Effect of Oil-in-Water Emulsions on 5-Aminolevulinic Acid Uptake and Metabolism to PpIX in Cultured MCF-7 Cells

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Purpose. To identify the optimal vehicle for fast and efficient cellular production of the photosensitizer, protoporphyrin IX (PpIX), upon administration of 5-aminolevulinic acid (ALA).

Methods. ALA in various oil/water o/w emulsions was applied to the human mammary epithelial cell line (MCF-7) cultured in microplates. Upon incubation for 1–4 h, the accumulated amount of PpIX was determined by fluorescence spectroscopy. Variables such as the pH and concentration of the emulsions, the temperature and duration of incubation were examined along with the importance of ALA concentration and the presence of endocytosis inhibitors.

Results. An increase in the amount of produced PpIX was observed with an increase in extracellular pH, incubation temperature, and ALA concentration. A saturable mechanism of PpIX accumulation was evident, mainly as a result of the uptake mechanism for ALA. Some of the o/w emulsions increased the amount of intracellular PpIX, and the results indicated that this was not due to an increased k_m of the extracellular ALA to intracellular PpIX conversion, but to the increased endocytotic uptake in the presence of the emulsions. In general, the increase in PpIX in the presence of emulsions relative to the control was more pronounced after 1 h as compared to after 2–4 h.

Conclusions. The formation of PpIX in MCF-7 cells exposed to ALA is improved by the presence of certain o/w emulsions, which could be explained by endocytosis.

KEY WORDS: aminolevulinic acid uptake; endocytosis; MCF-7 cells; oil-in-water emulsions; photodynamic diagnosis and therapy; protoporphyrin IX.

INTRODUCTION

Photodynamic diagnosis and therapy is a noninvasive technique well suited for light-accessible superficial neoplastic cells (1). A range of studies have shown that topically applied 5-aminolevulinic acid (ALA)—a heme precursor—in photodynamic diagnosis and therapy of oncologic (2) and non-oncologic pathologies (3) is superior to conventional treatments. Under physiologic conditions, the synthesis of ALA from glycine and succinyl CoA is inhibited by a negative feedback mechanism caused by heme, but if exogenous ALA is introduced, the amount of the metabolite protoporphyrin IX (PpIX) increases (1,2). PpIX induces apoptosis, is the main endogenous photosensitizer derived from ALA, and it tends to accumulate more easily in tumor tissue compared to normal tissue. The reason for this is not precisely known, but probably depends on a variety of factors, such as an increased uptake or increased affinity of ALA caused by the neoplastic cells, a faster metabolism to PpIX due to slightly higher temperatures in tumor cells as compared to normal cells, or a decreased transformation of PpIX to heme (1,2).

The main clinical limitations with ALA are its instability at physiologic pH as well as its hydrophilicity, which result in relatively low cellular uptake. ALA is relatively stable at lower pH values (4,5), as dimerization—and further formation of metabolites—depends on the amount of anionic ALA. The pK_a values of ALA in an aqueous solution are approximately 4 and 8 (5); thus, at pH values below 4, ALA is quite stable. Other parameters, which strongly influence ALA stability, encompass the ALA concentration in a formulation, as well as the viscosity and storage temperature of the formulation (6). Approaches taken to increase the cellular uptake have mainly dealt with increasing the passive diffusion of ALA across the plasma membrane by using more lipophilic ALA prodrugs (7,8). Especially ALA-methylester was effective in a range of clinical trials (1). When using ALA-hexylester, PpIX formation was 2.5–3.5 times higher for up to 24 h of incubation in cell cultures (8). *In vivo*, the increase was, however, merely 1.3 (8) and in organ cultures, PpIX formation was not increased with the hexylester (7). Another approach is to target ALA to the mitochondria by increasing the affinity for the mitochondrial membrane, which might be achieved by the use of specific lipids in the formulation (9). Topical application of ALA in formulations with increased ALA stability and/or permeability has been investigated using gels (10) and films (11). Furthermore, encapsulation of ALA in nanoparticles and application as colloidal lotion was highly effective, probably due to increased chemical stability of ALA (12). Water-in-oil and oil-in-water formulations have been used in clinical studies, all with therapeutic success (13), which probably is due to increased stability of ALA as well as improved permeability of ALA across the biological barrier. However, a more recent study showed that the accumulation of ALA in a murine subcutaneous adenocarcinoma *in vivo* model was higher when applied topically in saline (with or without DMSO) as compared to a w/o-based cream, vaseline or liposomes (14). Furthermore, incorporation of ALA into liposomes did not increase the rate of PpIX synthesis, neither in tissue explant culture nor in the M2 mammary adenocarcinoma cell line (7).

