ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2): 163-168
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Endogenous production of protoporphyrin IX induced by 5-aminolevulinic acid in leukemia cells[1]

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KEY WORDS 5-aminolevulinic acid; protoporphyrin; photosensitization

ABSTRACT

AIM: To explore the photosensitization of 5-aminolevulinic acid (ALA) in myeloid leukemia cell line. **METHODS**: Using the technique of fluorescence spectra, the ALA induced protoporphyrin IX (PpIX) was measured in myeloid leukemia JCS cells. Cofocal laser scanning microscopy (CLSM) combined with fluorescence organelle probe was used to detecte the localization of PpIX in JCS cells at the subcellular levels. MTT assay was used to measure the cell survival after light irradiation. **RESULTS**: ALA successfully produced endogenous PpIX in leukemia JCS cells. PpIX was observed to be distributed in the cytoplasm and mitochondria was exhibited as the one of binding sites of PpIX. As a photosensitizer, PpIX initiated photodynamic reaction after light irradiation and effectivily photodamaged leukemia cells. **CONCLUSION**: ALA-based photosensitization could be used for inactivation of leukemia cells.

INTRODUCTION

Worldwide, photodynamic therapy (PDT) is being evaluated as a new and promising treatment modality for neoplastic diseases[1]. The treatment is based on injection of a photosensitizer, followed by exposure of the tumor area to high dosage of light at an appropriate wavelength, the photosensitizer accumulated in the tumor then initiates a photodynamic reaction and produces active oxygen to destroy the tumor. Nevertheless, the remanent skin photosensitivity for several weeks makes the PDT treatment limited in clinical application. Recently, there has been a considerable interest in a new approach to PDT in which a non-phototoxic prodrug, 5-aminolevulinic acid (ALA) is administered resulting in the endogenous synthesis of the photosensitizer, protoporphyrin IX (PpIX). ALA is a precursor of porphyrin. In the first step of heme biosynthetic pathway, ALA is formed from glycine and succinyl coenzyme A (CoA). The whole pathway has been shown in Fig 1. The last step is the incorporation of iron into PpIX to form heme under the action of enzyme ferrochelatase. It has been found that ferrochelates activity is low in tumor cells. With the addition of the exogenous ALA, PpIX accumulates more in tumor cells than in normal cells in which the ferrochelates activity is normal[2]. It was reported that the ALA-derived PpIX contrast ratio between the tumor and non-malignant organs reached to 30 at 4-6 h post ALA injection[3], and in most normal tissues the ALA-derived PpIX vanished within 24 h following systemic adminstration of ALA[4]. So, the skin photosensitivity could be greatly improved in ALA-PDT treatment[2]. Based on this remarkable advantage of ALA-PDT, several clinical trails were carried out and results were very encouraging for treatment of basal cell carcinoma, squamous cell carcinoma, and some other skin disease[5]. As ALA-PDT was successful in superficial solid tumor treatment, it was tested in leukemia in this work. Leukemia cells also could be inactivated by PDT method using photosensitizer merocyanine 540 (MC540). MC540-PDT holds promise in the extracorporeal purging of bone marrow for leukemia treatment[6]. However, as an exogenous photosensitizer, the binding mechanism of MC540 to leukemia cells is still not clear[7]. Here, in this paper, we explore the possibility of ALA-PDT application in leukemia cells.

MATERIALS AND METHODS

Chemicals ALA was obtained from Sigma

[1]Project supported by the National Natural Science Foundation of China, No 39970186.
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Received 2000-07-12 Accepted 2000-11-26

