Research article

In vitro AND in vivo MATRIX METALLOPROTEINASE EXPRESSION
AFTER PHOTODYNAMIC THERAPY WITH A LIPOSOMAL
FORMULATION OF AMINOLEVULINIC ACID
AND ITS METHYL ESTER

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Abstract: Photodynamic therapy (PDT) is a well-known method for the
treatment of malignant tumors, and its principles have been well established over
the past 30 years. This therapy involves the application of a chemical called
a photosensitizer and its subsequent excitation with light at the appropriate
wavelength and energy. Topical photodynamic therapy with aminolevulinic acid
(5-ALA) is an alternative therapy for many malignant processes, including non-
melanoma skin cancers such as basal-cell carcinoma (BCC). Our novel approach
for this study was to use a liposomal formulation of 5-ALA and its methyl ester
(commercially available as metvix) both in vitro and in vivo, and to check
whether the liposome-entrapped precursors of photosensitizers can induce the
expression of metalloproteinases (MMPs) in animal tumor cells and in other
tissues from tumor-bearing rats and in selected cell lines in vitro. We also
checked whether the application of tissue inhibitors of matrix metalloproteinases

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Abbreviations used: 5-ALA – aminolevulinic acid; BCC – basal cell carcinoma; CNV –
choroidal neo-vascularization; CPI – coproporphyrin I; GM-CSF – granulocyte-
macrophage colony stimulating factor; HNSCC – head and neck squamous cell carcinoma;
IL-1 – interleukin-1; met-ALA – methyl ester of aminolevulinic acid; MMPs – matrix
metalloproteinases; m-THPC – meso-tetra(hydroxyphenyl)chlorin; PDT – photodynamic
therapy; PPIX – protoporphyrin IX; ROS – reactive oxygen species, TIMPs – tissue
inhibitors of matrix metalloproteinases; VEGF – vascular endothelial growth factor
TIMPs) has any effect on MMPs in the above-mentioned experimental models, and if they can cause complete inhibition of MMP expression. Immunohistochemical studies revealed that after the PDT, the intensity of expression of MMPs in healthy animals was very low and seen in single cells only. After the PDT in tumor-bearing rats, MMP-3 was expressed in the tumor cells with the highest intensity of staining in the tissues directly adjacent to the tumors, while MMP-2 and -9 were not found. In the control groups, there was no observed expression of MMPs. In vitro studies showed that MMP-3 was expressed in MCF-7 cells after PDT, but MMP-9 was not observed and MMP-2 was only seen in single cases. Our studies confirmed that the application of an MMP-3 inhibitor may block an induction of MMP-3 expression which had previously been initiated by PDT. The preliminary data obtained from cancer patients revealed that new precursors are effective in terms of PDT, and that using MMP inhibitors should be considered as a potential enhancing factor in clinical PDT.

Key words: Photodynamic therapy, Aminolevulinic acid, Methyl aminolevulinate, Liposomes, Metalloproteinases

INTRODUCTION

Photodynamic therapy (PDT) is a well-known method of treatment of malignant tumors with principles that have been well established over the past 30 years [1]. The therapy involves the application of a chemical called a photosensitizer, and its subsequent excitation with light at the proper wavelength and energy [2-4]. Such excitation of the compound using a laser or non-coherent light results in the production of singlet oxygen and reactive oxygen species (ROS). Both singlet oxygen and ROS destroy cellular targets with a selective predilection for rapidly growing cells such as cancer cells, because photosensitizers localize in malignant cells; thus, healthy cells remain unaffected.

The mechanism of PDT is in general well known, and depends on the induction of necrosis and/or apoptosis, and on vascular and immunological alterations. Although it has been extensively studied, there are still many unanswered questions about this mechanism. The photosensitizers used in the past were mainly from the porphyrin or phthalocyanine families, and for the last 20 years, aminolevulinic acid (5-ALA) and its derivatives have also proven to be effective, mainly in topical therapy [5-7]. Since the late 80s, much effort has been made in the synthesis and application of new photosensitizers for PDT, including novel porphyrins synthesized at the University of Wroclaw [8]. These photosensitizers turned out to be very effective in both in vitro and in vivo studies. There are many enhancing compounds that have been used to increase the efficacy of local PDT, e.g. glycolic acid, azone, and liposomal formulations [9-14]. There were also different ideas used for improving systemic PDT, e.g. using systemic administration of a photosensitizer coupled to a specific antibody [15-16]. One important modification of PDT is known as photodynamic diagnostics.
PDD) where the principles are similar to those of PDT, but the parameters of the applied photosensitizers and light are different.

In this study, we examined the efficacy of liposomal formulations of aminolevulinic acid and its methyl ester, commercially known as metvix. Liposomes consist of spherical phospholipid bilayers with a number of properties that make them useful for the topical application of drugs. Liposomes can be used as carriers for hydrophilic and lipophilic therapeutic agents because they possess some amphiphilic character. Over the last two decades, liposome research has expanded, and it is now possible to form a wide range of liposomes varying in size, phospholipid content and surface characteristics to suit specific applications for which they are constructed.

They can improve the stabilization of some drugs by encapsulating them, and they can serve as penetration enhancers, facilitating the transport of compounds that otherwise cannot penetrate the skin or penetrate the skin less effectively. Liposomes reduce skin irritation by sustaining the release of drugs and by hydrating the epidermis.