An increased active uptake of ALA could improve its therapeutic effect. ALA is recognized as a substrate by several membrane transporters. For example, PEPT2 translocates ^{3}H-ALA by means of a saturable transport mechanism (15). The authors of that study concluded that for neutral compounds, such as ALA, extracellular pH only alters the maximal transport rate, but not the affinity to the transporter (15). The PEPT2 transporter seems to be expressed in mammary gland epithelium (16), and RT-PCR screening of rabbit

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mammary gland also showed the presence of mRNA for the PEPT2 transporter (15). Recently, PEPT1 was found to transport ALA into epithelial cells of the extrahepatic biliary duct with an apparent affinity constant of 2.1 mM (17). In intestinal Caco-2 cells, mainly the PEPT1 and the basolateral peptide transporter with the k_m values of 1.6 and 2.4 mM, respectively, translocated ALA (18), and the uptake of ALA was only slightly inhibited by GABA. On the contrary, in a murine mammary adenocarcinoma cell line, the uptake of ALA was mediated by a BETA transporter, as inhibition was seen with GABA and not with substrates for the PEPT1 and PEPT2 transporters (19); here, the ALA efflux was diffusion controlled (20). Also in a human colonic adenocarcinoma cell line, ALA was found to be a substrate for the BETA transporter (21).

Because an increased uptake of ALA may both reduce the amount of drug to be applied as well as shorten the time of incubation before diagnosis or therapy, the objective of the present study was to optimize uptake and/or metabolism of ALA in MCF-7 cells, that is, to assess the overall kinetics of PpIX formation. The MCF-7 cell line is derived from pleural effusion from a metastatic breast carcinoma. The cells are of human origin and presumed to conserve some differentiated mammary epithelial cell function (22), that is, potentially the PEPT2 transporter. MCF-7 cells have previously been used in studies with photosensitizers (23). In particular, the effect which different oil/water (o/w) emulsions have on the amount of PpIX formed was evaluated. Different endocytosis inhibitors were investigated to assess mechanistic features associated with the o/w formulations.

MATERIALS AND METHODS

Materials

The MCF-7 cells (no. HTB-22) were from ATCC. Fetal bovine serum (FBS) was from BioChrom (Berlin, Germany), whereas Optimem 1, penicillin/streptomycin, trypsin-EDTA, phosphate-buffered saline free of $MgCl_2$ and $CaCl_2$ (PBS), RPMI 1640 with L-glutamine but without phenol red (RPMI), and Hanks balanced salt solution (HBSS) were from Gibco (Paisley, UK).

96-well Optilux black with transparent polystyrene bottom (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) were used for the uptake experiments and transparent polystyrene 96-well plates (TPP, Trasadingen, Switzerland) were used for the dehydrogenase activity assays.

5-Aminolevulinic acid HCl (ALA) was synthesized by Sochinaz (Vionnaz, Switzerland) and kindly provided by ASAT (Zug, Switzerland). Intralipid 10% (IL10) and Lipovenös 20% (LV20) were from Fresenius Kabi (Stans, Switzerland). Squalene, glycerol and L-α-phosphatidyl-L-serin sodium salt were from Fluka (Buchs, Switzerland), Synperonic F68 from ICI (Middlesborough, UK), Lipoid E PC from Lipoid (Ludwigshafen, Germany), and phosphatidylglycerol (1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol sodium salt) from Sygena (Liestal, Switzerland). Ammonium chloride (NH_4Cl), D-mannitol, monensin sodium salt, brefeldin A, cytochalasin D, and Triton X-100 were from Fluka (Buchs, Switzerland). Protein assay kit [bicinchoninic acid (BCA) solution and 4% $CuSO_4(H_2O)_5$] and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and CellTiter 96 AQueous MTS Reagent Powder was from Promega (Madison, WI, USA).

Preparation of O/W Emulsions

The emulsifiers, glycerol and water were mixed and stirred magnetically until dissolution, which took a few minutes at room temperature for the mixture with Synperonic F68, and 1 h at 50°C for the mixtures containing the phosphatidyl compounds (Table I). The oil phase was then added and the mixtures emulsified by high-speed mixing at 5000 rpm for 10 min at 50°C (Polytron, Kinematica, Littau-Lucerne, Switzerland). Then, the emulsions were homogenized through 5 passages in a Microfluidizer (M-110T, Microfluidics, Newton, MA, USA). The obtained nanoemulsions (Table I) were stored at 4°C until further use.

The droplet size, measured by laser light diffraction (Zetasizer 3, Malvern Instruments, Worchester, UK), was in the range of 350–400 nm for the commercial emulsions and for SOPG(0.6), SOPG(0.2) and SOPS(0.2), while it was in the range of 220–240 nm for SQF68. The zeta potential of the O-droplets was measured in triplicate by electrophoretic mo-

Table I. Composition of the O/W-Emulsions

Component	IL10	LV20	SQF68	SOPG (0.6)	SOPG (0.2)	SOPS (0.2)
Quantity (%)	(w/v)	(w/v)	(w/w)	(w/w)	(w/w)	(w/w)
Soya oil	10.0	20.0		10.0	10.0	10.0
Squalene			10.0			
Synperonic F68			1.2			
Egg lecithin	1.2	1.2[a]		0.6[b]	1.0[b]	1.0[b]
Phosphatidylserine						0.2
Phosphatidylglycerol				0.6	0.2	
Glycerine 98%	2.2		2.2	2.2	2.2	2.2
Water	86.6	78.8	86.6	86.6	86.6	86.6
Zeta potential (mV)[c]	−30.3 ± 1.0	−28.2 ± 0.8	−0.3 ± 2.3	−15.3 ± 2.3	−9.6 ± 2.3	−15.5 ± 2.3

[a] 3-sn-phosphatidylcholin egg lecithin.
[b] Lipoid E PC.
[c] Mean ± SD, n = 3.

bility (Zetasizer 3 equipped with electrophoretic cell AZ-55, Malvern Instruments, Worchester, UK) (Table I). All emulsions were isoosmolar and had a pH within the range of 7–8.

