# Optimization of 5-aminolevulinic acid derivatives-mediated photomedicine: new strategies, models, and applications

Fotinos, Nicolas

**UNIVERSITÉ DE GENÈVE**

Section des sciences pharmaceutiques

Laboratoire de pharmacie galénique
et de biopharmacie

**FACULTÉ DES SCIENCES**

Professeur Robert GURNY

Docteur Norbert LANGE

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# Optimization of 5-aminolevulinic acid derivatives-mediated photomedicine: new strategies, models, and applications

THÈSE

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par

**Nicolas FOTINOS**

de

Chavannes-près-Renens (VD)

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## ABBREVIATIONS

| | |
|---|---|
| 5-ALA | 5-aminolevulinic acid |
| 5-ALA DGME | 5-aminolevulinic diethylenglycol monomethylether ester |
| x-CP | x-carboxyporphyrin (x = 8, 7, 6, 5, 4, 2) |
| ADAG | 2-(5-ALA)-1,3-diacetylglyceride |
| ADBG | 2-(5-ALA)-1,3-dibutyrylglyceride |
| AK | actinic keratosis, solar keratosis |
| AOMM | 3-amino-3-oxohenadioic-1-methyl-6-methylester |
| BAL | 5-aminolevulinic acid butylester |
| BBB | blood brain barrier |
| BzAL | 5-aminolevulinic acid benzylester |
| CAM | chorioallantoic membrane |
| CFU | colony forming unit |
| CIN | cervical intraepithelial neoplasia |
| CIS | carcinoma *in situ* |
| CNS | central nervous system |
| CP I & III | coproporphyrin I & III (structural isomers) |
| CR | complete response |
| DGME | diethylenglycol monomethylether |
| DMSO | dimethylsulfoxide |
| EDD | embryo development day |
| EDTA | ethylenediamine tetraacetic acid |
| EPR | enhanced permeation and retention |
| FD | fluorescence diagnosis |
| GABA | γ-aminobutyric acid |
| HAL | 5-aminolevulinic acid hexylester |

| | |
|---|---|
| HPD | hematoporphyrin derivative |
| MAL | 5-aminolevulinic acid methylester |
| $N_0$ | CFU/ml in the initial bacterial suspension |
| $N_{DARK}$ | CFU/ml after incubation with the substrate in the dark |
| $N_{PDI}$ | CFU/ml after photoinactivation |
| OAL | 5-aminolevulinic acid octylester |
| PAL | 5-aminolevulinic acid pentylester |
| PAP | photoactive porphyrin |
| PBG | porphobilinogen |
| PDI | photodynamic inactivation |
| PDT | photodynamic therapy |
| PpIX | protoporphyrin IX |
| PS | photosensitizer |
| SC | stratum corneum |
| SCC | squamous cell carcinoma |
| TG | thiogylcolate |
| TSB | tryptone soya broth |
| UP I & III | uroporphyrin I & III (structural isomers) |
| WLC | white light cystoscopy |

# CHAPTER I

# Introduction

## Photodynamic Therapy and Fluorescence Diagnosis

In today's occidental society, medical cares have achieved a very high level of efficacy. Therefore, most of the formerly lethal diseases can now be treated successfully. However, effective treatments against the two major causes of death in the western world, *i.e.* cardiovascular diseases and cancer, are still missing. The treatment of tumors is in fact considered as one of the most challenging medical fields, with respect to the difficulty to target selectively cancer cells. Many drugs or treatment procedures on the market display the capacity to destroy diseased cells, but often lack selectivity leading to marked collateral damages of healthy tissues. Different strategies have been developed to deliver the anti-cancer agents directly and selectively to the tumor, including the coupling to tumor-specific antibodies, the use of an adapted carrier systems or the targeting of proteases expressed abundantly in tumor environment [1,2]. Since a few decades, one therapeutical methodology received increasing attention due to its outstanding selectivity: Photodynamic Therapy (PDT).

PDT was discovered in Germany, in the beginning of the 20$^{th}$ century. A PhD student, O. Raab, and his supervisor H. von Tappeiner studied the lethal effect of some dyes on paramecium. They discovered that light was strongly influencing the survival of the parasites incubated with acridine. A few years later, von Tappeiner observed that oxygen was also involved in this phenomenon and proposed the term "dynamic" to precise the contribution of oxygen, and to differentiate it from phototherapy [3].

PDT can be defined as followed. It is the combination of three individually non-toxic components, (i) a photosensitizing agent, termed photosensitizer (PS), (ii) light that activates the PS, and finally, (iii) oxygen that upon excitation by the activated PS will be transformed into highly toxic reactive oxygen species (ROS) [4]. The selectivity of PDT relies on a contribution of each of these parameters (Fig. 1). Firstly, the PS accumulates selectively into the target tissues due to physiological alterations in the pathological environment, like *e.g.* leaky vasculature, abnormal enzymatic activity, pH variations, or reduced lymphatic drainage. Secondly, the local irradiation of the diseased area, and finally, the very short

lifetime of the reactive oxygen species limits the damage to the target tissues by restraining a migration to the healthy surrounding tissues [5].

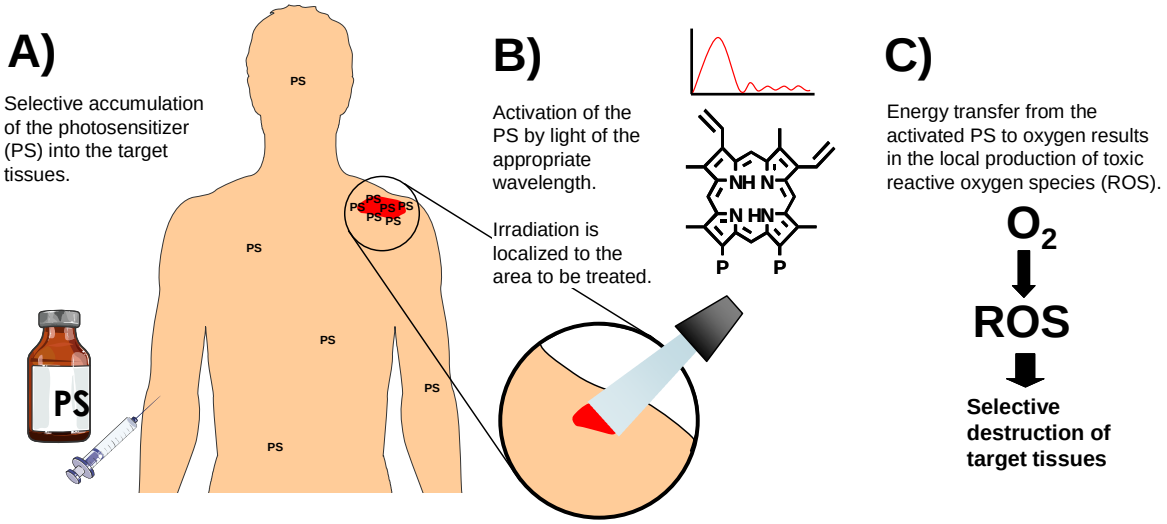


**Figure 1:** Principle of photodynamic therapy. The cumulative effect of a photosensitizer accumulated in the target tissues (A), a localized irradiation (B), and the formation of an effective, confined, toxic agent (C), is responsible for the outstanding selectivity encountered in PDT. (ROS = reactive oxygen species).

PDT raised interest due to numerous advantages. It is a non invasive technique, in contrast to surgery, and does not induce cumulative toxicity, which is a weakness of chemo- and radiotherapy. Furthermore, sensations of burning or itching, which are the most common adverse events observed during or after PDT, are usually well tolerated by the patients and do generally not require use of anesthetics.

To resume, the selectivity, repeatability, and low toxicity of PDT are relatively uncommon in oncological treatment, and are undoubtedly the strengths of this methodology. In counterpart, some drawbacks have to be mentioned, as many parameters have to be controlled for an efficient PDT treatment. Firstly, the PS has to accumulate sufficiently, selectively, and homogenously into the target tissues after administration, without inducing significant skin photosensitivity. Secondly, light should be able to reach and activate the PS deeply enough in the case of non superficial lesions. Finally, oxygen has to be present in sufficient amount within the tissues, which is not the case in some hypoxic conditions. The absence of one of these parameters will irremediably lead to the failure of the PDT treatment.

## Photosensitizers

Since the first PDT treatment with topical eosin, several breakthroughs with respect to the understanding of phototoxic mechanisms and development of optimized photosensitizers have been made [6]. In fact, PS can adopt various structures, but are generally composed of polycyclic aromatic rings (Fig. 2).

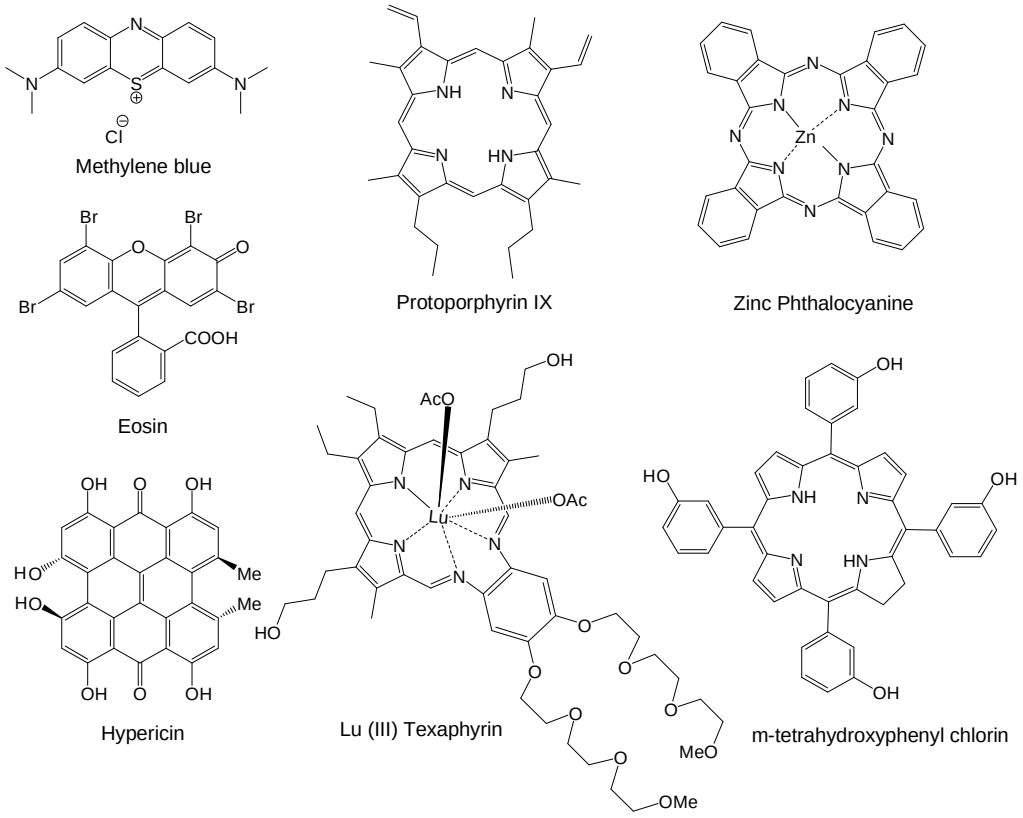


**Figure 2:** Examples of Photosensitizers. PS are generally composed of polycyclic aromatic rings, but can take various structures. Most of the clinically relevant PS are based on porphyrin (protoporphyrin IX) or expanded porphyrin (phthalocyanine, texaphyrin, chlorin) skeleton.

In this context, tetrapyrrolic macrocycles, like porphyrins or expanded porpyhrins, have shown to be extremely efficient molecules for light capture and triplet state quantum yield [7,8] and have therefore been extensively investigated in photomedicine. The first porphyrins used in PDT were inhomogeneous and badly defined mixes of different hematoporphyrins extracted from blood, that predominantly suffered from long-lasting skin photosensitization, high variability in the treatment response, unfavorable absorption spectra, and lack of selectivity [3]. Since this initial trial, numerous optimized porphyrin or porphyrin-like PS have been synthesized.

The ideal PS can be characterized by the following desired properties:

- chemically pure product, easy to synthesize
- easy administration, adapted formulation
- preferential accumulation in target tissues
- strong light absorption, ideally in the red part of spectrum (see below)
- high singlet oxygen quantum yield
- no or low dark toxicity
- no skin photosensitization
- fast elimination from the body

Plenty of newly designed PS have been patented and tested in preclinical studies, but only few of them gained marketing authorization, mainly due to the difficulty to reach all the requisites cited above.

## 5-aminolevulinic acid

In the development of new PS, apart from structural modifications of existing molecules, another strategy has attracted increasing interest; *i.e.* the induction of endogenous porphyrins by administration of porphyrin precursors. Almost every living cell synthesizes porphyrins through heme biosynthetic pathway. It was observed that exogenous administration of 5-aminolevulininc acid (5-ALA), overcomes endogenous regulation mechanisms and selectively induces an accumulation of photoactive porphyrins (PAP) within diseased cells [9] (Fig. 3). The main advantages of this method are the fast clearance of the induced PS, within 24-48 hours, and the high tolerance for this endogenous molecule. Another benefit of 5-ALA-mediated porphyrin induction is the high selectivity observed for pathological tissues, due to environmental, cellular and metabolic abnormalities in pathological tissues.

Neoplastic cells present an enhanced activity of pre-PpIX enzymes (*e.g.* PBG deaminase) and decreased activity of post-PpIX enzymes (*e.g.* ferrochelatase) compared to normal cells. This leads into a higher formation of photoactive porphyrins, and lower transformation of these PS into non-photoactive heme. The poorer iron pool measured in tumor cells amplifies this phenomenon, as the over-expression of benzodiazepine receptor, involved in the oxidation of coproporphyrinogen into protoporphyrinogen is also favorable for an accumulation of photoactive porphyrins [10].

5-ALA was rapidly promoted as clinical candidate for the fluorescence detection and the photodynamic therapy of various pathological conditions, amongst which are urology [11], pneumology [12], or brain surgery [13]. Nevertheless, 5-ALA received approval for only one dermatological application; the PDT treatment of actinic keratoses[14]. This somehow deceiving outcome can be mostly attributed to 5-ALA's low systemic and local bioavailability.

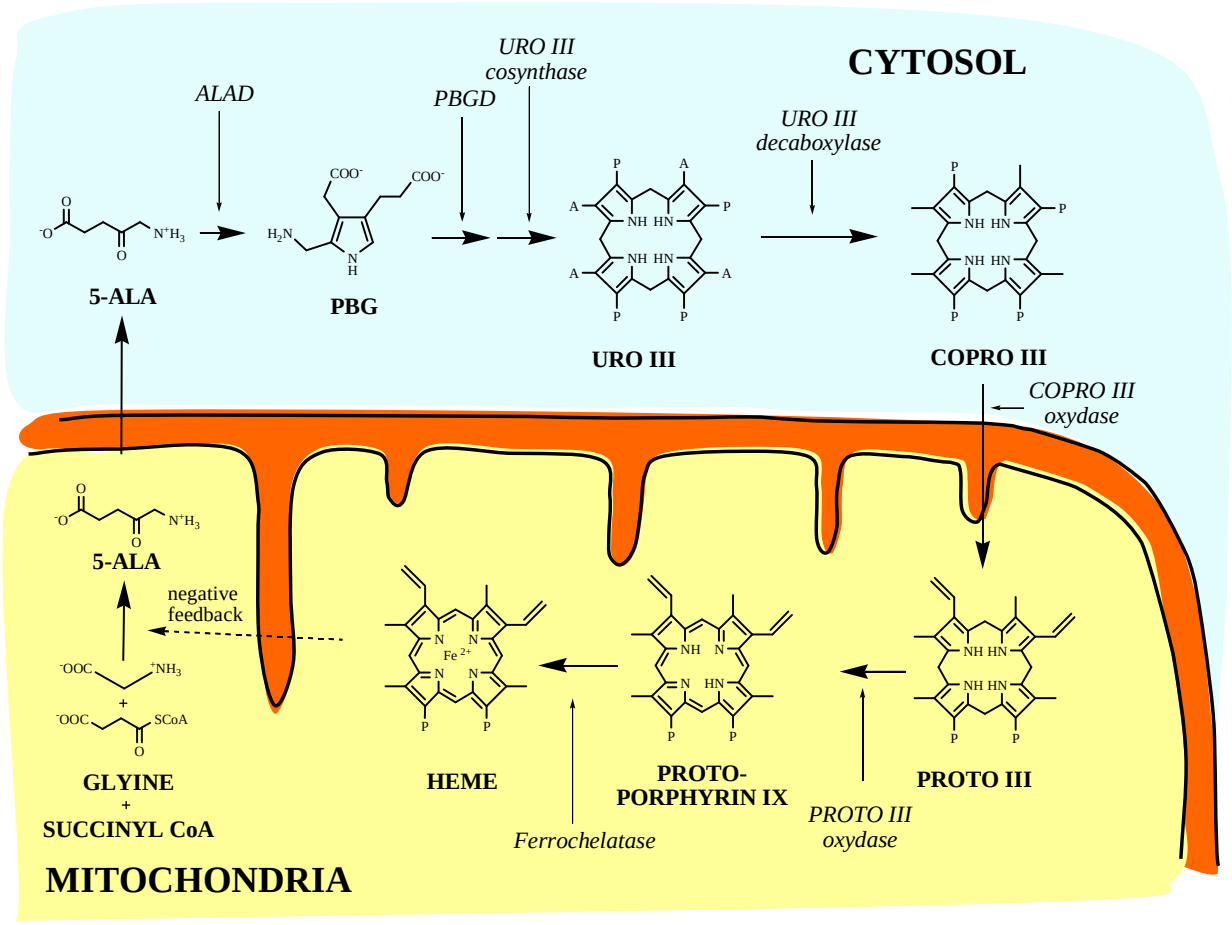


**Figure 3:** Heme biosynthesis cycle is present in almost every living cell. 5-aminolevulinic acid (5-ALA) is formed in mammals from the condensation of glycine and succinylCoA. Two molecules of 5-ALA are assembled to form porphobilinogen (PBG), and four molecules of PBG are linked to form a tetrapyrrole. After cyclization of the tetrapyrrole, uroporphyrinogen III (UROIII) undergo a series of enzymatic decarboxylations resulting into the formation of protoporphyrin IX (PpIX), the photoactive molecule desired in 5-ALA mediated PDT. The ferrochelatase, an enzyme less active in tumor cells, introduces an atom of iron into PpIX to form heme, the biologically active, but non photoactive moiety. Heme exerts a negative feedback on 5-ALA formation that is overcome by the exogenous administration of 5-ALA. (A=acetate, P=propionate)

## 5-aminolevulinic acid derivatives

The bioavailability of 5-ALA is strongly impaired by its zwitterionic nature under physiological conditions (Fig. 4), as charged hydrophilic molecules have low ability to cross biological barriers. Therefore, 5-ALA has to be internalized by active, energy consuming, transporter mechanisms [15]. In consequence, 5-ALA's low bioavailability led to the development of 5-ALA derivatives with optimized properties [16]. The derivatization of 5-ALA into more lipophilic molecules permits the passive internalization of PAP precursors, optimizing the induced porphyrin formation both *in vitro* and *in vivo* [17,18]. Apart from this enhanced bioavailability, other advantages toward the parent compound can be mentioned, *e.g.* the quasi absence of skin photosensitivity, and the less painful sensation during irradiation.

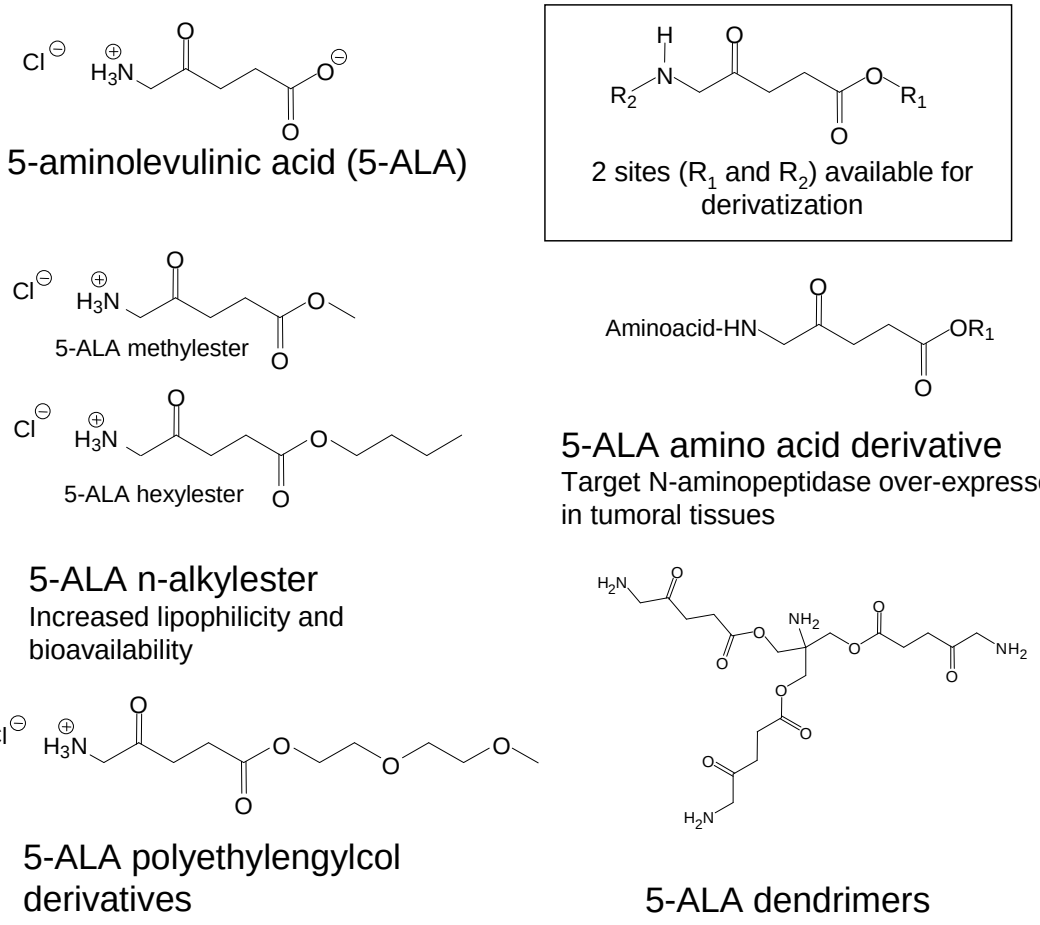


**Figure 4:** Structure of 5-ALA and some relevant 5-ALA derivatives; 5-ALA is presented here under its zwitterionic form, majority under physiological conditions. 5-ALA derivatives can be developed from site $R_1$ and/or $R_2$.

Chapter II reviews the general knowledge accumulated until today in the domain of 5-ALA derivative-mediated photomedicine. The following points are developed, (i) biochemistry of 5-ALA derivatives, (ii) topical and systemic bioavailability, (iii) chemistry and design of 5-ALA derivatives (Fig. 4), (iv) preclinical studies of the most promising compounds, and finally, (v) clinical studies with 5-ALA derivatives that achieved marketing authorization. Although considerable progress has been realized with 5-ALA derivative, some important questions remain unanswered. For example, the exact fate of 5-ALA derivatives after their entrance into the cell is still hypothetic. Furthermore the optimal derivatization strategy for a systemic administration of 5-ALA, in order to treat non superficial pathologies, has not been defined. These subjects are treated later in chapter III and IV, respectively.

## Irradiation

To obtain the most favorable PDT regimen, different parameters have to be optimized, both for the photosensitizer administration (route, time, conditions, formulation), and for the irradiation procedure. The choice of the light source, irradiation wavelength (Fig. 5), light dose and intensity are primordial for a successful PDT treatment [19,20,21]. To be exhaustive, the PDT irradiation protocol should also include drug administration-light irradiation interval, repetition of the PDT treatment, and light diffuser device (cylindrical, frontal, inflating balloon).

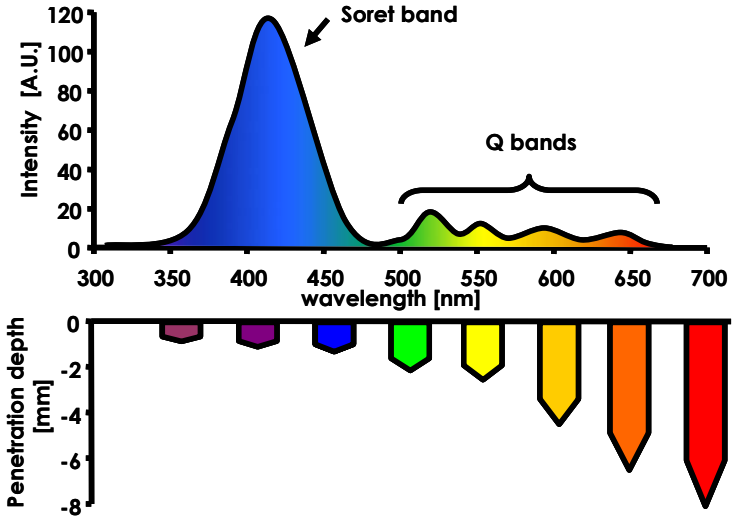


**Figure 5:** The upper part represents a classical absorption spectrum of a porphyrin, with a maximum peak (Soret band) at around 400 nm followed by four Q bands between 450 and 700 nm. The lower part shows the penetration depth of light as a function of wavelength. For optimal irradiation conditions in PDT, both parameters have to be taken into account

## Photodynamic therapy.

Subsequently to the excitation of the PS with light, two reaction mechanisms can occur (Fig. 6). While Type I reactions lead to damage through radical reactions by the activated photosensitizer generating potentially superoxydes, Type II implies the activation of oxygen molecules into highly toxic singlet oxygen. These reactive oxygen species (ROS) will exert cellular damage through oxidation process of cellular structures (like *e.g.* phospholipids, amino acids, cholesterol and nucleic acids), leading finally to cell necrosis and/or apoptosis [22,23].

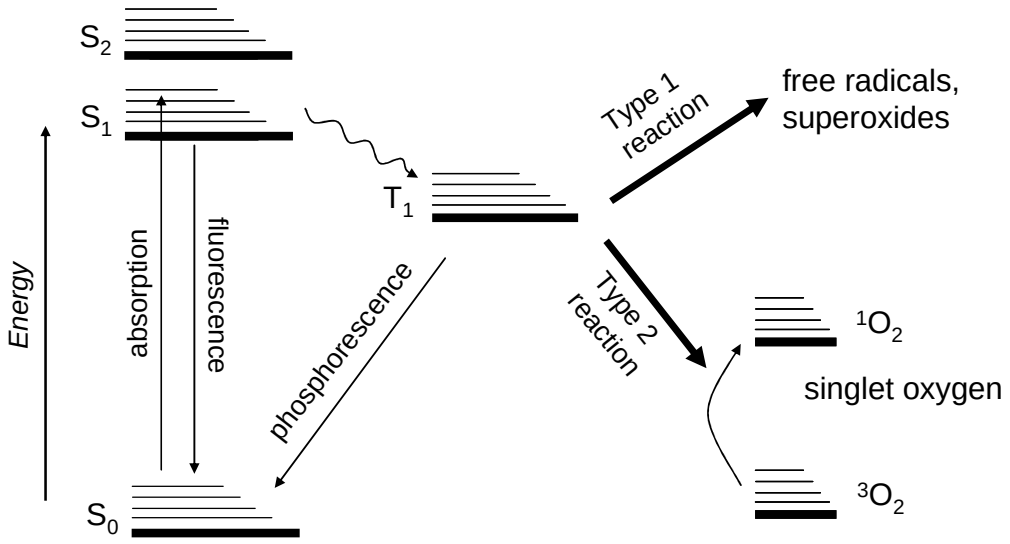


**Figure 6 :** Jablonski diagram. The photosensitizer is activated by light from its ground state $S_0$ to a high energy level ($S_1$ or $S_2$). A return to the ground state can result in the emission of fluorescence. Deactivation through an intersystem crossing into a triplet state ($T_1$) results in the generation of cellular damage through Type 1 (direct) or Type 2 (involving singlet oxygen) reactions.

Apart from these direct cellular effects, PDT is also involved in various physiological reactions [24]. PDT-mediated tumor destruction is the consequence of (i) an induced necrosis or apoptosis of tumor cells, (ii) the occlusion of tumor-associated vasculature, depriving these high demanding tissues of nutrient and oxygen supply, and finally, (iii) an activation of the immune system [25].

## Medical applications

Fluorescence detection and photodynamic therapy are used in diverse medical domains, mostly related to pathologies of the skin or hollow organs that are easily accessible to light. Dermatological pre-cancerous or cancerous disorders, like actinic keratoses [26,27,28] and basal cell carcinoma [29,30], can be successfully treated with PDT. In this context, 5-ALA methylester-mediated PDT, under the tradename Metvix® PDT, is now accepted in routine clinical practice (Fig. 7) for these indications.