5-ALA, used in this study, is a natural precursor of porphyrins, e.g. protoporphyrin IX, which is required in human cells for the synthesis of heme and subsequently hemoglobin. Previous clinical studies indicated that aminolevulinic acid (5-ALA) entrapped in liposomes improved the quality of fluorescence diagnosis using 5-ALA-induced porphyrins and optimized the results of photodynamic therapy [17].

Topical photodynamic therapy with aminolevulinic acid is an alternative therapy for many malignant processes including non-melanoma skin cancers such as basal-cell carcinoma. The major limitation of this therapy is the low permeability of 5-ALA through the stratum corneum of the skin. Therefore, many photosensitizers were tested in pre-clinical and clinical studies. For example, a liposomal formulation of meso-tetra(hydroxyphenyl)chlorine (m-THPC) was proposed for use with the aim to optimize photodynamic therapy [18]. An attempt at liposomalization of coproporphyrin I resulting in a compound with hydrophobic properties was also made in another study [19]. Oral carcinoma cells were used to evaluate chloroaluminum-phthalocyanine entrapped in liposomes as the photosensitizing agent in photodynamic therapy [20].

Liposomes were not solely used for oncological PDT. Antimicrobial photodynamic therapy is also a promising therapeutic modality for bacterial infections. To optimize the antibacterial activity of m-THPC, it was encapsulated in mixed cationic liposomes with a positive effect [21]. In another study, the objective was to characterize the ethosomal formulation of 5-ALA and to enhance the skin production of protoporphyrin IX (PPIX) [22].

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix proteins. Along with their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), they play an important role in the spreading of malignant tumors. MMPs are also thought to play a major role in angiogenesis, cell proliferation, differentiation, apoptosis and host defense.
Cytokines, growth factors, oncogenes, and reactive oxygen species are among the stimuli that activate MMP transcription. Therefore, several studies were carried out to check whether PDT induces MMP expression and whether PDT could be modified via the use of specific inhibitors of MMPs, i.e. TIMPs. Choroidal neo-vascularization (CNV) studies without previous therapy found MMP-2 and -9 in the RPE-Bruch’s membrane, vessels and stroma at different intensities. Three days after PDT with verteporfin, MMP-9 expression was found to be significantly weaker in the stroma, and at longer post-PDT intervals, a significant increase in the MMP-9 in the RPE-Bruch’s membrane, vessels and stroma was found. The MMP-2 expression was not significantly changed. Thus, it was assumed that PDT induced an early decrease in MMP-9 expression, and at longer intervals, an increase possibly associated with angiogenic processes responsible for recurrence after PDT [23].

In earlier studies, the role of photofrin-mediated PDT in eliciting the expression of MMPs and modulators of MMP activity and in the activation of vascular-endothelial growth factor (VEGF) and COX-2-derived prostaglandins was evaluated. The in vivo activity of MMPs was regulated in part by endogenous tissue inhibitors of MMPs or TIMPs. The efficacy of a synthetic MMP inhibitor, prinomastat, to enhance tumoricidal activity after PDT was examined using a mouse tumor model, and the results indicated that PDT-treated tumors have increased expression of MMPs and that pharmacological inhibition of MMPs using prinomastat could selectively increase in vivo PDT tumoricidal activity [24, 25]. Unfortunately, the results of Phase III clinical trials using MMP inhibitors performed within the last 6 to 8 years were rather disappointing.

In our study, the main aim was to use a liposomal formulation of 5-ALA and its methyl ester (commercially available as metvix) both in vitro and in vivo and to check whether liposome-entrapped precursors of photosensitizers may induce MMP expression in an animal tumor model, in other tissues from tumor-bearing rats, and in selected cell lines. In the preliminary study, we also checked whether using tissue inhibitors of matrix metalloproteinases (TIMPs) has any effect on MMPs in the above-mentioned experimental models, and whether they can cause complete inhibition of MMP expression.

MATERIALS AND METHODS

Chemicals
The phosphatidyl choline preparation Phospholipon 90 was purchased from Lipoid (Germany). All the other chemicals were from Sigma-Aldrich and were of analytical grade. All the solutions were prepared in nanopure water. TIMPs were purchased from Calbiochem-Merck, Poland, and reconstituted with distilled water prior to use and addition to the media.

