantified with a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA), and 10,000 cells were analyzed. The ALA-induced photosensitizer, PpIX, was excited by an argon ion laser emission at 488 nm and collected by a photomultiplier tube after passing through a 670-nm long-pass filter.

Photodynamic treatment

For photodynamic treatment, cells were seeded in 96-well plates (at about 6000 cells/well) and grown overnight in complete medium. After incubation with 1mM ALA with or without 0.05% CP971P for 3 hours, cells were exposed to light at doses of 0–32 J/cm². The light source was a light-emitting diode at 60 mW, with a wavelength emission of red light at 630 ± 5 nm. After irradiation, cells were incubated with fresh complete medium for another 24 hours until further analysis.

Cell viability assay

The PDT-induced phototoxicity of test cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each individual phototoxic experiment was performed in triplicate. The MTT assay is based on the activity of mitochondrion dehydrogenase, which can reduce a water-soluble tetrazolium salt to a purple insoluble formazan product. The resulting formazan crystals were dissolved by the addition of dimethyl sulfoxide, and analyzed spectrophotometrically at an absorbance of 570 nm. Cells exposed to ALA but without light were used as the control. The cell survival rate (%) was calculated as: (mean optical density value of treated cells/mean optical density value of control cells) × 100%.

Determination of ALA uptake

[¹⁴C]ALA hydrochloride was purchased from New England Nuclear (Boston, MA, USA) and used in this part of the study. Unlabelled ALA was dissolved in phosphate-buffered saline (PBS), and [¹⁴C]ALA was added so that the final solution contained 0.0222 MBq/mL. Cells were washed twice with 0.5 mL PBS at 37°C and incubated with 0.3 mL radiolabeled ALA solution in the culture medium without FBS. At certain time intervals, the reaction was stopped, and cells were washed four times with 0.5 mL ice-cold PBS. Cells were then disrupted in 0.2mM NaOH and transferred to vials containing 5 mL cocktail scintillation fluid (Beckman Ready Protein; Beckman Instruments, Fullerton, CA, USA). The radioactive content of the samples was determined by a Beckman LS 1801 counter (Beckman Instruments, Karlsruhe, Germany)16. Data are expressed as the apparent ALA concentration in pmol/mg of protein, calculated from the amount of radioactivity in cells.

Statistical analysis

Data are presented as the mean ± standard error of the mean. At least three independent experiments were performed for each experiment. An unpaired t test was used to establish the significance of differences between groups. Differences were considered statistically significant at P<0.05.

Results and Discussion

The effect of CP971P on the relative fluorescence of ALA-converted PpIX in CL1-0 cells is shown in Fig. 2. The fluorescence intensity was measured by flow cytometry after incubation with 1mM ALA in the presence or absence of CP971P for 3 hours. As shown in Fig. 2, in the presence of CP971P, the fluorescence intensity increased in a concentration-dependent manner until it reached a plateau at 0.05%. A further increase in the CP971P concentration to 0.1% did not further increase the fluorescence intensity. In fact, the PpIX fluorescence intensity of cells treated with 0.05% CP971P was almost double that without CP971P. A similar effect of CP971P on the formation of PpIX was found with oral epithelial carcinoma cells, and the results are given in Table 1. For KB cells, even 0.001% CP971P showed 23% enhancement of the PpIX fluorescence. The higher fluorescence intensity indicated that
more ALA-converted PpIX was present in the presence of CP971P, suggesting that more reactive oxygen species were produced to kill cancer cells after light irradiation. However, it should be noted that KB cells were shown to be contaminated by HeLa cells. In this regard, it is necessary to perform more studies in other representative cell lines derived from oral squamous cell carcinoma.