Cell Culture

Cultured human mammary gland carcinoma cells, MCF-7, were rinsed with PBS, trypsinized for 2–3 min, resuspended in Optimem 1 supplemented with 10% FBS, 90 U/ml penicillin, and 90 μg/ml streptomycin. Then, 200 μl of a cell suspension with 10,000 cells/ml were seeded in each well of a 96-well plate. Passages 188–196 were cultivated at 37°C in a humidified atmosphere of 5% CO_2 until 90–100% confluency (6 days) before use. The medium was exchanged on the day before the experiment.

Dehydrogenase Activity Assay (MTS/PMS assay)

As an indirect measure of cytotoxicity, decrease in dehydrogenase activity was measured. In this test, 180 μl of medium was removed from each well and replaced by 200 μl of test solution. The test solutions consisted of 20 μl of a 10 mg/ml stock solution of ALA in RPMI or in emulsion mixed with 180 μl RPMI (pH 5.7, 6.6, or 7.4) immediately before use. For all experiments, the pH of the 10 mg/ml stock solution was adjusted to 5.5 for stability reasons. The concentration of ALA was 1 mg/ml and the final pH in the test solutions corresponded to the pH of the buffer, that is, 5.7, 6.6, or 7.4.

The cells were protected from light by aluminum foil and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 h. The test solutions were removed, and the wells rinsed with 3 × 300 μl HBSS of 37°C. Then, 125 μl MTS/PMS reagent mixture diluted in HBSS (240 μg/ml MTS and 2.4 μg/ml PMS) (24) were added, followed by incubation at 37°C under shaking (50 rpm) for 1 h. The absorbance was read at 492 nm using a $THERMO_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA, USA).

The cytotoxicity of ALA in RPMI was examined for a concentration range of 1 μg/ml to 50 mg/ml (n = 8). The o/w emulsions with or without 1 mg/ml ALA were used at a concentration of 10% (v/v) in RPMI (n = 8). For endocytosis inhibition, D-mannitol and NH_4Cl were tested in a concentration range of 10 μM to 1 M; monensin in the range of 0.1 μM to 5 mM; brefeldin A in the range of 0.036 to 3.6 μM; and cytochalasin D between 0.011 μM and 1.1 mM. These tests were all done with and without 1 mg/ml ALA (n = 4).

In each experiment, RPMI was used as negative control and 2%(v/v) Triton X-100 in RPMI as positive control. As a blind reference, the assays were performed without cells.

Uptake of ALA and Metabolism to PpIX

For all cell experiments, ALA was added from freshly prepared stock solutions or emulsions which contained 10 mg/ml of ALA and had a pH-value adjusted to 5.5 for stability reasons. In the test solutions, the ALA preparations were generally diluted in the appropriate buffer to a concentration of 1 mg/ml and the emulsion generally to 10% (v/v). The pH was adjusted to the desired value for the experiment.

Effect of the pH Value of the Incubation Medium

Initially, 180 μl of the medium was removed and replaced by 180 μl of RPMI (pH-adjusted to 5.7, 6.6, or 7.4), or 20 μl of the o/w emulsions without ALA was mixed with 160 μl of pH-adjusted RPMI and added to the cells. Then, 20 μl of a stock solution of ALA in RPMI was added to each well. Before each reading, mixing was done for 2 s in the fluorescence plate reader (FluoroCount plate reader, Packard Instrument, Meriden, CT, USA). The settings (λ excitation: 400 nm; λ emission: 620 nm; PMT: 1100V; gain: 12) were kept for all readings. The standard curve of PpIX was linear up to 400 nM. The final pH value of the test mixture (ALA stock solution in RPMI) was comparable to the initial value of the RPMI, that is, 5.7–5.8, 6.6–6.7, and 7.4 for the experiments referred to as pH 5.7, 6.6, and 7.4, respectively. The cells were protected from light with aluminum foil and incubated for up to 4 h. Readings of PpIX fluorescence were taken after 0 and 30 min as well as after 1, 2, 3, and 4 h. After 4 h, the viability of the cells was examined by rinsing carefully with 3 × 300 μl HBSS of 37°C, and adding MTS/PMS reagent mixture to each well. The number of repetitions was 8.

Effect of Emulsion Concentration

At pH 5.7 and 7.4, the concentration of LV20 and SOPS(0.2) in the incubation medium was increased 2 and 3 times, respectively, keeping the total volume in each well constant at 220 μl. Further, LV20 and SOPS(0.2) were mixed in equal volumes of 10 μl or 20 μl each and tested. The experiment was carried out as described as described in "Effect of the pH Value of the Incubation Medium," above.

Effect of Temperature and Duration of Preincubation

The cells were kept in RPMI at either 4°C or 37°C for 30 min or 2 h. The test formulations (20 μl), with or without 1.0 mg/ml ALA, were mixed with 200 μl of RPMI and applied to the cells. The cells were then washed with ice-cold HBSS (3 × 300 μl), 220 μl RPMI was added, and incubation was continued at 37°C for 4 h, regardless of whether the cells had previously been incubated at 4°C or 37°C. As a control, MCF-7 cells were incubated at 37°C for 30 min or 2 h with ALA-free formulations after which ALA in RPMI (1 mg/ml) was added, and the fluorescence was then measured over 4 h. Throughout the experiments, protection from light was maintained, and the fluorescence was measured every hour. The final pH of the test mixtures was 7.2-7.5. At the end of the experiment, the test mixtures were removed, and the MTS/PMS assay for cell viability was performed.