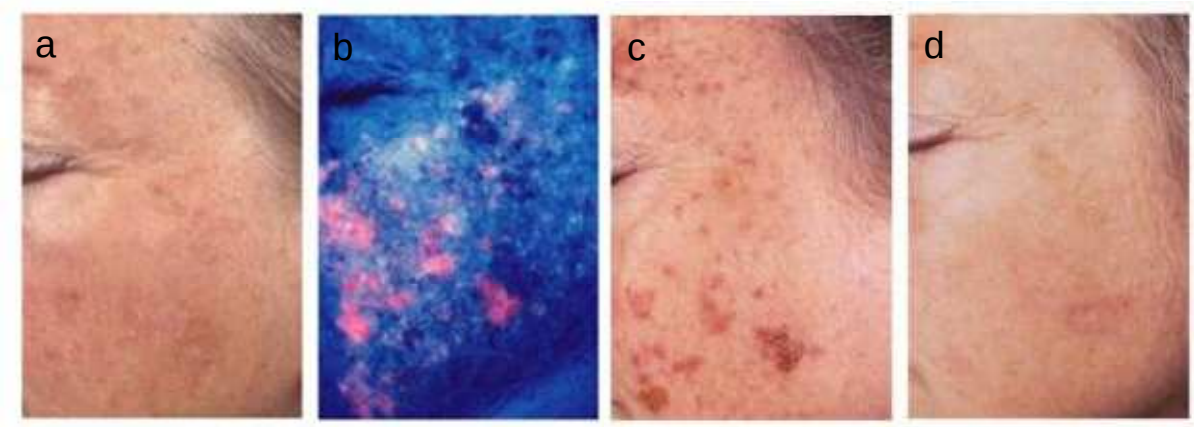


**Figure 7 :** Patient, 57 year old, diagnosed with actinic keratoses and treated with 5-ALA methylester-mediated PDT (Metvix®). a) before treatment, b) fluorescence diagnosis by the selective accumulation of induced-photoactive porphyrins in the lesions, c) 5 days after treatment, selective crusting and destruction of the lesions, d) 6 weeks after treatment, complete response to the PDT treatment and excellent cosmetic outcome. (Photos courtesy of Photocure ASA, Oslo, Norway)

Furthermore, PDT of gynecological [31,32], oesophagal [33], bronchial [34] and bladder cancers is experimentally evaluated in different preclinical or clinical trials [35]. For instance, the use of 5-ALA hexylester or Hexvix® for the enhanced fluorescence detection of bladder cancer is approved as a tool to improve surgical resection. It permits the detection of flat lesions named carcinoma *in situ*, often missed under white light cystoscopy, and responsible of recurrences.

PDT of tumors located in the brain [36,37], liver [38], and prostate [39], is more challenging as light has to be brought into the body, through special optical devices. Nevertheless, clinical trials have demonstrated their feasibility.

Although photodynamic therapy and fluorescence diagnosis are mostly mentioned in oncology, they have been tested for numerous non-oncologic medical indications, such as cutaneous abnormalities (warts, psoriasis, acne) [40,41,42], endometriosis [43], rheumatoid arthritis [44], choroidal neovascularization secondary to age related macular degeneration [45], or microbial infections [46]. The latter domain comprise the photoinactivation of viruses [47,48,49], bacteria [50,51,52,53], fungi [54,55] and parasites [56], and will be developed in chapters V and VI.

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# CHAPTER II

# 5-Aminolevulinic Acid Derivatives in Photomedicine: Characteristics, Application and Perspectives

Nicolas Fotinos, Marino A. Campo, Florence Popowycz, Robert Gurny and Norbert Lange

Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, 1211 Geneva 4, Switzerland

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## Abstract

The introduction of lipophilic derivatives of the naturally occurring heme precursor 5-aminolevulinic acid (5-ALA) into photomedicine has led to a true revival of this research area. 5-ALA-mediated photodynamic therapy (PDT) and fluorescence photodetection (FD) of neoplastic disease is probably one of the most selective cancer treatments currently known in oncology. To date, this method has been assessed experimentally for the treatment of various medical indications. However, the limited local bioavailability of 5-ALA has widely prevented its use in daily clinical practice. Although researchers were already aware of this drawback early during the development of 5-ALA-mediated PDT, only recently have well-established concepts in pharmaceutical science been adapted to investigate ways to overcome this drawback.

Recently, two derivatives of 5-ALA, methylaminolevulinate (MAL) and hexylaminolevulinate (HAL), gained marketing authorization from the regulatory offices in Europe and Australia. MAL is marketed under the trade name Metvix® for the treatment of actinic keratosis and difficult-to-treat basal cell carcinoma. HAL has recently been launched in Europe under the trade name Hexvix® to improve the detection of superficial bladder cancer.

This review will first present the fundamental concepts underlying the use of 5-ALA derivatives in PDT and FD from a chemical, biochemical and pharmaceutical point of view. Experimental evidences from preclinical data on the improvements and limits observed with 5-ALA derivatives will then be introduced. The state-of-the art from clinical studies with 5-ALA esters will be discussed, with special emphasis placed on the process that led to the development of MAL in dermatology and HAL in urology. Finally, we will discuss promising medical fields in which the use of 5-ALA derivatives might potentially lead to further application of this methodology in photomedicine.

## Introduction

After Kelly *et al.* [1] clinically demonstrated the selective accumulation of exogenously applied hematoporphyrin derivative (HPD) in human bladder cancer, photodynamic therapy (PDT) gave rise to a growing interest in the medical community. This alternative treatment modality consists of the administration of a so-called tumour localizing photosensitizer, followed by the irradiation of the target tumour site with light of an appropriate wavelength. The mechanism of PDT action has been the subject of numerous investigations [2,3]. Depending on the photosensitizing agent, light activation results in the generation of highly active reactive oxygen species that exert damaging action to cellular structures, such as the cell membrane, mitochondria, lysosomes and nuclei. After this initial photodamage, PDT then results in selective tumour eradication through a complex cascade of photochemical, immunological and physiological reactions [4]. Unlike ionizing radiation, PDT can be applied repeatedly at the same site and is characterized particularly by its minimally invasive character. Among the main advantages of PDT are its cost-effectiveness and simplicity of use. Furthermore, conventional treatment strategies, such as chemotherapy, ionizing radiation and surgery, do not preclude PDT.

In addition to the therapeutic role of PDT, the selective accumulation of photosensitizers in neoplastic tissues can be used for diagnostic purposes. In such procedures, often referred to as “fluorescence diagnosis” (FD) or “fluorescence photodetection”, sensitive imaging devices are used to permit the specific detection of the fluorescence characteristic of the given photosensitizers. The clinical use of FD and its relevant benefits in patient care have been recently reviewed by Wagnières *et al.* [5].

Despite considerable efforts of the scientific and medical community only a few photosensitizers gained marketing authorisation for use in oncological therapy. The limited clinical use of PDT in this medical area has been mostly attributed to intrinsic drawbacks of

conventional photosensitizers, such as poor selectivity, prolonged skin photosensitization, reduced absorbance in the red part of the visible spectrum and difficulties in the development of appropriate formulations. However, the fact that PDT can be a powerful tool from a therapeutic as well as a commercial point of view is demonstrated by the tremendous success of Visudyne®-mediated PDT of choroidal neovascularisation secondary to age-related macular degeneration.

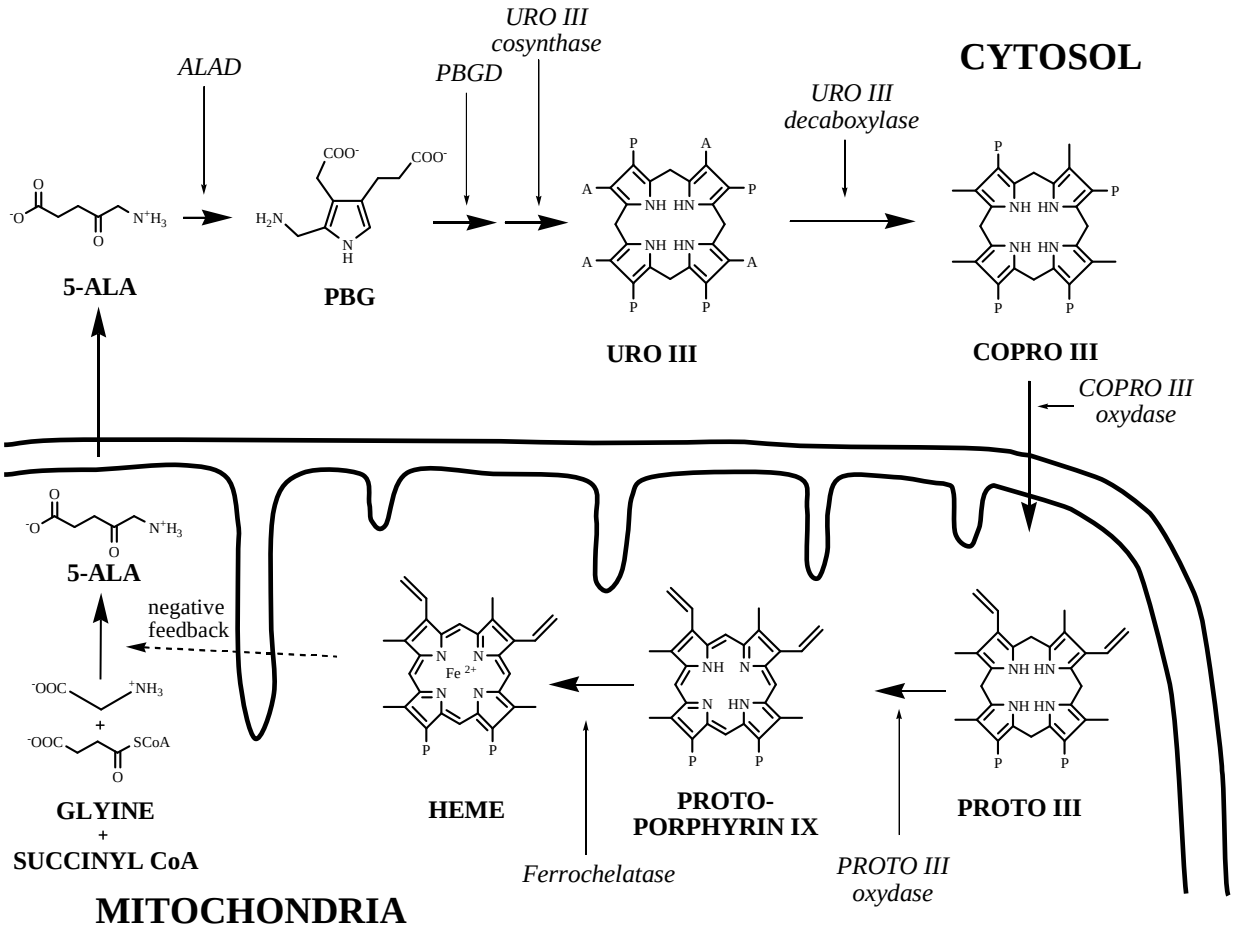



**Figure 1**: The heme biosynthetic pathway in mammals. The first enzyme 5-aminolevulinate synthase (ALAS) catalyses the conversion of glycine and succinyl CoA into one molecule of 5-aminolevulinic acid (5-ALA), which is followed by the assymetric condensation of two 5-ALA molecules by 5-aminolevulinate dehydratase (ALAD) into one molecule of porphobilinogen (PBG). An enzymatic cascade then converts four molecules of PBG into a tetrapyrrol ring that undergo decarboxylations, leading to the formation of protoporphyrin IX (PpIX). Heme is produced when the ferrochelatase inserts a ferrous iron into PpIX.

A, acetate; P, propionate; URO, uropophyrinogen; COPRO, coproporphyrinogen; PROTO, protoporphyrinogen; PBGD, PBG deaminase

An alternative approach to the direct administration of a photosensitive agent is offered by the prodrug concept. According to Albert [6] a prodrug is a nonactive compound that, after administration, is metabolically converted to pharmacologically active compound. One such prodrugs, 5-aminolevulinic acid (5-ALA), was introduced into PDT by Kennedy *et al.* in 1992 [7]. They showed that, after exogenous administration of 5-ALA in aqueous solution to patients with superficial cutaneous disorders, photosensitization with protoporphyrin IX (PpIX) is observed that is mainly confined to diseased epithelium. Indeed, both 5-ALA and PpIX are naturally occurring intermediates in heme biosynthesis taking place in nearly all aerobic cells in mammals (Fig. 1). PpIX, in contrast to heme (its iron-containing counterpart), is a photoactive substance with a singlet oxygen yield of approximately 56% [8] and a reasonable fluorescence quantum yield. Normally, heme inhibits the endogenous formation of excess 5-ALA by a negative-feedback control mechanism, thereby avoiding natural PpIX photosensitization [9,10]. However, the presence of exogenous 5-ALA bypasses this regulatory mechanism and results in the transient formation of excess PpIX that takes place preferentially in neoplastic cells. The factors underlying this phenomenon have been the subject of profound debate in the literature and have been recently reviewed by Collaud *et al.* [10]. However, it seems that apart from differences of metabolic origin in neoplastic cells, environmental and morphological factors may have an impact on the selective generation of PpIX.

Today, 5-ALA-mediated PDT can be considered as one of the most selective treatments for neoplastic disease. The growing interest in this treatment modality is reflected by an increase in the number of articles in the scientific literature (Fig. 2). In addition to its good tumor selectivity 5-ALA-induced PpIX has several supplementary advantages over conventional photosensitising agents, including limited systemic toxicity and low skin photosensitization 24-48 hours after administration [11]. Despite promising results in several medical studies of 5-ALA-mediated PDT and FD [12], these treatment modalities still lack the wide acceptance in the medical community. Today the only exception in this context is the 1999 US Food and Drug Administration approval of 5-ALA for the treatment of actinic keratosis (AK).

The failure of 5-ALA to gain marketing authorisation for the treatment or diagnosis of other medical indications can indeed be ascribed to a multitude of reasons. However, one of the main reasons relies on the physical-chemical properties of the PpIX precursor itself. Under physiological conditions more than 90% of all 5-ALA molecules are present as zwitterions and carry a positive charge at the amine terminal and a negative charge at the carboxylic terminal.

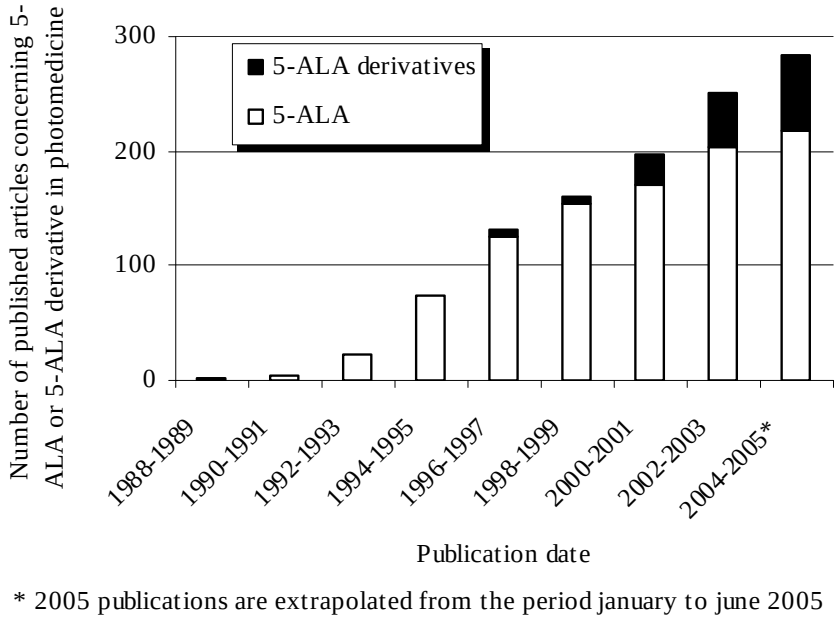



**Figure 2**: Results of a PubMed search showing the number of published articles with 5-ALA only (white) or 5-ALA derivatives (black).

Such compounds have limited capacities to reach and ultimately enter the target cell within a biological environment. This deficiency results in a low penetration depth and a nonhomogeneous distribution of 5-ALA-induced PpIX after topical application and may well explain the low response rate of 5-ALA mediated PDT of noduloulcerative basal cell carcinomas (BCCs) to 5-ALA mediated-PDT [12,13]. The limited bioavailability of 5-ALA after topical administration is even more pronounced when given parenterally. In fact 5-ALA has been shown to be rapidly eliminated from the human body, with a plasma half-life of 50 min when given intravenously and 45 min when given orally [14]. The small volume of distribution of only 8.3 L indicates that a large portion will be excreted unchanged in the urine and trapped by first-pass metabolism. Studies involving dogs revealed that > 50 % of the applied drug dose will end up in the liver and that approximately 15% will end up in the kidneys [15]. Its poor pharmacokinetic profile is therefore highly unfavourable with respect to the generation of photodynamically efficient doses of PpIX after systemic administration of 5-ALA.

Apart from its limited bioavailability the parenteral use of 5-ALA is associated with considerable adverse in humans. In addition to nausea, vomiting and transcient abnormal liver functions, significant decreases in systolic and diastolic blood and pulmonary pressure have been reported [16,17]. Because of drawbacks associated with the therapeutic use of

5-ALA, research has focused on improving local delivery of this compound. In addition to modification of 5-ALA containing formulations (Table 1) such as *i.e.* liposomal formulation methods, the use of penetration enhancers and heme biosynthesis modifying agents have been developed. Although these methods have had promising results in experiments, the implementation of these techniques into daily clinical practice has failed.

**Table 1**: Overview of methods other than derivatization that enhances 5-ALA-mediated PpIX production.

| Method | | Reference |
|---|---|---|
| Penetration enhancer | *DMSO* | Malik *et al.* [18], Casas *et al.* [19], De Rosa *et al.* [20] |
| | *Glycolic acid* | Ziolkowski *et al.* [21] |
| Iron chelator | *EDTA* | Malik *et al.* [18], De Rosa *et al.* [20], Tsai *et al.* [22] |
| | *Desfferioxamine* | Tsai *et al.* [22], Choudry *et al.* [23] |
| | *CP94* | Chang *et al.* [24], Bech *et al.* [25] |
| Vehicle formulation | *Lotion, film, ointment, gel* | Casas *et al.* [26,19], Lieb *et al.* [27], Turchiello *et al.* [28] |
| | *Liposomes* | Casas *et al.* [29], Auner *et al.* [30], Pierre *et al.* [31] |
| | *Nanoparticles* | Hürlimann *et al.* [32] |
| Physical method | *Iontophoresis* | Rhodes *et al.* [33], Lopez *et al.* [34,35] |
| | *Erbium:YAG laser* | Fang *et al.* [36] |
| | *Tape-stripping* | van den Akker *et al.* [37], Moan *et al.* [38] |
| | *Temperature* | Juzeniene *et al.* [39], van den Akker *et al.* [40] |
| | *Ultrasound* | Ma *et al.* [41] |
| | *Microdermoabrasion* | Fang *et al.* [36] |

A real breakthrough with respect to the improved production and accumulation of endogenous porphyrin, however, has been achieved by the introduction of lipophilic derivatives of 5-ALA [42,43]. Increasing the lipophilicity of a pharmacologically active compound when high hydrophilicity limits the effective delivery to the target site is a well-known concept in pharmaceutical sciences. The impact of lipophilic derivatives on 5-ALA-related research is revealed by a literature survey recently performed in our group. Figure 2 shows the number of publications on PDT-related research involving 5-ALA and its derivatives.

After the initial proposal of Kennedy *et al.* [7] the number of publications on 5-ALA-induced PpIX steadily increased until 2000. Since then the number of publication of research solely related to 5-ALA remained nearly unchanged. In contrast the constantly increasing number of publications about 5-ALA derivatives reflects the huge impact of this research area on PDT (Fig. 2). In principle the use of 5-ALA derivatives in PDT promises several advantages over 5-ALA, among which are higher generation of photoactive compounds, improved penetrations depth into deeper tissue layers, more-homogenous distribution of photoactive porphyrins, shorter application times, lower drug doses, reduced number of adverse effects and improved stability.

Since the initial proposal of increasing the lipophilicity of 5-ALA to circumvent its limited local bioavailability, several different derivatives, many of which are even somewhat exotic, have been proposed. However, most of the clinical and preclinical data available are for simple 5-ALA n-alkylesters. Two of these "5-ALA esters", methylaminolevulinate (MAL) and hexylaminolevulinate (HAL), have successfully finished multicenter phase-III trials for different diseases. MAL gained marketing authorization for the treatment of AK and BCC in Europe and Australia and HAL has been used to improve the detection of superficial bladder cancer. In addition, Hexvix® was recently approved in 27 countries in the European Union including Norway and Island. In view of this success, which has been achieved in a short period, the present review will summarize the available data on 5-ALA derivatives. We provide a brief introduction into concept underlying this methodology, followed by an extensive review on preclinical and clinical data. In addition, we discuss how concepts of controlled drug delivery can be applied to 5-ALA-PDT to further improve the specificity of this technique and treat pathological conditions that are not currently accessible.

## The biochemistry of 5-ALA and its derivatives

### Heme biosynthesis

Although it is far beyond the focus of the present review to provide a detailed overview of heme biosynthesis, knowledge of some features of this important metabolic cycle is essential to appreciate the improvements achieved by lipophilic 5-ALA derivatives. For profound explanations of heme biosynthesis and the mechanisms underlying the preferential accumulation of PpIX in neoplastic tissue after exogenous administration of 5-ALA, the reader is referred to the recent reviews of Fukuda *et al.* [44], Peng *et al.* [9], and Collaud *et al.* [10].

In addition to its function as a prosthetic group in numerous hemoproteins, such as hemoglobin, myoglobin and cytochroms, heme plays an essential role in the regulation of protein synthesis and cell differentiation [45]. Almost all nucleated cells in mammals exhibit the ability to produce heme. Heme biosynthesis is tightly regulated by various mechanisms at a cellular level. One of the most important of these mechanisms is the negative-feedback control that heme exerts on the first enzymatic step in heme biosynthesis. Indeed, heme may regulate the 5-ALA synthase-catalyzed condensation of glycine and succinyl CoA by decreasing the enzyme's mRNA half-life and/or by blocking the transport of the enzyme into the mitochondria (Fig. 1)

After entry of 5-ALA into the cytosol seven consecutive enzymatic reaction occur; four are cytosolic and the others mitochondrial. In the cytosol, 5-ALA dehydrase induces the asymmetric condensation of two molecules of 5-ALA to form porphobilinogen (PBG). Subsequently, PBG-deaminase and uroporphyrinogen cosynthase catalyse the cyclization of four PBG molecules that comprise the tetrapyrrolic skeleton of a porphyrin. Finally, a series of decarboxylations and oxidations inside the cytoplasm and the mitochondria most occur before the PpIX is formed by the protoporphyrinogen oxidases-catalyzed removal of six hydrogen atoms from the Protoporphyrinogen IX. Heme biosynthesis is then completed by the ferrochelatase-mediated insertion of ferrous iron, which takes place in the inner mitochondrial membrane.

Exogenous provision of excess 5-ALA circumvents the regulatory mechanism that heme exerts on endogenous 5-ALA formation, thus allowing for the production of heme and its intermediates at rates that are primarily limited by the activity of involved enzymes and the amount of available intracellular 5-ALA. In this context 5-ALA derivatives have the potential to increase the pool of intracellular substrate molecules, because of their modified physical chemical properties.

However, at present there is no clear experimental evidence whether molecules such as. 5-ALA esters have to be converted into 5-ALA before entering the heme biosynthesis cycle or whether they may act directly as a substrate for the enzymes involved in this pathway (see below). This is partly due to the fact that the PpIX dialkylesters or monoalkylesters that may be created have essentially the same spectral properties (Fotinos *et al.*, unpublished). Therefore, we would like to introduce the term "photoactive porphyrins" (PAP) instead of PpIX to describe 5-ALA derivative-mediated PDT and FD.

## Enhanced uptake by chemical modification

As mentioned above, enhanced lipophilicity induced by chemical modification is the most important benefit offered by 5-ALA derivatives. Because the reader of this journal might not be familiar with the underlying pharmaceutical concepts associated to this process, we will explain the fundamentals of drug absorption with respect to 5-ALA derivative-mediated photomedecine.

To exert a therapeutic effect,5-ALA or one of its derivatives must reach the intended target at a sufficient concentration and be transformed intracellularly into PAP with a high specificity and sensitivity. This process is mainly affected by the release of these compounds from the formulation in which they are introduced, subsequent transport from the application site to the directly adjacent compartment (*i.e.* absorption), transport to the deeper compartment (*i.e.* distribution), transport across cellular membranes (*i.e.* uptake), biotransformation, and its elimination from the body [46]. Together these steps determine the local bioavailability of the particular compound. The overall process can be divided into two main phases: (1) liberation of the 5-ALA derivative from it's formulation; and (2) a so-called pharmacokinetic phase, which describes the course of the drug in the body. As a result of these processes a biological response with a therapeutic effect may be obtained.

Transport through biological membranes can occur through different mechanisms, including active transport, facilitated diffusion, filtration, and passive diffusion through paracellular and transcellular pathways. Passive diffusion is the most significant transport mechanism for the majority of compounds. It is controlled primarily by the physicochemical properties of both the drug and the biological barriers (*e.g.* membranes and tissues).

According to the so-called fluid-mosaic-model, biomembranes are composed of a double layer of lipids [47]. This double layer is the result of the orientation of amphiprotic lipids (phospholipids, glycoproteins and cholesterol) in an aqueous environment. Proteins that perform different functions are present in this membrane. Interaction between membrane proteins at the contact surfaces between single cells form so called tight junctions. In most biological membranes these tight junctions are fenestrated and can be regarded as water-filled pores. The number and size of these pores depend on the type and localization of the particular biomembrane. In the small intestine tight junctions amount to approximately 0.01% of the surface area [48]. Therefore, most of the surface area of biomembranes is lipophilic and has a higher affinity for lipophilic uncharged compounds. The key characteristics that control the optimal delivery of a drug compound through the biomembrane are the molecular size and shape of the compound, its solubility, its lipophilicity, its polarity and its charge.

The movement of a compound from its formulation environment into a membrane is most commonly expressed by Fick's first law, as follows.

$$\frac{dQ}{dt} = -D \cdot A \cdot \frac{dC}{dh} \quad \text{(Eq.1)}$$

where *dQ* is the amount of the compound that diffuses during interval *dt* across area *A* under the influence of concentration gradient $dC \cdot dh^{-1}$ [49]. *D* is known as the diffusion coefficient and depends on the physicochemical properties of the membrane, the drug and the drug formulation (see below). This diffusion coefficient does not obey the familiar Stokes-Einstein equation describing the motion of spherical particles in a continuous fluid medium. Unlike Stokesian diffusion, models of membrane diffusion show an extremely sensitive dependence on molecular size [50].

To distinguish between the partition coefficient $K_m$ of the drug in its formulation environment and the membrane and its diffusivity $D_m$ in the membrane, (Eq. 1) can be modified as follows:

$$\frac{dQ}{dt} = J = \frac{K_m \cdot D_m \cdot A}{h} \cdot \Delta C_v \quad \text{(Eq.2)}$$

According to this relationship transmembrane movements can be increased by increasing the drug's diffusivity and partitioning in the membrane and/or by increasing the concentration gradient. However, in the case of 5-ALA derivatives one should consider that these compounds must reach the cytosol to exert their activity. Therefore, the particular derivative must be able to distinguish between the biomembrane and the hydrophilic cytoplasm. 5-ALA derivatives with an affinity that is too high for biomembranes may therefore be trapped in this lipophilic environment rather than enter the cytosol. It is generally accepted that the oil-water partition coefficient $P_{oil/water}$ of a compound is crucial to its ability to penetrate biomembranes. The partition coefficient *P* between a membrane and its environment is usually expressed as follows:

$$P = \frac{C_{mem}}{C_{env}} \quad \text{(Eq.3)}$$

where $C_{mem}$ and $C_{env}$ are the concentrations of the drug in the membrane and the solution, respectively, under equilibrium. The experimental determination of partition coefficient in a

biological environment is a very difficult task. Therefore, model systems simulating biological membranes have been proposed.

Although other solvent systems have been proposed for this purpose and certainly deserve attention as well, the system 1-octanol/water has been widely accepted as a relatively good predictor [51]. Additional reasons to use 1-octanol/water partition coefficients, usually expressed in their logarithmic form (log *P*), are the fact that large compilations of experimental log*P* values are available and various theoretical approaches exist to estimate log *P* values. In addition to these advantages a further practical reason to use the 1-octanol/water system is that 1-octanol, unlike many other organic solvents, is a reasonably good solvent for many organic compounds.

There is a limited number of comprehensive studies in which membrane permeability has been related to the corresponding *P*. These "structure–activity" relationships have a characteristic pattern. First, they all reveal an essentially linear region in which, according to Fick's law, the permeability increases with log *P*. Second, as log *P* becomes large the relationship reaches a plateau after which, in some cases, permeability decreases. Uehlinger *et al.* [52] have determined the log *P* values of a homologous series of 5-ALA n-alkylesters. They have shown that, by simple esterification, *P* can be varied by more than four orders of magnitude between 5-ALA (log *P* = -1.5) and 5-ALA octylester (log*P* = 2.6).

**Table 2**: Molecular weights, experimental and calculated octanol-water partition coefficient (*P*) and stratum corneum-water partition coefficient $K_{sc/w}$ for 5-ALA and 5-ALA n-alkyl esters.

| Compound | MW [g/mol] | Experimental log $P_{(o/w)}$ * | Calculated clog *P* † | Calculated xlog *P* ‡ | Experimental Log $K_{sc/w}$ § |
|---|---|---|---|---|---|
| 5-ALA | 167.6 | -1.52 | -4.08 | -2.03 | -1.37 |
| methyl- | 181.6 | -0.94 | -0.64 | -1.23 | 0.21 |
| butyl- | 223.8 | 1.42 | 0.94 | 0.12 | 0.30 |
| hexyl- | 251.8 | 1.84 | 2.00 | 1.26 | 0.91 |
| octyl- | 279.6 | 2.62 | 3.06 | 2.40 | 1.02 |

* Adapted from Uehlinger *et al.* [52]. † Calculated values for ionized species (available at: http://www.daylight.com). ‡ Calculated values for ionized species (available at: http://146.107.217.178/lab/alogps). § Adapted from De Rosa *et al.* [53]

As mentioned above, a multitude of different theoretical approaches exist to predict log *P* values. Table 2 shows the results of these calculations, as compared to experimental values of Uehlinger *et al.* [52] obtained by the shake-flask method.