To examine the efficacy of PDT, an MTT assay was performed to evaluate the cell-killing effect. As shown above, 1 mM ALA combined with 0.05% CP971P showed the strongest PpIX fluorescence. Therefore, the possible increased efficacy of PDT by CP971P was evaluated using this condition, and the results are shown in Fig. 3. In the absence of CP971P, the cell survival rate decreased in a light-dose relationship as expected. In the presence of 0.05% CP971P, the cell survival rate still showed a decreasing tendency with an increasing light dose. However, inclusion of 0.05% CP971P in the ALA PDT study resulted in a more significant enhancement of cell death, especially at the light dose ranges of 8 and 16 J/cm². Cell survival rates without CP971P were around 80% and 60% under light doses of 8 and 16 J/cm², respectively. However, in the presence of CP971P, the cell survival rate was down to 50% and 20% under light doses of 8 and 16 J/cm², respectively. The result is not surprising, because the presence of CP971P was found to increase the formation of PpIX. An increase in the photosensitizer is expected to increase the phototoxicity of cells where the photosensitizer resides. The results of the increased PpIX fluorescence and enhanced killing effect by adding a trace amount of a common polymer (such as Carbopol) are encouraging and of high potential in drug development. If PDT’s efficacy can be improved simply by adding a much cheaper polymer concurrently with the photosensitizer, the formulator can utilize such information and design a less expensive drug for PDT.

The conversion of ALA to PpIX was correlated with several steps, including the amount of ALA transported into cells and the activity of several enzymes involved in the heme synthesis pathway (Fig. 4). CP971P is a macromolecular polymer with an average molecular weight of 10⁶ kDa, and its penetration through cell membranes is almost impossible. Therefore, the enhancement of PpIX fluorescence, as well as ALA-PDT’s effect, could have been due to an increase in the passive diffusion of ALA into cells or the active uptake of ALA by cells in the presence of CP971P. By tracing the intensity of labeled ALA in cells after 3 hours of incubation and removal of the culture medium, it was found that the uptake of ALA by CL1-0 cells was greatly enhanced in the presence of CP971P in the system as shown in Fig. 5. In the presence of CP971P, ALA
uptake increased in a concentration-dependent manner. This result is consistent with the increased PpIX fluorescence intensity, where the PpIX fluorescence also showed an increasing tendency in the presence of CP971P (Fig. 2). However, there was almost no difference in the PpIX fluorescence with 0.05% or 0.1% CP971P, but the uptake was higher with 0.1% CP971P. The presence of macromolecules in the culture medium may have been responsible for this. Since PpIX can only be formed in cells, there is no need to replace the culture medium after ALA incubation. In this study, after culturing with ALA (whether or not in the presence of CP971P), cells along with the culture medium were subjected to flow cytometric measurement without pre-washing or replacing the medium. Therefore, the added CP971P was in the culture medium throughout the study. The effect of pharmaceutical excipients interfering with the measured absorbances of a drug was reported by Fuerte and Maldonado,18 and predictions of the drug concentration in the analyzed samples can be misleading because of the scattering effect of light. Since light scattering can affect both the absorbance and the emission of light, one possible reason could be due to a light-scattering effect in the presence of macromolecules such as CP971P.

The cellular PpIX amount derived from ALA depends on ALA uptake and the activity of enzymes involved in ALA PpIX’s conversion. ALA PDT-induced selective destruction of neoplastic lesions was attributed to an aberration of heme biosynthesis, such as the reduction of ferrochelatase activities or upregulation of porphobilinogen deaminase, in tumor cells.16 As shown in Fig. 5, CP971 significantly increased the uptake of ALA in a concentration-dependent manner. Although 0.05% CP971 significantly increased the PpIX amount (Fig. 2), an increased concentration of CP971 did not further increase PpIX, which might
have been due to the maximum enzyme activity having already been achieved.

In conclusion, PDT is a new treatment modality for surface cancers such as skin cancers and oral cancers, and has shown potential in treating externally approachable sites. However, photosensitizers that can be used with PDT are extremely expensive. Although ALA, a photosensitizer precursor, is much less expensive than other photosensitizer drugs, it generally requires a larger dose and the cost is still rather high. To reduce the costs of PDT drugs, the field of excipient application is becoming more attractive. In this study, we proved that a mucoadhesive polymer, CP971P, can increase ALA uptake by cancer cells and the formation of PpIX.

Acknowledgments

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References

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