

Cell specific effects of polyunsaturated fatty acids on 5-aminolevulinic acid based photosensitization

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The purpose of this study was to examine whether the dietary components *n*-6 and *n*-3 polyunsaturated fatty acids (PUFAs) may potentiate the effect of photodynamic therapy (PDT) in human cancer cell lines by enhancing the lipid peroxidation. The effects of the porphyrin precursor 5-aminolevulinic acid (5-ALA) and light ($320 < \lambda < 440$ nm, 33 W m^{-2}), with or without docosahexaenoic acid (DHA) or arachidonic acid (AA), were tested in the colon carcinoma cell lines SW480 and WiDr, the glioblastoma cell line A-172 and the lung adenocarcinoma cell line A-427. The production of endogenous protoporphyrin IX (PpIX) varied substantially between the cell lines and was ~ 4 -fold higher in WiDr as compared with SW480. Cell killing by 5-ALA-PDT also varied between the cell lines, but without clear correlation with PpIX levels. Treatment with DHA or AA (10 or 70 μM , 48 or 72 h) in combination with 5-ALA-PDT (1 or 2 mM) enhanced the cytotoxic effect in A-172 and A-427 cells, but not in SW480 and WiDr cells. While 5-ALA-PDT alone increased the lipid peroxidation in A-172 and WiDr cells only, 5-ALA-PDT plus PUFAs increased the lipid peroxidation substantially in all four cell lines. Interestingly, α -tocopherol (50 μM , 48 h) strongly reduced lipid peroxidation after all treatments in all cell lines, while cytotoxicity was only reduced substantially in A-427 cells. This demonstrates that induction of lipid peroxidation is not a general mechanism responsible for the cytotoxicity of 5-ALA-PDT, although it may be important in cell lines with an inherent sensitivity to lipid peroxidation products. Thus, the mechanisms of cell growth inhibition/cell killing by PDT are complex and cell specific.

1 Introduction

Application of the natural precursor in the heme synthesis, 5-aminolevulinic acid (5-ALA), has been shown to induce accumulation of the photosensitizer protoporphyrin (PpIX) in many cell types.¹⁻⁶ Photochemical reactions occur after exposure of PpIX to light, and the cytotoxic effect involves singlet oxygen and free oxygen radicals.^{7,8} One disadvantage of exogenously added 5-ALA, is its poor ability to diffuse across biological membranes because of its low lipophilicity.⁹ Several studies have been carried out in order to improve the 5-ALA uptake and penetration through tissue including the use of 5-ALA esters^{2,4,10-12} iontophoresis¹³ and penetration enhancers. Another possibility, assessed in this study, is to attempt to increase the 5-ALA uptake and the level of lipid peroxidation by modulating the lipid structure of the plasma membrane.

The transport of molecules across the cell membrane depends on the chemical and physical properties of the lipid bilayer. By increasing the concentration of polyunsaturated fatty acids (PUFAs), the molecular movement will result in a higher degree of fluidity and may also influence the biochemical properties of transporter proteins.^{14,15} PUFAs may increase the lipid peroxidation since they are easily oxidized and the photodynamic effect on cell killing may thus be improved. Both epidemiological and experimental studies conducted over the last few decades, have shown a trend towards a tumour growth promoting effect of *n*-6 PUFAs, while *n*-3 PUFAs seem to inhibit tumour growth

and metastasis.¹⁶⁻¹⁸ Detailed studies on several human cancer cell lines have shown that some tumour cells are more sensitive to the cytotoxic and the antiproliferative effects of PUFAs than normal cells, and these cells would seem well suited for modulation of membranes.¹⁹⁻²² Measurement of malondialdehyde (MDA) has been the most common method used to assess lipid peroxidation in cells for many decades.^{23,24} MDA is one of the decomposition products from a variety of unsaturated fatty acids after the free radical mediated lipid peroxidation process and can be formed by oxidation of PUFAs or by decomposition of pre-existing lipoxides in the sample.²⁵

In the present study four different human cancer cell lines have been used to examine the effects of pre-incubation with PUFAs and α -tocopherol on 5-ALA-PDT. The following parameters were determined: PpIX accumulation, cell growth (MTT-assay), production of malondialdehyde (MDA) and PpIX content correlated to the effects of the PUFAs.

Our results show that differential sensitivities were not correlated to the level of PpIX and the MDA-production after treatment with PUFAs and 5-ALA-PDT. All together the results indicate that the tested human cancer cell lines respond quite differently to 5-ALA-PDT and that mechanisms other than lipid peroxidation were of major significance for cell killing or growth inhibition.