Recently, Brunner *et al*. [54] screened a large variety of 5-ALA esters (see below) for their ability to induce PAP in colonic and urothelial carcinoma cell lines. In brief, human adenocarcinoma cell line HT29 and urothelial carcinoma cell line J82 were incubated with the different 5-ALA derivatives at a concentration of 0.12 mM. After 3 hours the cellular fluorescence was quantified by a fluorescence-activated cell sorter. Of interest, when plotting PAP fluorescence intensity induced by different 5-ALA esters as a function of calculated lipophilicity, a clear biphasic relationship that accords with the considerations discussed above becomes apparent (Fig. 3).

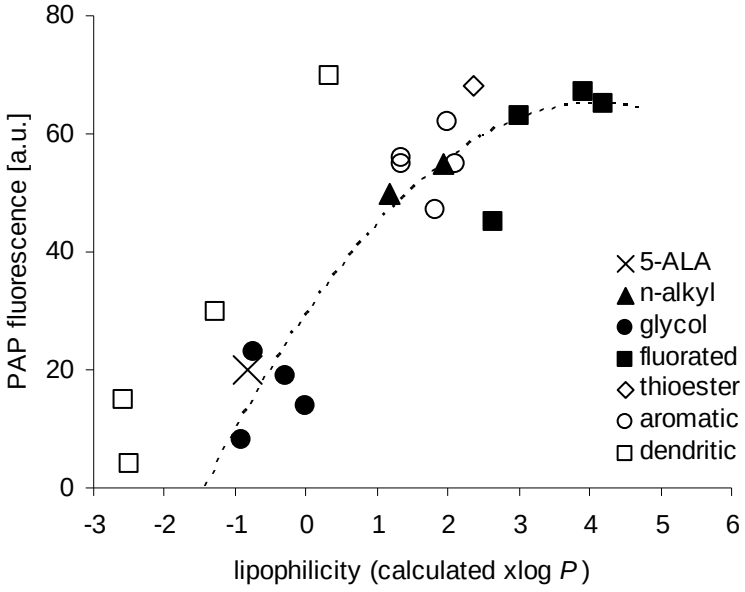



**Figure 3**: PAP fluorescence in J82 urothelial cells exposed to various 5-ALA derivatives in function of the calculated lipophilicity xlog *P* of the incubated compound [54].

However, lipophilicity seems not to be the sole characteristic responsible for the efficacy of 5-ALA derivative-induced PAP formation. It has been shown by Uehlinger *et al.* [52] that 5-ALA hexylester and 5-ALA cyclohexylester displaying essentially the same log *P* yet differ widely with respect to their activity. These results where confirmed by Whitaker *et al.* [55]

in investigations involving a series of straight-chained, branched and cyclo- 5-ALA alkylester in a pancreatic tumor cell line.

The advantage of 5-ALA derivatives over 5-ALA can be mainly attributed to the increases in the following independent processes: the rate at which these compounds reach the target site, the rate at which they reach the intracellular space and the rate of their enzymatic conversion into photoactive compounds The rates of these processes vary significantly, depending on the nature of the particular compound.

Today it is well accepted that 5-ALA gains access to the intracellular space via active-transport mechanisms that depend on the $Na^+$ and $Cl^-$ concentration [56]. Whitaker *et al.* [55] have identified dipeptide and tripeptide transporters, such as PEPT1 transporters, as potential carriers for 5-ALA. Moreover, in an epithelial cell line Döring *et al.* [57] observed that the PEPT2 transporter system appears to be involved in the transmembrane transport of 5-ALA. Despite the structural similarity of 5-ALA and γ-aminobutyric acid (GABA), 5-ALA but not GABA was found to compete with the active uptake of PEPT1 and PEPT2.

However, Berg *et al*. [56] have shown in human adenocarcinoma cell line that GABA inhibits effectively the uptake of 5-ALA and, thus, PpIX production. Furthermore, other structurally related compounds mainly transported by β system transporters impeded 5-ALA uptake. With the exception of valine, methionine and threonine, zwitterionic and basic amino acid were found to inhibit the uptake of 5-ALA.

In contrast to 5-ALA, the cellular uptake of MAL has been shown to be independent on the $Cl^-$ concentration [58]. Furthermore, the latter compound neither competes with the uptake of GABA nor inhibits the facilitated transport of 5-ALA. On the other hand the transport of MAL can be inhibited by the presence of nonpolar amino acids, such as alanine, methionine, tryptophan and glycine. Uptake of more-lipophilic compound was essentially unaffected by inhibitors of the transport of 5-ALA and MAL was the HAL. An apparent working hypothesis for this phenomenon might be that a passive diffusion or endocytosis are the main mechanisms contributing to the uptake of moderately lipophilic 5-ALA derivatives. The absence of significant resistance to the uptake of lipophilic 5-ALA esters is further supported by the observation that exposure of cells [59] or living pig bladder mucosae [60] for short periods was sufficient to maintain PAP formation over long periods. In the latter case it was shown that application durations as short as 10 min provided an intracellular pool of HAL sufficient to maintain PAP accumulation for at least 5 h. The significant differences in the cellular uptake mechanisms between 5-ALA and its esters might have an important impact on the clinical use of PAP-mediated PDT. It has been hypothesized that these differences

might be the basis for less pain during MAL-based treatment of AK, compared 5-ALA based treatment.

Another important observation with respect to the bioavailability of 5-ALA derivatives is based on some initial experiments of Kloek *et al*. [43] using Jurkat lymphoma cells. They allowed 5-ALA derivatives to penetrate the cell membrane during an incubation period (0-30 min). Then, after washing the cells, PAP synthesis was observed. These experiments showed that, when the incubation period was kept to a minimum, the differences between 5-ALA derivative and 5-ALA-induced porphyrin synthesis were maximised. These observations can mainly be attributed to the fact that 5-ALA esters enter the intracellular space without significant resistance compared to 5-ALA, thereby rapidly providing pool of PAP precursors that is sufficient enough to maintain PAP synthesis over long periods. Such short application times might be advantageous in clinical applications, such as the fluorescence photodetection of Barrett's esophagus [61], in which good contact between the drug and the mucosa can only be maintained for several minutes after topical administration.

## Bioconversion of 5-ALA derivatives

Below, it can be seen that there are nearly no limitations with the respect to the creativity of the medicinal chemist. Therefore, a nearly unlimited number of different 5-ALA derivatives can be produced that have different chemical, physical, biological and pharmacological properties. However, during the design of such compounds one should bare in mind the fundamental ideas (*i.e.* improvement of bioavailability and increased PAP formation) on which the use of these compounds in PDT and FD relies. Therefore, several considerations should be taken into account before planning the synthesis of new 5-ALA derivatives by means of concepts in rational drug design [62,63].

The tetrapyrrolic structure of porphyrins implies the cleavage of 5-ALA amides before initiation of heme biosynthesis. This aspect is supported by the experimental observation that 5-ALA amides and their esters generally fail to induce large amounts of PAP *in vitro* [43,64,65,66,67,68] and *in vivo* [68,43] in the absence of specific peptidases. Furthermore, Moan *et al.* have recently shown that an *N*-formyl 5-aminolevulinic acid derivative neither induced porphyrin synthesis nor inhibited the formation of PpIX induced by 5-ALA [69]. However, these characteristics might be advantageously used to further increase the selectivity of 5-ALA by targeting specific proteases found in abundance in some tumors [64,70,71,72]. Not only *N*-substituted 5-ALA derivatives must be cleaved, and dendrimeric 5-ALA derivatives, such

as described in the concerned section, must be able to release 5-ALA in order to be potent substrates for heme biosynthesis. Although some researchers hypothesize that the same holds true for modified 5-ALA at the C-terminal function [73,64,65,74,67], the situation is less clear for these esterified compounds from a (bio) chemical point of view.

To the best of our knowledge there is currently no direct experimental evidence that 5-ALA esters cannot act as substrate for the enzymes involved in heme biosynthesis. Knowledge of the mechanisms of 5-ALA ester-mediated PAP formation at the cellular level might, however, be mandatory for the medicinal chemist for the synthesis for more potent porphyrin precursors. The huge difficulties in elucidating differences between 5-ALA and 5-ALA derivative-induced porphyrin formation might be partly explained by the fact that there are no differences in the absorption or emission spectra between, *e.g.* PpIX or PpIX dimethyl ester (a potential PAP following exposure cells to MAL). Furthermore, Gaullier *et al.*, and Vena *et al.* have observed no differences in fluorescence localization after exposure to 5-ALA or 5-ALA esters at the cellular level [75,76].

Assuming that 5-ALA esters enter heme biosynthesis without undergoing hydrolysis, the metalloprotein 5-ALA dehydrase will be the first obstacle of such compounds towards PAP biosynthesis. As mentioned above this enzyme catalyzes the nonsymmetrical condensation of two 5-ALA molecules. Recently, Jarret *et al.* [77] showed that the initial C-N bound formation is the main pathway for the final dimerization of two 5-ALA molecules to give porphobilinogen (PBG). Indeed, sterically hindered or extremely large 5-ALA derivatives would not be able to act as substrate for this enzyme without cleavage prior to this enzyme-catalyzed reaction.

In this context, one way to test for the ability of 5-ALA esters to act as direct precursors for PBG monoester or diester would be the use of the purified enzyme and the determination of the corresponding Michaelis-Menten kinetics for various 5-ALA esters in the presence and absence of nonspecific esterases. Using TLC methods Taylor *et al.* [78] showed that exposure of 5-ALA dehydrase to a mixture of 5-ALA and 5-ALA ethylester resulted in the appearance of a second peak in chromatograms, which they attributed to the formation of PBG monoethylester. Furthermore, we have recently shown that the exposure of MAL to 5-ALA dehydrase resulted in a product susceptible to undergo a characteristic reaction with Ehrlich's reagent.

Interestingly, topical or intravenous administration of 5-ALA esters to tumour-bearing mice resulted in a greater porphyrin accumulation in skin, compared with tumor tissue. Because esterase activity in these normal tissues was found to be higher than in tumor

tissue, Tunstall *et al*. [66] concluded that 5-ALA esters have to be cleaved before inducing porphyrin synthesis. However, one should keep in mind that the skin posses a multitude of different enzymes by which topically applied drugs can be metabolized [79]. Furthermore, the findings of Tunstall *et al*. [66] do not reveal whether the *in vivo* metabolism of 5-ALA esters is dominated by hydrolysis of the ester function followed by entering heme biosynthesis, direct entry of the compound into this biosynthetic pathway or other degradation reactions. Furthermore, there might be considerable differences between the intracellular and extracellular metabolisms of these compounds.

In chemical processes, esters are often used to protect the carboxylic function of organic acids against unwanted reactions. Under normal conditions the ease of ester cleavage decreases with increasing chain-length of aliphatic alcohol substituent. As a consequence it can be assumed that once MAL is present intracellularly it will be the most efficient porphyrin precursors of a homologous series of 5-ALA n-alkylesters. Kloek *et al.* [59] tested this hypothesis in cell lysates exposed to various n-alkyl esters. As predicted the rate of 5-ALA alkylester conversion decreased with increasing chain-length up to the n-butlyester. It is interesting that longer-chained 5-ALA esters (<C8) have shown a similar activity than 5-ALA butyl ester. Furthermore, 5-ALA hexylester has shown a significantly increased porphyrin induction capacity in these experiments, indicating that there might be other mechanisms underlying the improved PAP formation after administration of long-chain 5-ALA esters.

In this context, Perotti *et al.* [80] recently analyzed the intracellular accumulation of 5-ALA and its derivatives in a LM3 murine mammary carcinoma cell line. Cells were exposed to equimolar concentrations of 5-ALA and three of its alkyl ester derivatives. Three hours following administration they determined the intracellular concentration of both 5-ALA and its corresponding derivative. In these experiments Perotti *et al*. [80] found that three times more HAL was delivered to the cytoplasm, compared with the parent compound. However, approximately 20% of HAL and 40% of 5-ALA tetrahydrofuranyl ester have been converted into 5-ALA, resulting essentially in the same amount of porphyrins under the conditions used. Finally, it has to be mentioned that MAL induced a substantially different profile of porphyrins in gram negative bacteria [81].

In conclusion, although the knowledge of the bioconversion of 5-ALA derivatives is of fundamental interest for the medicinal chemists for further improvements in this field, this area remains largely unexplored.

## Bioavailability and local Bioavailability of 5-ALA derivatives

In addition to the enzymatic conversion of 5-ALA or one of its derivatives into porphyrins within the cells, the efficacy of this treatment modality largely depends on the local concentration of the particular compound at the target site (*i.e.* on its ability to penetrate the tissue and reach the lesion). The resulting local concentration after topical administration depends on characteristics such as drug permeability and diffusion coefficients in the tissue, which are in turn functions of drug structure, formulation, drug release, and local drug clearance.

Despite the recent approvals of MAL for the photodynamic treatment of skin conditions and HAL for diagnostic purposes in urology [82,83,74], the ability of 5-ALA derivatives to induce more porphyrins after topical administration *in vivo* still remains controversial. In cell culture several 5-ALA derivatives have been shown to increase PAP formation, compared to the parent compound, even when they are applied at significantly lower concentrations [43,52,54]. As such, the magnitude of porphyrin production and the optimal substrate concentration depended on the used cell lines that were used. Furthermore, some 5-ALA esters have been shown to induce higher PAP levels when applied topically to a pig bladder model that had intact morphologic architecture [84]. This is in agreement with clinical studies [73,74] showing the superiority of HAL over the parent compound with respect to porphyrin formation and drug penetration.

Using an alcoholic solution of 5-ALA ethyl ester, 5-ALA butylester and 5-ALA (hydroxymethyl)tetrahydrofuranly ester (10% wt/vol), Kloek *et al.* [43] showed that PAP formation increased between two- and three-fold *in vivo*, compared with 5-ALA, 6 hours after topical administration. This is in agreement with results of a later study in which higher PAP levels were observed 14 hours after of nude mice were exposed to creams containing short-chained 5-ALA esters. However, results obtained after topical administration to the skin *in vivo* as well as *in vitro* are somewhat contradictory. In this context other investigators observed that MAL applied to the skin of nude mice for 24 hours induced lower porphyrin levels with a delayed $t_{max}$ compared with that for 5-ALA under the same conditions [85].

In a more recent study, when 5-ALA and its hexylester were formulated in a standard, lipophilic ointment similar porphyrin levels were observed when applied to the skin of nude mice for 24 h or 10 min after removal of the stratum corneum [86]. In this study, short term application, however, resulted in lower fluorescence intensities for HAL than for 5-ALA. These results have been confirmed under similar experimental conditions with 5-ALA methyl, hexyl, octyl, and diethylenglycol monomethylether ester [87,88]. In hairless mouse skin with

UVB-induced early skin cancer, the pentyl ester derivative of 5-ALA induced slightly more PAPs than did 5-ALA when formulated in a hydrogel. However, fluorescence microscopy revealed no significant difference in the displastic layers of the epidermis. The biodistribution measured by fluorescence intensities after topical administration of 5-ALA and MAL in human AK have been compared [89]. Although PAP formation was found to be more efficient with 5-ALA, the selectivity for the diseased tissue was higher with MAL.

As discussed above, these somewhat confusing results may be mainly attributed to the disregard of two basic principles in drug delivery: the role of biological barriers (in the case of the skin, the stratum corneum [SC]), and the important role of the vehicle for the optimized delivery to the target site. Therefore, unambiguous conclusions cannot be deduced. However, it can be concluded from studies in which the SC was removed by either shaving [43] or tape stripping [86,37,90] that 5-ALA esters were at least as efficient as 5-ALA. On the other hand, without pretreatment preparation of the application area of long-chained 5-ALA esters, a significant lag time before the onset of the porphyrin fluorescence signal can be observed [86,88,53]. This lag time is characteristic for the “reservoir function” of the SC for lipophilic compounds [91].

Recently, the partition coefficient between the SC and water ($K_{sc/w}$) was determined by de Rosa *et al.* [53] (see Table 2). In this study, the log $K_{sc/w}$ of 5-ALA esters was significantly higher than the log $K_{sc/w}$ of 5-ALA. The highest measurable log $K_{sc/w}$ was approximately 1 for HAL and 5-ALA octylester. In the same study an oil-in-water emulsion containing different 5-ALA derivatives (2% w/w) was used to determine the penetration of the compounds into different compartments of dermatomed skin of mice. In contrast to most other studies using porphyrin fluorescence as indirect measure to assess 5-ALA uptake, this study directly quantified the active compound by means of precolumn derivatization fluorescence HPLC [92,93]. It was shown that the movement of 5-ALA across full thickness skin tissue remained nearly constant over 12 h. However, the movement of HAL constantly increased in a sigmoidal manner over the same period. After 6 h the flux of HAL was significantly higher than for 5-ALA. The movements of other esters tested in this study were substantially lower, but had the same sigmoidal flux-time profile than 5-ALA hexylester.

This might indicate differences in permeation mechanisms between 5-ALA esters and the parent compound. The permeation of 5-ALA octylester through the skin was found to be significantly less than that of the hexylester derivative, both differing only slightly in their $K_{sc/w}$ values. However, a low permeation across the skin does not necessarily mean low penetration into the skin. The increased lipophilicity of 5-ALA esters might decrease the partition between the lipophilic environment of the skin and the hydrophilic environment of

the receptor compartment (*in vivo*, the blood stream). Such a situation would result in the accumulation of moderately lipophilic compounds in the stratum corneum, epidermis and dermis. This has been shown by de Rosa *et al.* [53], who reported that retention of HAL and 5-ALA octylester in the epidermis and the dermis was 2.5-3 times higher than that of 5-ALA. The results of this study are in slight disagreement with a similar study that was performed by van den Akker *et al.* [90], who showed less permeation of HAL through mouse skin. However, in this study significantly higher drug doses and a different formulation were used. Furthermore, the pH was significantly higher thus, further increasing the lipophilicity of the compounds. Finally, an aqueous solution has been employed in the study performed by de Rosa *et al.*, revealing the important impact of the vehicle in the controlled drug delivery of 5-ALA and its derivatives. Indeed, optimization of the vehicle is a crucial step for transdermal and dermatological drug delivery. The appropriate choice of the formulation not only involves delivery of the compound to the target tissue but also maximizes therapeutic efficacy by considering the etiology of the disease and the mechanism of action of the drug. In this context only few studies have addressed this optimized formulation of lipophilic 5-ALA derivatives.

From a very simplified point of view, one has to maximize the partition coefficient $K_{m/veh}$ (membrane/vehicle) in order to maximize the flux of a particular compound from the vehicle into the tissue. For relatively lipophilic compounds such as HAL an aqueous solution might be the optimal choice [73]. From a practical point of view, the intravesical administration of an aqueous solution to the bladder presents no major problem. However, compounds that are too lipophilic might tend to precipitate in an aqueous environment, thereby impeding the tissular uptake of the active ingredient. Furthermore, in other medical indications where topical administration is desired, such as dermatology or gynaecology, a liquid might not fully guarantee the retention of the compound at the target site. Recently, we have shown, that the release of 5-ALA derivatives from a hydrogel into a lipid matrix increases with increasing lipophilicity [94]. Furthermore, release nearly vanished after use of standard lipophilic ointments such as unguentum or a “cold cream” formulation [87,66,95,37,96].

Another factor strongly influencing the release profiles of a topically applied compound is its localization within the vehicle. For example, in oil-in-water emulsions more-lipophilic compounds such as HAL will tend to dissolve in the internal phase, whereas hydrophilic compounds such as 5-ALA or MAL will dissolve preferentially in the external phase. Thus, it will take more time for lipophilic 5-ALA esters to migrate to the interface between vehicle and tissue, resulting in substantially different release profiles than observed for the parent compound under the same conditions [53].

Batlle *et al.* has expended much effort to optimize formulations for 5-ALA esters [97,98,29,19]. Cell culture experiments showed no increased porphyrin buildup in murine tumor cells with liposomal formulation of HAL, compared with 5-ALA [29]. In another report [97], they sowed that a lotion containing DMSO and ethanol was favourable with respect to PpIX buildup when using 5-ALA, whereas a cream seemed to be more advantageous for topical administration of HAL. Experiments on tumor-bearing mice showed no significant differences between 5-ALA and HAL-mediated porphyrin formation with these optimized formulations. In a subsequent study [98], a lotion was identified to be optimal for the delivery of HAL to the skin. In tumor bearing-mice this formulation led to the induction of substantially higher PAP levels compared with the cream formulation. Furthermore, fluorescence surpassed that induced by 5-ALA 5 h after administration.

Recently, it was shown that the addition of penetration enhancers, such as ibuprofen acid or medium-chain triglycerides, to hydrophilic formulations increases the penetration of 5-ALA butlyester through the SC by a factor of 10 [99,100]. A liposomal formulation of HAL, however, failed to increase the porphyrin formation in murine tumor cells, compared to liposomal 5-ALA [29]. In contrast, a liposomal formulation of 5-ALA substantially increased its systemic bioavailability, compared with an aqueous solution.

Another important factor that potentially influences the release of the drug and its permeation through biological membranes is its physical appearance (*i.e.* state) within the delivery vehicle. Using Fourier-transform Infrared spectral measurements, Chen *et al.* [101] have found that HAL in solution exists principally in its monomeric form whereas 5-ALA, similar to other organic acids, is present as a mixture of monomers and dimers.

In addition to penetration enhancers, iontophoresis has been proposed to further increase the bioavailability of 5-ALA derivatives after topical administration. Because 5-ALA esters have a positive charge at the *N*-terminal under physiological conditions, they are supposed to be transported by electromigration through the SC. It has been demonstrated that 50 times more MAL and 15 times more HAL can be transported through the skin than 5-ALA by iontophoresis [102]. Furthermore, Gerscher *et al.* [103,104] have used iontophoretic methods to administer 5-ALA, 5-ALA butyl ester, 5-ALA pentyl ester, and HAL to healthy volunteers. In agreement with the findings of van den Akker *et al.* [86] they found that HAL-mediated PAP fluorescence peaked earlier compared to 5-ALA-induced PpIX under similar conditions. Furthermore, HAL was found to be slightly more phototoxic and more homogenously distributed than 5-ALA. A more homogenous distribution of PAP fluorescence after exposure to HAL, as compared to 5-ALA, was also reported by other authors after *in*

*vitro* and *in vivo* experiments [84,73,53,105]. In these experiments HAL was formulated in an aqueous solution or in a rather hydrophilic vehicle.

On the basis of the considerations mentioned above it can be deduced that, apart from an optimal concentration (see below), 5-ALA derivatives have to be formulated with respect to their lipophilicity, their physicochemical properties and the specific medical application.

Another important difference between 5-ALA and 5-ALA derivatives in terms of bioavailability is the systemic uptake of these compounds after topical administration. Several studies involving topical 5-ALA application to mice have shown that PpIX production is not limited to the administration area [42,85,19,67,95,37,106]. Substantial PpIX fluorescence was found not only in the flank colateral to application site, but also in other body compartments, such as blood, liver, spleen and kidneys [26] and liver, intestinal tract and lungs [106]. Topical administration of 5-ALA for 24 hours to nude mice showed fluorescence intensities at other sites (including the ears and the collateral site, but lower in the liver, muscle and brain) that were similar to fluorescence intensities at the application site. Although 5-ALA methyl, ethyl, propyl or hexyl ester can induce similar or higher fluorescence levels at the application sites of nude mice, such systemic effects were not observed. This can presumably be attributed to differences in metabolic degradation (de Rosa *et al.*)[53], binding to plasma proteins and limited capacity to overcome the endothelial layers.

## Chemistry and Design of 5-ALA derivatives

### 5-ALA alkylester

The simpliest way to modify the lipophilicity of an organic acid is the derivatization with various alkyl alcohols into the corresponding ester derivative. Generally, 5-ALA esters are prepared using standard reactions for esterification between 5-ALA and the corresponding alcohol in the presence of thionylchloride or hydrochloric acid [43] (Fig. 4). In this way several linear (**compounds 2-9**), branched (**10-18**), cyclic (**19-32**) as well as ethylenglycol derivatives (**33-38**) have been prepared. However, in some cases it is advantageous to use tert-butylcarbonyl (BOC) chemistry to prevent cross reactivity when using carbodiimide coupling to activate the carboxylic function.

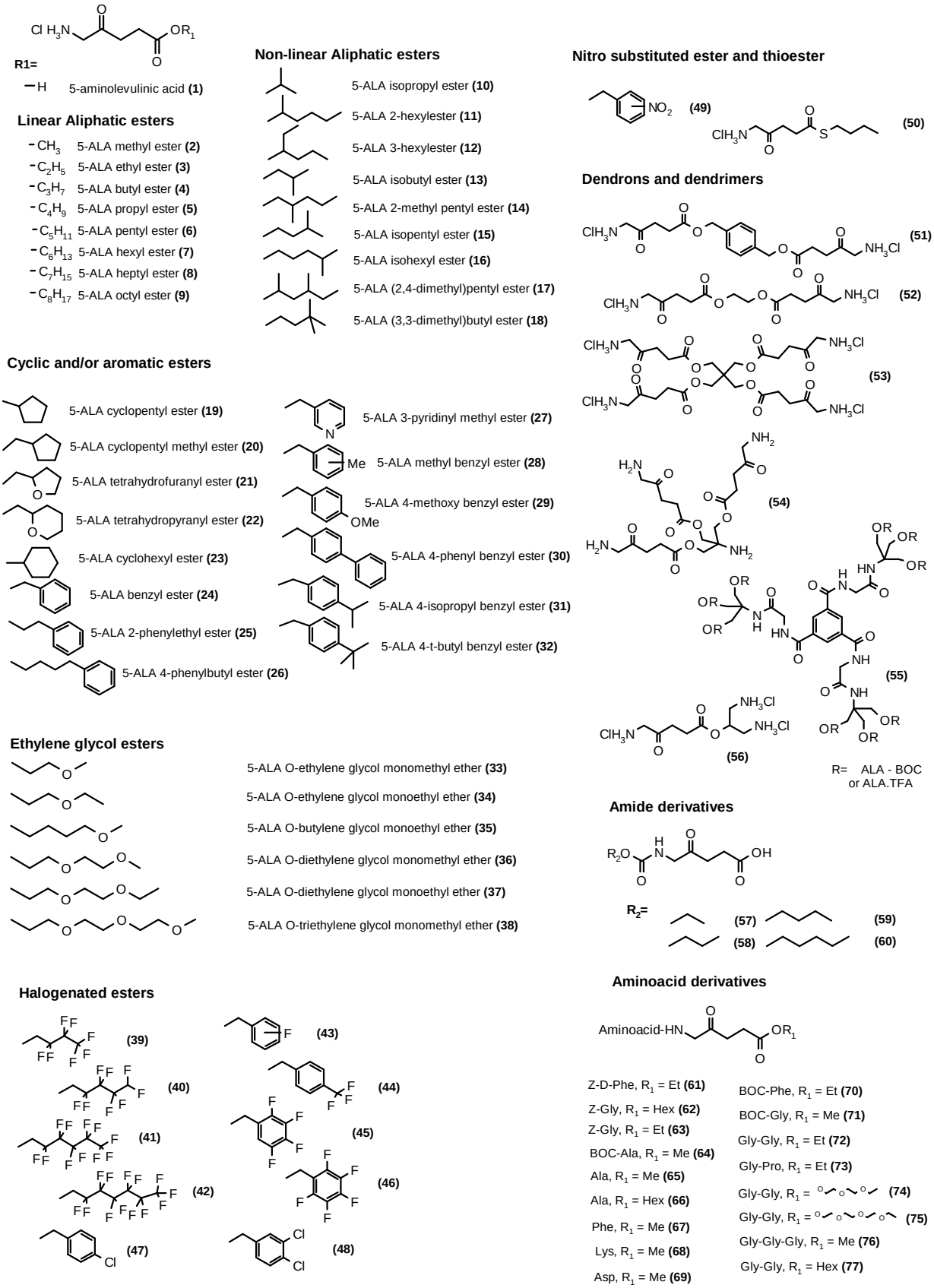



**Figure 4**: High diversity of 5-ALA derivative and conjugate structures, ranging from simple n-alkyl esters to dendrimers or aminoacid derivatives.

However, care should be taken about the acid used for deprotection of the BOC function, since the resulting salts (*e.g.* HCl or TFA) might substantially alter the pharmacokinetics of the active compound [107].

For example 5-ALA thionylester derivatives have been prepared according to this procedure. These compounds maintained biological activity *in vitro* [54]. Furthermore, halogenated alcohol moieties have been introduced that enhanced the lipophilicity, compared with their hydrocarbonyl counterparts (**39-48**). In this way the lipophilicity of 5-ALA can easily be modified over several orders of magnitude [52]. Furthermore, the electron withdrawing effect of the halogenosubstituents might have additional influence on the porphyrin production capacity of such compounds.

The general approaches to assess the different derivatives with respect to their porphyrin induction capacity are mainly *in vitro* tests. These can be done in cell culture [108,75,109], cell culture on hen egg model [110], 3D cell culture [105,111], skin explants [90 99] or other organ culture models [60,112,113]. To which extend these *in vitro* tests reflect the real *in vivo* situation in terms of bioavailability remains unclear. However, some basic conclusions may already be drawn from these experiences.

First, in tests in which dose-response curves have been determined [52] a characteristic biphasic relationship was found. In these dose-response curves PAP generation is positively correlated up to an optimal concentration $C_{opt}$ at which highest fluorescence intensities can be observed; beyond this value PAP formation sharply decreases. This decrease of PAP formation at higher doses can most likely be attributed to cytotoxic effects generated by the particular compound itself or its metabolic products. Furthermore, disruption of the cell membrane following exposure of high doses of lipophilic 5-ALA esters has been reported [84]. Thus, good equilibrium among the capacity to penetrate cell membrane, the ability to induce, PAP and cytotoxicity has to be found for each application.

Second, the absolute value of $C_{opt}$ varies with the type of precursor and the particular cell line. For most long-chained 5-ALA esters (C5-C8) $C_{opt}$ has been found to be 30-150-fold lower that for 5-ALA. At these low concentrations similar or higher fluorescence intensity levels have been reached with 5-ALA esters. In terms of phototoxicity no differences have been observed after 5-ALA and 5-ALA ester-mediated PDT at similar porphyrin levels *in vitro* and *in vivo*. The same behavior was observed in a series of fluorinated 5-ALA alkylesters [54]. For these compounds, the short-chain C4 derivative did not achieve high PAP levels, whereas the long-chained C7 derivative did. Finally, the best 5-ALA derivative for optimal PAP formation seems to vary from cell line to cell line.