Preparation of liposomes containing aminolevulinic acid or its methyl ester
The liposomes containing aminolevulinic acid or its methyl ester were prepared according to the following protocol. A weighed-out amount of phosphatidyl
Choline was placed in a glass test tube or small bulb and dissolved in a small volume of chloroform. The solvent was evaporated under a stream of nitrogen to the moment when a lipid film appeared on the tube/bulb wall. Next, drying of the sample was continued under a vacuum for at least two hours. The dried phospholipid was hydrated with an appropriate volume of 250 mM solution of aminolevulinic acid or 200 mM solution of its methyl ester in 10 mM phosphate buffer, pH 7.4. The final concentration of the phosphatidylcholine in the prepared suspension was 100 mg/ml. The suspension was intensively mixed and shaken for 10 min at 60-62°C to yield liposomes containing aminolevulinic acid or its methyl ester. The liposome dispersions were extruded through a polycarbonate membrane filter with 100 nm size pores (Corning Costar Corporation, MA, USA) with a 10-ml thermostated extruder at 60°C. The obtained preparation was dialysed exhaustively against 10 mM phosphate buffer, pH 7.4, at room temperature. Next, the content of aminolevulinic acid or its methyl ester was determined in the liposomes and in the buffer used for dialysis, using the method described by Tomokuni and Ogata [26] and Tomokuni and Ichiba [27]. We found that 15-19% of aminolevulinic acid or its methyl ester was always encapsulated in the liposomes prepared according to the above described method. The liposomes were unilamellar, homogeneous and 105-130 nm in diameter. The liposome size was determined using a Zetasizer Nano series Model ZEN 3600 (Malvern Instruments). Preparations of the liposomes were finally concentrated to the point when 5-ALA or its methyl ester achieved the required concentration (usually 110 mM). The process did not change the size of the liposomes or their stability. The properties of the concentrated liposomes were unchanged even after a four-week storage period. Preparations of free 5-ALA or its free methyl ester for topical applications were made as 20% (w/w) cream with eucerin.

**Cell lines**

We used two different, well-established and commercially available human cell lines: LoVo, a colon adenocarcinoma cell line, and MCF-7, a breast cancer cell line. The cells were set on plastic 48-well plates (10⁴ cells per well) in EMEM medium (Lonza, Belgium) supplemented with 5% fetal bovine serum (Lonza, Belgium) and with a stabilized solution of L-glutamine-penicillin-streptomycin (Sigma) in proportion of 1 ml per 100 ml of medium. Cells were cultured at 37°C in a gas phase of air with 5% CO₂.

**The concentrations of 5-ALA and its methyl ester in the cell culture medium**

For the experiments with cell cultures, one concentration of studied precursors was selected: 6.5 mM. 5-ALA and its methyl ester were prepared as a stock solution in 10 mM phosphate buffer, pH 7.4. Free or liposomal formulations of the precursors were added to the cell culture medium directly before administration to the cell-containing wells.
Immunocytochemical study of MMPs in cells

Cells were incubated without (control cells) or with free or encapsulated precursors (5-ALA or its methyl ester) for 2, 6 or 24 h. After the incubation, the medium was replaced with serum, phenol-red and precursor-free Eagle medium, and the irradiation was performed. Immediately after that, the medium was replaced again with EMEM medium without precursors, and cells were incubated for 2, 18, 24 or 48 h. In the experiments, we used two groups of control cells. The first group was cultured in DMEM medium but without any precursors. The second group was treated in the same manner as the irradiated cells but without irradiation. At that time, the control cells were kept in the dark out of the incubator in the same temperature and atmosphere as the irradiated cells. After the incubation, the cells were counted with regard to their survival rate and stained immunocytochemically for MMP-2, -3 and -9 using monoclonal antibodies (Calbiochem-Merck, Poland).

Cell survival test

The number of living cells was determined using the colorimetric sulphorhodamine B (SRB) test evaluating the amount of SRB bound by living cells. The cells were fixed with 50% trichloracetic acid and subsequently stained with a 0.4% solution of SRB in 1% acetic acid for 30 min. The unbound dye was removed by washing in 1% acetic acid and the cell protein-bound dye was extracted with 10 mM unbuffered Tris solution. The optical density of the solution was read in an Elx 800 microplate reader (Bio-Tek, Instruments Inc, Vinooski, USA) at 562 nm. All of the reagents were purchased from Sigma-Aldrich Chemie.

Another in vitro experiment involved PDT on MCF-7 cells under the same conditions as above with one exception: the cells were subjected to the action of 100 μM of the tissue inhibitor of matrix metalloproteinases for MMP-3 directly after light irradiation for 15 min. Then the cells were counted, fixed and stained for MMP-3 using immunocytochemical methods. We studied the expression of MMP-3 exclusively because it was the only metalloproteinase which was found to be induced by PDT in our experiments. Since the preliminary results of in vitro studies performed on LoVo cells did not show a significant effect of PDT on the survival rate and MMP expression, we focused on MCF-7 cells. The slides with immunostainings were assessed under a light microscope (Olympus BX40) and the positive reactions were documented using a digital camera (Olympus DP10).

Animals

We used inbred female Wistar rats, aged 3 to 4 months and with an established weight of 200 g. They were kept in plastic cages, at room temperature, and with moderate humidity according to the Guidelines for the Care and Use of Animals.
Tumor model
Mammary solid adenocarcinoma was used in all the in vivo studies. The tumor was obtained from the Institute of Oncology, Gliwice, Poland. Tumors were inoculated subcutaneously into the left abdominal region of the rat. The experiments started when the tumor had a volume of approximately 0.5 cm³ (the diameter was 1 cm and was measured using a slide caliper, and the volume = \(\frac{4}{3}\pi r^3\)), i.e. 7 days after the s.c. inoculation. In healthy rats, the analogous skin area was treated using the same protocol as for tumor-bearing animals.

Dosage of 5-ALA and its methyl ester
Liposomal formulations of 5-ALA or its methyl ester and 20% (w/w) mixtures of free 5-ALA or its methyl ester with eucerin were topically applied on the surface of shaved skin of the rats in an appropriate amount containing 0.33 or 0.66 \(\mu\)mol of the precursor, i.e. respectively 0.5 or 1.0 mg per rat. The place was then covered with a round piece of aluminum foil (11-15 mm in diameter) and a 20-mm wide adhesive tape and left for 24 h. Since the animals weighed 200 g each, the final dose of the precursor was 2.5 or 5.0 mg/kg.