2 Materials and methods

2.1 Chemicals

Minimum essential medium with Earle's salts without L-glutamine (MEM), Dulbecco's Modified Eagle's Medium

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(DMEM), RPMI-1640 medium, L-glutamine, fetal calf serum (FCS), sodium pyruvate, non-essential amino acids, trypsin and Dulbecco's phosphate buffered saline (DPBS) were obtained from Gibco BRL, Life Technologies (Inchinnan, Scotland). Gentamicin sulfate was from Schering Corp (Kenilworth, NJ) and absolute ethanol was from Arcus A/S (Oslo, Norway). 2-Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), α -tocopherol and Bio-Rad protein assay were from Sigma Chemicals Co. (St. Louis, MO, USA). *cis*-4,7,10,13,16,19-Docosahexaenoic acid (DHA) and arachidonic acid (AA) were obtained from Cayman Chemical (Ann Arbor, MI, USA) as solutions in ethanol. 5-Aminolevulinic acid (5-ALA) and protoporphyrin IX (PpIX) were from Porphyrin Products (Logan, UT, USA). Other chemicals were of the highest quality commercially available. Ultra-purified water, Milli-Q PLUS (Millipore, Molsheim, France) was used throughout.

2.2 Cell cultures

The human cancer cell lines SW480 (colon adenocarcinoma), A-172 (glioblastoma) and A-427 (lung carcinoma) were obtained from the American Type Culture Collection (Rockville, MD). The SW480 cells were cultured in Leibovitz's L-15 medium supplemented with gentamicin (45 mg l⁻¹) and 10% (v/v) FCS. The A-172 cells were cultured in DMEM with 4.5 g l⁻¹ glucose supplemented with 10% (v/v) FCS, gentamicin (50 mg l⁻¹), L-glutamine (80 mg l⁻¹) and sodium pyruvate (1 mM). The A-427 (lung carcinoma) cells were cultured in MEM supplemented with 10% (v/v) fetal calf serum (FCS), gentamicin (45 mg l⁻¹), L-glutamine (80 mg l⁻¹), sodium pyruvate (1 mM) and non-essential amino acids (100x, 10 ml l⁻¹). The cell line WiDr was derived from a human primary adenocarcinoma of the rectosigmoid colon²⁶ and was cultured in RPMI 1640 medium containing 10% (v/v) FCS, L-glutamine (80 mg l⁻¹) and gentamicin (50 mg l⁻¹). All cell lines were grown in an atmosphere of 95% air and 5% CO₂ at 37 °C and sub-cultured approximately twice a week.

2.3 Treatment with PUFAs, 5-ALA and α -tocopherol

For the MTT-assay 3000 cells (well)⁻¹ were seeded in 96 well microtiter plates (Costar 3598). The cells were allowed to adhere for 4 h before adding: PUFAs, PUFAs together with α -tocopherol or α -tocopherol alone. Stock solutions of DHA, AA and α -tocopherol were prepared in ethanol, and the final ethanol concentration in the serum-enriched medium (10%) never exceeded 0.38% (v/v), which had no effect on the cell survival.²² In experiments with DHA or AA, the 5-ALA incubation (3.5 h) was also done in serum-enriched medium (10%) and started immediately after 48 or 72 h incubation with PUFAs. The concentration of the PUFAs has been 70 μ M (72 h) during the incubation of the cells, except in the experiments with the A-427 cells (10 μ M, 48 h). The optimal concentration and incubation periods of DHA, AA or α -tocopherol, selected for the presented data, were based on earlier reports^{21,22} and unpublished results by S. A. Schönberg. Because of the comparison between the cell lines, the effect of PUFAs and α -tocopherol (50 μ M, 48 h) on PpIX production was carried out using the same concentrations of PUFAs (10 μ M, 48 h).

After treatment with 5-ALA (1 mM in A-427 cells and 2 mM in SW480, A-172, and WiDr cells) the wells were washed three times with DPBS before illumination (in DPBS), as described in section 2.4. The cells were then incubated for 24 h with serum-enriched medium (10%) in 5% CO₂ at 37 °C. This was the standard procedure unless otherwise described.

For the MDA measurements, 1 \times 10⁶ cells were seeded in 98 mm cell culture dishes (Nunclon), and incubated with PUFAs or α -tocopherol for 48 or 72 h in serum-enriched medium (10%) at 37 °C. Thereafter the cells (in the nearly confluent layers) were washed three times before 5-ALA incubation (1 mM in A-427

cells and 2 mM in SW480, A-172 and WiDr cells) for 3.5 h at 37 °C in the respective serum-enriched (10%) cell culture medium. The MDA measurements and the PpIX experiments are further described in sections 2.6, 2.7 and 2.8.

2.4 Light exposure

The cells were illuminated with a Waldmann lamp (PUVA, D7220VS, Swenningen, Germany) containing 14 fluorescent tubes (Sylvania F8T5, USA). The culture dishes were positioned at a distance of 10 cm from the light source. Approximately 70% of the emission energy of these lamps is between 340 and 380 nm. The rest of the emission energy is distributed between 310 and 340 nm (10%) and between 380 and 440 nm (20%). The light intensity at the level of the cells was 33 W m⁻², measured with an Optometer UDT model 161, radiometer-photometer (United Detector Technology, Culver City, CA, USA), giving a total light dose of 1.98 kJ m⁻² on the cells during a 1 min illumination period.²⁸ All illuminations were done in DPBS.