The *in vitro* fluorescence intensity time profiles may also vary substantially. Uehlinger *et al.* [52] have reported a sigmoidal profile over a of 24 h period in which cells of urothelium origin exposed to HAL in wide concentration range. This is in agreement with Perotti *et al.* [80] which showed a similar pharmacokinetics in a murine cell line. However, recently, Brunner *et al.* [109,54] reported a very fast increase in porphyrin fluorescence intensity, reaching a plateau already after approximately 10 minutes in HT29 cells.

Whitaker and coworkers [55] have tested a set of linear and branched 5-ALA esters of the same number of carbon atoms. Their results agreed with those of other investigators [52,67,114] that a branching point in position 1 from the carboxylic function impedes porphyrin synthesis *in vitro*. However, we have recently tested a number of 1,3-diacylglycerides of 5-ALA that have shown a similar *in vivo* activity than 5-ALA following systemic administration (see below). However, the corresponding 5-ALA–CH-$(CH_2$-$NH_2)_2$ (**56**) has not shown a promising biological activity *in vitro* [54]. Although the screening of new 5-ALA derivatives in cell culture might give a preliminary indication for promising drug candidates, such studies cannot answer the major questions in this area of research, including improved bioavailability and selectivity. Furthermore, the reduced water solubility of some 5-ALA derivatives might further impede such screening approaches, because precipitation in the incubation medium might reduce the total available concentration of porphyrin precursor or because the used solvent system can alter the pharmacokinetics and heme metabolism.

On the basis of a previous study reported by Krieg *et al.* [115], Brunner *et al.*[54] have proposed a screening approach for various 5-ALA derivatives with respect to PAP induction capacity and selectivity. In their studies, they separately incubated a human cell line of cancerous origin and their corresponding “normal” counterparts with different 5-ALA esters and determined the resulting porphyrin contents. In their bladder cancer model (J82/UROtsa cells) they found a maximal ratio of tumor tissue to normal tissue (T/N) of 2.1 with a fluorinated hexylester derivative of 5-ALA (**41**). This T/N ratio corresponds approximately to the lower end that can be observed with HAL in humans (Collaud *et al.* submitted). However, using a similar system consisting of a human adenocarcinoma cell line and a colonic fibroblast, they found T/N ratios as high as 55. However, it must be taken into account that in the latter case the number of tumor cells was five times higher than in the case of their normal counterparts. Nevertheless, such systems represent a valid approach for the preliminary screening for better 5-ALA derivatives. Even closer to the real *in vivo* situation are tumoral coculture models. Very recently, we developed such a system for bladder cancer (Fotinos *et al*. unpublished results). For this purpose, GFP-transfected T24 bladder cancer

cells were coincubated with fully differentiated, multilayer urothelium of neonatal origin. Three to five days after incubation, cells of tumor origin developed clearly visible foci, migrating towards the basement membrane. Incubation with appropriate doses of HAL showed a selective accumulation of PAP in GFP-labeled cells. Such models will not only enable to test local bioavailability and selectivity of new 5-ALA derivatives but also to determine optimal conditions with respect to PDT.

Although the introduction of 5-ALA esters into the field of fluorescence photodiagnosis can be considered as success story, their application fields will stay restricted to organs that are accessible by topical administration. Simple 5-ALA esters have been conceived for topical administration in creams, gels, ointments or solutions rather than for systemic administration. Although intraperitoneal administration of HAL has been shown to increase porphyrin production in mouse brain compared with 5-ALA, approaches to use 5-ALA esters systemically have shown less drastic effects on the formation of PAP *in vivo*. This is presumably a result of their limited stability, their binding to plasma proteins and, thus, their limited bioavailability, as well as to a pronounced acute toxic potential when given intravenously. [116, 66].

After oral administration a large fraction of 5-ALA esters might be hydrolyzed or otherwise degraded under the highly acidic conditions present in the stomach. Therefore, at equimolar doses, the initial advantage over 5-ALA is lost for this administration route. However, encapsulation in controlled release formulations protecting the respective derivative against the acidic attack in the stomach might circumvent this problem. Perotti *et al*. [116] hypothesized that, once 5-ALA esters reach the blood circulation, because they are too lipophilic they might not cross the endothelial barrier of the vascular system and might not be able to reach their target. Another feature that may hamper the intraperitoneal use of simple 5-ALA n-alkylesters is the acute toxicity observed at doses of approximately 1000 $mg \cdot kg^{-1}$ that has been observed in mice [116]. However, intravenous doses of up to 30 $mg \cdot kg^{-1}$ or intraperitoneal doses of up to 800 $mg \cdot kg^{-1}$ have been reported to be safe in mice [66] and rats [117], respectively. In this context the binding of 5-ALA n-alkylesters to plasma proteins might play an important role with respect to both toxicity and bioavailability. After intravenous bolus administration the amount of free 5-ALA n-alkylester can temporarily exceed the toxic dose, whereas if it is slowly infused (which favors binding to plasma proteins) it can reduce the toxic potential of 5-ALA derivatives. However, the irreversible binding to plasma proteins can strongly reduce the systemic bioavailability of lipophilic compounds such as HAL. However, to the best of our knowledge neither clinical nor animal studies provide consistent answer with regard to the efficacy of 5-ALA ester after systemic administration.

In addition to the advantageous us of 5-ALA derivatives for internal use in oncology, the treatment of pathological conditions (such as rheumatoid arthritis [118]) might also benefit from the advantages offered by 5-ALA derivatives in this context. However, the huge potential of other 5-ALA derivatives with respect to parenteral applications remains largely unexplored. Important life-threatening diseases including prostate cancer, mammary carcinoma, breast cancer, lymphoma and brain cancer can thus hardly be treated by this approach, although 5-ALA esters have been demonstrated to induce high PAP concentrations in many cell cultures models of these diseases [80,119,52].

The drawback with respect to 5-ALA derivative related research can be mainly attributed to the fact that investigators in this research area have largely ignored advances made in recent prodrug research, until today [120,121,122].

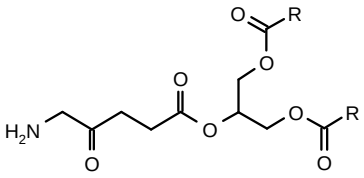
For example. in the context of 5-ALA-mediated treatment of brain cancer, it is generally assumed that increasing lipophilicity increases blood brain barrier permeability in a systematic fashion. The successful design of lipophilic esters with the ability to enhance parent drug concentration in the brain in the context of 5-ALA more than likely requires optimization rather than maximization of lipophilicity and bioconversion rate. The factors contributing to the existence of an optimal lipophilicity for CNS delivery have been reviewed recently and several biophysical kinetic models have been developed in this context [123,124,125]. Brain uptake of highly lipophilic compounds is limited by their interactions with plasma proteins and by unfavorable partition from the endothelium into the underlying tissue, both of which increase with increasing lipophilicity. There are now a number of instances in which poor brain uptake of prodrugs designed with increasing lipophilicity has been attributed to plasma protein binding or fast cleavage by carboxyesterases in the blood. Apparently, similar mechanisms are also responsible for the failure of 5-ALA ester to induce porphyrins when applied intravenously.

Despite evidence mentioned above there are examples of extremely lipophilic properties for efficient CNS delivery. One of these approaches that takes advantage of the metabolic pathway of lipids is the triglyceride prodrug approach. It benefits from the metabolic fate of glycerides to improve intrinsic drawbacks of the present compound when the latter is covalently coupled to position 2 of the glycerolic backbone. Triglycerides are major constituents of dietary fats. Their absorption involves the hydrolysis by lipases to monoglycerides and fatty acids. The following three enzymes participates to the catabolism of lipids: pancreatic lipase, phospholipase A2 and nonspecific esterases. For the degradation of triglycerides the pancreatic lipase plays the most important role. It hydrolyses the esters in positions 1 or 3. Hydrolysis in position 2 occurs very slowly. After being released, the

resulting monoglycerides and fatty acids enter the enterocytes, where their fate depends on the nature of the remaining acid in position 2. Short-chain fatty acids pass through the cells into the blood without re-esterification, medium-chain fatty acids are mainly oxidized, and long-chain fatty acids are used for re-esterification into triglycerides.

The use of triglycerides to increase the brain penetration of drugs began with a report by Jacob *et al.* [126] who synthesized triglyceride derivatives of GABA. A dramatic increase in brain penetration was observed, with up to 127 times more of the applied drug found, compared to the parent compound. In these studies it was shown that the introduction of unsaturated fatty acid chains was favorable with respect to the activity, despite lower lipophilicity. Because of the similarity between these compounds and natural components of lipid bilayer membranes, Jacob *et al.* suggested that these compounds may become associated with brain lipid membranes, serving as a reservoir for sustained release. However, the detailed mechanisms by which these agents release GABA to the brain are still unclear. Because 5-ALA is structurally closely related to GABA, one can suggest that similar pathways for 5-ALA diacylglycerides derivatives would favor the brain uptake of these compounds when applied systemically.

**Table 3**: Estimated log *P* values for various 1,3 diacylglycerides

|  | clog *P* (ionized form) | Compound number |
|---|---|---|
| R = $CH_3$ | -0.38 | **(78)** |
| R = $(CH_2)_2CH_3$ | 1.74 | **(79)** |
| R = $(CH_2)_4CH_3$ | 3.85 | **(80)** |
| R = $(CH_2)_6CH_3$ | 5.97 | **(81)** |
| R = $(CH_2)_8CH_3$ | 8.09 | **(82)** |
| R = $(CH_2)_{10}CH_3$ | 10.17 | **(83)** |
| R = $(CH_2)_{14}CH_3$ | 14.43 | **(84)** |

We have recently explored this approach for the improved systemic delivery of 5-ALA. For this purpose it was necessary to develop a synthetic route to prepare a huge pool of different 1,3-diacyl-2-ALA glycerides because of the lack of commercially available 1,3-diacyl glycol-2-ol.

Table 3 shows the estimated log *P* values of such compounds. Because the testing of such moderate to highly lipophilic compounds *in vitro* is difficult, we have adapted the chick's chorioallantoic membrane model for drug screening purposes (unpublished results). This model not only allows us to show the PAP formation capacity of these derivatives, compared with that of 5-ALA but it also shows the biodistribution of PAP formation. Furthermore, it allows the easy evaluation of acute toxicity of newly prepared 5-ALA derivatives following intravenous administration.

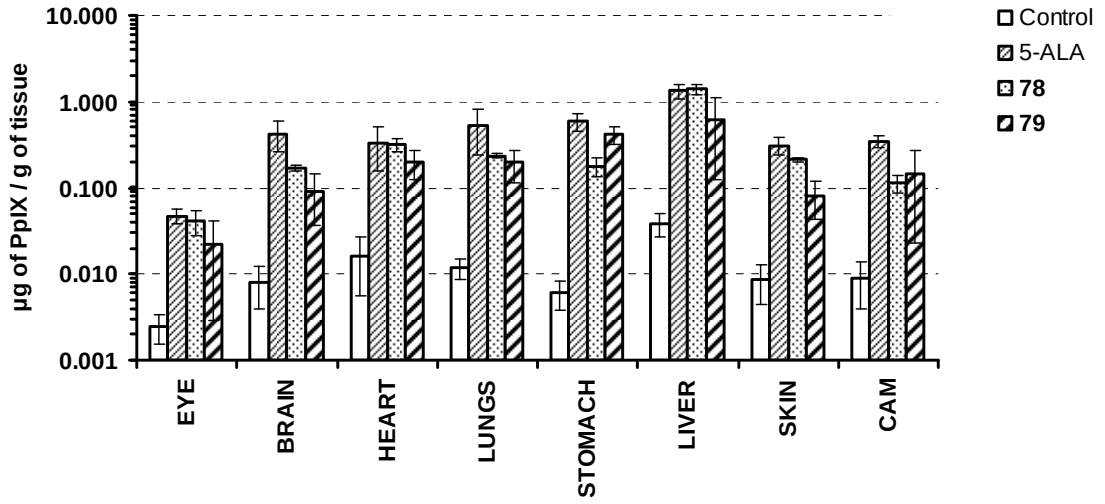



**Figure 5**: Biodistribution of porphyrins after administration of solvent control, 5-ALA and two 1,3-diacylglyceride derivatives at equimolar doses to the CAM model.

Figure 5 shows an example of the biodistribution of porphyrins after administration of 5-ALA and its 1,3-diacylglyceride derivative at equimolar doses. It can be seen that similar concentration of PAP can be obtained with these simple 5-ALA derivatives, compared with 5-ALA. None of these compounds have shown an acute toxicity in the CAM model (dose >400 $mg \cdot kg^{-1}$) whereas HAL was found to be toxic at doses as small as 10 $mg \cdot kg^{-1}$ after intravenous administration. Therefore, such 1,3–diacylglyceride 5-ALA derivatives represent a promising class of porphyrinic precursors for the treatment of pathological conditions for

which topical administration is not appropriate, too complicated and/or might run counter to clinical practice. In addition to this approach other derivatives, such as dendrimers (see below), might be of potential interest for their use in the treatment of internal diseases.

## 5-ALA amides and pseudopeptides

Another approach to alter the lipophilicity of 5-ALA by a simple derivatization is its conversion of the 5-aminogroup into an amide (Fig. 4). In its most simple approach this can be achieved by the condensation of 5-ALA or its ester with acetic anhydride or acetylchloride under basic conditions (**57-60**). Kloek *et al.* [43] were the first to follow this approach. However, simple *N*-acetyl 5-ALA derivatives largely failed to induce porphyrins *in vitro* and *in vivo*. This observation can be predominantly attributed to the reduced ability of cells to cleave the *N*-acetyl function which is mandatory for the induction of porphyrin synthesis (see above). Interestingly, Casas *et al.* [67] found that the carbobenzyloxy-D-phenylalanyl 5-ALA ethylester showed considerably high capacity to induce porphyrins in human and rat skin explants (**70**).

However, after these initial studies some groups became aware that this approach has the potential to further improve the specificity of 5-ALA-mediated PpIX by targeting elevated levels of aminopeptidase activity observed in certain human tumor cell lines and mammalian metastatic tumor cells.

The use of aminoacyl derivatives of anticancer drugs addresses these altered enzymatic activities has been exploited experimentally [127,128,129]. Furthermore, altered expression of some acidic, basic and neutral aminopeptidases in tumor associated vasculature is a further indication for this approach.

Berger *et al.* [64] pioneered this approach in 5-ALA related research. In a first attempt, they have prepared some BOC-protected and deprotected pseudopeptide derivative of 5-ALA and 5-ALA esters (Fig. 4). On a cellular level the best PAP precursor was the Phe-5-ALA methylester (**67**) derivative in all cell lines independently of the level of expression of aminopeptidases in the particular cell line. The Lys-5-ALA methylester (**68**) has not shown a significant biological activity in human endothelial cells (HCECs), despite a high expression of APB peptidases in the cell line. No PAP formation was observed with *N*-BOC protected prodrugs, which demonstrates the importance of the involvement of peptidase activity for such derivatives. In a second investigation the same group [65] has prepared some dipeptide 5-ALA derivatives in order to increase the specificity of above mentioned peptidase activity and/or to specifically target cellular peptide transporters. In HCECs both the BOC protected as well as the deprotected equivalent of Gly-Pro-5-ALA ethylester (**73**) were more efficient

than 5-ALA itself. Furthermore, in the same cell line the BOC protected dipeptide derivative was more efficient than its more hydrophyilic deprotected counterpart in terms of dose response. More importantly BOC-Gly-Pro-5ALA ethylester has been found to very selectively induce PAP in human endothelial cells. However, relatively high doses of these derivative are necessary to induce porphyrins in A549 cells as compared to simple n-alkylesters [52].

### From dendrimers and other derivatives

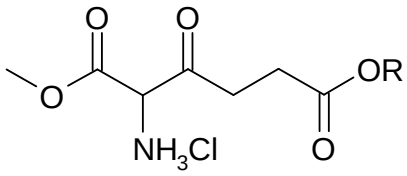
Another way to circumvent the poor systemic bioavailability of 5-ALA and its n-alkylester derivatives might rely on the use of dendrimeric structures. In particular, such polymeric drug delivery system might represent an attractive method in cancer therapy, because with different mechanisms the enhanced perfusion and retention effect [130] and the 5-ALA PAP pathway are combined. Furthermore, polymers will also allow the delivery of several drug molecules simultaneously. In the past, most research of such polymeric drug delivery systems was somewhat hampered due to the high polydispersity and poorly defined structure. However, recent advances in polymer chemistry now allow the synthesis of structurally defined hyperbranched polymers (dendrimers), to which the drug moiety can be coupled in a controlled way. The minimal structure of a dendrimer is called dendron. Both Brunner *et al.* [54] and Di Venosa *et al.* [131] have evaluated the efficacy of such dendron 5-ALA conjugates *in vitro* (see Fig. 4).

In an *in vitro* screening procedure Brunner *et al.* found the dendron 5-ALA conjugate (**51**) was more effective than the tetramer (**53**) in terms of PAP induction capacity at equimolar doses. Furthermore, compound **51** has been shown to induce fluorescence intensity in J82 and HT29 cells that is respectively 3 and 5 times higher than that induced by 5-ALA. In contrast, Di Venosa *et al.* [131] found similar *in vitro* activity of a tetrameric 5-ALA dendron, compared with 5-ALA, in LM3 cells. However, they have found that 3 h after administration to the cells on average only one-third of the 5-ALA moieties was released from the dendrons present intracellularly.

On the other hand dendrons containing as much as 18 5-ALA residues [132] have been shown to substantially improve the intracellular PAP fluorescence intensity and the photodynamic efficacy in PAM212 cells, compared with 5-ALA.

However, whether such approaches are potentially interesting must be invetigated *in vivo*: derivatives that poorly perform *in vitro* might show a totally different performance in *in vivo* studies. We have recently prepared a homologous series of 3-amino-3-oxohexandioic-1-methyl-6-alkylester (Fig. 6). These compounds have shown essentially no biological *in vitro*

activity, compared with 5-ALA or its methylester. Only 10 h after incubation some fluorescence was observed *in vitro*. However, by far more activity with respect to PAP formation was observed *in vivo* (CAM model). Furthermore, the most active compound AOMM has shown the tendency to increase PAP fluorescence in most organs of the chick's embryo while MAL induced PAP fluorescence decreased.




R= Methyl AOMM
Ethyl AOME
Butyl AOMB

**Figure 6**: Structure of homologous series of 3-amino-3-oxohexanedioic-1-methyl-6-alkylesters.

## Clinical evaluation

MAL is the first 5-ALA derivative authorized for commercialization and is now marketed under the trade name Metvix®. It is used to treat thin or nonhyperkeratotic and nonpigmented actinic keratoses (AK) on the face and scalp when other therapies are considered nonappropriate and to treat superficial and/or nodular basal cell carcinoma, BCC (such as lesions on the mid-face or ears, lesions on severely sun damaged skin, large lesions, or recurrent lesions) for which other available therapies are inappropriate because of the potential for treatment-related morbidity and poor cosmetic outcome. Recently, a second 5-ALA derivative, HAL, received marketing authorization under the trade name Hexvix® for the improved detection of bladder cancer.

### Initial studies with MAL

In contrast to the random clinical evaluation of 5-ALA based PDT in the treatment of AK, all studies involving MAL used the same standard protocol in order to obtain comparable and meaningful results under conditions established as optimal in terms of drug concentration and application time. There is, however, an exception in a European study in which the second PDT procedure was performed only on lesions not located on scalp or face (Table 4).

**Table 4**: Metvix® photodynamic therapy treatment protocol

| | | | |
|---|---|---|---|
| A | Diagnostic | Lesion analysis:<br>*-Localization*<br>*-Size*<br>*-Number* | |
| B | Lesion preparation | Crust removal<br>Surface roughen<br>In the case of nodular BCC intratumoral debulking was performed to reduce tumour thickness | *Not a therapeutical curettage procedure* |
| C | Drug application | Application of the Metvix® cream 160 mg·g$^{-1}$ in a 1 mm thick layer, under occlusive dressing for 3 h | *Minimize light exposure* |
| D | Irradiation conditions* | The light dose depends on the light source<br>• 37 J/cm$^2$ when using Actilite LED 16 or 128<br>• 75 J/cm$^2$ when using a broadband source | *Up to the clinician, an anaesthetic pre-treatment can be applied.* |
| F | Repetition of the treatment after 7 days | | *Exception:*<br>*-In the European AK study [133](Szeimies et al.) only the lesions not localized on face and scalp were retreated.*<br>*-Soler et al. [134] treated 287 (93%) BCC lesions only once* |
| (G) | Repetition of the cycle after 3 months | The lesions with non complete response after 3 months are retreated with the same 2 PDT session cycle. | *Only in the studies of Horn et al.[135], Rhodes et al.[136], and. Vinciullo et al. [137] on BCC* |

*VARIATION: Soler *et al.* [134] light dose 50-200 J·cm$^{-2}$

To find these optimal conditions, a dose finding study involving 112 patients with a diagnostic of AK was designed to compare the efficacy of 4 treatment conditions with different MAL concentration (80 mg·g$^{-1}$ *versus* 160 mg·g$^{-1}$) and application durations (1 *versus* 3 h) [138]. Before every MAL administration the lesions were prepared using a curette, in order to remove scales and crust. After red light illumination (600-700 nm, 75 J·cm$^{-2}$), of the lesions (n=380), those treated with the 160 mg·g$^{-1}$ MAL cream for 3 h showed slightly better results than those treated under other conditions, with complete responses in 67% and 57-66 % of the lesions, respectively. Treatment was reapplied 3 months later on residual lesions, with comparable results: the 160 mg·g$^{-1}$ MAL cream applied for 3 h produced a superior complete response (90% of lesions) compared with a response of 80-87% obtained with the other treatment regimens.

Another study examined whether or not treatment efficacy could be improved by increasing drug application time [139]. In this study 141 patients clinically diagnosed with BCC received a Metvix® cream (160 mg·g$^{-1}$) that was applied for either 1, 3, 5, or 18 h. Extended exposure to the drug did not improve treatment efficacy and the highest complete response (88% of the treated lesions) was observed 3 h after application. No significant difference was

observed after retreating the residual lesions 3 months later and lesion complete response rates remained between 92% and 96%. Te investigators concluded that application of the drug for durations longer than 3 h did not result in an improved clinical response.

In the specific case of nodular BCC, in which drug penetration depth and homogeneous distribution plays an important role for the successful treatments, Peng *et al.* [140] tested different MAL cream concentrations (16, 80 and 160 $mg \cdot g^{-1}$) and application times (3 or 18 h) and concluded that the best results were obtained with the 160 $mg \cdot g^{-1}$ MAL cream that was applied for 3 h.

**Aktinic keratoses**

AKs are known to be the most common type of premalignant skin lesion. In some areas of the world with high local incident UV radiation, such as Australia, approximately 60% of predisposed persons over 40 years of age have at least one AK, with up to 90% of persons older than 80 years of age having at least one AK [141]. Some studies have shown that 40% of all metastatic squamous cell carcinoma originate from AKs, whereas others suggest that AK is itself an early form of carcinoma *in situ* [142,143]. Most AK lesions are located on sun-exposed areas, such as the face, scalp, ears and forearms. Treatment of these particular areas tend to be complicated, because of the limited available treatment options. Although there are some treatments that are effective, as in the case of AK and BCC, can cure these lesions, the price to pay in terms of disfigurement in the facial area might be rather high.

A commonly used treatment for AK is cryotherapy, which gained popularity due to its high effectiveness [144]. Other ablative procedures, such as dermoabrasion or surgery, are sometimes used to treat multiple lesions or to evaluate suspicious lesions [145]. Topical treatments with imiquimod, diclofenac or 5-fluorouracil (5-FU) are also used in relation to the type of lesions [144]. In this context, PDT has enormous potential to demonstrate its numerous advantages in terms of curing efficacy, noninvasiveness, multiple lesion treatment and repeatability, with excellent cosmetic outcome.

In a pioneer study Fritsch *et al.* [89] compared the fluorescence intensity arising after 5-ALA or MAL application on AK lesions in patients who had undergone surgery. A cream containing 20% of the active drug was applied over a period of 1 or 6h on AK lesions and on normal skin samples and the resulting porphyrin formation was determined by fluorescence measurements. Although 5-ALA seemed to produce the more porphyrins, the specificity of MAL for AK lesions appeared to be 70% higher than that for 5-ALA. Therefore, one can

assume that when applied properly, MAL PDT would be more effective in terms of collateral damage and induced pain than for instance, 5-ALA PDT.

**Table 5**: Reports of Metvix®-mediated treatment of AK

| Author | N°of patient (lesions) | Comparison | Follow up | Lesion complete response | Cosmetic outcome † |
|---|---|---|---|---|---|
| Pariser *et al.* (2003) [146] | 80 (502) | Placebo | 3 months | 89% MAL 38% placebo | Excellent or good 97% MAL |
| Freeman *et al.* (2003) [147] | 204 (855) | Cryotherapy & placebo | 3 months | 91% MAL 68% cryo 30% placebo | Excellent 83% MAL 51% cryo |
| Szeimies *et al.* (2003) [133] | 202 (732) | Cryotherapy | 3 months | 69% MAL 75% cryo | Excellent or good 96% MAL 81% cryo |
| Dragieva *et al.* (2004) [148] | 17 (129) * | Placebo | 4 months | 90% MAL 0% placebo | N.A. |

* AK in transplant recipients; † investigator evaluation

A total of 4 clinical trials of MAL-mediated treatment of AK have been reported [133,146,147,148]. The results are summarized in Table 5. In all studies the MAL treatment were compared to cryotherapy or placebo treatments with respect to response rate, cosmetic outcome and patient satisfaction. Complete response rates ranged from 69% to 90% for MAL, compared with 70% for cryotherapy and 30-38% for placebo. In some studies the cosmetic outcome was evaluated in those patients with more than 75% of the lesions displaying complete response [133,147]. The results indicate a significant advantage of MAL-mediated PDT: 83-96% of the investigators and 76-98% of the patients graded the cosmetic outcome as good or excellent, whereas in the case of cryotherapy only 51-81% of the investigators and 56-91% of the patients graded the cosmetic outcome as good or excellent. Most adverse events reported for MAL-treated patients (burning, pain and crusting) were of mild or moderate intensity.

As shown by the results of the three main studies (Table 6), methylaminolevulinate-mediated PDT effectively treated 716 (82%) out of 875 AK lesions, and 429 (88%) out of 489 focusing on thin or mild lesions. The lowest response rate obtained by the European study

can be explained by the single PDT session applied on the face and scalp lesions, which suggests that a protocol with multiple sessions is highly desirable.

**Table 6**: Complete response rates of AK lesions after Metvix®-mediated PDT in function of lesion grade

| | **Lesion grade** | | | | | |
|---|---|---|---|---|---|---|
| | **Overall*** | | **Mild** | | **Moderate** | |
| | *n°lesions* | *CR* | *n°lesions* | *CR* | *n°lesions* | *CR* |
| Pariser *et al.* | 236 | 209 | 179 | 161 | 57 | 48 |
| Freeman *et al.* | 295 | 267 | 167 | 161 | 128 | 106 |
| Szeimies *et al.* | 344 | 240 | 143 | 107 | 201 | 133 |
| **TOTAL** | **875** | **716** | **489** | **429** | **386** | **287** |
| **CR rate** | **82%** | | **88%** | | **74%** | |

* Thick lesions reported in the the Szeimies *et al*. study were not taken into account

This point was confirmed by the recent study of Tarstedt *et al*. [149] who compared two treatment regimens of MAL-PDT, one with a single application of PDT and the other with two application of PDT 1 week apart. The single-treatment regimen appeared to be as effective as the dual regimen for classical lesions but less effective in the case of thicker lesions. The cosmetic outcome was graded as excellent or good for 83–91 % of the patients treated, which is significantly superior to the standard treatment cryotherapy. Painful skin sensation, burning, erythema and crusting were the most common local adverse events reported but had generally mild or moderate intensity. Furthermore, allergies to 5-ALA or MAL have been mentioned [150,151].

More recently, Dragieva *et al.* [148] compared the efficacy of MAL PDT for transplant recipients known to have an increased tendency to develop multiple AKs or cancer due to immunosuppression. Because ulceration and infection may be life threatening for immunodepressed patients, these adverse events are uncommon in PDT, which thus makes PDT the treatment of choice. These particular lesions responded well to PDT: 56 (90%) of the 62 lesions had a complete response. However, three patients had only a partial response

and one had no response. This non response was explained by the ineffectiveness of the immune system to survey and eradicate residual tumor cells after PDT.

### Basal Cell Carcinoma

BCC is the most common skin cancer, with an incidence of half a million new cases in the United States every year. Conventional treatments are either superficial ablative techniques (electrodesiccation, curettage and cryotherapy), which are used primarily for low-risk tumors, or full-thickness techniques (Mohs surgery, excisional surgery and radiotherapy) [152]. However, because of the very poor cosmetic outcome after surgical therapy, PDT may represent an excellent alternative to effectively treat these lesions.