Immunohistochemical study of MMPs in animal tissues
Four separate experiments were performed to investigate the influence of PDT on MMPs in the tissues of rats when the following precursors were used: 1) free 5-ALA, 2) a liposomal formulation of 5-ALA, 3) the free methyl ester of 5-ALA, and 4) a liposomal formulation of the methyl ester of 5-ALA. The rats were treated with a topical application of the precursor and a light dosage of 100 J/sq.cm. The six groups of rats were treated:
A. with 5-ALA (1 mg) or methyl ester (1 mg) without a liposomal formulation and without light;  
B. with a liposomal formulation of 5-ALA (1 mg) or methyl ester (1 mg) without light; 
C. with light only (100 J/sq.cm); 
D. with liposomes only; 
E. with liposomes and light (100 J/sq.cm); and 
F. without any procedure.
Each group consisted of 8 animals. The above experiment was repeated with healthy animals. The rats were killed at the following time points after light irradiation: 2, 24 and 48 h. Samples of the skin, tumor, kidney and liver were excised, fixed in formalin, embedded in paraffin and cut into 5 \(\mu\)m slices. Skin samples were taken from above the tumors, and this skin was exposed to light. The skin was less than 1 mm thick. The tumor samples were taken directly from underneath that skin. Then they were stained using the mouse monoclonal antibodies (Calbiochem-Merck, Poland) for MMP-2, -3 and -9 and the appropriate kit (mouse UniTect ABC, Calbiochem-Merck). We did not count all the cells expressing the MMPs in each histological preparation. Two team members who are pathologists independently evaluated whether the staining
occurred in tumor cells and not in other cells, like neutrophils, lymphocytes and macrophages, and whether the staining showed a diffuse pattern throughout the whole specimen or was limited to single cells.

**Fluorescence detection and measurement**

After the application of the above liposomal formulations of 5-ALA or its methyl ester in the amount of 0.5 or 1.0 mg on the skin of the examined healthy animals, we checked whether the PPIX fluorescence was present or not. This was done using a special detector (AccuALA) to collect fluorescence values. The device was designed and built at the Wroclaw University of Technology, Poland. It consisted of a block for exciting the photosensitizer (the source of light was a 405 nm LED), and a second block for measuring the fluorescence value after photosensitizer excitation (using light detector bandpass filters, which only allow the red light characteristic for porphyrine fluorescence to pass, and amplifiers). AccuALA compares the value of fluorescence of normal skin without a photosensitizer versus that for skin excited by the photosensitizer. **In vivo** observations (fluorescence induced by UV lamp) and measurements were performed at 2, 6, 24 and 48 h after the administration of the precursor. We used five animals per time point. We used one-way ANOVA to estimate any statistical differences between the groups. We assumed a value of > 0.05 as significant.

**Light source**

All of the irradiations were performed using a halogen lamp (Penta Lamps, Teclas, Switzerland) at an excitation wavelength of 630 +/- 20 nm selected with a bandpass filter, and a total light dose of 100 J/sq.cm in the animal studies (24 h after the administration of the precursor), and 10 J/sq.cm in the **in vitro** experiments (after 2, 6 or 24 h incubation with the precursor). All of the irradiations were performed using an irradiance of 100 mW/sq.cm.

**RESULTS**

**In vitro experiments**

Tab. 1 shows the effect of PDT on MCF-7 cells when 6.5 mM of pure or liposome-entrapped precursors (aminolevulinic acid or its methyl ester) were used. The cells were incubated with a precursor, and after its removal, were irradiated and incubated again (see the Materials and Methods section). The precursor concentrations used in the experiments were optimal for the cells. Under these conditions their viability in non-irradiated samples incubated with the precursor for 6 h and next without the precursor for 24 h was over 90%. Such results proved that the high mortality of the cells observed after irradiation was the consequence of photocytotoxic effects. Some positive results were also obtained when the incubation time after irradiation was 18 h. The effects of PDT obtained for MCF-7 cells were slightly better for metALA than for 5-ALA, particularly for its encapsulated form. Generally, the effectiveness of the pure
forms of the precursors was higher in comparison with their encapsulated forms when the incubation time with cells was 6 h or less. If that incubation time was 24 h, the results turned out to be reversed. Interestingly, the liposomal formulations after 24 h of incubation without light showed toxic effects (see Tab. 1). LoVo cells were much more resistant to PDT. The precursor concentration of 6.5 mM was then not effective. When the concentration increased twice and the incubation times with the precursor and after irradiation were 6 and 24 h, cell mortality was still low, i.e. 10-15%.