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

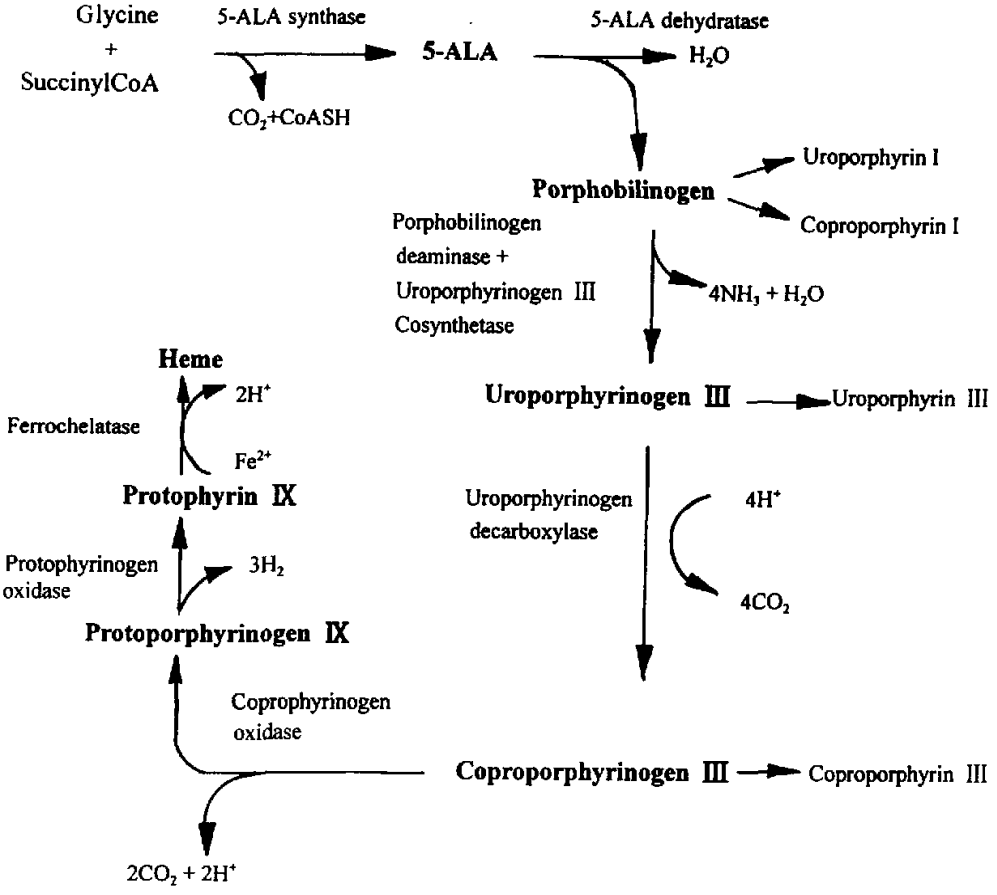

Fig 1. The heme biosynthetic pathway. The synthesis steps are indicated by arrows.

Chemical Co (Missouri, USA). A stock solution of 1 g/L (6 mmol/L) was prepared by dissolving the ALA powder in the PBS with PH 7.0. The fluorescence probe MitoTracker Green FM (M-7514) specific for mitochondria staining was purchased from Molecular Probes (Eugene, OR) with an emission peak at 517 nm and the width about 40 nm. Under suitable excitation, M-7514 can show the mitochondria localization in cells by fluorescence emission.

Cells and photodynamic treatment The murine WEHI-3B (JCS) myeloid leukemia cells obtained from John Curtin School of Medical Research, Australia, were maintained in RPMI 1640 medium, supplemented with 10 % fetal calf serum (FCS, Gibco) and antibiotics PSN (benzylpenicillin 50 000 U/L, streptomycin 50 mg/L, and neomycin 100 mg/L). Cells were incubated at 37 ℃ in a humidified 5 % CO_2 incubator. Cells in the exponential growth phase were used in each experiment. The doubling time of cell dividing for JCS was 8 h. In the experiments, leukemia cells ($2 \times 10^8 - 3 \times 10^8$/L) were incubated in ALA (1 mmol/L) containing RPMI 1640 medium with no serum. The cells were kept in the dark in the incubator at 37 ℃ and incubated for a few hours. After incubation, the cells were washed three times with fresh medium and resuspended in serum-free medium for fluorescent measurement. In the photodynamic treatment, leukemia cells were then irradiated with white light as described previously[8], and then cell survival of each group under different treatment conditions was measured by MTT assay (see below).

Spectral measurements The fluorescence spectra were measured with a luminescence spectrometer

(Perkin Elmer, Model-LS50B). In the spectral measurements, the densities of ALA-loaded cells were $1 \times 10^9 - 5 \times 10^9$/L.