**Table 7**: Report of clinical studies concerning Metvix® treatment of BCC

| Author | No° of patient (no. of lesions) | Compa-rison | Follow up | Lesion complete response after 3 months | Recurrence after 12 months | Recurrence after 24 months | Cosmetic outcome rated excellent or good by investigator |
|---|---|---|---|---|---|---|---|
| Soler *et al.* (2001) | 59 (350) | None | 24-48 months | 89% * | | | 98% |
| Horn *et al.* (2003) | 94 (123) | None | 24 months † | 87% clinical<br>77% histological | | 18% | 76% 3 months<br>94% 24 months |
| Rhodes *et al.* (2004) | 103 (118) | Surgery | 24 months | 91% MAL<br>98% surgery | 13% MAL<br>2% surgery | 19% MAL<br>4% surgery | 24 months<br>83% MAL<br>41% surgery |
| Vinciullo *et al.* (2005) | 95 (148) | None | 24 months ** | 89% | 18% | 24% | 65% 3 months<br>79% 12 months<br>84% 24 months |

* CR after 3 – 6 months. † Follow-up observation is continuing for a total of 5 years

A summary of the results of clinical studies reporting on MAL-mediated PDT for BCC can be seen in Table 7. The first work, published in 2001 by Soler *et al.* [134], is a retrospective, noncomparative, long term follow up study that evaluated the clinical response of BCC to MAL PDT. This study, which had a follow up period of 2-4 years, included 59 patients with 350 BCC lesions treated with MAL PDT. A total of 310 lesions (89%) showed a complete response after 3 months. Only 11% of these 310 lesions recurred after 24 to 48 months. Fourteen percent of the lesions were especially thick and nodular, and therefore more difficult to treat, which lowered the long-term complete response to 79%. The cosmetic outcome was assessed as good or excellent in 98% of the cases.

In a more recent multicenter study Horn *et al.* [135] analyzed MAL PDT effectiveness in 94 patients who presented 123 difficult-to-treat BCC lesions. Lesions with large surface area and those located in the middle of the face are at high risk for morbidity or disfigurement and were thus selected as good candidates for PDT. After 3 months the overall clinical response rate was 87% but is decreased to 77% after histological control. Complete response rates were slightly better for superficial BCC than for nodular BCC (92% *versus* 87%). Recurrence after 24 months was observed in 18 % the cases and the cosmetic outcome was judged as excellent or good in 94% of all cases, even for large lesions. It is interesting that, investigations for systemic effects of MAL application showed no effect on liver, kidney or bone marrow function. These results are in agreement with results of a similar study recently published by an Australian group.

In contrast to the previously cited studies, all of which are noncomparative, a European study [136] directly compared MAL PDT with standard excision surgery in a group of 103 patients diagnosed for primary nodular BCC. Lesions with incomplete remission after first treatment underwent one additional course of PDT. Both PDT and surgical treatments produced similar rates of short-term complete response (91% and 98%, respectively). Surgery had slightly better long-term results than MAL PDT with respect to recurrence (2% *versus* 13% after 12 months and 4% *versus* 19% after 24 months). On the other hand, in terms of cosmetic outcome investigators noted a clear benefit of PDT over surgery. Although half of the patients who received PDT reported adverse effects, such as burning sensation, pain and erythema, these were only mild or moderate in nature and the condition resolved itself without any medical treatment. On the other hand, only a third of the surgical patients complained of adverse events but more serious cases (*e.g.* skin infection) were reported.

In summary, MAL-mediated PDT has been shown to be a very competitive treatment for BCC in terms of clinical response rate and cosmetic outcome, compared with conventional protocols. Nevertheless, some drawbacks are encountered when treating large lesions or lesions located in the trunk and neck, but in such cases multiple PDT treatments might be a reasonable option. A recent study [153] evaluating the economic potential of this treatment modality revealed that Metvix®-PDT represents an economically efficient intervention in the therapy of difficult-to-treat BCC, because of the lower total treatment costs than surgery and greatly reduced need for reconstructive surgery.

However, although the CR rates of MAL PDT 3 months after treatment are highly encouraging, one must await the results of the long term studies to fully assess this method.

### Additional indications

Today there are several other exploratory clinical PDT trials of MAL underway for treatment of conditions such as acne, oral squamous cell carcinoma. However, the outcomes of these studies have not been disclosed in the scientific literature until today.

## HAL and diagnosis of bladder cancer

Bladder cancer, which is the ninth most common cancer, had an incidence of >350'000 persons in 2002 and killed 145'000. The prevalence in 2002 was approximately three times higher among males. Today, the main therapy for superficial bladder cancer still remains surgical resection, chemotherapy and BCG (Bacillus Calmette-Guerin) therapy. However, some clinical studies using 5-ALA-mediated PDT have shown encouraging results in terms of safety, effectiveness and lack of major adverse effects in the treatment of recurrent bladder cancer and may thus become a useful tool in the clinician's therapeutic arsenal [154,155,156]. Despite advantages toward the parent compound, no clinical trial using HAL for the PDT treatment of recurrent bladder cancer has been reported.. However, HAL has been found to be a valuable tool in tumor detection for the fluorescence guided transurethral cystoscopy and has consequently gained official marketing authorization in Europe and has been recently recommended in the guidelines of the European Association of Urologists. We will briefly summarize the development of HAL-mediated FD from the first clinical study (in 1994) that tested 5-ALA [157] to the very recent approval of HAL in Europe (Table 8). For more information about fluorescence detection or treatment of bladder cancer, the reader is referred to some reviews of particular interest [158,159,160].

Since its initial use in 1992 [161] 5-ALA-mediated fluorescence cystoscopy, has quickly spread worldwide and a vast body of knowledge and experience is now available to the clinician. The general sensitivity achieved by 5-ALA FD in different studies is generally very high, with values 87-97% [162], 5-ALA-mediated FD was shown to detect two times more tumors than standard white light cystoscopy [163].

From 1997 through 1999 a pilot study investigated the possibility of enhancing 5-ALA-mediated bladder FD by the use of HAL, with the goal of increasing the PAP fluorescence intensity, decreasing the drug dose and decreasing instillation time, which is critical for patient compliance and higher tumor specificity [74]. HAL was selected over other n-alkyl-chained derivatives because of its satisfactory water solubility and capacity to induce high amounts of PAP at lower doses than 5-ALA. The optimal drug dose required to obtain the highest ratio of tumor to healthy tissue fluorescence was established in 25 patients. The

results obtained with papillary tumors were very clear and showed that fluorescence increased as a function of time, with the highest value obtained when with the 8 mM HAL solution. Interestingly, a 2 h resting period after the 2 h instillation gave a fluorescent value that was 2 times that for a simple 4 h instillation period.

**Table 8**: Report of clinical studies concerning Hexvix® fluorescence detection of bladder cancer

| Author | N° of patient | Comment |
|---|---|---|
| Lange *et al.* (1999) [74] | 25 | Pilot study: Determined the optimal time and concentration in terms of fluorescence production. |
| Marti *et al.* (2003) [73] | 18 | Compared HAL and 5-ALA in terms of fluorescence production. |
| Jichlinski *et al.* (2003) [164] | 52 | Observed a clear improvement in the detection of CIS. |
| Schmidbauer *et al.* (2004) [83] | 282 | Large multicenter study. Confirmed the superiority of HAL-mediated FD for CIS lesions. |
| Witjes *et al.* (2005) [167] | 20 | Observed the superiority of rigid towards flexible Fluorescence Cystoscopy. |
| Loidl *et al.* (2005) [165] | 45 | Observed the superiority of rigid towards flexible Fluorescence Cystoscopy. |
| Jocham *et al.* (2005) [168] | 146 | Confirmed the significant contribution of HAL-mediated fluorescence cystoscopy in terms of improved treatment. |
| Collaud *et al.* (submitted) | 12 | Pharmacokinetic study. |

In a subsequent study, Marti *et al.* [73] directly compared 5-ALA with HAL FD to confirm the presumed advantages of HAL over the parent compound in terms of induced fluorescence, lower effective dose, higher selectivity for tumoral tissues and drug penetration. A total of 18 patients were separated in 3 groups that received either 180 mM 5-ALA for 6 h, 8 mM HAL for 4 h or 8 mM HAL for 2 h plus a 2 h resting period (HAL 2+2 group). Although all conditions produced higher fluorescence in malignant cells, the mean ratio of tumor to normal urothelium appeared to be 70 % higher in the HAL 2+2 group. The advantages of HAL over 5-ALA were confirmed and included lower efficient concentration,

shorter administration time (2 h *versus* 6 h), and deeper penetration into the tissue. Furthermore, no PAP fluorescence was detected in the basement membrane or the chorion.

Despite of the high fluorescence obtained with the optimal incubation time of 2+2 (or even with 4 hours), in terms of clinical practice 1 h of instillation is recommended currently [164,83,165]. Furthermore short instillation times may be beneficial with respect to selectivity [166]. These conditions result in the minimum necessary amount of porphyrins in tumors for proper FD, with sensitivities (97%) and specificities (74%) [164] that are similar to longer instillation times.

The improved FD of carcinoma *in situ* (CIS) is one of the most remarkable outcomes of clinical studies involving HAL. A large multicenter clinical trial [83] was conducted that involved a total number of 286 patients, 83% (39%) of whom had CIS, an aggressive, high risk and easily overlooked tumour [169]. The results revealed that HAL FD detected 97% of all lesions, compared with only 78% by conventional white light cystoscopy (WLC). The superiority of the technique becomes evident when focusing on difficult to detect CIS, with detection rates of 97% for HAL FD and 58% for WLC. Furthermore, there were only two patients with adverse effects directly related to HAL FD, which included burning sensation after illumination and mild bladder spasm. This high tolerance was confirmed by Collaud *et al.* (Drugs R D, in press) who proceeded in a safety study concluding on the absence of serious adverse events and negligible systemic uptake of the drug. Although the use of HAL FD allowed for improved resection of CIS in 25% more patients (this lesion type was detected in 80 patients with HAL FD and only 64 with WLC), only a long term observational study can confirm the real benefits of this methodology in terms of patient survival rate and tumor free resection survey. Nevertheless, recent studies [170,171,172] have shown a direct correlation between improved detection using 5-ALA-mediated fluorescence detection and increased tumor-free resection.

The final two clinical studies [165,167] compared HAL FD involving a flexible cystoscope (the gold standard for detection of bladder cancer [173,174]) and a rigid, cystoscope with respect to convenience and compliance for the patient. Both studies concluded that the rigid cystoscopy had higher detection rate (85-94% *versus* 70-89%).

### HAL and other clinical domains

Because photomedicine is limited to light-accessible organs, gynaecological cancer like ovarian or cervical cancers and the gastrointestinal tract may be good candidates for photodetection and photodynamic therapy.

The early stages of ovarian cancer are generally asymptomatic, and it diagnosed at a late stage in the majority of cases, when it is incurable, making it the fourth leading cause of cancer-related death among women. The 5 year survival rate for this type of cancer is 94% when the tumors are localized but decrease dramatically to 29% when metastasis has occurred [175]. Encouraging results were obtained by a HAL FD on Fisher rats implanted with NuTu-19 cells (rat epithelial ovarian cell line), resulting in the detection of twice as many cancer lesions, compared to white-light inspections, and showing a clear advantage in terms of porphyrin accumulation, compared with 5-ALA [117]. Loning *et al.* [176] have used 5-ALA-mediated laparoscopic FD for the detection of ovarian carcinoma metastases and observed a very high sensitivity (92%), allowing for significant improvement in metastasis detection. However, to the best of our knowledge, no clinical trials using 5-ALA derivatives are scheduled.

Another gynaecological malignancy for which PDT might be useful is cervical cancer, which is the second most common cancer among women worldwide [177]. The standard treatment for cervical intraepithelial neoplasia (CIN) is surgical excision. However, this invasive procedure has many complications, including bleeding, infections, cervical stenosis, preterm delivery and high recurrence rates demand problematic repetition of the treatment[178]. Because of its increased patient tolerance, high selectivity and low tissue destruction PDT effect on CIN was investigated using different photosensitizers. Ichimura *et al.* [179] used hematoporphyrins-mediated PDT and obtained high response rates of approximately 90%, but patients had to avoid light exposure because of the prolonged skin photosensitivity of these drugs. Additional clinical studies using 5-ALA-mediated PDT showed contradictory results in terms of efficacy. Thus Bodner *et al.* [180] obtained responses similar to cold-knife conization, whereas Keefe *et al.* [181] observed no difference from the placebo group. Recently, a Swiss group [182] examined the dependence of PAP formation as a function HAL topical application time. They determined that application duration of 100 min for HAL cream was optimal to obtain the highest ratio of epithelium to underlying lamina propria fluorescence. However, the results showed a surprisingly low selectivity for tumor cells *versus* normal tissues. More information will become available as clinical studies continue.

Apart from bladder and gynaecological cancers, HAL-mediated FD has been investigated for the diagnosis of rectal adenoma and cancer. For this purpose, a pilot study evaluated the efficiency of an instilled HAL solution to induce selective fluorescence of rectal tumors (adenoma and malignant tumors) [183]. The study included 10 patients who received an enema composed of a 3.2 mM HAL solution preceded by an initial treatment with *N*-acetylcystein to eliminate the mucus layer at the surface of the rectal epithelium and thus

enhance contact between the drug and the target tissue. One purpose of this study was to determine the optimal drug application conditions to achieve the best ratio of tumor to healthy tissue fluorescence. The investigators looked at different application times of a 3.2 mM HAL solution ranging from 30 to 60 min and also evaluated the effect of a resting period after drug application. After direct endoscopical observation after 30, 45 and 60 min of HAL application that gave unsatisfactory results, characterized by weak tumor fluorescence signal, low tumor selectivity and high background fluorescence, it was decided to follow a 30 min application period with a 30 min resting period. Under these conditions fluorescence appeared to emanate selectively from the tumors and a significant difference was observed between adenomas that had homogenous fluorescence and moderately differentiated carcinoma that had fluorescence only at the tumor edge. More data are required to determine the exact improvement of HAL FD in this domain.

## Conclusion

5-ALA derivatives have proven to substantially improve the local bioavailability of 5-ALA *in vitro* and *in vivo*. Depending on the respective derivatives drug doses as low as two order of magnitude less than 5-ALA are necessary to achieve similar or sometimes even better therapeutic results. In some selected cases 5-ALA n-alkylesters induced porphyrins more selectively in the target tissue. In some cases the increased production of porphyrins led to a substantial shortening of the interval between drug administration and irradiation. The superiority of 5-ALA esters over 5-ALA has been recently confirmed by the approval of Metvix® in Europe and Australia for the treatment of AK and Hexvix® for the improved FD of bladder cancer.

However, today it seems to be clear that the formulation has to be carefully adapted with respect to the lipophilicity of the respective 5-ALA derivative and medical indication. In terms of viscosity the appropriate formulation for the treatment of cervical cancer might be substantially different from those were other rheological properties are necessary. Although a moderately lipophilic formulation might be still appropriate for MAL, it will have a tremendous effect on the local bioavailability of lipophilic 5-ALA esters, such as HAL. In this context, principle consideration of pharmaceutical technology and biopharmacy might give further rise to a similar success for 5-ALA derivatives in other clinical indications in which topical administration is appropriate and convenient. Particular challenges are represented by indications such as Barett's esophagus or colon cancer.

Cosmetics are another interesting area of application for 5-ALA esters. The outcome of current studies of the treatment of acne is of particular interest. Indications of purely cosmetic interest, such as hair removal or skin rejuvenation, are of interest, at least from a commercial point of view. 5-ALA derivatives could have therapeutic indication in the area of bacterial infection and have shown a huge potential for the photodynamic inactivation of gram-positive and gram-negative bacteria in preliminary studies. In this context, 5-ALA esters might be of some interest as adjuvant treatment for the improvement of wound healing or of bacterial infestation in the oral cavity.

However, the huge opportunities underlying 5-ALA derivative-related research with respect to compounds that show enhanced activity following systemic administration remain largely unexplored until today. At present, the treatment or diagnosis of some life-threatening diseases, such as brain tumors, breast cancer and ovarian cancer, can hardly be achieved by simple 5-ALA derivatives. In the future it might be favorable for researchers working in the field to adopt lessons learned from rational prodrug design in their investigations of 5-ALA derivatives in order to further increase the impact of this research area.

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# CHAPTER III

## On the metabolism of 5-aminolevulinic acid derivatives

Nicolas Fotinos, Marino Andres Campo, Robert Gurny and Norbert Lange

Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, CH-1211 Geneva 4, Switzerland.

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## Abstract

Today, derivatives of 5-aminolevulinic acid (5-ALA) are commonly used in photomedicine as porphyrin precursors for the fluorescence detection or photodynamic treatment of cancerous and pre cancerous lesions. Although they have gained marketing authorization, and thus possess a high degree of security and effectiveness, research in this domain is still active in order to optimize the pharmacokinetics of these molecules. The main challenges, with respect to the development of 5-ALA derivatives, are optimized formulations, increased stability, and newly designed derivatives for specific applications. For this purpose, a better understanding of the exact fates of these molecules after their administration is needed.

In this context, we synthesized a 2-amino-3-oxohenadioic-1-methyl-6-methylester (AOMM), in which a carboxymethyl moiety is attached next to the amino group of 5-ALA methylester (MAL). This functionalization was designed to follow the accepted metabolism route of 5-ALA derivatives, and was performed to avoid a dimerization of the substrate, the main degradation mechanism occurring in aqueous environments. Therefore, this molecule has to be activated by an esterase to liberate 5-ALA or its methylester.

We observed that AOMM was efficiently hydrolysed into MAL and 5-ALA when incubated with porcine liver esterases. Surprisingly, human bladder cancer cells incubated with AOMM were totally unable to produce any type of porphyrins, in contrast to those incubated with 5-ALA or MAL. However, *in vivo* experiments showed that after intravenous injection of AOMM into chick embryos, a slight increase in porphyrin production compared to untreated controls can be observed. These results question the exact involvement of the enzymatic hydrolysis of 5-ALA derivatives within cells, and suggest the need for further investigation in this area, in order to optimize 5-ALA derivatization strategies.

## Introduction

Today, the use of prodrugs has become a key step in the development of pharmaceutical products, aiming at the improvement of crucial parameters like the solubility, tolerability, stability, and bioavailability of active compounds [1]. In the particular domain of photodynamic therapy (PDT), the use of 5-aminolevulinic acid (5-ALA) as a precursor of photoactive porphyrins (PAP) results in a drastic decrease of adverse events such as skin photosensitization compared to first generation photosensitizers (PS). Furthermore, an outstanding selectivity of 5-ALA induced PAPs for neoplastic cells was observed [2].

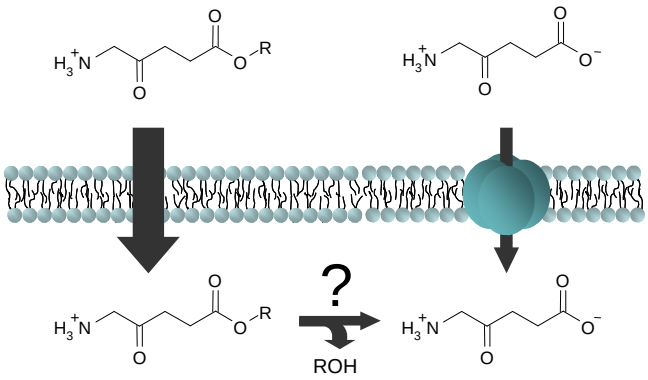


**Figure 1:** 5-ALA derivatives enter the cell by passive diffusion, in contrast with the parent compound, which has to be actively transported into the cytosol by non-specific peptide transporters. 5-ALA derivatives may then be hydrolyzed intracellularly by non-specific esterases to liberate 5-ALA.

Nevertheless, the double opposite charges carried by 5-ALA under physiological conditions seriously limit its ability to cross biological membranes, consequently restraining its bioavailability. One successful strategy adopted to overcome this low bioavailability was the derivatization of 5-ALA into more lipophilic n-alkylchained esters [3]. Several experiments have confirmed that these simple derivatives induced equivalent or higher porphyrin accumulation than the parent compound, but at significantly lower concentrations *in vitro* [4,5,6].

The explanation of this increased potency is related to the drug internalization process (Fig. 1). It was demonstrated that active transporters are involved in the entrance of 5-ALA or 5-ALA methylester (MAL) into the cells [7,8]. In contrast, lipophilic 5-ALA derivatives can cross biological barriers by passive diffusion or endocytosis, as incubation in the presence of transport competitive inhibitors have shown limited influence [8,9,10]. This non-energy dependent penetration of moderately lipophilic 5-ALA esters through cellular membranes is directly correlated to the lipophilicity of the molecule, enabling a faster incorporation of porphyrin precursors into the target cells.

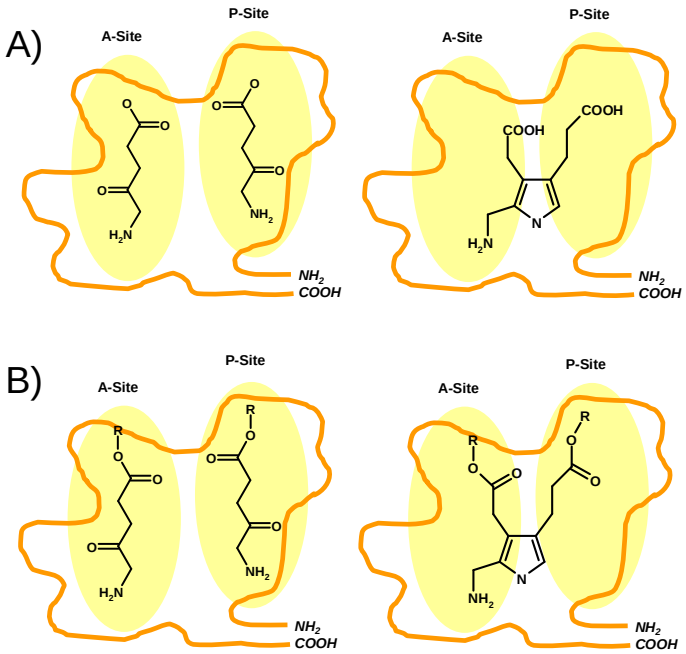


**Figure 2:** A) 5-ALA dehydratase, the second enzyme involved in the heme biosynthetic cycle, catalyzes the production of one molecule of porphobilinogen from two molecules of 5-aminolevulinic acid. B) As the carboxylic group of 5-ALA is not involved mechanistically in this enzymatic conversion, 5-ALA esters may be potential substrates, leading to the formation of esterified porphobilinogen.

After internalization, it is assumed that lipophilic derivatives of 5-ALA are cleaved intracellularly by non-specific esterases to liberate 5-ALA, the natural heme precursor. In this context, different experiments have evaluated the hydrolysis rate of 5-ALA derivatives, for example using lysed cells [11] or tissue homogenates [12]. Nevertheless, it is important to note that the esterified carboxylic groups are not involved in the enzymatic transformation of 5-ALA into porphobilinogen (PBG) [13]. It may, therefore, be in principle a direct substrate of the porphobilinogen synthase (Fig.2).

The aim of the present study was to explore possibilities to increase drug stability and bioavailability by taking advantage of the 5-ALA derivative metabolism. Therefore, we synthesized 3-amino-3-oxohenadioic-1,6-dimethyl ester (AOMM) (Fig. 3), which presents the following particularities, (i) physicochemical properties similar to MAL, (ii) stability towards dimerization due to the attachment of a methoxycarbonyl function next to the amino group, (iii) has to be expressly hydrolyzed and activated by esterases in order to undergo a rapid decarboxylation into MAL.

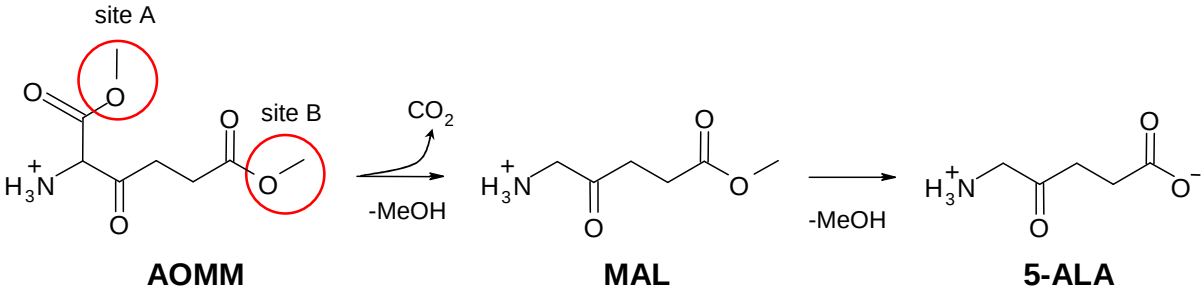


**Figure 3:** In the presence of intracellular esterases, AOMM will undergo enzymatic hydrolysis at two sites. Hydrolysis at site A will instantly release $CO_2$ through direct decarboxylation to form MAL, while cleavage at site B will finally lead to the liberation of 5-ALA.

## Material and Methods

**Chemicals.** All commercial reagents were used as obtained. D-MEM+GlutaMAX I, Penicillin-Streptomycin and D-PBS were obtained from Gibco (Invitrogen, Basel, Switzerland). Heat inactivated fetal-bovine serum and porcine liver esterase were purchased from Sigma (Buchs, Switzerland). 5-aminolevulinic acid was provided by Fluka (Buchs, Switzerland), and 5-ALA methylester from Organix (Colchester, UK). Protoporphyrin IX standard was obtained from Porphyrin products (Logan, USA).

**AOMM Synthesis.** In order to prepare AOMM, commercially available 1,6-dimethyl-3-oxo-hexanedioic acid was first nitrosilated at the 2-position with nitrous acid to yield 1,6-dimethyl-2-hydroxyimino-3-oxo-hexanedioic acid. The resulting hydroxyimino functionality was transformed to a Boc protected amine in a single step by hydrogenating over palladium on carbon in the presence of Boc anhydride. Subsequently, the Boc protecting group was removed under acidic conditions to yield the final product as a hydrochloride salt. 1,3-dihydroxy acetone and 1,6-dimethyl-3-oxo-hexanedioic acid were purchased from Fluka (Buchs, Switzerland).

1,6-Dimethyl-2-(hydroxyimino)-3-oxo-hexanedoic acid synthesis. $NaNO_2$ (0.95 g, 13.8 mmol) in water (1.5 ml) was added dropwise over 1h to a solution of 1,6-dimethyl-3-oxo-hexanedioic acid (2.16 g, 11.5 mmol) in acetic acid (5.0 ml) at 0 ºC. The resulting solution was stirred for an additional 2 hours at this temperature. The reaction was then diluted with ethyl acetate (200 ml) and washed with saturated NaCl solution (20 ml). The organic phase was separated, dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on a silica gel column using ethyl acetate/hexanes (1:2) to yield 2.17 g (87% yield) of the indicated compound as a clear oil. All spectral properties were identical to those previously reported [14].

Methyl 5-methoxycarbonyl-5-(*N*-(tert-Butoxycarbonyl)amino)levulinate synthesis. 1,6-dimethyl-2-(hydroxyimino)-3-oxo-hexanedoic acid (2.45 g, 11.3 mmol), $Boc_2O$ (4.94 g, 22.6 mmol), 10% Pd/C (0.23 g), and methanol (110 ml) were placed in a 250 ml round bottom flask, and the resulting mixture was stirred under a hydrogen atmosphere for 2.5 h at room temperature. The reaction mixture was filtered through Celite, and the solvent was removed under reduced pressure. The crude was purified by flash chromatography on a silica gel column using ethyl acetate/hexanes (1:3) to yield 2.74 g (80% yield) of the indicated compound as a clear oil. $^1$H NMR (300 MHz, $CHCl_3$): 8.70 (br. s, 1H), 5.76 (d, J=6.6Hz, 1H), 5.08 (d, J=6.6Hz, 1H), 3.78 (s, 3H), 3.55 (s, 3H), 2.98-3.17 (m, 1H), 2.80-2.95 (m, 1H), 2.60-2.80 (m, 2H), 1.43 (s, 9H) ppm. $^{13}$C NMR (75.5Hz, CHCl3): 199.9, 177.7, 166.9, 155.1, 80.9, 63.6, 55.6, 53.4, 34.8, 28.3, 27.7 ppm, respectively.

Methyl 5-methoxycarbonyl-5-aminolevulinate hydrochloride synthesis. i-$Pr_3SiH$ (0.43 ml, 2.6 mmol) was added to a solution of methyl 5-methoxycarbonyl-5-(*N*-(tert-Butoxycarbonyl)amino)levulinate (0.61 g, 2.0 mmol) in anhydrous THF (20 ml), and then dry HCl gas was bubbled through the solution until saturation (determined by the evolution of white fumes). The resulting solution was left to stand at room temperature for 6 h in a closed container. The solvent was then removed under reduced pressure, and the remaining solid was washed several times with diethyl ether to yield 0.41 g (89% yield) of the desired

compound as a white solid. $^{1}$H NMR (300 MHz, DMSO): 9.01 (br. s, 3H), 5.41 (s, 1H), 3.86 (s, 3H), 3.62 (s, 3H), 3.15-3.26 (m, 1H), 2.93-3.03 (m, 1H), 2.61-2.66 (m, 2H) ppm. $^{13}$C NMR (75.5Hz, DMSO): 198.4, 172.3, 164.6, 61.5, 60.7, 54.3, 52.1, 35.5 ppm.

**Enzymatic hydrolysis.** Freshly prepared AOMM aqueous solutions were incubated in the dark with porcine liver esterase (Sigma, Buchs, Switzerland) at a concentration of 14 U·µl$^{-1}$ in borate buffer pH 7.6 at 25 °C. These conditions were determined as the best compromise for efficient enzymatic activity (optimal under alkaline conditions) and reduced drug degradation (more stable at acidic pH). The reaction was stopped by the addition of 200 µl of acetone to 100 µl of a reaction sample. The hydrolysis was followed by the fluorescence dosage of AOMM and its different metabolites, 5-ALA and MAL, after pre-column derivatization of the amino groups with fluorescamine (Fluka, Buchs, Switzerland) [15]. For this purpose, the sample was diluted in 5 ml borate buffer pH 8.5, and an excess of fluorescamine (300 µl, 1 mg·ml$^{-1}$ in anhydrous acetone) was added prior to agitation. Analysis was performed by fluorimetric-HPLC. Samples were injected into a LaChrom D7000 system (Merck-Hitachi, Tokyo, Japan) equipped with an L7100 high pressure pump. Separation was carried out on a reverse phase column 125/4 Nucleodur 3 µm C18 gravity (Macherey-Nagel, Oensingen, Switzerland), protected with the corresponding precolumn. Elution was performed at a flow rate of 0.7 ml·min$^{-1}$ using an isocratic mode with a solvent composed of borate buffer 0.1 M pH 8.5 and methanol in a ratio of 1:1. Detection was performed with a fluorescence detector (Merck-Hitachi, La Chrom L-7480) set to an excitation wavelength of 390 nm and an emission wavelength of 470 nm.