2.5 Mitochondrial dehydrogenase activity (MTT-assay)

Cell survival was determined in 96-well microtiter plates (Costar 3598), using the colorimetric methylthiazoldiphenyl-tetrazoliumbromide assay (MTT), measuring the mitochondrial dehydrogenase activity by the reduction of tetrazolium salts as described by Carmichael *et al.*²⁹ The cells were seeded out (3000 cells (well)⁻¹) as described in section 2.3. Cell survival was measured 24 h after 5-ALA incubation and light treatment, measuring the absorbance at 588 nm on a Titertek Multiscan Plus Reader, as described by Brekke *et al.*³⁰ All experiments were performed at least three times with eight parallels.

2.6 Malondialdehyde measurements using TBARS assay

After treatment with PUFAs, α -tocopherol or 5-ALA, the cells were washed three times in nominally calcium and magnesium free DPBS and illuminated in DPBS using a PUVA lamp as described in section 2.4. After incubation (37 °C) for 24 h in fresh medium, the cells were harvested by scraping, centrifuged (5 min, 800 \times g) and washed twice in DPBS. The cells were resuspended in 800 μ l NaCl (0.9%, w/v in H₂O) at 4 °C. Aliquots (2 \times 25 μ l) were taken for protein analysis (Bio-Rad),³¹ and the cells were lysed and the proteins precipitated with 200 μ l 20% (w/v) trichloroacetic acid (TCA). After 10 min on ice (0 °C) 250 μ l thiobarbituric acid (0.67%) was added to the rest of the cell suspension. The thiobarbituric acid reactive substances (TBARS) were measured according to Brekke *et al.*³⁰ and Chow *et al.*³² using reduced reagent volumes to increase the sensitivity. The compound 1,1,3,3-tetramethoxypropane (0–10 μ M) was used as an external standard. Measurements were expressed in terms of malondialdehyde (MDA) relative to the cell protein content. The measurements were performed in duplicate and the results are based on 3–4 separate experiments.

2.7 High-pressure liquid chromatography (HPLC) assay of TBA-MDA

The reaction of malondialdehyde with 2-thiobarbituric acid was performed as described in section 2.6. The TBA-MDA complex (from the TBARS assay) was described by Bird *et al.*²⁴ Briefly, the HPLC system (1100 series) consisted of a Hewlett Packard 1100 gradient pump equipped with an automatic injector, a 1100 Hewlett Packard diode-array absorption detector (532 nm) and a personal computer using Chem Station Software from Hewlett Packard. Aliquots (50 μ l) of the TBARS samples were injected on a 5 μ m Supelcosil LC-18 reversed phase column (30 cm \times 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μ m filter (Millipore, Bedford, MA). The flow rate was 1 ml min⁻¹. The absorption spectra of standards and samples were identical with a characteristic peak at 532 nm.

2.8 Measurements of the cellular protoporphyrin IX (PpIX) content

Approximately 1×10^6 cells were seeded into 57 cm² cell culture dishes (Nunc) for PpIX content measurements. After treatment with PUFAs, α -tocopherol or 5-ALA (described in section 2.3) the cells were washed three times (DPBS) and harvested by scraping in DPBS (1.5 ml). Aliquots (200 μ l) were taken for protein analysis, and to the rest of the cell suspension were added 3 ml of ethyl acetate–acetic acid (3 : 1, v/v). The pH was adjusted to pH = 2.4 in cell suspensions as well as in standards. Cell debris was removed by centrifugation (5 min, 800 \times g) and the PpIX content of the supernatants was detected spectrofluorimetrically on a Perkin Elmer LS50B spectrofluorimeter, using external PpIX standards (0–50 nmol l⁻¹). It was supposed that the PpIX concentration was proportional to the fluorescence at 635 nm. The fluorescence emission was measured between 550 and 700 nm and the PpIX molecules were excited at 405 nm (the excitation/emission slits were 5/5 nm). A long pass cut-off filter (470 nm) was used during the measurements.

2.9 Protein determination

The protein content was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA)³¹ with bovine serum albumin (Sigma) as a standard.

2.10 Statistical analysis

Each experiment ($n \geq 3$) was run at least in duplicate or triplicate, and the data are presented as mean \pm SD. The figures presented are of representative experiments unless otherwise stated in the legend of the figures. The groups were compared by Student's *t*-test and Mann–Whitney test; $p < 0.05$ was accepted as significant.