Tab. 1. The influence of PDT on the viability of MCF-7 cells with regard to the form of the precursor.

| Precursors         | 6.5 mM 5-ALA (n = 4) | 6.5 mM metALA (n = 4) | 6.5 mM 5-ALA (n = 12) | 6.5 mM metALA (n = 8) | 6.5 mM 5-ALA (n = 4) | 6.5 mM metALA (n = 4) |
|--------------------|----------------------|-----------------------|-----------------------|-----------------------|----------------------|-----------------------|
| Incubation time with precursors (h) | 2                    | 2                     | 6                     | 6                     | 6                    | 24                    |
| Incubation time after irradiation (h) | 2                    | 2                     | 24                    | 24                    | 18                   | 24                    |
| Viability of MCF-7 control cells; control* | 100 ± 5.1            | 100 ± 6.4             | 100 ± 4.2             | 100 ± 4.2             | 100 ± 2.3            | 100 ± 2.7             |
| Viability of MCF-7 cells + free precursor (% of control) | 82.2 ± 3.2           | 91.8 ± 3.1            | 92.4 ± 5.2            | 90.2 ± 3.7            | 97.6 ± 1.5           | 78.2 ± 2.2            |
| Viability of MCF-7 cells + free precursor + irradiation (% of control) | 59.2 ± 4.6           | 77 ± 4.1              | 38.3 ± 2.9            | 32.8 ± 3.0            | 39.5 ± 5.6           | 39.5 ± 3.3            |
| Viability of MCF-7 cells + encapsulated precursor (% of control) | 86.5 ± 3.7           | 76 ± 6.7              | 90.7 ± 8.8            | 91.1 ± 4.4            | 97.5 ± 1.9           | 42.2 ± 1.8            |
| Viability of MCF-7 cells + encapsulated precursor + irradiation (% of control) | 80.5 ± 2.5           | 63.8 ± 14             | 45.8 ± 3.8            | 40.1 ± 3.8            | 38.7 ± 5.8           | 16.9 ± 3.8            |

*Since there was no difference in the viability of the non-irradiated and radiated MCF-7 control cells, the data for the latter was omitted.

Fig. 1 shows the staining effect for MMP-3 in the cytoplasm of MCF-7 cells after 24 h incubation with 6.5 mM of the methyl ester of 5-ALA (liposomal formulation) and light irradiation with 10 J/sq.cm at 24 h after PDT. The staining pattern, i.e. cytoplasmic, was the same in all of the cells that revealed a positive reaction to the anti-MMP-3 antibody. We did not find MMP-3 expression in the MCF-7 cells in the control groups, e.g. in cells which were neither exposed to the precursors nor to the light (Fig. 2). We did not observe any positive post-PDT staining for MMP-9 in the MCF-7 cells and control groups. The staining for MMP-2 appeared to be very weak, and it was found in a very low number of the treated and control cells (data not shown).

Both the pure and liposomal formulations of the precursors yielded cytoplasmic expression of MMP-3 with comparable intensity. We did not observe any significant difference in the MMP-3 expression with relation to the incubation
Fig. 1. MCF-7 cells treated with a liposomal formulation of 6.5 mM of the methyl ester of 5-ALA (24 h incubation) and irradiated with light (10 J/sq.cm). The expression of MMP-3 is seen in the cytoplasm of the cells 24 h after PDT. Immunohistochemical staining, ABC method. Magn. 400x; counterstained with hematoxylin.

Fig. 2. MCF-7 cells which were neither exposed to the precursor nor to light. No MMP-3 expression was not seen in these cells. Immunohistochemical staining, ABC method. Magn. 400x; counterstained with hematoxylin.

time with the precursor, i.e. a shorter incubation time for the MCF-7 cells with the precursors did not yield a remarkably more intense immunohistochemical expression of MMP-3. No expression of MMP-3 was observed 48 h after PDT. Preliminary studies revealed that after the PDT and the application of the MMP-3 inhibitor, the survival rate of MCF-7 cells did not vary from that observed in other experiments performed without inhibitors. The survival rate was reduced to 20-30% in comparison with the control cells. The immunohistochemical stainings for MMP-3 turned out to be negative in all of the examined samples after the application of the MMP-3 inhibitor. Thus, the use of photodynamic therapy together with TIMPs prevented the tumor cells from undesired production of MMPs. Further studies are needed in order to elucidate the role of such inhibitors in photodynamic therapy in vitro and in vivo including clinical experiments on human patients.

In vivo fluorescence. The fluorescence in the skin was found to be very weak early in the progress of the experiment, i.e. directly after the application of the liposomal formulation of 5-ALA or its methyl ester, and at the next time points, 2 and 6 h, but it was noticeable. The fluorescence significantly increased at 24 h and decreased until 48 h. At 48 h, it was not observed. The values of
fluorescence in the skin after the administration of the various precursors are shown in Fig. 3. We used a special detector (AccuALA) to collect the fluorescence values. The control tissue test was performed on the skin without any photosensitizer. The maximum value of fluorescence (1.217) was obtained in the case where free 5-ALA was applied, and a slightly lower value was obtained after using the liposomal formulation of 5-ALA (1.190). The liposomal formulation of the methyl ester of 5-ALA yielded a fluorescence value at the level of 1.162. The smallest fluorescence value was obtained after using free methyl ester (1.134) and this was close to the control value. We did not see any statistical differences in the fluorescence for the six groups at the level of significance of 0.05 ($p > 0.05$).

Fig. 3. The fluorescence values for the various precursors in the skin 24 h after their application; the mean from 5 animals per time point is shown. Methyl ester of ALA – met-ALA; liposomal formulation of met-ALA – L-met-ALA; free aminolevulinic acid – 5-ALA; liposomal formulation of 5-ALA – L-ALA; skin – control (skin without any photosensitizer).