**Cell culture experiments.** The T24 human bladder carcinoma cell line was kindly provided by Meddisovery SA, and was grown as a monolayer and routinely maintained by serial passage in D-MEM+GlutaMAX I medium supplemented with 10% FCS, 100 µl·ml$^{-1}$ streptomycin, and 100 IE·ml$^{-1}$ penicillin in a regulated atmosphere of 5% $CO_2$ 95% air at 37 °C. Ninety-six well plates were prepared by seeding approximately 5,000 cells in medium, which were 90% confluent after 3 days. Prior to experimentation, the medium was removed, cells were washed two times with D-PBS and incubated with freshly prepared substrate solutions in D-PBS. Porphyrin fluorescence was monitored over 24 hours using a Saphir (Tecan, Männedorf, Switzerland) multi-plate reader, set to an excitation wavelength of 405 nm and an emission wavelength of 630 nm.

**Chick Embryo Model.** Fertilized hen eggs (Animalerie universitaire, University of Geneva, Geneva, Switzerland) were placed in an incubator set at 37 ℃ and a relative humidity (RH) of 65%. Until embryo development day (EDD) 4, eggs were rotated twice a day. Then, a 3 mm hole was drilled into the eggshell at the narrow apex and covered with adhesive tape. Eggs were then incubated without rotation until the assay on EDD 12. Then, the hole in the eggshell was enlarged to a diameter of 2–3 cm, allowing access to the chorioallantoic membrane (CAM) vasculature. Substrate solutions in a mixture of PEG400, ethanol and water (30:20:50) (v/v/v) were injected into one of the principal blood vessels of the CAM through a 33-gauge needle. After 3 hours, eggs were frozen to -80 ℃ until analysis. Before dissection, the chick hen egg was allowed to thaw for 4 hours at 4 ℃, and the following full organs or samples were removed: eyes, brain, lungs, liver, stomach, skin, CAM. Porphyrins were extracted by adding 10 µl of extraction solvent (ethanol/dimethylsulfoxide/acetic acid 80:20:1) per milligram of tissue followed by five sonication cycles of 5 seconds at 0 ℃. After 5 minutes centrifugation at 12,000 × *g*, the supernatant was collected, and protoporphyrin IX (PpIX), the main porphyrin produced, was measured by reverse phase chromatography using the same system as mentioned previously. Elution was performed using an isocratic mode with a solvent mixture composed of methanol, water, and acetic acid in a ratio of 90:10:0.1 (v/v/v). Detection was performed with a fluorescence detector (Merck-Hitachi, La Chrom L-7480) set to an excitation wavelength of 405 nm and an emission wavelength of 630 nm.

## Results

Figure 4 shows a chromatogram drawn after 20 minutes of AOMM incubation with porcine liver esterase. Retention times for 5-ALA, MAL and AOMM were 1.6, 2.4, and 3.5 minutes, respectively, consistent with their lipophilicities. By simultaneously monitoring the disappearance of AOMM and the appearance of 5-ALA and MAL (Fig. 5), it can be seen that AOMM is a substrate for this non-specific esterase.

Furthermore, the A site seems to be the preferred target for enzymatically catalyzed hydrolysis. As expected, the subsequent instantaneous decarboxylation led to the formation of MAL in solution. While AOMM was transformed into MAL within less than 40 minutes, the latter was hydrolyzed only slowly into 5-ALA under the action of porcine liver esterases. Only five percent of the initial AOMM dose was converted to 5-ALA after 6 hours of incubation

(Fig. 5). Since AOMM showed the capacity to be efficiently converted into MAL and 5-ALA *in vitro*, we proceeded to its assessment in cell culture

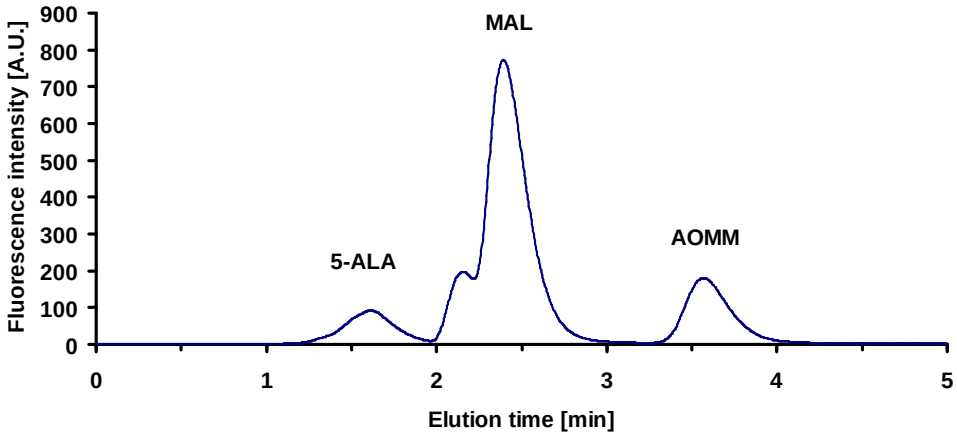


**Figure 4:** HPLC profile of AOMM and its two metabolites 5-ALA and MAL. Detection was performed by fluorescence ($\lambda_{excitation}$ 390 nm, $\lambda_{emission}$ 470 nm) after pre-column derivatization with fluorescamine and reverse phase separation.

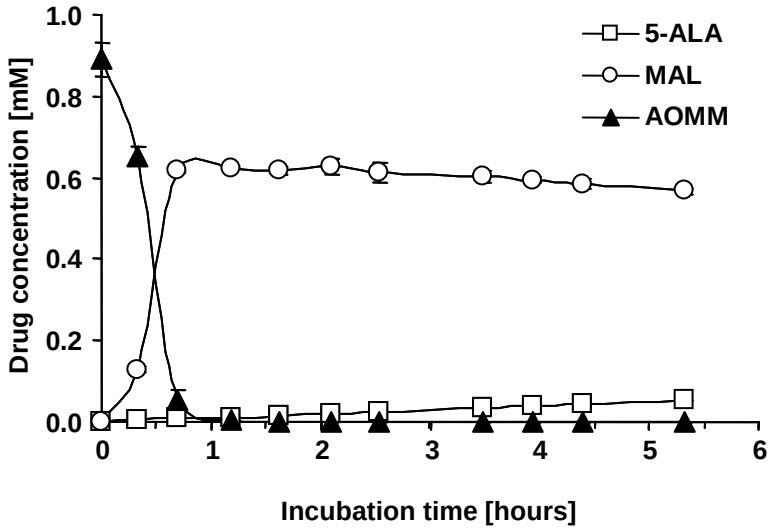


**Figure 5:** Enzymatic conversion of AOMM into MAL and 5-ALA (Porcine liver esterase 14 $U \cdot \mu l^{-1}$ in borate buffer pH 7.6, 25 °C).

*In vitro* cell culture experiments consisted of the monitoring of porphyrin fluorescence in the T24 human bladder cancer cell line upon incubation with 5-ALA or 5-ALA derivatives (Fig. 6). Strong porphyrin fluorescence was observed after incubation with exogenous 5-ALA.

Effective substrate concentrations ranged from 0.1 to 10 mM, with a maximum fluorescence intensity observed around 3 mM. 5-ALA methylester induced similar porphyrin fluorescence intensities, at slightly higher concentrations than the parent compound, which is in agreement with previously published data [4]. In contrast, AOMM induced no porphyrin production at any tested concentration during the first 6 hours of incubation. However, a slight porphyrin fluorescence was detected when increasing the incubation time over 10 hours (data not shown).

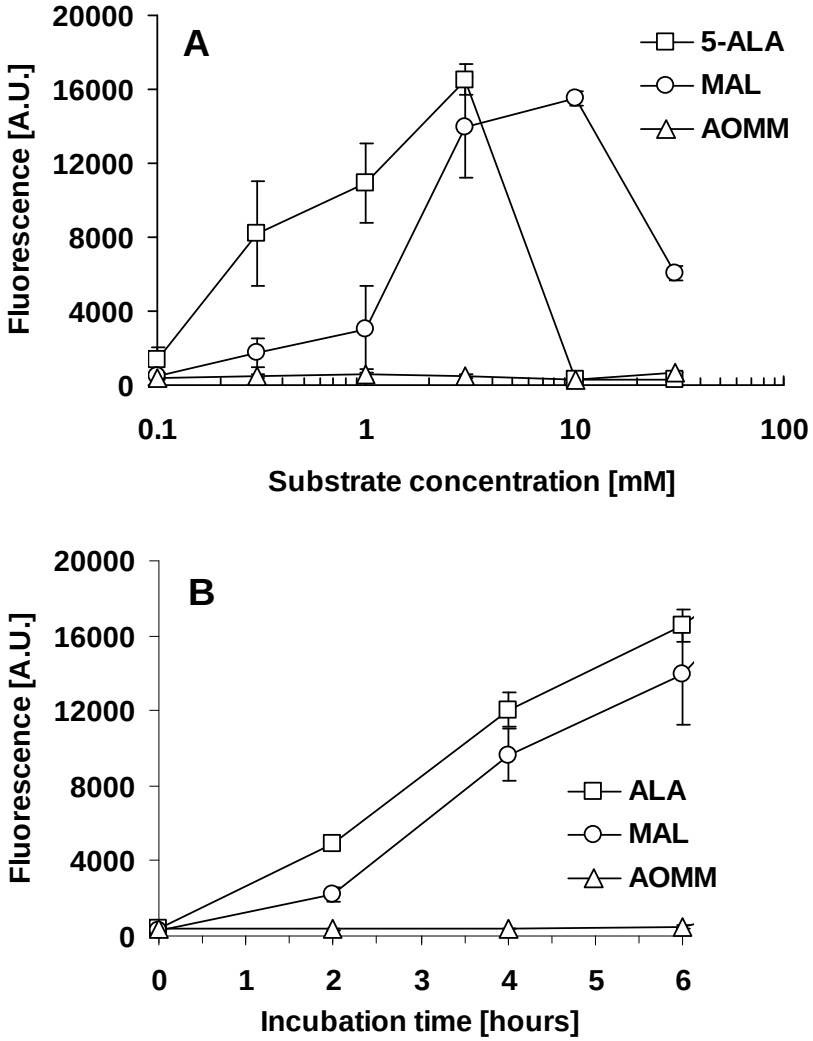


**Figure 6:** A) Porphyrin fluorescence of T24 human bladder cancer cells after 6 hours incubation with increasing concentrations of 5-ALA (□), MAL (○) and AOMM (Δ). B) Porphyrin fluorescence of T24 human bladder cancer cells incubated with 5-ALA (□), MAL (○) and AOMM (Δ) at 3 mM as a function of time.

As it was demonstrated that AOMM was enzymatically metabolized into MAL and 5-ALA, and despite its limited ability to induce porphyrin formation *in vitro*, we evaluated its capacity to induce porphyrin accumulation *in vivo* using the chick embryo *in ovo*. Three hours after intravenous administration in the CAM, we observed that AOMM induced a 3-fold increase in PAP production in the chick embryo organs as compared to the untreated control. However, the porphyrin induction was still inferior to 5-ALA and MAL, which induced 40- and 12-fold increases, respectively (Fig. 7).

Nevertheless, a prolonged incubation time of 6 hours increased the formation of photoactive porphyrin induced by AOMM, while a decrease was observed with MAL (results not shown). Therefore, AOMM has the capacity to act as a PAP precursor *in vivo*, although activation times appear to be delayed when compared to MAL.

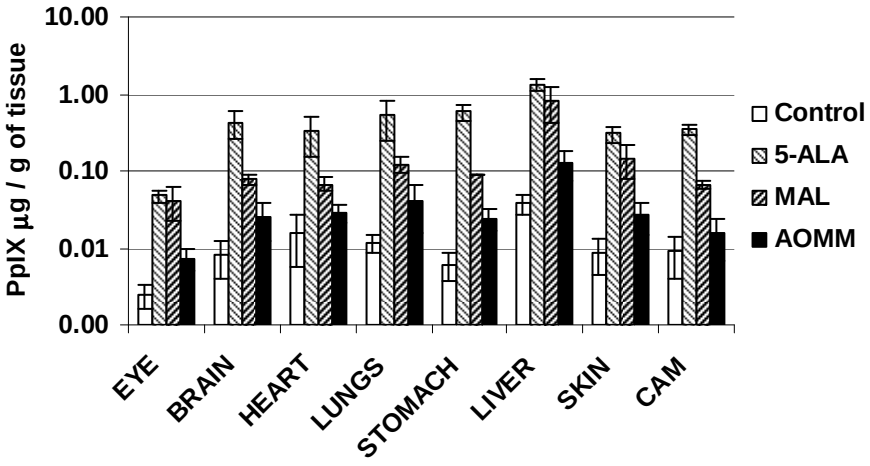


**Figure 7:** PpIX biodistribution in the chick embryo model after injection of equimolar doses of 5-ALA, MAL, and AOMM compared to the control (injection of solvent). Experimental conditions: 3 hours incubation time; 350 µmol·Kg$^{-1}$ dose.

## DISCUSSION

Today, the expanding clinical use of 5-ALA derivative-mediated photomedicine has triggered intense research efforts in this domain. Investigations currently focus on (i) the optimized formulation of available 5-ALA derivatives for increased bioavailability and stability [16,17], (ii) the development of new 5-ALA derivatives with optimized properties or

targeting intentions [18,19,20], and (iii) the fundamental investigation of the uptake [21], metabolism [8,12], and degradation [22,23] processes involved after the administration of 5-ALA derivatives.

In this context, we decided to focus on novel strategies for the design of 5-ALA derivatives. Since the stability of 5-ALA derivatives plays an important role in the process of drug development, we explored different options to prevent degradation. Today, other than lowering pH to subphysiological values, no method has obtained satisfying results. Therefore, extemporaneous formulation or shortened shelf life are recommended [23]. Unfortunately, too acidic pH values can potentially harm the ester function. The main degradation mechanism observed with such compounds in aqueous solutions is the dimerization of two 5-ALA or 5-ALA derivative molecules into pyrazines. The rate of this reaction increases with increasing substrate concentration and pH [24].

An apparently promising method to overcome this undesirable degradation process is the synthesis of 5-ALA derivatives with a protected amino group. Unfortunately, this approach leads to unsatisfactory PAP induction *in vitro* [25,26]. However, the fact that this strategy failed, presumably due to the absence of adapted lysis enzymes, does not necessarily imply the inability to “protect” the amino moiety against intermolecular condensations. An alternative approach to this problem may consist of the addition of a protective group next to the amino moiety that impedes intermolecular condensation.

According to the literature, esterases are the ideal target enzymes for prodrug activation. It is commonly accepted that 5-ALA derivatives, after passive entrance into the cell, are cleaved by non-specific esterases into free 5-ALA, which enters the heme biosynthesis pathway. The addition of a methoxy-carbonyl moiety next to the amino group is, in theory, a promising approach considering that (i) it will not significantly modify lipophilicity or molecular weight, which could significantly modulate cell penetration, (ii) the dimerization into pyrazine is sterically hindered, and (iii) esterases will induce a direct decarboxylation upon hydrolysis of the methoxy moiety.

Our observations confirm that, as expected, AOMM is efficiently hydrolysed by porcine liver esterase, and undergoes spontaneous decarboxylation into MAL. The latter is then slowly converted into 5-ALA. It is known that enzyme affinity for the substrate depends on chain length and lipophilicity. In the case of AOMM, for both methyl ester functions, a similar cleavage rate would have been expected. The fact that the decarboxylation subsequent to ester cleavage at site A is irreversible may explain the preferential hydrolysis of the protecting group.

Reports of the metabolism of 5-ALA derivatives are somewhat controversial. For example, the apparition of porphyrin fluorescence was taken as proof of 5-ALA derivative hydrolysis. Nevertheless, it should be considered that 5-ALA esters can be a potential substrate for heme biosynthesis (Fig. 2) [27].

Different studies investigated the metabolism of 5-ALA derivatives, especially 5-ALA n-alkyl esters, regarding their hydrolysis rate and enzymatic transformation into the parent compound [12,28,11]. In a recent study, Di Venosa *et al.* [28] evaluated the regulation of porphyrin synthesis and hydrolysis from 5-ALA derivatives. The intracellular accumulation of 5-ALA, 5-ALA derivatives, PBG, and porphyrins under different conditions was measured. These authors concluded that at high substrate concentrations, porphyrin synthesis from 5-ALA and 5-ALA derivatives was regulated by porphobiligenase and esterase, respectively. Furthermore, (i) only a small fraction of 5-ALA hexylester (HAL) was converted into 5-ALA intracellularly, (ii) at very low concentrations of HAL, only 18.7 pmol/$10^5$ cells was found, but porphyrin synthesis was already saturated, and (iii) at these intracellular concentrations, porphyrin synthesis was not saturated when using 5-ALA in the same cell line.

However, these experiments did not reveal whether the *in vivo* metabolism of 5-ALA esters is dominated by hydrolysis followed by heme biosynthesis or by the direct entry of the 5-ALA esters into this biosynthetic pathway. The fact that AOMM failed to induce porphyrin formation in cells could be explained by several different causes. A less efficient cell uptake of this compound as compared to MAL seems improbable, since the charge distribution, lipophilicity, and molecular weights are similar. The small increase of molecular weight should not inhibit passive transport through biomembranes, considering the *in vitro* porphyrin induction of porphyrin induced by 5-ALA derivatives of much higher molecular weights [20].

Consequently, it has to be questioned at which stage during heme biosynthesis 5-ALA derivative cleavage occurs. It was demonstrated that in the presence of tissue homogenates, long chained esters like 5-ALA hexylester were better substrates for enzymatic hydrolysis than short chained derivatives [12]. In contrast, Kloek *et al*. incubated 5-ALA esters of increasing chain length with lysed cells to minimize the influence of cellular uptake. Under their conditions, porphyrin production decreased with increasing substituent chain length until 5-ALA butylester. However, increasing porphyrin production was again observed with 5-ALA pentyl-, hexyl-, and octylester.

However, if MAL has to be converted into 5-ALA prior to entering heme biosynthesis, then, the presence of intracellular esterase activity is given in our *in vitro* studies by the MAL induced fluorescence in cells. Since our experiments with porcine liver esterases showed

that AOMM is efficiently converted into MAL, then AOMM would be thought to be converted into MAL in cells, and then into a substrate for heme biosynthesis. However, this is not the case, even at very high concentrations. Therefore, we hypothesize that esterase activity is not the sole parameter that dominates the conversion of 5-ALA derivatives into porphyrins.

## Conclusions

Our observations on the studied methoxycarbonyl-5-ALA methylester underline that cellular enzymatic cleavage of 5-ALA derivatives may not be mandatory. Focusing on enzymatic affinity, we observed that AOMM was a more preferred substrate for porcine liver esterase than MAL. However, cells incubated with MAL but not AOMM were able to accumulate fluorescent porphyrins. These results are not consistent with the commonly accepted metabolism route of 5-ALA derivatives, and encourage supplemental investigation in this area. A deeper understanding of the exact fate of 5-ALA derivatives after their administration will allow for the development of better targeting strategies for 5-ALA derivatives in the future.

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# CHAPTER IV

# The chick embryo model for the evaluation of 5-aminolevulinic acid derivatives

N. Fotinos, M.A. Campo, F. Popowycz, R. Gurny, N. Lange

Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, CH-1211 Geneva 4, Switzerland

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## Abstract

5-aminolevulinic acid (5-ALA) has arisen as a leading candidate for photodynamic therapy and fluorescence detection due to its numerous advantages over first generation photosensitizers, including tolerability, high selectivity for the diseased tissue and rapid elimination. However, the bioavailability of 5-ALA is structurally limited by its zwitterionic character at physiological pH, limiting its passage through biological barriers. More lipophilic 5-ALA derivatives, predominantly designed to increase local bioavailability after topical administration, have been developed to overcome this drawback. Nevertheless, several life threatening pathologies such as brain, breast or ovarian cancer cannot be efficiently treated using these compounds.

In this context, we synthesized a series of derivatives adapted to systemic administration. However, the insufficient aqueous solubility of these compounds makes their screening in conventional *in vitro* cell culture systems difficult. Therefore, we have used chick embryos for the evaluation of the biodistribution of 5-ALA derivative induced porphyrins. The molecules to be tested were dissolved in a solvent mixture of PEG, ethanol and water, and injected into a large vessel of the chorioallantoic membrane. After a defined exposure time, the chick embryo survival was evaluated, and then the embryos were frozen to -80 °C. After dissection, different organs were collected, and photoactive products were extracted by sonication and quantified by HPLC.

This model allowed for the determination of the most promising compounds in terms of their ability to induce the accumulation of photoactive porphyrins in different organs and the absence of acute toxicity after intravenous administration. Furthermore, the present system might be a cost effective alternative for initial biodistribution and toxicity studies.

## Introduction

Over the last decade, the use of photodynamic therapy (PDT) and fluorescence detection (FD) in various medical fields has become increasingly widespread. These non-invasive techniques combine the use of a photosensitizer (PS) accumulated into the target tissues with activation by light to induce fluorescence in the case of FD or initiation of the formation of reactive oxygen species in the case of PDT, ultimately leading to the destruction of targeted pathological tissues.

Different photosensitizer types have been tested in photomedicine, usually derived from a porphyrinic skeleton [1]. However, one endogenous molecule, 5-aminolevulinic acid (5-ALA), has raised particular interest in this field, since it acts as a pro-photosensitizer when administered exogenously. In fact, the formation of 5-ALA catalyzed by 5-ALA synthase is the first step of heme biosynthesis, and can be observed in all nucleated cells. The limited accumulation of photoactive porphyrins (PAP) in tissues is physiologically regulated by a negative feedback induced by heme on 5-ALA synthase. However, an exogenous administration of 5-ALA overcomes this negative feedback, leading to the temporary accumulation of PAP within cells, especially those with high metabolic turnover like cancer cells [2].

One major drawback of 5-ALA-mediated PDT is directly related to the structure of the molecule, which is present as a zwitterion under physiological conditions. This highly charged molecule cannot easily cross biological membranes, and has to be internalized by active transporters [3]. To overcome this problem, more lipophilic derivatives have been proposed [4]. Two of them, 5-ALA methylester and 5-ALA hexylester, achieved marketing authorization and are indicated for PDT treatment of basal cell carcinoma and actinic keratosis and improved detection of bladder cancer, respectively. However, these two molecules are adapted for topical administration, but are suboptimal for utilization in a systemic approach. In this context, life threatening diseases like breast cancer, prostate cancer, or brain cancer cannot be optimally targeted.

When targeting brain cancer, the ability to cross the blood brain barrier (BBB) has to be considered a major prerequisite. This ability can be obtained by targeting specific transporters or by increased lipophilicity [5]. Different derivatization strategies are conceivable, but should be performed preferentially on the carboxylic function, as amide derivatives usually fail to induce sufficient PAP formation in cell culture [6].

However, in opposition to the desired increased lipophilicity, some derivatives do not show sufficient water solubility to be screened in cell culture. Their solubility can be enhanced by the use of cosolvents, but their potential cytotoxicity often makes them unsuitable for cell culture. Furthermore, other solubilizing molecules (cyclodextrines) or specific formulations (liposomes, nanoparticles, micelles) are not suitable for the screening phase of drug development due to their potential interference with PAP formation [7].

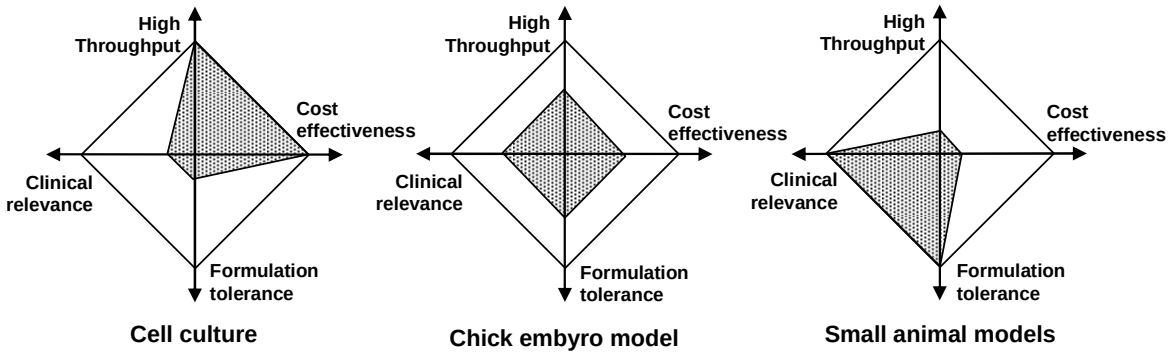


**Figure 1**: Advantages and drawbacks of the different models available for the evaluation of 5-ALA derivatives, expressed in terms of high throughput ability, cost effectiveness, clinical relevance and formulation tolerance.

We therefore evaluated hens' chick embryos for the initial assessment of the biodistribution of systemically administered 5-ALA derivatives. The model of the chick embryo chorioallantoic membrane (CAM) has been used for several years in the domain of photomedicine for the evaluation of the vaso-occlusive properties of PDT regimens [8,9]. This model is relatively easy to use and set up, it supports the use of mixtures of solubilizing cosolvents, and it therefore allows for an easy evaluation of the potential of non water-soluble PAP precursors. Furthermore, it may fill a gap between *in vitro* models and the cost and time prohibitive preclinical evaluation on small animals (Fig. 1).

## Materials and Methods

**Chemicals.** 5-aminolevulinic acid was obtained from Fluka (Buchs, Switzerland) and 5-ALA hexylester from Organix (Colchester, UK). Protoporphyrin IX was purchased from Frontier Scientific (Logan, USA). All other solvents and chemicals used were of analytical grade and used without further purification.

**Chick Embryo Model.** Fertilized hen eggs (Animalerie universitaire, University of Geneva, Geneva, Switzerland) were placed into an incubator set at 37 °C and a relative humidity of 65%. Until embryo development day (EDD) 4, eggs were rotated twice a day. Then, a 3 mm hole was drilled into the eggshell at the narrow apex and covered with adhesive tape. Eggs were then incubated without rotation until the experiment on EDD 12.

On EDD 12, the hole in the eggshell was enlarged to a diameter of 2–3 cm to allow access to the CAM vasculature. Substrates to be evaluated (Fig. 2) were solubilized with a solvent mixture composed of PEG400, ethanol and water (30:20:50; v/v/v), and injected into one of the principal blood vessels of CAM through a 33-gauge needle. After defined incubation times, survival of the embryos was evaluated, and eggs were frozen at -80 °C until analysis.

Before dissection, the chick egg was allowed to thaw for 4 hours at 4 °C, and the following full organs or samples were collected (eye, brain, lung, liver, stomach, skin, CAM) [10]. Porphyrins were extracted from the different organ samples after membrane disruption, according to a modified method described by Peng *et al.* [11]. Five sonication cycles of 5 seconds at 0 °C were conducted using a probe sonicator (Branson digital sonifier, Danbury, USA) after the addition of 10 µl of extraction solvent (ethanol/dimethylsulfoxide/acetic acid; 80:20:1) per milligram of tissue. The samples were centrifuged at 12,500 × *g* for 5 minutes, the supernatant was collected, and protoporphyrin IX was dosed by reverse phase chromatography with a 125/4 Nucleodur C18 gravity column (Macherey-Nagel, Oensingen, Switzerland) using an isocratic elution with methanol/water/acetic acid (90:10:0.1; v/v/v). Detection was performed with a fluorescence detector (Merck-Hitachi, La Chrom L-7480; excitation wavelength: 407 nm and emission wavelength: 630 nm). A calibration curve was performed with protoporphyrin IX standard solutions.

(a) The toxicity of the solvent, (b) the toxicity of the different substrates, and (c) the biodistribution of protoporphyrin IX formation in the different organs of the chick embryo were assessed.

**5-ALA diacylglyceride derivative synthesis**. 1,3-diacetyl acetone, 1,3-diacetyl-2-hydroxy glyceride, and 1,3-dibutyrate-2-hydroxy glyceride were prepared according to Paris *et al*. [12].

15-((*N*-tert-butoxycarbonyl)amino)levulinic acid synthesis: A solution of 5-aminolevulinic acid (20.9 mmol) in methanol (50 ml) was added slowly to a solution of Boc anhydride (6.84 g, 31 mmol) and diisopropyl ethyl amine (5.4 g, 2 equiv.) in methanol (40 ml), with stirring. The reaction was allowed to proceed for 1.5 h, and then the solvent was evaporated under reduced pressure. The compound of interest was obtained after chromatographic purification on silica gel using ethyl acetate/hexane (3:2) as a clear oil weighing 4.2 g. $^{1}$H NMR (300 MHz, $CDCl_3$): 1.46 (s, 9H), 2.71 (br s, 4H), 4.07 (br s, 2H), 5.25 (br s, 1H), 8.93 (br s, 1H) ppm. $^{13}$C NMR (75.5Hz, $CDCl_3$): 27.8, 28.5, 34.4, 50.5, 80.3, 177.4, 204.5 (missing one $sp^2$ carbon due to overlap) ppm.