3 Results

3.1 Protoporphyrin IX accumulation

(a) **Emission spectra after 5-ALA incubation.** The effects of 5-ALA incubation (2 mM, 3.5 h) on the protoporphyrin (PpIX) synthesis in the cell suspension from WiDr, A-172, A-427 and SW480 cells are shown in Fig. 1. All the cell lines showed an emission maximum at about 635 nm after excitation at 405 nm. The fluorescence intensity of the WiDr cell line was found to be more than 2.5 times that of the A-172 cells, corresponding to 138 pmol PpIX mg(protein)⁻¹ and 49 pmol PpIX mg(protein)⁻¹, respectively (Fig. 2). The cell lines SW480 and A-427 have approximately identical fluorescence intensities, corresponding

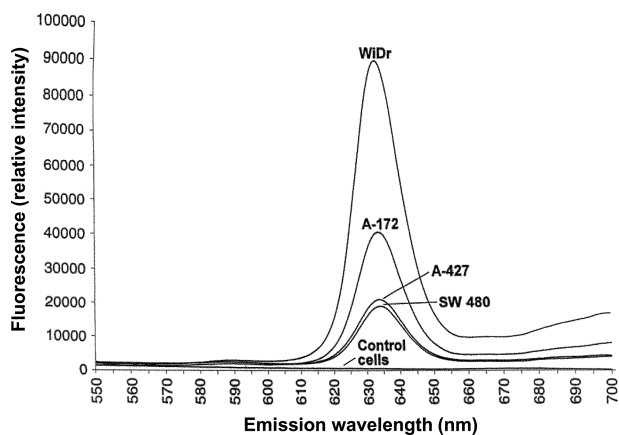


Fig. 1 The fluorescence emission spectra (relative intensity) of cell extracts from the human cancer cell lines WiDr, A-172, A-427 and SW480 after incubation with 5-ALA (2 mM, 3.5 h). The cells were cultured as described in 'Materials and methods'. Extracts of WiDr cells (without 5-ALA) were used as controls. The pH in the cell extracts was adjusted to pH 2.4, and the excitation was performed at 405 nm. The spectra are one of three representative experiments.

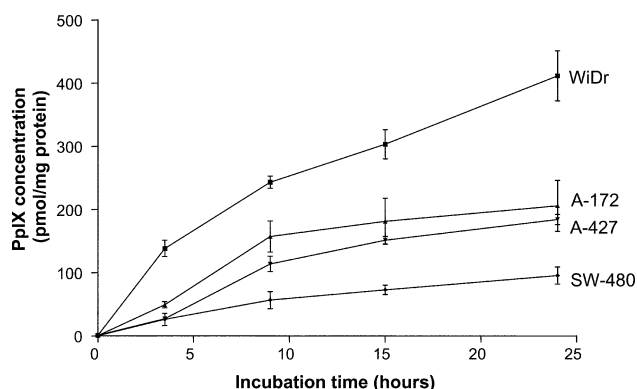


Fig. 2 Protoporphyrin (PpIX) formation (pmol mg(protein)⁻¹) in the human cancer cell lines WiDr, A-172, A-427 and SW480 after treatment with 5-ALA (2 mM, 0–24 h) in serum-enriched medium. The cells were cultured in an ordinary medium 72 h before incubating with 5-ALA as described in 'Materials and methods'. The data are the mean value \pm SD from two independent experiments.

to 26 and 27 pmol PpIX mg(protein)⁻¹, after incubation with 5-ALA for 3.5 h. Control cells from the four cell lines (without 5-ALA incubation) did not show detectable PpIX fluorescence.

The endogenous PpIX concentration increased with the time of incubation in the presence of 5-ALA (Fig. 2). Already after 3.5 h of incubation with 5-ALA, there is a considerable difference in PpIX production between the cell lines. After 24 h of 5-ALA incubation, WiDr cells have the highest production of PpIX (400 pmol mg(protein)⁻¹) while the SW480 cells have the lowest (95 pmol mg(protein)⁻¹). A-427 and SW480 cells produced quite similar amounts of PpIX; after 3.5 h of 5-ALA incubation, 27.4 and 26.0 pmol mg(protein)⁻¹, respectively (Figs. 1 and 2).

3.2 Effect of PUFAs and α -tocopherol on PpIX accumulation in the human cancer cell lines

Measurements of the PpIX production after incubation with DHA (10 μ M, 48 h) or AA (10 μ M, 48 h) or α -tocopherol (50 μ M, 48 h) followed by 5-ALA incubation (2 mM, 3.5 h), were performed in the four human cell lines (Table 1). All cell lines were incubated with the same concentration of PUFAs (10 μ M, 48 h) and α -tocopherol (50 μ M, 48 h) to exert only moderate toxic effects. PUFAs were found to have a negligible effect on the PpIX production in SW480, A-172 and A-427 cells, but caused a moderate, yet statistically significant decrease of PpIX in WiDr cells after addition of DHA ($p < 0.001$). Furthermore, α -tocopherol together with 5-ALA resulted in a significant increase in PpIX formation in A-427 cells ($p < 0.001$).

3.3 Effects of 5-ALA-PDT on cell survival after treatment with DHA, AA or α -tocopherol

The effects of illumination (0–10 min) on survival of the four 5-ALA-treated cell lines are shown in Fig. 3, and the more detailed studies after 3 min of illumination (SW480, A-172 and WiDr) or 0.5 min of illumination (A-427) are shown in Fig. 4. The studies were performed after pre-incubation with PUFAs (10 μ M, 48 h or 70 μ M, 72 h) and 5-ALA (1 or 2 mM, 3.5 h) as described in 'Materials and methods'. The incubation with 5-ALA alone without illumination resulted in a small, but significant reduction of the cell survival as measured by the MTT-assay; a reduction of 14% and 19% could be observed for WiDr and A-427 cells, respectively (Fig. 3, time 0). However, treatment with 5-ALA followed by illumination for 10 min reduced the cell survival of all cell lines by 80–97%. The cell survival was not influenced by illumination alone, as demonstrated by the controls that were not 5-ALA-treated (Fig. 3). Furthermore, PUFAs and light (0–10 min) had no influence on the cell survival in any of the cell lines relative to PUFAs alone (data not shown).