Effect of ALA Concentration

The formation of PpIX after administration of 0–1.0 mg/ml ALA in RPMI at pH 5.7 and pH 7.4 was evaluated. Identical ALA concentrations were also applied in the presence of LV20 and SOPS(0.2). The experiment was carried out as described in "Effect of the pH Value of the Incubation Medium," above.

The protein content per well was determined after lysis of cells treated with RPMI (n = 16). Cell lysis was achieved with 100 μl 0.1% Triton®X-100 in HBSS for 4 h, and the amount of protein was assessed by a BCA assay (25).

Effect of Endocytosis Inhibitors

The endocytosis inhibitor NH_4Cl was added to the RPMI in concentrations of 20 and 50 mM. Monensin was used at a

concentration of 50 μM, cytochalasin D at a concentration of 4.9 μM, and brefeldin A at a concentration of 8.9 μM. Incubation at 37°C for 30 min with the inhibitors in RPMI was done before the test formulation and ALA were added. Otherwise, the experiment was carried out described in "Effect of the pH Value of the Incubation Medium," above.

Data Analysis

The PpIX standard solution (1 μM) in the 96-well plate was scanned using a CARY Eclipse fluorescence spectrophotometer (Varian, Zug, Switzerland). At an excitation wavelength of 400 nm, an emission maximum of 610 nm was observed.

The data was corrected for background (ALA-free test mixture applied to cells) and normalized relative to the first measurement (t = 0).

For kinetic fitting, Eq. 1 was used to estimate V_{max} and k_m:

$$\frac{d(PpIX)}{dt} = \frac{V_{max} \times C_{ALA}}{k_m + C_{ALA}} \quad (1)$$

ANOVA was used to evaluate differences, which were considered statistically significant if $p < 0.05$.

RESULTS

Dehydrogenase Activity

Dehydrogenase activity of MCF-7 cells exposed to up to 5 mg/ml ALA in RPMI at pH 7.4 (97 ± 21%) was not decreased relative to the control (100 ± 21%), whereas exposure to 10 mg/ml ALA was significantly more cytotoxic (57 ± 7%) ($p < 0.001$). In general, dehydrogenase activity in the cells was the same at pH 7.4 and 6.6, but decreased to approximately 60% when the pH of the incubation medium was changed to 5.7. For all the examined pH values, however, none of the ALA-containing test solutions showed differences from the RPMI, which had been adjusted to the same pH value, even when higher emulsion concentrations were used indicating that a decrease in enzyme activity was only due to changes in the pH-value and not to the formulation.

The endocytosis inhibitors D-mannitol and NH_4Cl were not cytotoxic at concentrations up to 100 mM in RPMI of pH 7.4 (between 90 ± 7% and 103 ± 11%), and the concomitant presence of 1 mg/ml ALA exerted no influence ($p > 0.05$). Monensin up to 500 μM (120 ± 23%) and 50 μM cytochalasin D (93 ± 13%) could also be used without any measurable toxic effects. Brefeldin A did not decrease the cell viability at 3.6 μM (116 ± 22%), but did so at 17.8 μM (20 ± 15%)($p < 0.001$).

Uptake of ALA and Metabolism to PpIX

Effect of the pH Value of the Incubation Medium

When the pH of the final test formulation applied to the cells was increased from pH 5.7 to 7.4, the amount of PpIX formed increased significantly (Fig. 1). Compared to the RPMI-control, the o/w emulsion enhanced the PpIX formation during the first hour of incubation. However, this difference became less important with increasing pH and with longer exposure time. After 4 h incubation, the amount of PpIX in the control cells was mostly comparable to that of the cells exposed to the o/w-emulsions.

Effect of Emulsion Concentration

The amount of PpIX formed upon an increase in the concentration of LV20 and SOPS(0.2) in RPMI at pH 7.4, in the presence of 1 mg/ml ALA, is shown in Fig. 2. At a 3 times higher LV20-concentration, the amount of PpIX observed after 1 and 4 h appeared to be higher. On the contrary, increasing amounts of SOPS(0.2) emulsion did not result in more PpIX. In RPMI at pH 5.7, the amount of PpIX was not increased when the concentration of LV20 and SOPS(0.2) was increased. Mixing of LV20 and SOPS(0.2) resulted in a decrease in the amount of PpIX (approximately 44 nM PpIX), as compared to LV20 alone (52 ± 3 nM PpIX), and to a decrease as compared to SOPS(0.2) alone (26 ± 6 nM PpIX).

Effects of Temperature and Duration of Preincubation

ALA metabolism to PpIX was examined over 4 h after the cells were preincubated with 1 mg/ml ALA in different formulations for 30 min or 2 h, which was subsequently rinsed off. When preincubation was conducted at 4°C, no uptake of ALA occurred as no PpIX formed during the subsequent incubation for 1 and 4 h in ALA-free RPMI at 37°C. Fig. 3 depicts the result after 2 h preincubation. However, the amount of PpIX seemed to be independent of the time of preincubation. Preincubation at 37°C resulted in kinetic profiles that were all quite similar suggesting that the uptake of ALA during the pre-incubation was comparable for the RPMI and emulsion formulations.