1,3-dibutyrate-2-(*N*-tert-butoxycarbonyl)ALA glyceride synthesis: EDC (0.238 g, 1.5 equiv.) was added to a solution of 1,3-dibutyrate-2-hydroxy glyceride (0.288 g, 1.24 mmol), 5-((*N*-tert-butoxycarbonyl)amino)levulinic acid (0.19 g, 0.827 mmol), and DMAP (5 mg) in DCM (2.5 ml), and the solution was stirred for 5 h at room temperature. Solvent was then removed under reduced pressure, and the mixture was purified by column chromatography on a silica gel using ethyl acetate/hexane (1:2). The desired product was obtained as a clear oil weighing 0.204 g (55% yield). $^{1}$H NMR (300 MHz, $CDCl_3$): 5.10-5.01 (m, 1H), 4.16-3.88 (m, 6H), 2.73 (t, J=6.5Hz, 2H), 2.54 (t, J=6.5Hz, 2H), 2.15 (t, J=7.3Hz, 4H), 1.49 (h, J=7,6Hz, 4H), 1.29 (s, 9H), 0.79 (t, J=7.3Hz, 6H) ppm. $^{13}$C NMR (75.5Hz, $CDCl_3$): 13.7, 18.4, 27.8, 28.4, 34.3, 35.9, 50.3, 61.9, 69.7, 171.6, 173.2 (missing two $sp^2$ and one $sp^3$ carbons due to overlap) ppm.

1,3-dibutyrate-2-ALA glyceride TFA salt synthesis: 1,3-dibutyrate-2-(*N*-tert-butoxycarbonyl)-ALA glyceride was dissolved in dichloromethane/TFA (4:1), and the reaction was allowed to proceed for 2 h at room temperature. Solvent was then removed under reduced pressure to obtain the desired compound as a thick oil in quantitative yield. $^{1}$H NMR (300 MHz, $CDCl_3$): 8.11 (br. s, 3H), 5.04-4.97 (m, 1H), 4.17-3.95 (m, 6H), 2.76 (t, J=6.3Hz, 2H), 2.52 (t, J=6.3Hz, 2H), 2.15 (t, J=7.4Hz, 4H), 1.47 (h, J=7,5Hz, 4H), 0.78 (t, J=7.4Hz, 6H) ppm. $^{13}$C NMR (75.5Hz, $CDCl_3$): 13.7, 18.4, 27.5, 34.8, 35.9, 47.9, 61.8, 69.7, 171.8, 173.3, 202.4 ppm.

1,3-diacetyl-2-(*N*-tert-butoxycarbonyl)ALA glyceride synthesis: This compound was prepared in a fashion similar to 1,3-dibutyrate-2-(*N*-tert-butoxycarbonyl)ALA glyceride. $^{1}$H NMR (300 MHz, $CDCl_3$): 5.28-5.21 (m, 1H), 4.33-4.05 (m, 6H), 2.75 (t, J=6.3Hz, 2H), 2.68 (t, J=6.3Hz, 2H), 2.09 (s, 6H), 1.46 (s, 9H) ppm.

1,3-diacetyl-2-ALA glyceride TFA salt synthesis: This compound was prepared in a fashion similar to 1,3-dibutyrate-2-ALA glyceride TFA salt. $^{1}$H NMR (300 MHz, $CDCl_3$): 8.01 (br. s, 3H), 5.28-5.23 (m, 1H), 4.40-4.02 (m, 6H), 2.88 (t, J=6.3Hz, 2H), 2.70 (t, J=6.3Hz, 2H), 2.08 (s, 6H) ppm.

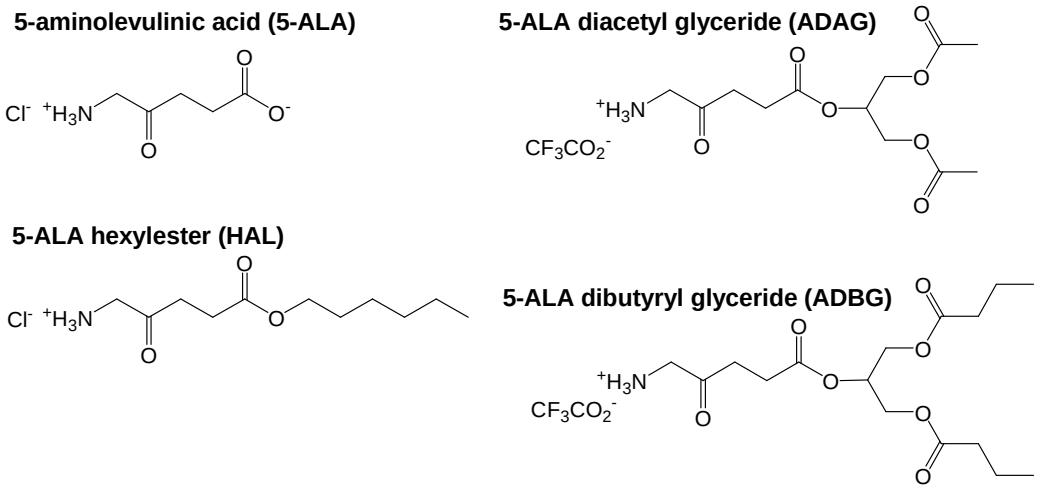


**Figure 2**: Chemical structures of the different pro-photosensitizers used for the evaluation of the chick embryo model.

## Results

**5-ALA-mediated porphyrin formation**. The chick embryo model was evaluated with respect to 5-ALA-mediated porphyrin formation as a function of increasing drug doses. The 5-ALA solutions were injected into a large vessel of the CAM, and the incubation time was set to 3 hours. Survival was monitored, and all embryos survived using 5-ALA doses ranging from 0 to 120 mg·Kg$^{-1}$, while injection of 240 mg·Kg$^{-1}$ was lethal to the embryos in all cases. The reasons for this toxicity are unknown, but some abdominal haemorrhages were observed that could be related to hepatotoxicity.

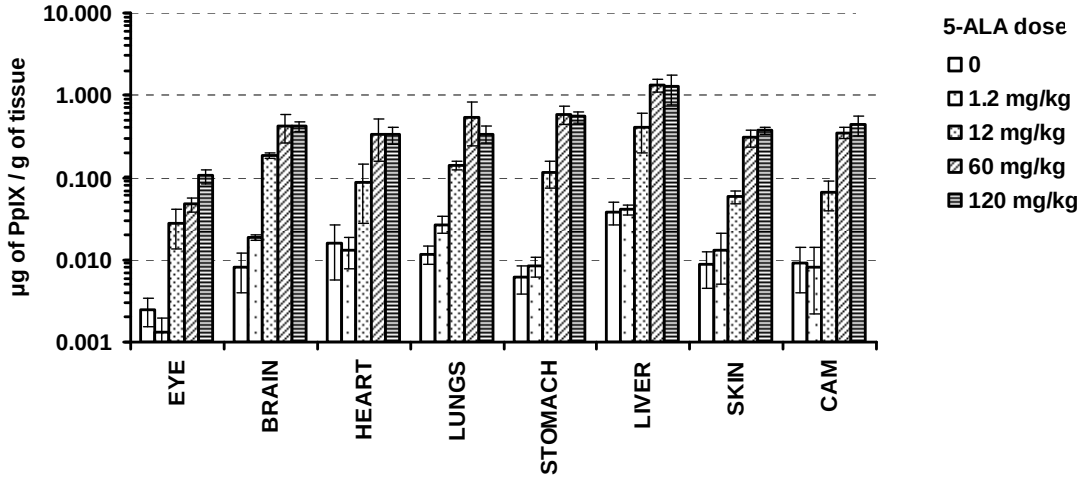


**Figure 3**: Porphyrin biodistribution after the injection of increasing doses of 5-ALA into the chick embryo model. Incubation time was 3 hours.

The control consisted of an injection of 10-50 µl of solubilizing vehicle, and showed no death was observed in any of the chick embryos. Samples taken from these control experiments showed a low basal porphyrin concentration with high inter-organ variation. The injection of low doses of 5-ALA (1.2 mg·Kg$^{-1}$) did not induce a significant increase in PpIX production. At 5-ALA doses of 12 mg·Kg$^{-1}$, the porphyrin accumulation in the different organs was 10-20 times higher than the control, and a plateau was reached at 60 mg·Kg$^{-1}$. At these optimal concentrations, the porphyrin content for all examined organs was increased by 1-2 orders of magnitude compared to the control, inducing a strong fluorescence in all tissues (Fig. 3).

**5-ALA derivative-mediated porphyrin formation**. 5-ALA hexylester, a molecule adapted for optimal topical application induced an immediate arrest of blood flow after doses of ≥ 60 mg·Kg$^{-1}$. Significantly different results were obtained with the 5-ALA diacylglyceride derivatives after administration to the chick embryo model. No mortality was observed after the injection of drug doses up to 240 mg·Kg$^{-1}$ for either 5-ALA diacetylglyceride or dibutyrylglyceride. The distribution showed an induction of porphyrin accumulation in various tissues, comparable to 5-ALA at equimolar doses (375 µmol·Kg$^{-1}$) (Fig. 4).

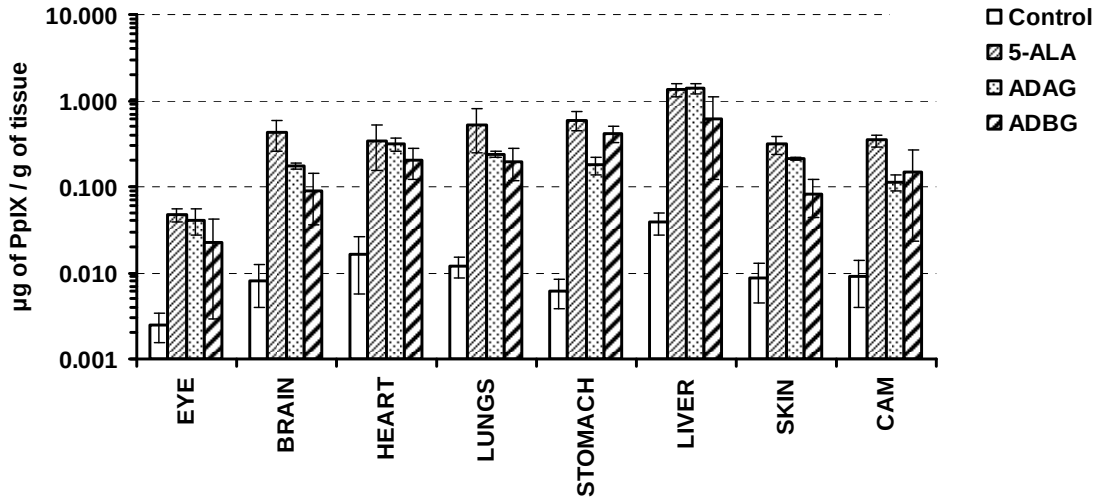


**Figure 4**: Porphyrin biodistribution after the injection of equimolar doses (375 µmol·Kg$^{-1}$) of 5-ALA, ADAG and ADBG into the chick embryo model, after 3 hours of incubation.

## Discussion

Different tools are currently available for the evaluation of the capacity of 5-ALA or 5-ALA derivatives to induce the formation of photoactive porphyrins. Cell culture is an easily accessible, economically attractive model that allows the screening of numerous molecules in multi-well plates [13]. However, the simplicity of such models, with single cell layers, largely fails to mimic the complex interactions of tissues in the organism. Such drawbacks can be improved by the use of three dimensional models such as spheroids [14,15]. *Ex vivo* experiments on specific animal tissue samples have also been used, *e.g.,* porcine bladder for the optimization of 5-ALA hexylester mediated FD and PDT [16], but this method is not as easy to use as *in vitro* methods, and it presents supply complications.

Furthermore, the high lipophilicity of some molecules in order to enable them to effectively cross the BBB can be an insurmountable obstacle for facilitated *in vitro* experimentation. In fact, the evaluation of molecules with limited water solubility has always been a problem in pharmaceutical research. Although numerous possibilities exist to overcome this problem during the formulation phase, such as co-solvents, solubilizing agents, surfactants, or specific formulations (nanoparticles, emulsions, micelles) [17], their use is either not compatible with cell culture or not appropriate during a screening phase for optimized compounds.

Furthermore, the *in vivo* screening of new compounds directly on expensive animal models is not conceivable, either practically or ethically. In this context, we considered the use of chick embryos. This "*in vivo*-like" model is easily accessible and cost effective. Furthermore, the injection of small volumes of co-solvents are well tolerated, and allow for the evaluation of non-water soluble molecules [8]. This method consists of the injection of the drug in a large vessel of the chorioallantoic membrane, and subsequent monitoring of the formation of the photoactive protoporphyin IX in the different tissues of the chick embryo.

We first evaluated this model with respect to the injection and incubation with different concentrations of 5-ALA dissolved in a solvent mixture. The porphyrin generation was analyzed by reverse liquid chromatography coupled to fluorescence detection. PpIX was the main component produced, while uroporphyrin III and coproporphyrin III were detected only in trace amounts. In the future, the analysis step could be simplified by a high throughput technique using a multi-plate reader to measure PpIX fluorescence, avoiding the time consuming separation. After dissection [10], the low resistance of the chick embryo organs allows for their fragmentation by sonication, leading to efficient extraction of 90-99% of the porphyrin. The lowest extraction yield was obtained in liver samples, which contain a high natural porphyrin content. An extraction solvent composed of ethanol, dimethylsulfoxide and acetic acid has been chosen due to its optimal solubilization of both hydrophilic and lipophilic porphyrins.

Although the results cannot be directly translated into other *in vivo* animal models, our results are on the same order of magnitude as porphyrin accumulation in tissues and optimal concentrations observed by Di Venosa *et al*. in mice after intraperitoneal administration of 5-ALA [18]. In mice, a plateau of porphyrin production was achieved at 5-ALA concentrations around 120-320 $mg \cdot Kg^{-1}$, and the maximal porphyrin amount accumulated in the different organs was around 1-10 µg·per g of tissue.

It is evident that some factors differentiate the chick embryo model from small animal models. At this time of the embryogenesis, the blood brain barrier is not totally effective, the connections with the central nervous system are not completed, the immunological system is immature and the elimination process of xenobiotics is certainly not totally functional. However, the results obtained are far more clinically relevant than those from cell culture, since the model takes into account parameters like metabolism, binding to plasma proteins, and biodistribution. Moreover, the early information about the approximate lethal doses of the tested product *in vivo* is highly desirable.

Peterka *et al.* [19] used chick embryos to evaluate the toxicity of 5-ALA and PpIX with and without light irradiation. No lethality or malformation was observed after systemic administration of 5-ALA with concentrations up to 300 µg (approximately 60 mg/kg), which is in accordance with our results. This preliminary indication about the toxicity of the tested precursors obtained with the chick embryo model is oversimplified, since it relies on the survival or not of the embryo after the injection. It does not give any information about different adverse events that may occur after systemic administration of 5-ALA. However, as these toxicity results are comparable to those obtained with small animals (see below), they may provide useful information when considering selection of drug candidates.

Furthermore, in order to improve the model, it is possible to implant different tumor tissues or tumor cells directly onto the CAM, inducing the growth of tumors fully supplied by the CAM vascularization [20,21]. Other routes of administration besides intravenous injection can also be evaluated, such as intraperitoneal injection into the chick embryo or topical application onto the CAM [22,21].

In the present study, we investigated novel 5-ALA derivatives intended for use in brain cancer. As mentioned above, the derivatization of 5-ALA into more lipophilic compounds to overcome its low bioavailability has been successfully employed in the case of topical applications [23,13]. 5-ALA n-alkylesters were effectively adapted for topical application in the cases of skin cancer [24] and bladder cancer [25]. However, to achieve increased systemic bioavailability, a very different approach must be considered. The case of 5-ALA mediated FD or PDT of brain cancer can be taken as an example.

Although it is currently under evaluation in clinical trials [26], the oral administration of 5-ALA may be suboptimal in terms of the ability of the porphyrin precursor to reach the central nervous system. First, studies in dogs have shown a moderate bioavailability (about 40%) after oral administration due to poor absorption and the first-pass metabolism in the intestines and the liver [27]. Secondly, a small distribution volume and rapid elimination through the liver and kidneys result in a short plasma half-life (40-50 minutes) of 5-ALA after systemic administration [28]. Finally, as mentioned previously, 5-ALA is a highly charged molecule, and it is well accepted that these types of hydrophilic compounds have a very limited capacity to cross the BBB [5]. This has been confirmed *in vivo,* notably by Van den Boogert *et al.*, who found a 5-ALA concentration in the brain between 1 and 2 orders of magnitude lower than in other organs, after intravenous or oral administration [29].

Nevertheless, an increased lipophilicity alone may not be an adequate solution, since apart from the improved passage through the BBB, a non selective accumulation in other tissues will also occur. For instance, 5-ALA hexylester, considered as the optimal n-alkyl derivative in terms of the lipophilicity-water solubility ratio, was unsuccessful in the improvement of brain cancer fluorescence detection due to its high systemic toxicity. This was shown by Perotti *et al*. [30], who administered equimolar solutions of 5-ALA or 5-ALA hexylester to mice intra-peritoneally. Although they measured more than 6 times the amount of 5-ALA hexylester in the brain than the parent compound, illustrating an increased transport through the BBB, mice treated with HAL did not survive the injection. Comparable systemic toxicity was observed after the injection of low amounts of HAL to chick embryos. Therefore, other derivatization strategies compatible with systemic administration must be considered for optimal targeting of the central nervous system (CNS).

One conceivable strategy for 5-ALA targeting to the CNS is lipidization [31], as has been shown for brain delivery of GABA (γ-aminobutyric acid), which is structurally very closely related to 5-ALA. A derivatization of GABA into glyceryl lipid prodrug increased GABA delivery to the brain by a factor of 200 when injected into mice [32]. We therefore set up a synthetic route to allow the production of a series of different 5-ALA diacylglycerides. However, it was reported that 5-ALA esters with a branch point in position one from the carboxylic group have only low capacities to induce the formation of porphyrin *in vitro* [33].

As glyceryl derivatives undergo a particular route of metabolism [31], it was therefore essential to establish the capacity of 5-ALA glyceride derivatives to liberate substrates able to induce the formation of PAPs *in vivo*. Here, we have demonstrated that after intravenous injection of the 5-ALA diacetylglyceride into the chick embryo model, porphyrin concentrations similar to those of the equimolar injection of the parent compound can be observed.

The more lipophilic 5-ALA dibutyrylgylceride, with a log *P* around 2, failed to increase the production of porphyrins in the brain. Nevertheless, the end goal of these modified compounds is not to increase porphyrin production in normal brain tissues, but to achieve a higher brain penetration index, permitting the administration of lower drug doses, thus limiting the undesirable adverse effects. As these 5-ALA diacylglycerides have shown the capacity to induce porphyrin formation in different tissues, the influence of different fatty acid types on delivery should be investigated. Furthermore, this concept will be validated using a rat glioma model [34], in order to confirm the feasibility and efficiency of such a lipidic strategy for the delivery of 5-ALA to the brain.

As we have shown that the chick embryo can be used as a model for the evaluation of lipophilic molecules that were previously difficult to test *in vitro*, other derivatization strategies can now be considered for the targeting of tumors localized in the CNS. Lipidization was discussed above, and its feasibility was demonstrated with 5-ALA diacylglycerides that induced PAP *in vivo*. In this context, 5-ALA may also be linked to fatty acids or phospholipids.

The "lock-in" approach developed by Bodor *et al.* [35] involves lipophilic 5-ALA derivatives that can easily cross the BBB where they will be enzymatically metabolized into hydrophilic products. Here, selectivity does not depend on the regional presence of target enzymes, as can occur in other organs, but on the localization into or out of the CNS. Heme precursors released into the CNS will remain trapped due to their inability to cross the BBB, while those located elsewhere will be rapidly excreted from the body.

Finally, the Schiff's base strategy may facilitate the entrance of 5-ALA across the BBB [36]. This approach involves 5-ALA derivatives bearing either an imine or an enamine moiety linked to a lipophilic carrier, as amines have shown to be unfavorably cleaved.

## Conclusions

The chick embryo model can become a useful tool for the evaluation of 5-ALA derivatives in terms of their ability to induce the accumulation of photoactive porphyrins in different organs and the absence of acute toxicity after intravenous administration. This system tolerates the use of co-solvents, and therefore allows for the direct evaluation of poorly water soluble molecules. Furthermore, the present system might be a cost effective alternative for initial biodistribution and toxicity studies.

In this context, triglyceride esters of 5-ALA have been shown to induce porphyrin accumulation in different organs after systemic administration without presenting acute toxicity. Further investigations should be performed to assess this molecule as a candidate for brain targeted treatment.

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# CHAPTER V

## 5-aminolevulinic acid and 5-aminolevulinic acid Derivatives mediated Effects on Gram-Negative and Gram-Positive Bacteria

Nicolas Fotinos [1], Maruska Convert [2], Jean-Claude Piffaretti [2], Robert Gurny [1] and Norbert Lange [1]

[1] Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, 1211Geneva 4, Switzerland

[2] Institute of Microbiology, Via Mirasole 22A, 6500 Bellinzona, Switzerland

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## Abstract

Due primarily to the extensive use of antibiotics, the spread of multi-resistant bacterial strains is one of the most worrying threats to public health. One strategy to overcome potential shortcomings might be the inactivation of pathogenic microorganisms by photodynamic therapy (PDT). In the past, different photosensitizers have been assessed on various pathogenic microorganisms, including bacteria (*Escherichia coli*, *Staphylococcus aureus*), viruses (Papilloma, Herpes), fungi and parasites (*Plasmodium, Leishmania*).

5-aminolevulinic acid (5-ALA)-mediated PDT has attracted considerable attention over the last decade, presumably due to its numerous advantages over conventional photosensitizers. 5-ALA has no photoactive properties, but when given exogenously, acts as a precursor of photosensitive porphyrins. This occurs predominantly in tissues or organisms that are characterized by a high metabolic turnover, such as tumors, macrophages, or bacteria. However, 5-ALA's weak ability to cross biological barriers has lead to the introduction of more lipophilic derivatives, such as methylaminolevulinate or hexylaminolevulinate, which display an improved capacity to reach the cytoplasm.

Different studies have shown that gram-positive bacteria are significantly more sensitive to PDT than gram-negative strains. This has been attributed to a less sophisticated barrier of gram-positive organisms when compared to the complex multilayer barrier of gram-negative microorganisms. Starting from the hypothesis that more lipophilic compounds carrying a permanent positive charge under physiological conditions may cross the bacterial multilayer barrier more easily, we have tested the efficacy of some 5-ALA n-alkylesters in bacterial inactivation. In our studies, 5-ALA methylester and butylester were the most effective compounds with respect to the photodynamic inactivation of bacteria. We observed significant differences in terms of the optimal drug concentration, bactericidal activities and porphyrin production.

## Introduction

The discovery of penicillin by Fleming in 1928 ushered in the golden age of antimicrobial therapy. Since then, the rapid development of new classes of active antibiotics reinforced the opinion that the plague of infectious diseases would be quickly resolved. However, many decades later, infectious threats are still a part of our lives, and antibiotic multi-resistance slowly but constantly increases, primarily due to an irrational and inappropriate use of these drugs in humans or agriculture [1]. In 1998, the World Health Assembly voted on a resolution to classify antimicrobial resistance as one of the major threats against human health [2].

During the last few decades, only a few new drugs and even fewer new antibiotic classes have reached the market; the many promises of biotechnology and genetics failed to deliver results. Furthermore, antimicrobial research is no longer a priority for many pharmaceutical companies, presumably due to an unfavourable benefit/risk ratio [3]. In this context, photodynamic therapy (PDT) may be an interesting alternative. PDT is the result of the combinational use of three autonomously non-active elements: (i) a non-toxic photoactive molecule called a photosensitizer (PS), (ii) light of the appropriate wavelength to excite the PS, and, finally, (iii) oxygen that will be transformed into the highly reactive singlet oxygen species upon energy transfer from the light-activated PS.

The use of PDT for antimicrobial purposes is not recent, as it was the predominant step leading to the discovery of this treatment modality in the beginning of the 20$^{th}$ century [4]. However, PDT never achieved a real success in its native domain, in contrast to other

therapeutic fields where it is now used in routine clinical practice, including the treatment of some forms of cancer and age-related macular degeneration [5,6].

The regain of interest in antimicrobial PDT originates from two main factors: first, the promising results obtained by PDT in the abovementioned fields; and second, the critical need for new antimicrobial therapies that has arisen from the spread of multi-resistant microorganisms.

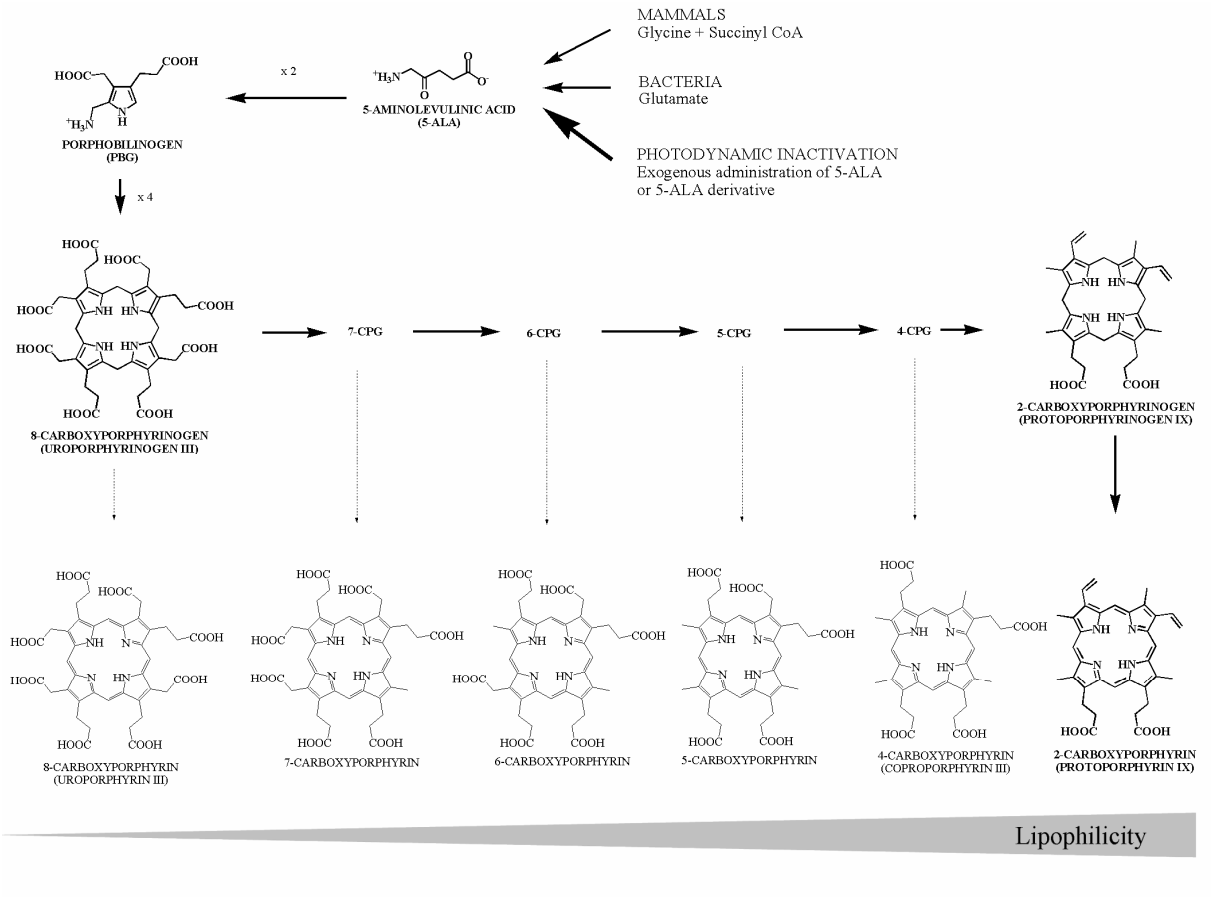


**Figure 1**: The heme biosynthesis cycle. In mammalian cells, 5-ALA is formed from the condensation of glycine and succinyl CoA, while most bacteria use glutamate as the substrate. Two molecules of 5-ALA condense to form porphobilinogen (PBG), catalyzed by 5-ALA dehydratase. Four molecules of PBG then condense to form a linear tetrapyrrole, which cyclizes to form 8-carboxyporphyrinogen (8-CPG, or uroporphyrinogen I & III). Plants and bacteria can use uroporphyrinogen III as an intermediate in the synthesis of various molecules (*e.g.*, chlorophyll, vitamin B12). Mammals exclusively use protoporphyrin IX obtained after numerous enzymatic decarboxylations and desaturations, while different carboxylated porphyrins (8-CP, 7-CP, 6-CP, 5-CP, 4-CP) can be found in bacteria.

One of the main observations obtained from the first attempts to photoinactivate bacteria with conventional photosensitizers was the relative sensitivity of gram-positive strains to photodynamic inactivation (PDI), whereas gram-negative strains were significantly more resistant [7]. To overcome the highly impermeable barrier of gram-negative bacteria, pre-treatment with EDTA or the use of polycationic polymers has been proposed. Furthermore, the use of positively charged porphyrins, phenotiazines, or phthalocyanines enabled the photoinactivation of both gram-positive and -negative strains without any permeabilizing pre-treatment [8].

Besides the classical photosensitizer (PS), one molecule of particular interest, 5-aminolevulinic acid (5-ALA), is gaining importance in the field of PDI. 5-ALA is not a PS by itself, but is an endogenous component in the heme biosynthesis pathway, and is ubiquitous in nearly all cells (Fig. 1). When provided exogenously, 5-ALA results in the accumulation of photoactive porphyrins (PAP) within the targeted cells. This accumulation of PAP is more pronounced in cells with high metabolic activity [9] like cancer cells, inflammatory cells or bacteria, resulting in high selectivity. However, the low capacity of 5-ALA to cross biological barriers led to the development of more lipophilic derivatives with improved local bioavailability [10]. As 5-ALA has been shown to be moderately efficient against gram-negative bacteria [11], these derivatives might also show a clear improvement in the photoinactivation of these microorganisms. In the present study, we compared bacterial PAP formation induced by 5-ALA to that of different 5-ALA derivatives in several gram-positive and gram-negative bacterial strains, and investigate whether the latter may be interesting candidates for the PDI of bacteria.