Table 1 The effect of PUFAs (DHA or AA) or α -tocopherol on PpIX accumulation in human cancer cell lines^a

	Protoporphyrin IX (PpIX)/pmol mg(protein) ⁻¹			
	SW480	A-172	WiDr	A-427
Control cells	0.51 ± 0.11	0.45 ± 0.15	0.78 ± 0.01	0.91 ± 0.04
5-ALA	26.01 ± 9.69	49.15 ± 4.87	138.30 ± 12.90	18.16 ± 2.29
5-ALA-DHA	22.60 ± 4.00	58.97 ± 0.97	104.00 ± 3.10 ^b	16.39 ± 3.31
5-ALA-AA	25.10 ± 3.27	53.43 ± 2.45	126.11 ± 8.40	17.34 ± 2.04
5-ALA-Tocopherol	25.09 ± 1.51	52.57 ± 2.33	134.76 ± 12.20	46.00 ± 1.57 ^b

^a Protoporphyrin IX (PpIX) concentrations in the human cancer cell lines SW480, A-172, WiDr and A-427 after pre-incubation with DHA (10 μ M, 48 h) or AA (10 μ M, 48 h) or with α -tocopherol (50 μ M, 48 h) before 5-ALA incubation (2 mM, 3.5 h). The value is the mean \pm SD from two representative experiments, both in triplicate. The groups were compared by Student's *t*-test and ^b $p < 0.001$ was accepted as significant.

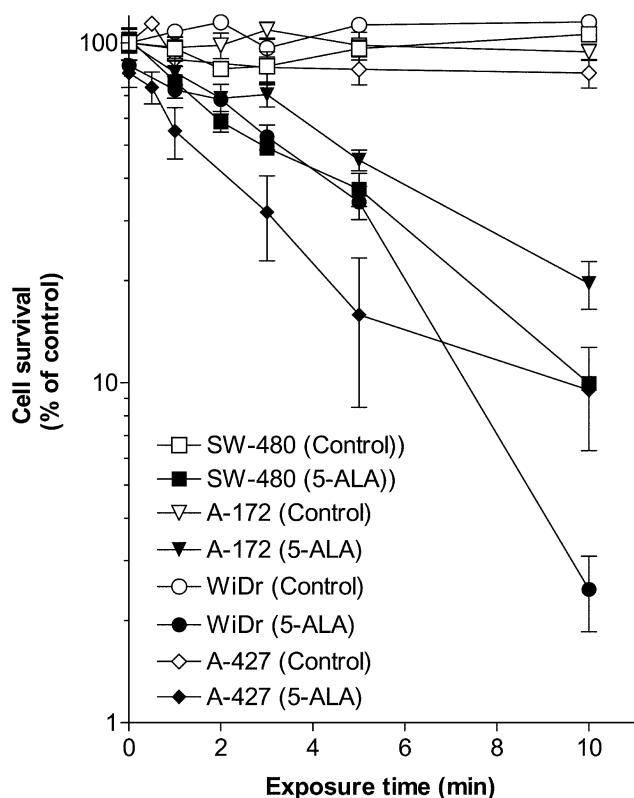


Fig. 3 Survival of the human cancer cell lines WiDr, A-172, A-427 and SW480 as a function of light treatment (0–10 min) using a Waldmann lamp (PUVA). The cells were incubated with 5-ALA (2 mM, 3.5 h) or illuminated at 20 °C before the MTT-assay. Error bars are \pm SD of minimum eight parallels in one of three representative experiments. The curves are normalized to 100% cell survival for the untreated cells.

Compared to 5-ALA-PDT alone, pre-incubation with DHA or AA prior to 5-ALA-PDT did not enhance cytotoxicity significantly in SW480 and WiDr cells. However, both DHA and AA significantly enhanced the cytotoxic effect of 5-ALA-PDT in A-172 and even more so in A-427 cells (Fig. 4). The enhancement by PUFAs of the cytotoxic effect of 5-ALA and light in A-172 and A-427 is probably mainly an additive effect. However, no clearly significant effect was seen in SW480 and WiDr cells (Fig. 4). In A-427 cells, α -tocopherol counteracted the growth-inhibitory or killing effect exerted by 5-ALA-PDT, or 5-ALA-PDT in combination with DHA, or 5-ALA-PDT in combination with AA. Only minor reversal of toxicity by α -tocopherol could be observed in the other cell lines, and this effect did not reach significance.