**In vivo MMP experiments**

*Healthy rats.* Immunohistochemical studies revealed that upon photodynamic therapy, MMPs were induced in healthy animals, but their expression in the cells of the irradiated tissues (inflammatory, connective tissue or endothelial cells) was extremely low. Thus, the photodynamic therapy alone could not be considered as an efficient agent for MMP induction in our study.

*Tumor-bearing animals.* Immunohistochemical studies revealed that after the application of PDT, the MMPs were expressed mainly in the tumor cells, where the intensity of staining was highest (Fig. 4, red arrows in the inset), whereas
a weak positive staining was also observed in the subcutaneous tissue directly adjacent to the tumors. In the latter location, the inflammatory cells (neutrophils, macrophages) and fibroblasts then also expressed MMPs. The strongest immunohistochemical reaction for MMP-3 was found 24 h after PDT. In the control groups, MMP expression was not observed: for example, in tumors treated with 5-ALA (or its methyl ester) alone or with light only (Fig. 5).

Fig. 4. Matrix metalloproteinase-3 expression in rat mammary solid adenocarcinoma cells 24 h after PDT with a liposomal formulation of 5 mg/kg of the methyl ester of 5-ALA and light at 100 J/sq.cm (irradiance: 100 mW/sq.cm). The inset shows the higher magnification (400x) taken from the same specimen (red arrows indicate MMP-3 within the tumor cells). Immunohistochemical staining for MMP-3; ABC method. Magn. 200x, inset 400x; counterstained with hematoxylin.

Fig. 5. Rat mammary solid adenocarcinoma from a control group. The tumor was treated with a liposomal formulation of 5 mg/kg of the methyl ester of 5-ALA without light and then this was stained for MMP-3 24 h later. The specimen shows in general an intact histological pattern. Result: no immunohistochemical staining for MMP-3 was seen 24 h after the methyl ester application. Immunohistochemical staining for MMP-3, ABC method. Magn. 200x; counterstained with hematoxylin.
We observed differences between the immunohistochemical expression of MMP-2, -3 and -9 in the tumor cells and adjacent subcutaneous tissue following photodynamic therapy. MMP-3 was strongly expressed in the tumor cells and in the skin above the tumors, whereas MMP-2 and -9 were not seen at all or were detected in single cases (see Tabs 2 and 3). The expression of MMPs in the internal organs like the kidney and liver in the tumor-bearing rats after PDT was observed in single cases only.

It has to be emphasized that we did not observe any differences in the intensity of expression of MMPs with relation to the dose of the precursor. In other words, using a 2.5 or 5.0 mg/kg dose of the precursor and subsequent treatment with light resulted in a similar expression of MMP-2 in only single tumor cells, and a more distinct expression of MMP-3 in the tumor cells and in the skin. MMP-9 expression was not observed.

Tab. 2. The expression of matrix metalloproteinases 2, 3 and 9 in an animal tumor (rat mammary solid adenocarcinoma) and in the skin from above the tumor following PDT with 5 mg/kg of four different preparations of a photosensitizer precursor (each value shows in how many of the eight rats in a group the staining was found to be positive). Measurements taken 24 h after PDT; light – 100 J/sq.cm.

|        | Tumor | Skin |
|--------|-------|------|
|        | met-ALA | L-met-ALA | 5-ALA | L-ALA | met-ALA | L-met-ALA | 5-ALA | L-ALA |
| MMP-2  | 0/8    | 2/8   | 1/8   | 1/8   | 0/8     | 0/8       | 1/8    | 0/8   |
| MMP-3  | 8/8    | 8/8   | 8/8   | 8/8   | 8/8     | 8/8       | 7/8    | 7/8   |
| MMP-9  | 0/8    | 0/8   | 0/8   | 0/8   | 0/8     | 0/8       | 0/8    | 0/8   |

Tab. 3. The expression of matrix metalloproteinases 2, 3 and 9 in two internal organs, i.e. in kidney and liver tissue taken from tumor-bearing animals (rat mammary solid adenocarcinoma) treated with PDT and 5 mg/kg of four different preparations of photosensitizer precursors (each value shows in how many of the eight rats in each group the staining was found to be positive). Measurements taken 24 h after PDT; light – 100 J/sq.cm.

|        | Kidney | Liver |
|--------|--------|-------|
|        | met-ALA | L-met-ALA | 5-ALA | L-ALA | met-ALA | L-met-ALA | 5-ALA | L-ALA |
| MMP-2  | 0/8    | 0/8   | 1/8   | 0/8   | 0/8     | 0/8       | 0/8    | 0/8   |
| 'MMP-3 | 3/8    | 0/8   | 2/8   | 1/8   | 1/8     | 1/8       | 0/8    | 0/8   |
| MMP-9  | 0/8    | 0/8   | 0/8   | 0/8   | 0/8     | 0/8       | 0/8    | 0/8   |

We did not observe any expression of MMPs after the latest time point used, i.e. 48 h after PDT, but 2 h after the therapy, MMP-3 (but not MMP-2 or MMP-9) was observed in a small number of tumor or inflammatory cells. However, the intensity of staining was lower than that found at 24 h.
DISCUSSION

Nanostructured drug delivery systems such as liposomes are a growing area in biomedical research. These microheterogeneous media can be used in many biological systems to provide appropriate drug levels with a specific biodistribution.