Similarly, when the cells were preincubated for 30 min or 2 h with ALA-free formulations, and the formulations were

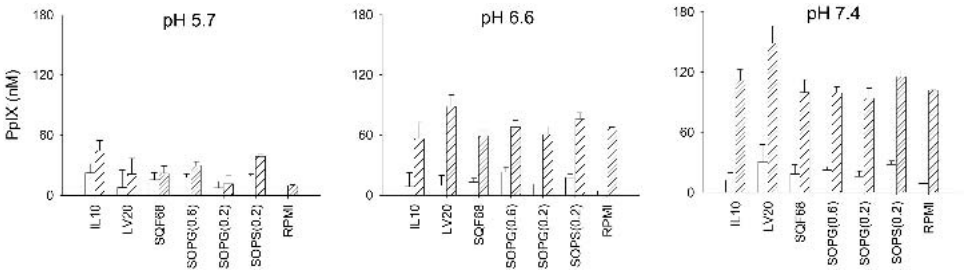

Fig. 1. Production of PpIX in MCF-7 cells during incubation at 37°C for 1 h (□) and 4 h (▨) with 1 mg/ml ALA formulations. The formulations (see Table I) were adjusted to pH values of 5.7, 6.6, and 7.4 immediately before addition to the cells. Mean ± SD (n = 8).

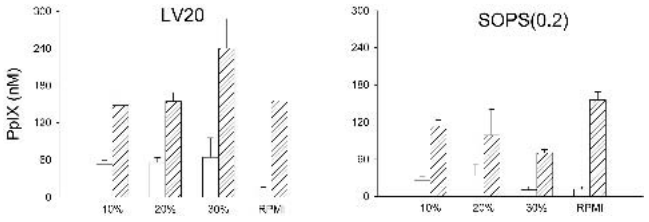

Fig. 2. Production of PpIX in MCF-7 cells during incubation at 37°C for 1 h (□) and 4 h (▨) with 1 mg/ml ALA in LV20 and SOPS(0.2). Here, different concentrations of LV20 or SOPS(0.2) emulsions were used in the medium at pH 7.4, with 10% being the standard concentration used. Mean ± SD (n = 8).

then rinsed off, and the cells finally incubated with ALA in RPMI, no significant effect of the pre-incubation modality was observed on the ALA uptake and metabolism over 4 h. For the other formulations, the same tendency was noted. No cytotoxicity was observed with any of the treatments.

Effect of ALA Concentration

The saturation profiles for the PpIX-formation upon incubation for 1 h and 4 h with ALA in RPMI of pH 5.7 and 7.4, with and without LV20 and SOPS(0.2), are depicted in Fig. 4. The process of PpIX formation seems to be saturated when 1 mg/ml ALA is applied to the cells, regardless of the test formulation used. The estimated k_m and V_{max} values are compiled in Table II.

After 1 h of incubation V_{max} is approximately 10 times higher in RPMI of pH 7.4 than in RPMI of pH 5.7. Interestingly, V_{max} and k_m values seemed to be unaffected by pH (7.4 or 5.7) when ALA was applied in LV20 or SOPS(0.2), but both increased when ALA was applied in RPMI. Furthermore, V_{max} was approximately 2–4 times and 20–40 times higher for the emulsions than for RPMI at pH 7.4 and 5.7, respectively. Statistical analysis of the data points after 1 h at ALA concentrations of 0.1, 0.5 and 1 mg/ml revealed a significantly higher PpIX formation in RPMI at pH 7.4 than at pH 5.4 ($p < 0.001$). Very interestingly, at ALA concentrations of 0.5 mg/ml ($p < 0.01$) and 1 mg/ml ($p < 0.001$), the PpIX formation was higher with LV20 or SOPS(0.2) than with RPMI at pH 7.4. For the results obtained after 4 h, the V_{max} in RPMI at pH 7.4 was comparable to RPMI at pH 5.7 and to V_{max} in either of the emulsions. At pH 5.7, however, the PpIX production seemed to reach a lower maximum when ALA was applied in emulsion. For both 1 h and 4 h results, the different formulations did not give substantial differences in the k_m values even though there was a tendency toward lower k_m values for the LV20 emulsion. However, it should be mentioned that with LV20, the fitting parameter is not optimal.

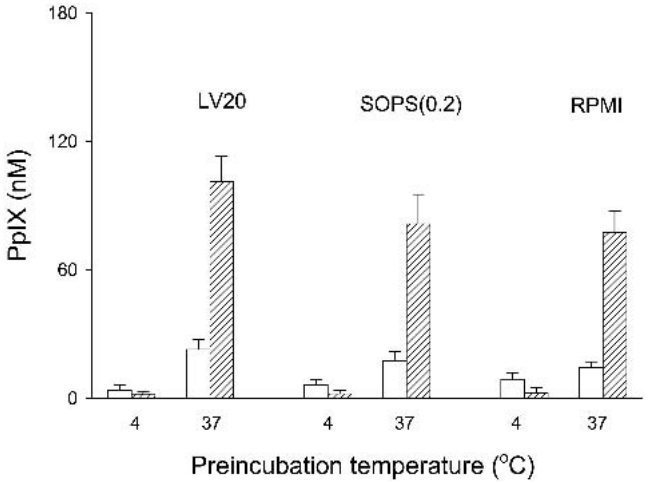

Fig. 3. Production of PpIX in MCF-7 cells during incubation at 37°C for 1 h (□) and 4 h (▨) with ALA-free RPMI (pH 7.4), after preincubation for 2 h with 1 mg/ml ALA in LV20 or SOPS(0.2) emulsion or RPMI at 4°C or 37°C (pH 7.4). Mean ± SD (n = 8).