3.4 Effects of 5-ALA-PDT on formation of MDA after pre-incubation with PUFAs and α -tocopherol

The generation of MDA in control cells, in cells treated with PUFAs, 5-ALA, α -tocopherol and/or light is shown in Fig. 5. All

the cell lines showed increased MDA generation after incubation with DHA or AA compared to control cells. A minor effect on MDA generation was seen for A-427 after DHA-treatment, although the effect of AA was as pronounced as in the other cell lines. α -Tocopherol nearly abolished the effects of fatty acids on MDA levels in A-172 and A-427 cells, but not in WiDr (both DHA and AA) and SW480 cells (AA). Treatment with 5-ALA alone did not induce an increase in the cellular MDA level under the present conditions.

Compared to the control, 5-ALA-PDT stimulated the MDA production considerably in both A-172 and WiDr cells, but not in SW480 and A-427 cells. However, the combination of pre-treatment with DHA and 5-ALA-PDT increased the MDA production several-fold in SW480 (8.6 times), WiDr cells (6.6 times) and A-427 cells (7.9 times), but less pronounced in A-172 (2.4 times). Similarly, the effect of pre-treatment with AA and 5-ALA-PDT on MDA production was distinctly increased in the cell lines SW480 (7.2 times), WiDr (5.3 times) and A-427 (4.7 times), and less pronounced in A-172 cells (2.0 times). All the tested cell lines showed a considerable reduction in the MDA production after pre-incubation of α -tocopherol in combination with PUFAs or 5-ALA-PDT. Pre-incubation with α -tocopherol alone also reduced the MDA concentration compared to the control cells.

4 Discussion

The aim of this study was to evaluate whether PUFAs could enhance the cytotoxicity of 5-ALA based PDT on human cancer cell lines. We have used several cell lines in an effort to determine possible cell specific effects, or whether effects were general and therefore indicating a common mechanism. The effects of the *n*-6 polyunsaturated fatty acid arachidonic acid (AA) and *n*-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) have been tested on the colon adenocarcinoma cell lines SW480 and WiDr, the glioblastoma cell line A-172 and the lung carcinoma cell line A-427 in combination with 5-ALA-PDT. The effects on protoporphyrin IX (PpIX) accumulation and the production of malondialdehyde (MDA) were also investigated.

The present results demonstrate that the wide range of sensitivity of different cells to growth inhibition and cytotoxic effects of PUFAs does not correlate to the PpIX production per mg protein, nor does cytotoxicity of PUFAs correlate to MDA levels. Earlier results from Berg,³³ Gaullier *et al.*⁴ and Moan *et al.*³⁴ show that PpIX accumulation varies from cell line to cell line, and that 5-ALA concentrations higher than 1 mM do not further increase PpIX formation.³⁵ Our data also indicate that the treatment of the cell lines with 5-ALA results in different intracellular concentration of PpIX, even when all PpIX measurements were done in solutions with stable pH (pH = 2.4).^{36,37}

The influence of PUFAs on the 5-ALA-based PDT effects in human cancer cell lines has not previously been investigated. However, effects of PUFAs on proliferation of different human cancer cell lines have been documented by several