Intracellular localization of endogenous production of PpIX by ALA in JCS cells Confocal Laser Scanning Microscopy-CLSM (Zeiss, Model LSM510) and the attached Zeiss software were used. The resolution on z direction of confocal images depended on the pinhole size. When it was around 100 μm in diameter, stray light from layers about 0.2 μm above and below the focused layer could be effectively eliminated. A pinhole size of 80 – 100 μm was used in this study. A magnification of ×630 was used to image an area of 140 μm × 140 μm onto 1024 × 1024 pixels. This represented a step-resolution of 0.14 μm in both x- and y-direction, which was less than the diffraction-limited resolution. Normally, the size of organelles, such as mitochondria, was larger than 0.2 μm × 1 μm. Given the current resolution along the three axes, organelles labeled with fluorescent probes could be detected easily. The emissions of PpIX in cells peaked at 630 nm while that of mitochondria-probe M-7514 peaked at 517 nm[3]. Since the emission spectral area of PpIX and M-7514 is not overlapped, their emission could be measured by different filter sets. To simultaneously detect endogenous PpIX and the mitochondrial probe in cells, detection channels channel 1 (red) of the CLSM was covered with a 590 nm long-pass filter for viewing the distribution of PpIX. Channel 2 (green) was covered with a 505 – 550 nm band-pass filter for viewing the localization of mitochondria in same cells. The 488 nm line of the argon-krypton laser was used for excitation.

Cell survival assay The 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltertrazolium bromide (MTT) tetrazolium reduction assay was used to monitor the cytotoxicity of ALA-PDT on leukemia cells[8,9]. The cell samples of untreated cells or ALA-PDT treated leukemia cells (5×10^7/L) were added into 96 well flat-bottomed tissue culture plates and the cells were incubated at 37 ℃ in a CO_2 incubator for 2 d. Viability was determined by adding 10 μL MTT (5 g/L) to each well and the mixture was incubated for another 3 h. To dissolve the formazan crystals, 150 μL of culture medium was replaced with dimethylsulfoxide. The optical density at 540 nm and 690 nm was determined using the iEMS analyzer (Labsystem). The cell survival of ALA-PDT treated cell samples was then obtained by comparing to the control samples (untreated).

Statistical Analysis Data were presented as $\bar{x} \pm s$ for all experiments repeated at least 3 times.

RESULTS

Endogenous production of photosensitizer PpIX by ALA in leukemia cells Based on previous report[10], ALA 1 mmol/L incubation concentration was selected in leukemia experiments. ALA does not fluoresce, while PpIX has typical fluorescence emission spectrum. So, if PpIX was being endogenously produced in cells, it could be detected by the fluorescence method. Fig 2 shows fluorescence spectrum of ALA-loaded leukemia cells. The typical emission spectrum of PpIX with the peak around 630 nm was seen in Fig 2, reflecting that PpIX was really produced in leukemia cells after ALA incubation. In contrast, the bottom curve of Fig 2 showed that no emission peak was found in control leukemia cells (no ALA added), reflecting that no PpIX existed in leukemia cells if unstimulated by ALA. Due to this characteristic of PpIX emission spectrum, the whole fluorescence emission of PpIX could be detected by a photo-detector with 590 nm long-pass filter as used in CLSM experiments.

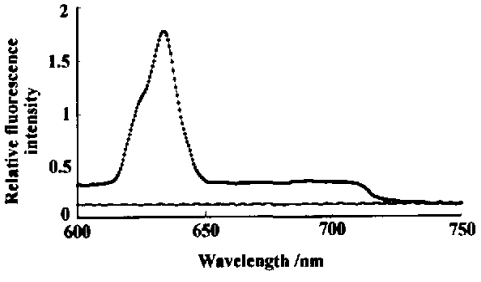

Fig 2. The fluorescence spectra of ALA-incubated cells and control cells. Upper curve: leukemia cells incubated with ALA 1 mmol/L for 4 h and then washed and resuspended in fresh medium; Bottom curve: control leukemia cells. Cell density: 2.5×10^9/L. Excitation: 400 nm. Slit width: 2 nm.

Fig 3 shows the kinetics of PpIX produced in cells by measuring the intensity of emission peak of PpIX at 630 nm at different ALA incubation times. It was seen that after 2 – 4 h, PpIX could be effectively produced in leukemia cells. However, the cell damaging with no light irradiation reached 15 % after a 4-h incubation of ALA 1 mmol/L, showing that long time incubation with

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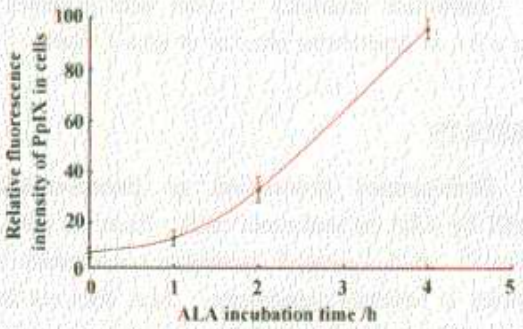

Fig 3. The kinetics of endogenously produced PpIX in leukemia cells. The different groups of cell samples were incubated with ALA (1 mmol/L) for different times and their fluorescence intensities at 630 nm (PpIX emission peak) were measured after cells were washed and resuspended in fresh medium at the same cell density (2.5×10^9/L). The datum of 0 h incubation represents the group of control cells (no ALA added). Each group at least had 3 cell samples. The fluorescence intensity was averaged from 3 individual measurements and the standard errors were obtained accordingly. Excitation: 400 nm.