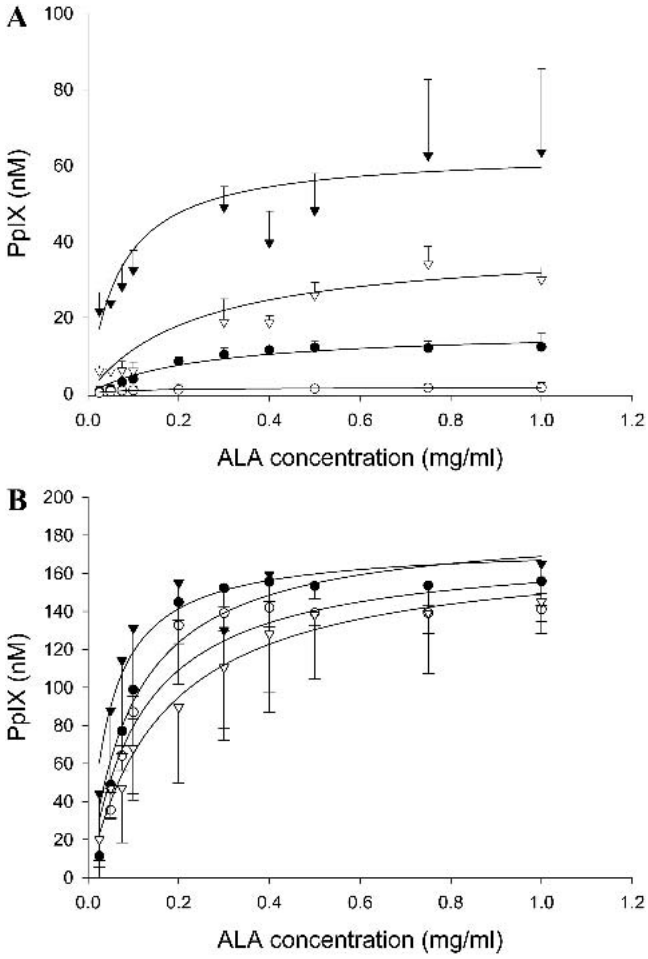

Fig. 4. PpIX production in MCF-7 cells after incubation at 37°C for 1 h (A) and for 4 h (B) with variable ALA concentrations in different emulsions and RPMI at pH 7.4 or 5.7. RPMI, pH 5.7 (○), RPMI, pH 7.4 (●), SOPS(0.2), pH 7.4 (▽), LV20, pH 7.4 (▼). Mean values and SD are presented (n = 8) and profiles fitted according to Eq. 1.

Effect of Endocytosis Inhibitors

The presence of PpIX found in MCF-7 cells exposed to ALA formulations and different endocytosis inhibitors is presented in Fig. 5. Some of the inhibitors decreased the amount of PpIX formed in the control cells (17 ± 2 nM/h) exposed to RPMI, that is, 50 mM NH_4Cl ($p < 0.001$), monensin ($p < 0.001$), and brefeldin A ($p < 0.05$). Cytochalasin D seemed to increase the intracellular amount of PpIX as compared to control cells (23 ± 6 nM/h) ($p < 0.05$) and to cause no change in the effect of the o/w-emulsions.

Table II. V_{max} and k_m Values for Production of PpIX in MCF-7 Cells after Incubation for 1 h and 4 h in RPMI or Emulsion at pH 5.7 or 7.4[a]

ALA formulation	pH of incubation medium	1 h			4 h		
		k_m (mg/ml)	V_{max} (nM)[b]	R^2	k_m (mg/ml)	V_{max} (nM)[b]	R^2
RPMI	5.7	0.10 ± 0.02	1.66 ± 0.09	0.9647	0.12 ± 0.04	173.53 ± 16.54	0.8981
LV20	5.7	0.09 ± 0.06	65.70 ± 11.73	0.5111	0.06 ± 0.04	74.35 ± 13.50	0.5683
SOPS(0.2)	5.7	0.17 ± 0.08	39.32 ± 6.53	0.7102	0.09 ± 0.04	67.97 ± 8.95	0.8056
RPMI	7.4	0.25 ± 0.07	16.80 ± 1.80	0.9478	0.10 ± 0.02	185.92 ± 12.70	0.9285
LV20	7.4	0.07 ± 0.02	63.64 ± 5.53	0.7718	0.05 ± 0.02	176.20 ± 15.52	0.8881
SOPS(0.2)	7.4	0.24 ± 0.09	39.44 ± 5.55	0.8910	0.17 ± 0.02	173.60 ± 6.33	0.9865

[a] Fitting according to Eq. 1. Mean ± SD (n = 8).
[b] The amount of protein per well was 1.82 ± 0.09 mg/well (n = 16).