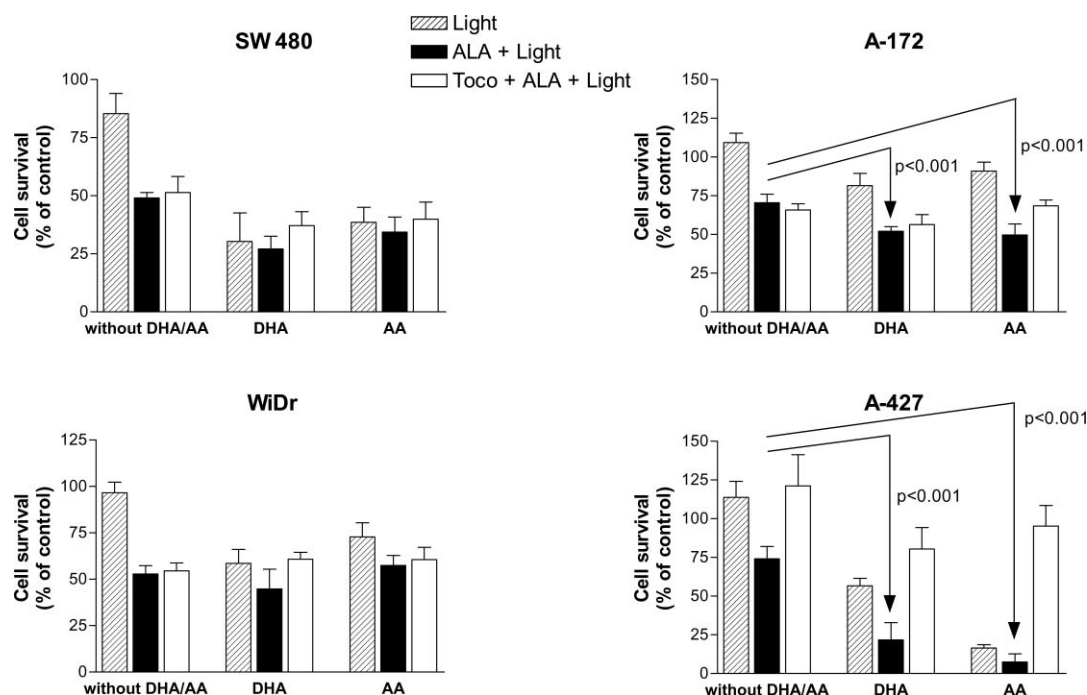


Fig. 4 The effect of 5-ALA-incubation and light treatment on the cell survival measured by the MTT-assay in the human cancer cell lines WiDr, A-172, A-427 and SW480. The cells were first cultured in the presence of docosahexaenoic acid (DHA), arachidonic acid (AA), α -tocopherol or a combination of PUFAs and α -tocopherol. The cell lines SW480, A-172 and WiDr were pre-incubated with DHA (70 μ M, 72 h) or AA (70 μ M, 72 h) or α -tocopherol (50 μ M, 72 h) before incubation in medium with or without 5-ALA (2 mM, 3.5 h) followed by illumination (3 min) when indicated, while the A-427 cells were pre-incubated with DHA (10 μ M, 48 h) or AA (10 μ M, 48 h) or α -tocopherol (50 μ M, 48 h) before 5-ALA incubation (1 mM) or illumination (0.5 min) as for the other cell lines. The groups of interest were compared using the Mann-Whitney test and indicated with arrows and p values. The groups of WiDr cells were also compared by Student's t -test and indicate the same tendency. The data are the mean value \pm SD in one of three representative experiments, all with a minimum of eight parallels.

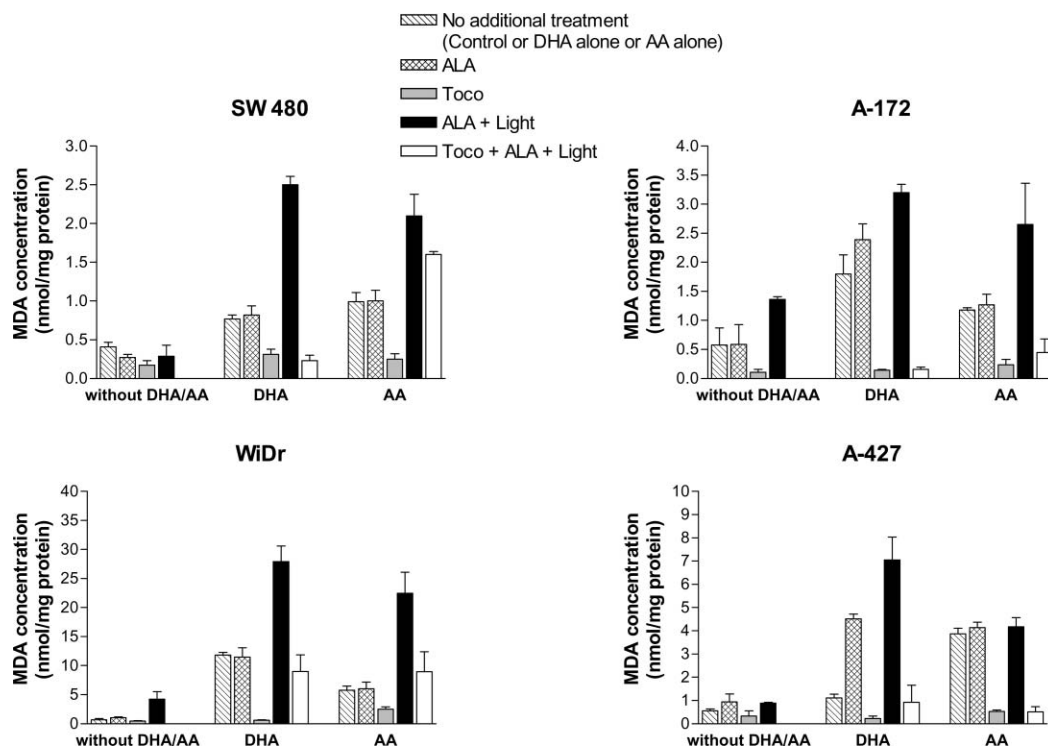


Fig. 5 Effects of 5-ALA and light on malondialdehyde (MDA) formation after treatment with docosahexaenoic acid (DHA) or arachidonic acid (AA). The MDA formation, measured by the TBARS assay, and illumination using a Waldmann lamp (PUVA) were described in 'Material and methods'. The cell lines SW480, A-172 and WiDr were pre-incubated with DHA (70 μ M, 72 h) or AA (70 μ M, 72 h) or α -tocopherol (50 μ M, 72 h) before incubation in medium with or without 5-ALA (2 mM, 3.5 h) followed by illumination (3 min) when indicated, while the A-427 cells were pre-incubated with DHA (10 μ M, 48 h) or AA (10 μ M, 48 h) or α -tocopherol (50 μ M, 48 h) before 5-ALA incubation (1 mM) or illumination (0.5 min) as for the other cell lines. The data are the mean \pm SD from three or four separate experiments.

studies.^{18,19,21,22,38,39} These agree with our results demonstrating a significant reduction in cell survival in two cell lines after treatment with DHA or AA alone and some further enhancement with 5-ALA-PDT in some cell lines. The toxicity of DHA or AA, together with 5-ALA-PDT, clearly seemed to be related to the generation of lipid peroxidation products in A-427 cells, as documented by the response to α -tocopherol. In the other cell lines, the lipid peroxidation products may have some influence, but the main effect seems to be independent of lipid peroxidation.