In our study, we examined the effectiveness of the liposomal formulation of well-known precursors of photosensitizers, i.e. aminolevulinic acid (5-ALA) and its methyl ester, in terms of photodynamic therapy. We compared their effects to those yielded by liposome-free precursors. We also evaluated the expression of matrix metalloproteinases after PDT and in control groups, both in vitro and in vivo. To the best of our knowledge this was the first such study. Therefore, we applied a novel approach, in which liposomal formulations of 5-ALA and its methyl ester were used in evaluating the expression of the above-mentioned matrix enzymes.

Both precursors are widely used in the treatment of numerous benign and malignant lesions [28-30]. The application of liposomes together with such precursors should result in a higher accumulation of the compounds in the targeted tissues and thus, in a higher efficacy of PDT. For example, two glycodendrimeric phenylporphyrins were synthesized, and their interaction with phospholipids was studied. Liposomes bearing glycodendrimeric porphyrins could form efficient carriers for drug targeting in photodynamic therapy [31]. The photophysical properties of a silicon derivative of tribenzonaphthoporphyrinrazinate (Si-tri-PcNc) incorporated into liposomes were studied and all the spectroscopic measurements performed allowed us to conclude that Si-tri-PcNc in liposomes is a promising system for PDT. The in vitro experiments with liposomal delivery systems showed that this was not toxic in darkness, but exhibited substantial photocytotoxicity for up to 80% of the cells [32]. In our studies, we observed that a liposomal formulation of 5-ALA or its methyl ester could induce significant photocytotoxic effects on MCF-7 cells, i.e. up to 60% in comparison to the control cells. Although very satisfactory, the in vitro killing ratio after PDT with liposomal formulations was not higher than that found with free precursors. Interestingly, the liposomal formulations after 24 h of incubation without light also showed toxic effects, and this was perhaps due to the prolonged time of incubation with the liposomes.

In our study, we observed that the in vivo fluorescence after the application of the liposomal formulation of 5-ALA or its methyl ester on the skin of animals was higher than that for the control group. Similarly, fluorescence measurements with different liposomes to m-THPC ratios demonstrated an increasing local m-THPC concentration, suggesting strong interactions between m-THPC molecules in the lipid bulk medium, and accumulation in the tissue [18]. Exposure to small light doses, i.e. below 50 mJ/cm², resulted in a substantial drop in fluorescence. This was attributed to photoinduced fluorescence quenching [18].
The weak fluorescence at early time points also observed in our studies could be explained by concentration quenching within the liposomes, as evidenced from the results of previously reported fluorescence polarization studies [33]. Progressive m-THPC redistribution from liposomes and its further incorporation into tumor tissue resulted in a fluorescence build-up over time with a maximum at 24 h post-injection. This correlated perfectly with the best therapeutic effect at this time point [33]. We observed in our study that the in vivo fluorescence intensity of treated skin was qualitatively higher at 6 h than that at 2 h following the local administration of liposomal formulations of 5-ALA or its methyl ester, and this increased, reaching its maximum 24 h after application. This was significantly later than in studies performed with a liposomal m-THPC formulation in HT29 human colon adenocarcinoma in NMRI nu/nu mice [34]. There the m-THPC localization was studied 2 to 8 h following the systemic administration of a photosensitizer, and the distribution within tumor and internal organs was investigated by means of e.g. in situ fluorescence imaging. For the tumor tissue, the m-THPC concentrations at 8 h were significantly higher than at 2 h [34]. In another study, the m-THPC was investigated for the first time as a gel formulation for topical application in connection with PDT of non-pigmented skin malignancies in humans. Intervals of 4 h between drug administration and light irradiation were used [35]. The liposomalization of some photosensitizers, e.g. coproporphyrin I (CPI), roughly depends on the buffers used. The CPI concentration in tumors was higher after PEG-CPI injection than after Lipo-CPI or CPI solution. The order of magnitude of CPI tumor cell uptake was PEG-CPI > Lipo-CPI > CPI solution. Thus, the PEG modification of CPI liposomes improved its tumor cell uptake [19].

Liposomes of different compositions were also designed to improve the delivery of aminolevulinic acid (5-ALA) and its esterified derivatives ALA-hexyl ester (He-ALA) and ALA-undecanoyl ester (Und-ALA) for its use in photodynamic therapy. Egg yolk phosphatidyl choline (PC), phosphatidic acid (PA) and phosphatidyl glycerol (PG) were employed in the preparation of these liposomes. In our study, in preparing precursors for in vitro and in vivo studies, we used similar substrates as other authors did with photofrin [36]. It is obvious that liposomalization may enhance phototoxicity, especially by PEG-modification, and this was significantly higher than that of photofrin in solution or photofrin in liposomes. However, photofrin-PEG-liposomes inhibited the uptake of photofrin into tumor cells [36]. In our studies, we also assessed the effectiveness of ethosomal formulation of 5-ALA, as the previous results showed that the average particle sizes of the ethosomes were less than those of liposomes, and indicated that the penetration ability of ethosomes was greater than that of liposomes [22]. Our results did not confirm these observations (data not shown).