ALA could still damage the cells. So, in our experiments, the ALA incubation time was kept not longer than 4 h.

Intracellular localization of PpIX in leukemia cells As shown above, PpIX could be endogenously produced in cells. However its localization intracellularly in leukemia cells is an interesting and important question. Though the rough fluorescence image with poor z axis resolution of porphyrin in cells can be obtained by conventional fluorescence microscopy, the cofocal laser scanning microscopy (CLSM) can produce a confocal fluorescence image with a much higher resolution in all three dimensions, providing a good way to measure the intracellular localization of the fluorescing drug[11]. Besides, with the help of fluorescence organelle probe, CLSM can detect the binding organelle of the photosensitizer in cells by two channels detection technique.

After the leukemia cells were incubated with ALA and stained with mitochondria-probe M-7514 and centrifuged and washed, these cells were then measured by CLSM using two channels detection. Fig 4a shows the confocal fluorescence image of PpIX in leukemia cells obtained from channel 1 (590 nm long-pass filter). It was observed that PpIX was diffusely distributed in the cytoplasm but not in the nuclear of the cells. From detection channel 2 (505 – 530 nm band-pass filter), the mitochondrial distribution of the same cells as in Fig 4a was imaged (see Fig 4b). Fig 4c shows the combination of the two images from the two channels. Here, the red color represented PpIX and green color was the mitochondria as shown in Fig 4a-4b. In Fig 4c, the yellow color (overlapping of red color and green color) represented PpIX overlaid with mitochondria in this area. The yellow granule appearance and almost no pure green color in Fig 4c clearly shows that most mitochondria of the leukemia cells was targeted by the endogenously produced

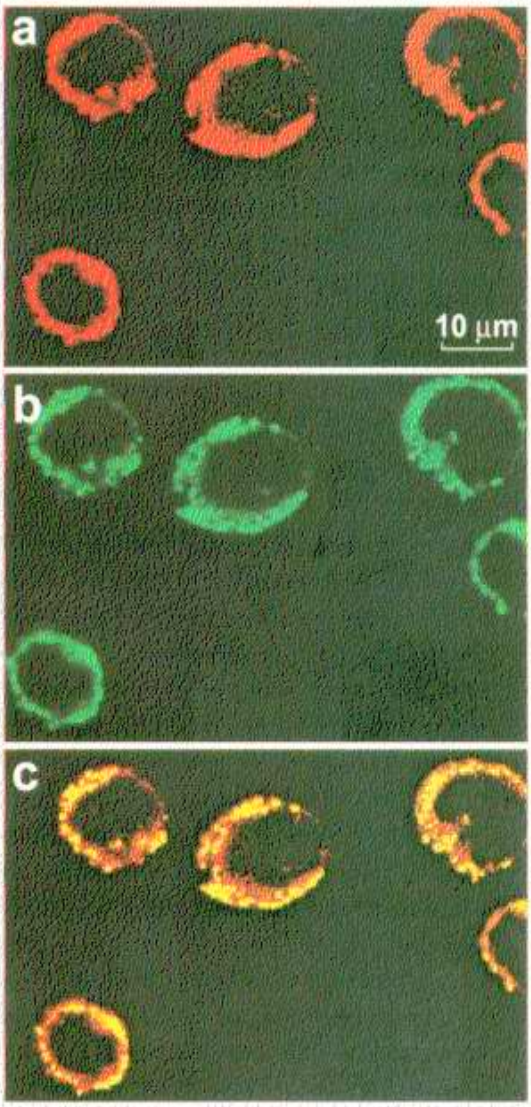

Fig 4. Confocal fluorescence images of cells. a): distribution of endogenously produced PpIX (red) in leukemia cells. b): localization of mitochondria (green) in the same cells as in "a". c): the combination of above two images (a and b). The leukemia cells were incubated with ALA (1 mmol/L) for 4 h and stained with M-7514 for half hour, washed and then measured by CLSM using 2 channel detection.