Generally, α -tocopherol in low concentrations (0–50 μ M) protects biosubstrates from oxidative damage. For higher concentrations (0.3–1.0 mM) the opposite has been reported by Melnikova and co-workers, who concluded that α -tocopherol becomes toxic and enhances the photo inactivation by *m*-tetra (hydroxyphenyl)chlorine (*m*-THPC) in human colon adenocarcinoma cells (HT-29).⁴⁰ In the present experiments the treatment with 50 μ M (48 h) falls within the range of α -tocopherol concentrations used in other studies for protection against oxidative damage.^{21,22,28,41,42} We observed that the antioxidant α -tocopherol reduced the lipid peroxidation, measured as MDA, in all cell lines after incubation with DHA, consistent with previous results with α -tocopherol and other antioxidants.^{21,22,27,43} However, in the present study this was not generally accompanied by reduced cytotoxicity, measured as cell growth, demonstrating that the lipid peroxidation alone is not responsible for the cytotoxicity. Previous studies have shown that some cell lines do not readily accumulate α -tocopherol in their cellular membrane.^{28,40} However, the substantial increase in the cell survival of A-427 cells after incubation with α -tocopherol together with PUFAs and 5-ALA-PDT, indicates that the lipid peroxidation is involved in cytotoxicity in this particular cell line. Processes induced by 5-ALA-PDT leading to oxidative damage increase the PpIX production in the cell lines, and our results indicate that the supplementation by α -tocopherol to 5-ALA-incubated cells (Table 1), stimulates the PpIX production significantly in A-427 cells ($p < 0.001$). The highly PUFA-sensitive cell line A-427 has a low level of selenium-dependent glutathione peroxidase activity and a weak antioxidant defence.²⁷ The α -tocopherol treatment strongly reduces the cytotoxicity and this may indirectly stimulate the PpIX production.

Although it is not clear how cells are inactivated by 5-ALA-PDT, it has been shown that apoptosis is induced in HL60 leukemic cells by 5-ALA-PDT,⁴⁴ while WiDr cells were killed only through necrotic cell death.⁴⁵ PUFAs may also induce apoptosis in some cells, e.g. the breast cancer cell lines MCF-7⁴⁶ and A-427⁴⁷ and the killing mechanism is concentration dependent.⁴⁶ Another study concluded that PUFAs inhibited cell multiplication in 11 out of 15 leukemia cell lines, and the reduction in cell number was caused by differentiation, decreased cell proliferation and/or induction of cell death.³⁸ Compared to the control, the stimulation of MDA production after 5-ALA-PDT was considerable in A-172 and WiDr cells but not in SW480 and A-427 cells. Further, the combination of DHA or AA and 5-ALA-PDT increases the MDA production more than 2-fold in all cell lines. Our results demonstrate that the effects of 5-ALA-PDT on cancer cell lines are quite complex and mechanisms other than the lipid peroxidation, or products that do not generate MDA, may be involved. Ehrenberg *et al.* concluded that photo-induced cell death may in part be caused by the damage to proteins in the cell, including membrane proteins and this may cause depolarization, loss of potassium ions and eventually cell death.⁴⁸

Further investigations may clarify whether the cell lines in our study undergo necrotic or apoptotic cell death after treatment with PUFAs and 5-ALA-PDT. Hypothetically, decomposition products other than MDA, such as hydroxy/epoxy derivatives, ketones, dimers or polymers of fatty acids, may be also formed during auto-oxidation of PUFAs after 5-ALA-PDT and these may be involved in cytotoxicity.^{49,50}

In conclusion, our studies strongly indicate that cytotoxicity from 5-ALA-PDT is not mainly caused by the lipid peroxidation, at least not in all cell lines. Furthermore, our data suggest that PUFAs may enhance the cytotoxicity of 5-ALA-PDT in some tumour cells and may therefore be of potential significance in cancer therapy.

Abbreviations

5-ALA, 5-aminolevulinic acid; PDT, photodynamic therapy; 5-ALA-PDT, photodynamic treatment based on exogenously added 5-ALA; PpIX, protoporphyrin IX; PUFAs, polyunsaturated fatty acids; DHA, docosahexaenoic acid; AA, arachidonic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric reactive substances; MTT, methylthiazoldiphenyl-tetrazoliumbromide; HPLC, high-pressure liquid chromatography; MDA, malondialdehyde; FCS, fetal calf serum.

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