Therefore, only the effect of 20 mM NH_4Cl and 20 and 50 mM D-mannitol was considered for PpIX production after incubation with the o/w emulsions. The PpIX formation was significantly decreased when the cells were incubated with ALA in SOPG(0.6), SOPG(0.2), and SOPS(0.2) in the presence of 20 mM or 50 mM D-mannitol ($p < 0.01$), or of 20 mM NH_4Cl ($p < 0.05$). Conversely, NH_4Cl and D-mannitol did not appear to affect the PpIX formation significantly when ALA was applied to the cells with less negatively charged emulsion droplets, that is, IL10, LV20 and SQF68, even though a slight tendency toward reduced PpIX formation was noticed for IL10 and SQF68 in the presence of 50 mM D-mannitol.

DISCUSSION

Uptake of ALA and Metabolism to PpIX

Effect of pH, Time, Temperature, and Concentration

The PpIX formation in cells exposed to ALA at pH 5.7 was generally higher when applied in the buffered emulsions than in the buffer solution (Fig. 1). This could indicate that the emulsions favored the extracellular stability or intracellular metabolism of ALA at this pH. However, in previous studies, o/w emulsions (including IL10 and LV20) did not increase the stability of ALA significantly as compared to a buffer solution (4). Furthermore, the 3- to 4-fold increase in the PpIX formation at pH 7.4 compared to pH 5.7 was similar regardless of whether ALA was applied in o/w emulsions or in buffer. This indicates that the effect of pH appears to be certainly important for metabolism although the importance for uptake should not be excluded. The optimal pH of 7.4 for incubation correlates well with the fact that the metabolic cascade from ALA to PpIX occurs in the mitochondria (9).

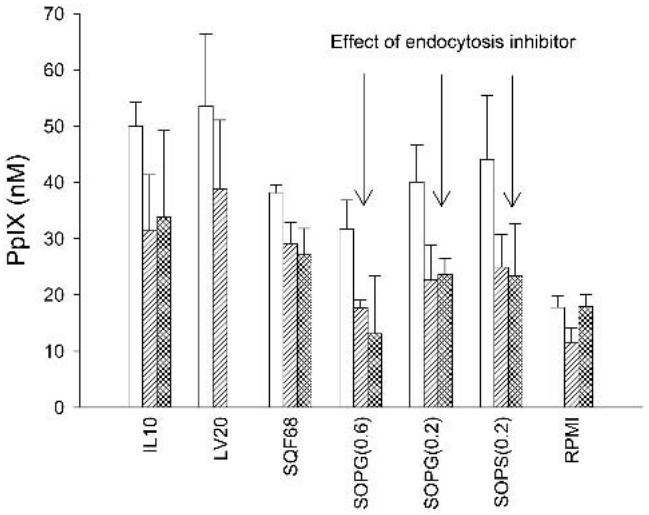

Fig. 5. Inhibition of PpIX production in MCF-7 cells after incubation at 37°C for 1 h with 1 mg/ml ALA in emulsion or RPMI, pH 7.4. No inhibitor (□), 20 mM NH_4Cl (▨), 50 mM mannitol (▩). Mean + SD (n = 8).

The amount of PpIX formed in the MCF-7 cells increased with longer exposure time to ALA. This could be due to both increased uptake and increased intracellular metabolism. The lack of intracellular PpIX formation at 4°C could simply be the result of a diminished metabolic activity of the cells. It has been shown that low temperature hinders PpIX production, but not the penetration of ALA into mouse and human skin (26). However, since no PpIX was formed in the cells incubated at 37°C for up to 4 h following pre-incubation with ALA for up to 2 h at 4°C, cellular uptake of ALA also appears to depend on temperature, which supports an uptake mechanism by transporter(s) or endocytosis. In accordance with previous findings, the intracellular accumulation of PpIX upon application of ALA occurs by saturable kinetics (7), with saturation manifesting in the concentration range of 0–1 mg/ml (Fig. 4). The conversion from extracellular ALA to intracellular PpIX is probably best described by a two-compartment model with Michaelis-Menten kinetics (27). It has previously been shown that the affinity of ALA for the receptor (k_m) is not altered by changes in the extracellular pH, whereas the maximal transport rate (V_{max}) is (15). A comparable observation derives from the present study, when ALA was applied in buffer. However, when o/w emulsions were used, V_{max} seemed to remain unaffected by a change in pH. This difference in the maximum amount of PpIX formed when applying the LV20 or the SOPS(0.2) emulsion for 1 h, as compared to buffer, could be due to either changes in the uptake mechanism or effects of emulsion components on the metabolism in terms of inducing or inhibiting certain enzymes of the heme synthesis pathway, or else effects of emulsion components on the rate of distribution to the mitochondria. The latter two mechanisms require that the cells ingest emulsion components, a phenomenon which deserves further investigations. Because V_{max} after 4 h in RPMI at pH 5.7 is

comparable to that after 4 h in RPMI at pH 7.4, it seems that during the first hour of incubation, the uptake of ALA is the rate-limiting step. The fact that the PpIX formation upon 4-h exposure to the emulsions is lower at pH 5.7 compared to pH 7.4 suggests that the emulsions limit the long-term accumulation of PpIX at low pH. Interestingly, this difference is not observed at pH 7.4, suggesting a different mechanism of action for the emulsions at different pH values.