A number of studies identified the role of matrix metalloproteinases (MMPs) in PDT. Sharwani et al. [37] observed in vitro the influence of PDT on the expression of these molecules on a series of human keratinocyte cell lines.
derived from human oral squamous cell carcinomas. Each cell line was subjected to a sublethal dose of PDT. The activities of MMP-2, -9 and -13 were evaluated at the protein level using zymography and ELISA on culture medium, and it was demonstrated that PDT causes the suppression of factors responsible for tumor invasion, which may be of therapeutic value [37]. PDT was found to be in turn responsible for increasing the expression of the anti-apoptotic and pro-angiogenic proteins such as survivin, Akt, HIF-1alpha, VEGF and MMP-2 [38]. Preclinical studies have demonstrated that head and neck squamous cell carcinomas (HNSCCs) express high levels of MMPs in vivo, and that inhibiting these enzymes in vitro and in mouse models decreases invasion and metastasis. Our in vitro and in vivo studies showed that MMP-2 and MMP-9 are not induced after PDT but that MMP-3 was induced, and was expressed in tumor cells both in vitro and in vivo. It was confirmed that three days after PDT with verteporfin, MMP-9 expression was significantly weaker in the stroma and at longer post-PDT intervals, a significant increase in MMP-9 in the RPE-Bruch’s membrane, vessels and stroma was disclosed, whereas MMP-2 expression did not significantly change. It was assumed that PDT induced an early decrease in MMP-9 expression and an increase at longer intervals [23]. In another study, it was shown that in keratinocyte supernatants, after PDT the levels of MMP-1 and MMP-3 were not significantly altered. Fibroblasts treated with keratinocyte-conditioned media after PDT showed an induction of MMP-1 and MMP-3 protein levels up to threefold in both models used, suggesting that 5-ALA-PDT modulates MMP-1 and MMP-3 production via indirect mechanisms [39]. In that study, Karrer et al. used an IL-1 antagonist to the keratinocyte-conditioned media and completely inhibited the induction of MMP-1 and -3 in the stimulated fibroblasts, suggesting that IL-1 plays an important role in these effects. Increased production of MMP-3 can also be associated with extracellular ATP administration [40]. Both MMP-2 and -9, called gelatinases, cleave collagen that has already been broken by MMP-1. Therefore, a lack of induction of MMP-2 and -9 after the PDT in our study could be regarded as a positive effect in the therapy of malignant tumors and a negative one in the resynthesis of new collagen. The increased expression of MMP-3 following PDT should be regarded as a negative effect, because many malignant tumors spread through the increase of MMP-3 production [41].

Inhibitors of MMPs, like prinomastat, were found to be active in the earliest stages of experimental choroidal neovascularization, and it was suggested to be used in combination with photodynamic therapy to inhibit the recurrence of choroidal neovascularization from temporarily closed new vessels [42]. The administration of prinomastat significantly improved the PDT-mediated tumor response.

Immunohistochemical analysis indicated that infiltrating inflammatory cells and endothelial cells were primary sources of MMP-9 expression after PDT, whereas negligible expression was observed in tumor cells, which remains in agreement with our observations on MCF-7 cells and our in vivo study in relation to MMP-9.
Our results indicate that PDT induces MMP-3 and in some cases MMP-2 \textit{in vitro} in MCF-7 cells, and that the adjunctive use of an MMP inhibitor results in a lack of MMP-3 expression. This in turn may result in a general improvement of the PDT tumor responses [24], but the clinical trials for MMP inhibitors have thus far failed to demonstrate a significant survival advantage in most cancer treatment. Perhaps the application of one or more MMP inhibitors will not result in any improvement in the PDT-mediated tumor response. Claudin-1 was found to up-regulate cancer invasion through the activation of membrane type-1 MMP (MT1-MMP) and MMP-2, which resulted in enhanced cleavage of laminin-5 gamma2 chains [43]. This was also evidenced by mouse model data and analysis of HNSCC tumor specimens which suggested that just membrane type-1 MMP (MT1-MMP) may be a critical enzyme in tumor cell invasion and survival \textit{in vivo}. Hypericin-mediated photodynamic therapy down-regulates MMP-9 expression via the inhibition of granulocyte-macrophage colony stimulating factor (GM-CSF) production, which in turn modulates AP1/NF-kappaB transcriptional activities [44]. Suppression of MMP-9 by hypericin-PDT may have therapeutic implications [44]. Altogether, this would rather indicate the applicability of selective MT1-MMP inhibitors as therapeutic agents in malignant tumors, and this should be evaluated in further PDT studies.

**SUMMARY**

We did not find significant differences between free ALA and liposomal formulations of ALA and its methyl ester. All were effective to a very similar degree. The strongest photocytotoxic effect \textit{in vitro} was found 24 h after irradiation with the liposomal formulations of 5-ALA or its methyl ester. The most frequently expressed metalloproteinase \textit{in vitro} and \textit{in vivo} was MMP-3. \textit{In vitro}, this was found 24 h after the PDT in the cytoplasm of MCF-7 cells that had been incubated with the precursors for 24 h. \textit{In vivo}, this expression of MMP-3 was mainly found in the tumor and skin after PDT. The strongest immunohistochemical reaction for MMP-3 \textit{in vivo} was found 24 h after PDT. In the control, untreated groups, the expression of MMPs was not observed; for example, in tumors treated with 5-ALA or its methyl ester alone.

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