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Biological activity of 5-aminolevulinic acid and its methyl ester after storage under different conditions

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Abstract

5-Aminolevulinic acid (ALA) is a natural precursor of protoporphyrin IX (PpIX) and heme in cells. Photodynamic therapy (PDT) utilizes a metabolic imbalance in cancer cells, leading to increased PpIX generation from exogenous ALA. Due to chemical instability of ALA in therapeutic concentrations at pH values larger than 5.0 and at high temperatures, it looses its activity by spontaneous dimerization to 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY). ALA esters are now supplementing ALA in PDT, but little is known about their stability.

We have studied the stability of ALA and its methyl ester (MAL) stored under different conditions (temperatures, pH values) by measuring their ability to generate PpIX. 100 mM solutions of both compounds were found to be stable at pH 4 and at 4 °C. However, at pH 5.5 they lost almost 10% of the initial activity during 5 days of storage at 4 °C. The fastest decay of ALA and MAL was seen at pH 7.4 and at 37 °C, and followed first order kinetics. At pH 7.4 and at 4 °C MAL lost its PpIX producing ability more slowly than at 37 °C. Our work shows that solutions should be prepared immediately before use and stored at low temperatures. The pH of stock solutions should not exceed 5.

Keywords: Photodynamic therapy; Stability; 5-Aminolevulinic acid; ALA esters; Protoporphyrin IX

1. Introduction

Photodynamic therapy with 5-aminolevulinic acid (ALA-PDT) is accepted world wide for treatment of skin cancers and non-cancerous diseases [1–3]. In normal and tumour cells enzymatic transformation of ALA, via the heme cycle, leads to production of protoporphyrin IX (PpIX) and then of heme. In some tumours this process is perturbed, and application of excess exogenous ALA induces preferential accumulation of PpIX that is a strong photosensitizer.

Due to the hydrophilic properties of ALA, its penetration through the plasma membrane of cells is slow. The penetration is expected to be better for lipophilic ester derivatives of ALA. These esters are supposed to be converted to a biologically active form of ALA, possibly ALA itself, by non-specific esterases [4–6].

ALA and its esters have significant advantages over exogenous photosensitizers. They give transient skin photosensitization, lasting only 24–48 h, compared to 6 weeks for photosensitizers [7]. Moreover, they can be applied topically.

ALA is an acid, and like methyl 5-aminolevulinate (MAL), it is applied in the form of its hydrochloric salt

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at a low pH value (about 2.5). For minimal discomfort of the patients the solutions are buffered to a pH value close to the physiological one. Under such conditions ALA undergoes dimerization, resulting in loss of activity. Two molecules of ALA form a cyclic pyrazine product [7–13].

The stability of ALA in different formulations has been investigated [14,15], while that of the commonly used esters is still scarce. In the present study we have investigated the influence of storage conditions on the stability of ALA and MAL. The stabilities were assessed by determining the abilities of the drugs to induce PpIX.

Gadmar et al. [16] presented data on the influence of storage conditions of ALA solutions on its ability to induce PpIX in cells. The method applied in the present work is similar to that applied by Gadmar et al. [16].

2. Materials and methods

2.1. Chemicals

5-Aminolevulinic acid hydrochloride (ALA, $ClNH_3CH_2CO(CH_2)_2COOH$, 167.6 g/mol), methyl 5-aminolevulinate hydrochloride (MAL, $ClNH_3CH_2CO(CH_2)_2COOCH_3$, 181.7 g/mol), RPMI-1640 medium, Penicillin/Streptomycin Solution, L-Glutamine, Trypsin-EDTA, phosphate-buffered saline (PBS), sodium phosphate dibasic, sodium phosphate monobasic and other chemicals were obtained from Sigma Chemical Co, St. Louis, MO. Foetal calf serum (FCS) was obtained from PAA Laboratories Gmbh, Linz, Austria. All chemicals were of the highest purity commercially available.

2.2. Stock solutions

Solutions (100 mM) were prepared by dissolving ALA or MAL in 0.4 M buffer made from Na_2HPO_4 and NaH_2PO_4. The pH of the solutions was checked with an electronic pH meter (Metrohm, Switzerland) and adjusted by addition of 3 M HCl or NaOH to pH 4.0, 5.5 and 7.4. During storage a slight decrease in pH was compensated by addition of 3 M NaOH. The resulting dilution was less than 3%. One set of samples, in closed vials, was stored at 4 °C, and another at 37 °C in an incubator. Stock solutions were diluted in medium without serum to a final concentration of 0.5 mM.

2.3. Cell cultivation

WiDr cells, derived from a primary adenocarcinoma of the human recto-sigmoid colon [17], were maintained in exponential growth in RPMI 1640 medium with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine. The cells were grown and incubated in cell culture flasks (Nunc, Roskilde, Denmark) at 37 °C in a humidified atmosphere 5% CO_2 and subcultured twice a week using 0.01% trypsin in 0.02% EDTA. Forty-eight hours before the experiments the WiDr cells were seeded in 6 well plates to a density of 2×10^5 cells per well and 3 ml of medium per well.

2.4. Determination of PpIX production in vitro

After 4 h incubation with ALA or MAL the cells were rinsed twice with 1 ml of ice cold PBS and one ml of porphyrin extracting solution of 1% sodium dodecyl sulphate (SDS) in 1 N perchloric acid – methanol (1:1 vol/vol) [18] was added per well. The cells were detached by means of a cell scrapper (Costar, Cambridge, MA) and collected into 1.5 ml tubes. The samples were kept frozen at −80 °C.

Just before PpIX measurements the samples were thawed, and the fluorescence of PpIX was measured in a 1 cm path length cuvette with a Perkin–Elmer LS-50B spectrofluorometer (Norwalk, CT). The excitation wavelength was set at 407 nm and emission was scanned from 550 to 750 nm, and showed maximum fluorescence at 606 nm. This corresponds to the maximum emission of PpIX in acidic medium [18]. The excitation and emission slits were set at 10 and 15 nm, respectively. A long-pass cut-off filter (515 nm) was used on the emission side to block scattered excitation light.

The effective ALA/MAL concentration was determined by comparing the fluorescence of PpIX of the sample with that of standard solutions.

2.5. Standard curve

WiDr cells were incubated for 4 h with 0–0.5 mM solutions of ALA and MAL in six well plates. After that the cells were rinsed twice with 1 ml of ice cold PBS and treated with 1 ml of porphyrin extracting solution. The cells were scraped and collected in 1.5 ml tube. Fluorescence was measured at $\lambda_{ex} = 407$ nm and $\lambda_{em} = 606$ nm.

2.6. Dark toxicity

About 10^4 cells were seeded out in 24 well plates. After 48 h the medium was removed, and 1 ml of medium without serum containing 0.5 mM ALA or MAL (from 100 mM solutions stored at different conditions) was added to each well. After 4 h incubation with the drugs the cells were washed with cold PBS, and fresh medium with serum was added for 24 h. The survival of the cells was measured using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl) assay [19].

MTT was dissolved in phosphate-buffered saline (PBS; pH 7.4) at 2 mg/ml, filtered to become sterile and stored at 4 °C. 50 μl of the stock solution was added to each well containing 1 ml of medium, and the well plates were incubated at 37 °C for 4 h. After that the medium was removed, and the cells were washed with ice-cold PBS. The formazan crystals were dissolved by adding 600 μl of isopropanol per well. Samples (75 μl) were transferred from each well into a 96-well micro plate with 200 μl of isopropanol. The optical density was read on a Multiskan MS (type 352, Labsys-