In general, the effects of pH, time, temperature and concentration observed in this study reflect a saturable uptake of ALA and future examination of the presence of specific transporters in the MCF-7 cell line is, therefore, of interest. Both the PEPT2 and BETA transporters have been suggested to transport ALA There is strong evidence for the presence of the PEPT2 transporter in mammary gland epithelium (15). Contrastingly, the BETA transporter, but not the PEPT2 transporter (16) has been identified in a mammary epithelial cell line (19,20).

Effect of O/W Emulsion

The accumulation of PpIX in MCF-7 cells was increased when the amphoteric ALA was formulated in certain o/w-emulsions—especially LV20 and SOPS(0.2) (Fig. 1). Previously, an o/w nanoemulsion has been shown to increase the cellular fluorescence very slightly after uptake of another photosensitizer, *m*THPC (*meta*-tetra(hydroxypropyl)chlorine), in macrophage-like cells (J774), but to decrease the uptake of the *m*THPC in HT29 adenocarcinoma cell line (28). DMSO in an o/w-emulsion has been demonstrated to improve the permeation of ALA across mouse skin *in vitro* (29) and *in vivo* (14) and also with increased PpIX synthesis (14).

The mechanisms responsible for the enhanced PpIX formation in the presence of emulsions could be explained by the present results. As ALA is an amphoteric molecule, it probably interacts strongly through ionic forces with the amphoteric lecithin used as emulsifier in most of the tested emulsions (Table I). An excess concentration of ALA at the o/w interface could have promoted the contact between ALA and the cell surface, resulting in an increased local concentration of ALA at the cell surface. Such locally increased ALA concentration at the epithelial surface could have favored cellular uptake of ALA, which probably occurred via endocytosis and not, as previously stated, by an increased affinity for (or velocity of) a transporter. In this context, it may be appropriate to consider the role of clathrin-coated pits in cellular plasma membranes. It has been shown that certain endogenous proteins/receptors become clustered in coated pits upon binding to soluble ligands (30), and that such clustering can result in an invagination to vesicles/endosomes, which are acidified. This hypothetic mechanism could explain the present results with some of the endocytosis inhibitors used, which decreased the amount of PpIX in the cells. Indeed, hyperosmolarity induced by mannitol as well as acidification of the cytoplasm induced by NH_4Cl decreased the formation of PpIX in a concentration dependent manner (Fig. 5). This coincides with previous observations that endocytosis mediated by coated pits is inhibited by hyperosmolarity (by mannitol) and acidification of the cytoplasm (by NH_4Cl) (31), whereas endocytosis via non-coated pits is not inhibited by hyperosmolarity (32).

The oil droplets of the o/w-emulsions did not interact with the cell plasma membrane in a harmful way, since none of the emulsions induced any decrease in dehydrogenase activity. Furthermore, no effect on plasma membrane integrity was noticed, since 30 min pre-incubation with ALA-free test formulations followed by incubation with ALA formulations for up to 4 h did not alter the accumulation of PpIX as compared to pre-incubation with buffer (Fig. 3).

The type or charge of the phospholipid in the o/w-emulsions seemed to be important to the amount of ALA endocytosed and PpIX formed, as LV20 yielded a higher V_{max} than SOPS(0.2) (Tables I and II). This is also supported by the data obtained from the inhibitor study (Fig. 5). Here, the IL10 and LV20 formulations produced higher amounts of PpIX than SOPG(0.6) and SOPG(0.2) in which up to 50% of the phosphatidylcholine/lecithin was replaced by the negatively charged phosphatidylglycerol. For the formulations which was added only 16% phosphatidylglycerol, SOPG(0.2), or 16% of negatively charged phosphatidylserine, SOPS(0.2), the difference in the amount of PpIX formed is less evident. Further support for the beneficial effect of the amphoteric phosphatidylcholine over the anionic phosphatidyl derivatives is provided by the increased PpIX formation with increasing concentrations of LV20 (10–30%) in the incubation medium on one side and by the lowered PpIX production with increasing SOPS(0.2) concentrations on the other side (Fig. 2). Whether the zeta potential played a significant role remains uncertain. The negatively charged oil droplets in LV20 should be repelled by the plasma membrane more strongly, at least in theory, than the less negatively charged oil droplets of SOPS(0.2) (Table 1). Furthermore, the fact that the amount of PpIX formed at pH 7.4 in the presence of ALA in the SQF68 formulation does not differ significantly from the SOPG and SOPS emulsions suggests that the type of oil, the droplet size and zeta potential play a minor role.

In conclusion, the present data show that the formation of PpIX in MCF-7 cells exposed to ALA depends on pH as well as time, temperature and concentration—both when ALA is applied in buffer and in o/w-formulations. By introducing ALA in an o/w-emulsion, it may be possible to use lower concentrations of ALA and shorten periods of incubation for accumulation of PpIX in epithelial cells in order to diagnose and treat superficial neoplastic cells. This could not only reduce the risk of side effects by the treatment, but also save time. The present results raise questions regarding the exact mechanisms involved and require further studies with organ cultures of normal cells and tumor cells or *in vivo* studies to ascertain tumor selectivity.

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