PpIX. In CLSM, two channels detection could simultaneously detect the fluorescing drug and probed organelle in the same cells. This technique has been reported to successfully measure the localization of some photosensitizers such as aluminum phthalocyanine (ALPCS) in cancer cells[12], and in this study also it has been observed that the mitochondria is one of the main binding target of endogenously produced PpIX in JCS leukemia cells.

The main active species of porphyrin sensitizers is singlet oxygen. The life time of singlet oxygen is very short in a biological environment, its migration distance being only about 0.1 nm[1], which means that the binding organelle of porphyrin is also the target of singlet oxygen attack.

ALA-PDT on leukemia cells When leukemia cells were exposed to ALA-PDT treatment, that included incubating with ALA (1 mmol/L) for 4 h and then washing and resuspending in fresh medium, irradiating with light (the irradiation spectrum used in experiments was basically flat from 500 nm to 900 nm[8]), endogenously produced PpIX caused photodynamic damage to the cells. As shown in Fig 5, under 45 kJ/m^2 light dose, almost all cells were destroyed. Comparing to the other cell line (HeLa cells)[13], leukemia cells showed similar sensitivity to ALA-PDT, demonstrating that leukemia cells also can be effectively inactivated by ALA-PDT. When the cells were incubated with exogenous PpIX, it mainly accumulated in plasma membrane of the cells, the membrane being the main target[13]. When cells were incubated with ALA, ALA endogenously produced PpIX, which distributed in cytoplasm and bound on mitochondria. This is the difference in mechanism of action of exogenous PpIX and endogenous PpIX on their intracellular localization. Compared to PpIX, ALA is a much smaller molecule. ALA can easily penetrate plasma membrane and get into the cells, while PpIX remains mainly limited on the plasm membrane due to its relatively big molecul weight and its lipid affinity. So that mitochondrial binding may be the typical characteristic of endogenous PpIX and this may be the advantage of ALA in PDT application. The importance of intracellular targetting of sensitizer has been noticed recently in PDT research[1]. It has been found that at the subcellular levels the mitochondria is the key target of sensitizers to initiate photosensitization. Here as we have shown, mitochondria was the main target of ALA-PDT in leukemia cells, which give the reason that how ALA-PDT could effectively damage leukemia cells.

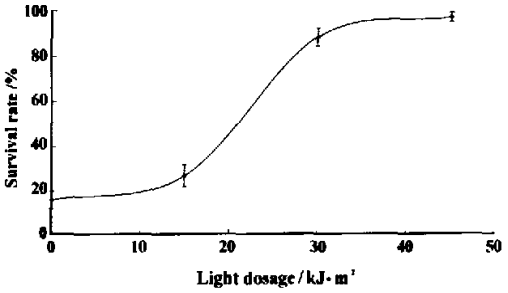

Fig 5. ALA-PDT effect on leukemia cells. Survival of ALA-loaded cells was measured individually at different doses by MTT method. The datum of 0 dose represents dark control cells (incubated with ALA but no light irradiation). At least 3 cell samples were repeated, and the data were then averaged.

In summary, ALA endogenously produced PpIX in leukemia cells and PpIX got distributed in the cytoplasm and targeted the mitochondria. ALA-PDT effectively damaged the leukemia cells. So, ALA-PDT showed potential in leukemia inactivation and its application will be further explored in extracorporeal purging of bone marrow for leukemia treatment.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
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氨乙酸丙酸在白血病细胞内内源生成光敏药物原卟啉 IX[1]

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关键词 氨乙酸丙酸；原卟啉；光敏反应

目的：探索用氨乙酸丙酸(ALA)对髓性白血病细胞进行光敏化灭活. **方法**：用荧光光谱技术测量 ALA 在白血病细胞中内源生成光敏药物原卟啉 IX；用共焦激光扫描荧光显微镜结合亚细胞器荧光探针探测其在 JCS 髓性白血病细胞中的分布；用 MTT 方法检测光敏化后的细胞存活. **结果**：ALA 成功的在白血病细胞中内源生成光敏药物原卟啉 IX；原卟啉 IX 分布在细胞核以外的细胞质中，线粒体是其的结合部位之一；经光照后此内源生成的原卟啉 IX 启动光敏化反应有效的灭活白血病细胞. **结论**：基于 ALA 的光动力反应有潜力用于对白血病细胞的光敏化灭活.

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