## Materials and methods

**Chemicals.** 5-ALA was obtained from Fluka (Buchs, Switzerland), and its methylester (MAL), butylester (BAL), pentylester (PAL), hexylester (HAL) and octylester (OAL) from Organix (Colchester, UK). The porphyrin standards were purchased from Frontier Scientific (Logan, Utah, US). All solvents and other chemicals were of analytical grade and used without further purification.

**Bacterial strains and growth conditions.** This study was conducted with three gram-negative and one gram-positive strain. The gram-negative strains were: (1) *Escherichia coli* K12, a non-pathogenic laboratory strain, (2) *E. coli* Ti05, a uropathogenic strain, and (3) *Pseudomonas aeruginosa*. The gram-positive strain was a *Staphylococcus aureus* MRSA.

All strains were provided by the Istituto Cantonale di Microbiologia (Bellinzona, Switzerland), and are described in Table 1.

**Table 1**: Characteristics of the bacterial strains tested in this study

| Strain | Gram | Identification | Source | Resistance |
|---|---|---|---|---|
| *E. coli* K12 | - | MG1655 | laboratory collection | none |
| *E. coli* Ti05 | - | 03-039705 | blood | Ampicillin, Cotrimoxazole, Trimethoprim, Streptomycin |
| *P. aeruginosa* | - | 04-022545 | expectorate | none of the *P. aeruginosa*-specific antibiotics [a] |
| *S. aureus* MRSA | + | 04-022798 | wound puncture | Penicillin, Ampicillin, Oxacillin, Cefazoline, Ciprofloxacine, Tetracycline, Augmentin, Trimethoprim/Sulfamethoxazole |

[a] Ceftazidine, Cefepime, Piperacillin, Tienam, Aztreonam, Gentamycin, Tobramycin, Amikacin, Ciprofloxacin

All strains were grown for 48 hours on Columbia plates (Columbia Agar Base, defibrinated sheep blood 5% (v/v); Oxoid, Basel Switzerland). Colonies were transferred into 100 ml of tryptone soya broth (TSB; Oxoid, Basel Switzerland), and incubated overnight at 37 °C. The bacterial suspension was diluted with TSB to an optical density of ~0.10 at 660 nm ($OD_{660}$). Bacterial suspensions were incubated at 37 °C to an $OD_{660}$ of ~0.30, and washed twice with phosphate buffer saline (PBS, 0.1 N, pH 6.5) before incubation with the substrate.

**Photosensitization**. Stock solutions of 5-ALA or 5-ALA derivatives MAL, BAL, PAL, HAL and OAL were freshly prepared by dissolving the different substrates in PBS (0.1 N, pH 6.5) and kept in the dark after proper dilution. The bacterial suspension was centrifuged 10 minutes at 2500 × *g*; the bacterial pellets were then resuspended in the substrate solutions and incubated in the dark for 4 hours at 37 °C in a shaking incubator (100 rpm). One fraction of the suspension was used for the PDI procedure, while the other was collected for the PAP analysis.

**Photodynamic inactivation**. 2.5 ml samples containing approximately $5 \cdot 10^7$ bacteria were introduced into sterile 35 mm-diameter Petri dishes and illuminated by white light for 40 minutes, corresponding to a light dose of 120 $J \cdot cm^{-2}$. The light source was a 400 W halogen lamp, and the illumination surface was controlled for a homogenous and constant light intensity.

**Bacterial cell survival assay**. Colony forming units (CFU) of a bacterial suspension were determined by plating appropriate dilutions (from $10^{-1}$ to $10^{-5}$) on Columbia plates. The survival fraction was calculated as $N_{PDI}/N_0$, where $N_{PDI}$ is the number of CFU per ml after photodynamic inactivation and $N_0$ is the number of CFU per ml in the initial sample. The dark toxicity of the substrates, defined as the intrinsic toxicity of the compounds in the absence of light, was monitored by evaluating the survival fraction of incubated but non-illuminated bacterial samples, and calculated as $N_{DARK}/N_0$, where $N_{DARK}$ is the number of CFU per ml of the non-illuminated samples. The results were expressed as mean values (n=4) with their standard deviation.

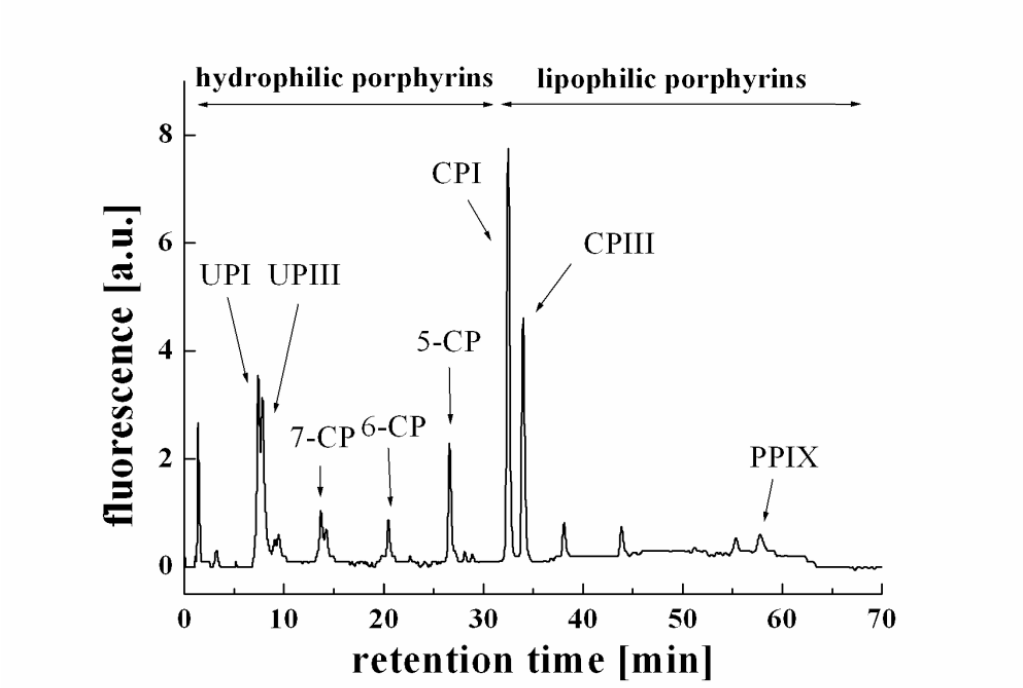


**Figure 2:** Chromatogram obtained with extracted bacterial porphyrins after gradient elution with solvent A (acetate buffer/acetonitrile 90:10) and solvent B (methanol/acetonitrile 90:10), and fluorescence detection ($\lambda_{em}$ 407 nm/ $\lambda_{ex}$ 630 nm). The elution order corresponds to the formation order in heme biosynthesis: 8-carboxyporphyrin (uroporphyrin I and III; UPI&III), 7-carboxyporphyrin (7-CP), 6-carboxyporphyrin (6-CP), 5-carboxyporphyrin (5-CP), 4-carboxyporphyrin (coproporphyrin I and III; CPI&III) and 2-carboxyporphyrin (protoporphyin IX; PpIX).

**HPLC analysis**. 10 ml bacterial suspensions were centrifuged for 10 minutes at 2500 × *g*. The supernatant was discarded, and the bacteria were resuspended in 1.0 ml of extraction solvent (ethanol/DMSO/acetic acid; 80:20:1; v/v/v) and stored at -80 ℃ until analysis. For the extraction of porphyrins, the bacterial wall was disrupted by 5 sonication cycles of 5 seconds at 0 ℃ using a sonicator probe (Branson digital so nifier, amplitude 30%, Danbury, USA). After centrifugation (4 minutes at 13,500 × *g*), the supernatant was collected and injected into the HPLC instrument.

Porphyrins were separated by reverse phase chromatography with a 125/4 Nucleodur C18 gravity 3 µm column (Macherey-Nagel, Oensingen, Switzerland) protected with the corresponding pre-column, using a gradient elution with solvent A (acetate buffer (pH 5.1 0.5 M)/acetonitrile; 90:10; v/v) and solvent B (methanol/acetonitrile; 90:10; v/v). Detection was performed by a fluorescence detector (Merck-Hitachi, La Chrom L-7480) with an excitation wavelength of 407 nm and emission wavelength of 620 nm (Fig. 2).

## Results

**Effect of 5-ALA PDI on *E. coli* K12**. Fig. 3A shows the results obtained after incubation of the laboratory strain *E. coli* K12 with increasing concentrations of 5-ALA. Without irradiation, bacterial survival was not affected until the 5-ALA concentration reached 40 mM. In contrast, irradiated samples reacted in a drastically different manner, as two phases could be observed. At the lowest 5-ALA concentration, a low PDI efficiency was observed, which increased to a maximal bacterial photoinactivation at the optimal concentration of 0.1 mM. Beyond this optimal concentration, PDI efficiency declined, resulting in a higher bacterial viability. In the second phase, high 5-ALA concentrations (40-100 mM) were used which resulted in a decrease in bacterial viability. This was presumably related to dark toxicity, a non-light-mediated process.

To understand this phenomenon, the total formation of the photoactive porphyrins (PAP) was analyzed in parallel to the PDI experiments (Fig. 3 A). The PAP formation profile seemed to be inversely correlated to bacterial survival after PDI, except at the higher concentrations. Low amounts of PAP were produced at low 5-ALA concentrations, increasing to a maximum of 7.5 pmol·$10^{-6}$ CFU at the optimal concentration of 0.1 mM, and then decreasing despite higher substrate concentrations. This particular bell-shaped profile is commonly observed in 5-ALA related photomedicine [12].

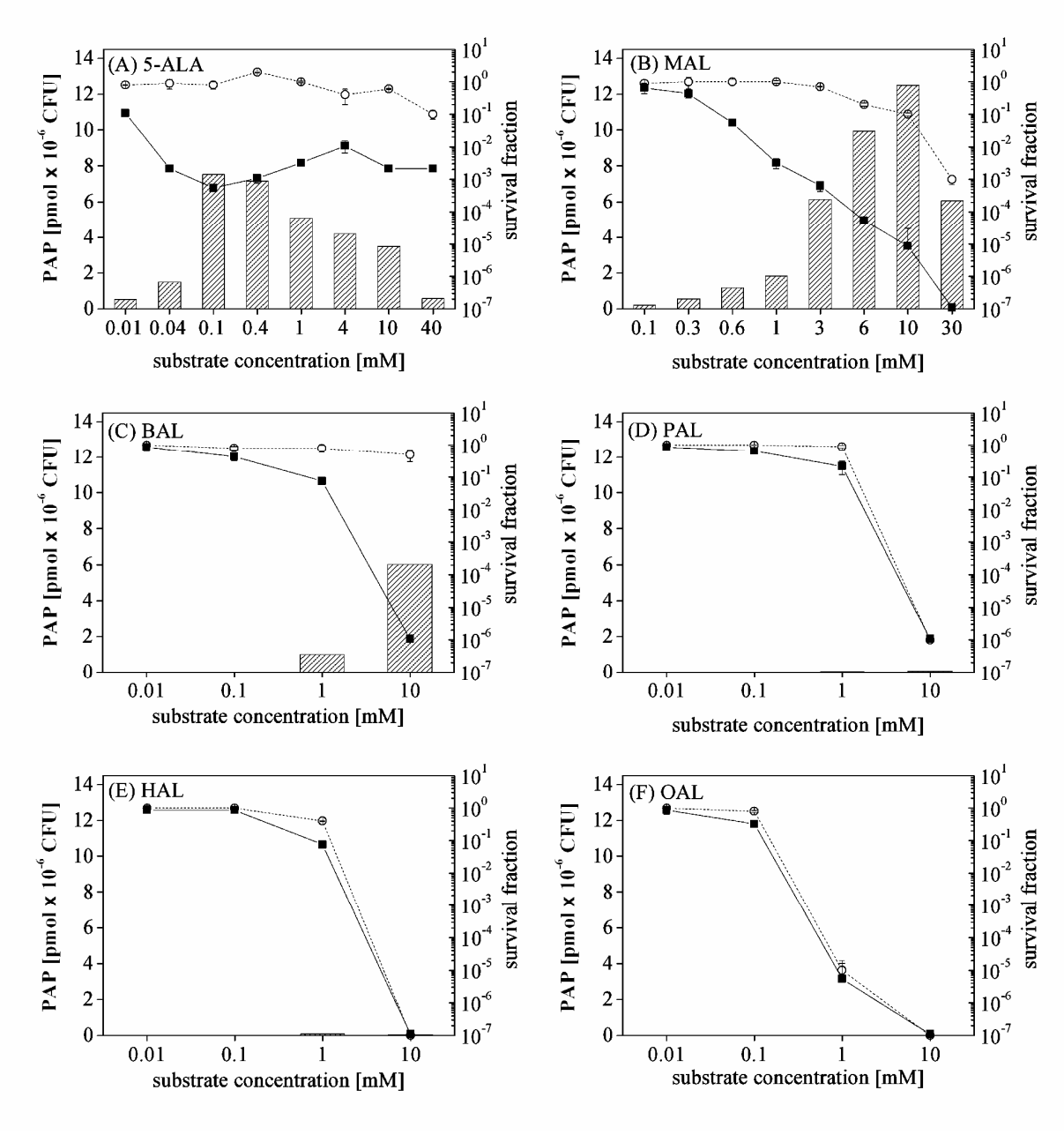


**Figure 3**: *E. coli* K12 incubated 4 hours in the dark with 5-ALA and different n-alkylesters (MAL, BAL, PAL HAL and OAL). Bars represent the total bacterial PAP produced, in pmol·$10^{-6}$ CFU. Dotted lines (-○-) represent the survival fraction after incubation without illumination (dark toxicity), while solid lines (-■-) represent the survival fraction after PDI (120 J·$cm^{-2}$).

Figure 4 shows, a direct correlation between PDI and the total amount of PAP produced in a range where no dark toxicity is observed.

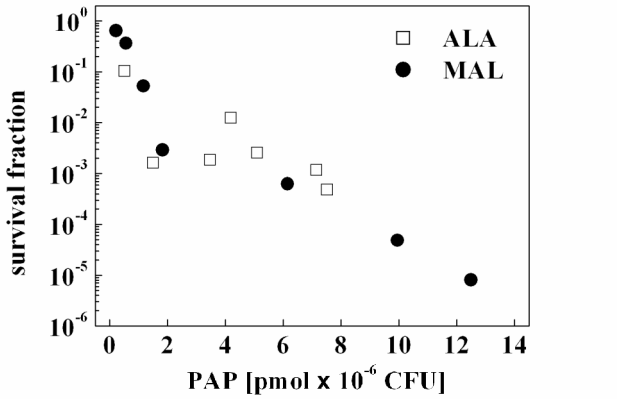


**Figure 4:** Survival of *E. coli* K12 after irradiation (120 J·cm$^{-2}$) decreases with the increase of the bacterial total PAP formation. 5-ALA ( □ ) and MAL ( ● ).

**Types of porphyrins produced by *E. coli* K12**. Analysis of the bacterial extracts revealed the presence of all of the porphyrins involved in heme biosynthesis, from uroporphyrin (UP) to protoporphyrin IX (PpIX). These porphyrins were present in varying proportions, and were dependent upon several factors like substrate type or substrate concentration (data not shown). The comparison of 5-ALA- and MAL-induced porphyrins at their optimal concentrations showed that the majority, when using 5-ALA, were lipophilic porphyrins (CP and Pp IX), while hydrophilic porphyrins (UP, 7-CP, 6-CP and 5-CP) were predominant in bacteria incubated with MAL.

Figure 5 represents the percentage of hydrophilic and lipophilic porphyrins as a function of total the PAP induced. When low amounts of porphyrin were induced, CP and PpIX were the most frequent types of PAP. In contrast, when higher amounts of PAP were produced, the proportion of hydrophilic porphyrins drastically increased.

**Effect of 5-ALA derivatives PDI on *E. coli* K12**. A series of 5-ALA n-alkyl esters with increasing chain length were tested for the photoinactivation of *E. coli* K12 under the same conditions. Major differences were observed in the behaviour of short- and long-chained derivatives. 5-ALA methylester (MAL) and butlyester (BAL) induced high PAP formation and efficient photoinactivation at concentrations of 10 mM (Fig. 3B, C). In contrast, 5-ALA pentyl (PAL), hexyl (HAL) and octylester (OAL) did not induce PAP formation or

effective photoinactivation, presumably due to the strong dark toxicity observed at concentrations ranging from 1 to 10 mM (see Fig. 3D, E, F). This intrinsic toxicity was also observed with 5-ALA and MAL when the concentrations were increased to 100 and 30 mM, respectively, suggesting the hypothesis that the dark toxicity of 5-ALA derivatives increases with chain length, and thus lipophilicity.

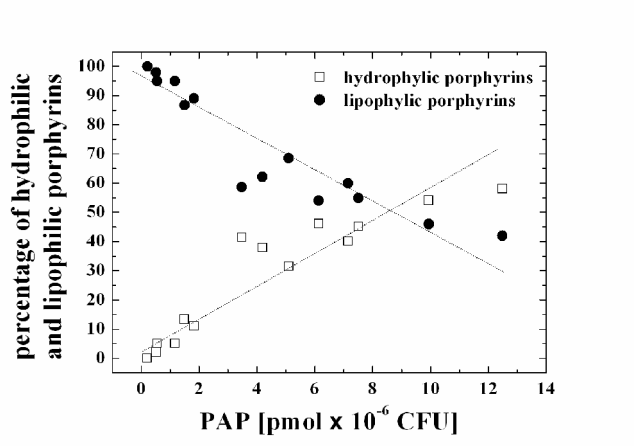


**Figure 5**: *E. coli* K12 5-ALA- and MAL-induced porphyrin distribution as a function of total PAP formation. The increased proportion of hydrophilic porphyrins ( ● ) toward lipophilic porphyrins ( □ ) when high amounts of PAP are produced may be explained by the saturation of decarboxylases involved in the heme biosynthesis cycle.

**Effect of 5-ALA and MAL on different gram-positive and gram-negative strains**. We investigated the efficacy of 5-ALA- and MAL-mediated PDI on two gram-negative strains, the pathogenic *E. coli* Ti05 and *P. aeruginosa*, and on the gram-positive multi-resistant *S. aureus*. The results obtained with *E. coli* Ti05 were very similar to those of the laboratory strain in terms of PDI and PAP induction after incubation with 5-ALA or MAL. However, as compared to *E. coli* K12, higher substrate concentrations (Fig. 6A, B) were needed to maintain optimal PAP formation.

Although *P. aeruginosa* produced 4-6 times higher amounts of porphyrins than *E. coli* K12 and Ti05 when incubated with 5-ALA, a proportional reduction in bacterial survival was not observed. A similar inactivation rate of 99.9% as compared to both *E. coli* strains was indeed obtained. In addition, at the tested concentrations, MAL did not induce a substantial PAP accumulation, although it still lead to a survival fraction of around $1.2 \cdot 10^{-2}$ upon irradiation (Fig. 6 C, D). Both substrates induced the accumulation of a majority of PpIX in *P. aeruginosa* for all experimental conditions (data not shown).

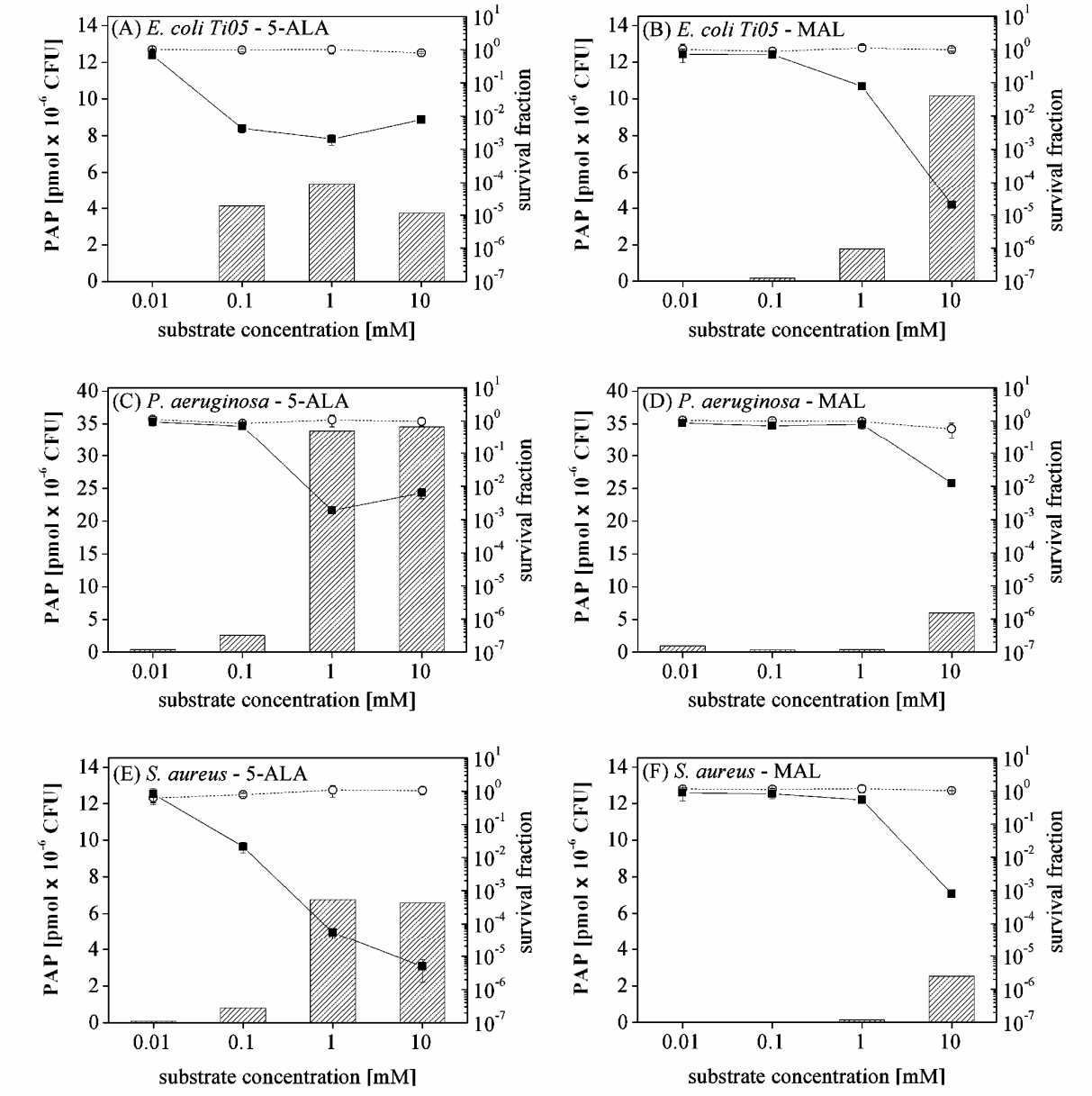


**Figure 6**: *E. coli* Ti05, *P. aeruginosa*, and *S. aureus* MRSA were incubated for 4 hours in the dark with 5-ALA and MAL. Bars represent total bacterial PAP produced, in pmol × $10^{-6}$ CFU. Dotted lines ( – ⊖ – ) represent the survival fraction after incubation (dark toxicity) while solid lines (–■–) represent the survival fraction after PDT (120 J × $cm^{-2}$). Please note the different scale used for *Pseudomonas*.

The gram-positive *S. aureus* appeared to be significantly more sensitive to 5-ALA-mediated PDI than the gram-negative strains (survival fraction $<10^{-5}$), although PAP production was comparable. MAL also induced sufficient PAP formation to achieve effective photoinactivation of > 99.9 % of this strain (Fig. 6 E, F). The different porphyrin extracts from *S. aureus* contained a majority of uroporphyrins (>80%).

An overview of these results can be seen in Table 2, which compares optimal concentration, survival and PAP formation for 5-ALA and MAL on the different tested strains.

**Table 2:** Comparison of 5-ALA- and MAL-mediated PDI on different bacterial strains. The optimal substrate concentration at which the highest PDI at 120 $J \cdot cm^{-2}$ is achieved, the survival fraction, and the total PAP formation induced are reported.

| | 5-ALA | | | MAL | | |
|---|---|---|---|---|---|---|
| Strain | Optimal concentration [mM] | Survival fraction | PAP [$pmol \cdot 10^{-6}$ CFU] | Optimal concentration [mM] | Survival fraction | PAP [$pmol \cdot 10^{-6}$ CFU] |
| *E. coli* K12 | 0.1 | $4.8 \cdot 10^{-4}$ | 7.5 | 10 | $8.1 \cdot 10^{-6}$ | 12.5 |
| *E. coli* Ti05 | 1.0 | $5.0 \cdot 10^{-5}$ | 5.4 | 10 | $2.0 \cdot 10^{-5}$ | 10.2 |
| *P. aeruginosa* | 1.0 | $1.9 \cdot 10^{-3}$ | 34.5 | 10 | $1.2 \cdot 10^{-2}$ | 6.0 |
| *S. aureus* | 10 | $5.0 \cdot 10^{-6}$ | 6.6 | 10 | $7.8 \cdot 10^{-4}$ | 2.5 |

## Discussion

The present study demonstrated the possibility of photodynamically inactivating gram-positive and gram-negative bacteria after incubation with 5-aminolevulinic acid or its methylester. The characteristic profile of 5-ALA-induced porphyrin production as a function of substrate concentration underlines the strong relationship between these two parameters, and the importance of determining the optimal effective concentrations of each substrate. The observed bell-shaped curve is in agreement with cell culture studies related to 5-ALA- and 5-ALA derivative-induced porphyrins [12].

It is known that, in addition to PpIX, porphyrins in general can inhibit different enzymes involved in the heme biosynthetic cycle in order to avoid accumulation of high amounts of intracellular PAP [13]. However, this negative feedback would result in the appearance of a plateau in terms of total porphyrin production with increasing 5-ALA concentration, and cannot explain the bell-shaped curve. Our results showed that at concentrations above the optimal concentration, 5-ALA itself appears to have an inhibitory effect on porphyrin formation, even though toxic drug concentrations were not reached. Furthermore, the same bacteria incubated with MAL produced significantly higher PAP than the parent compound, suggesting that the maximal PAP production capacities were not reached.

The differences in optimal concentrations observed for 5-ALA and MAL may result from different uptake mechanisms. It is well known that in animal cells, 5-ALA and MAL are transported into the cytosol via different active transport mechanisms [14]; however, more lipophilic derivatives enter via passive diffusion through the membranes or endocytosis. In gram-negative bacteria, the double membrane prevents penetration of exogenous molecules, while porins, which are transmembrane channels, allow only small hydrophilic molecules, usually nutrients, to enter the bacteria [15]. Thus, the entrance of 5-ALA into gram-negative bacteria is not problematic [16], as demonstrated by the low concentration necessary for optimal PAP induction. In the case of MAL, the addition of a methyl chain on the carboxylic group increases the log $P_{o/w}$ from -1.52 to -0.94 [12], which is still considered hydrophilic. Hence, this compound may also enter the bacterial cytosol through porin channels. However, the high concentrations (3-10 mM) necessary to induce sufficient PAP formation indicate that either the penetration or availability may vary from that of the parent compound.

The fact that more lipophilic derivatives, such as pentylester, hexylester and octylester, do not induce any significant PAP formation might be explained by the trapping of these highly lipophilic compounds in the bacterial membranes, or by the fact that the highly amphiphilic properties of these derivatives may induce membrane disruption, causing dark toxicity.

We have seen a strong correlation between PDI efficiency and the total amount of PAP formation in *E. coli* K12. However, this cannot be extrapolated to other strains due to the different types of porphyrin produced, protection mechanisms [17], and porphyrin localization. All types of porphyrins observed in our study have shown the capacity to absorb light and generate reactive oxygen species [18]. The main difference between these endogenous porphyrins is related to the number of carboxylic moieties, which influence

solubility, lipophilicity, aggregation and localization. In the case of *E. coli*, it was seen that the type of PAP depends on the quantity of the porphyrins produced. When low amounts of porphyrins are synthesized, the enzymatic machinery of the bacteria was able to proceed to the decarboxylation of a high proportion of these porphyrins, leading to the accumulation of mainly lipophilic porphyrins. When very large quantities of PAP are produced, the enzymatic process may be overwhelmed, leading into an increased proportion of hydrophilic porphyrins. This tendency was observed only in the *E. coli* strains (data not shown for the other species). In contrast, *S. aureus* produced uroporphyrins nearly exclusively and was efficiently photoinactivated, while *P. aeruginosa* produced 4-5 times more porphyrins, mainly PpIX, but was not more efficiently photoinactivated.

To obtain an efficient 5-ALA- or 5-ALA derivative-mediated PDI, the two main conditions necessary are sufficient PAP accumulation in the targeted bacteria, and effective irradiation to activate the PS. In fact, when using this type of photosensitizer prodrug, a PDI conducted with sub-optimal conditions may lead to contradictory results. For example, Karrer *et al.* [19] have shown that 5-ALA-mediated PDI induced significant inhibition of the growth of *S. aureus* but not *S. epidermis*. In contrast, Nitzan *et al*. [11] observed that 5-ALA mediated PDI was effective against both *S. aureus* and *S. epidermis* but not against gram-negative strains, including *E. coli* or *P. aeruginosa*. The latter strains were successfully photoinactivated by Szocs *et al.* [20] and Lee *et al.* [21], respectively, after incubation with 5-ALA.

In our study, both 5-ALA and MAL have been shown to induce increased PAP in all treated bacteria when optimal conditions were applied. As 5-ALA presented a biphasic inactivation curve, intermediate concentrations resulted in a suboptimal inactivation of the targeted bacteria. A more desirable behaviour was obtained with its methylester, as the bacterial survival continuously decreased with increasing MAL concentration. In this case, PAP formation, and thus PDI, was inhibited only when toxic drug concentrations (over 10 mM) were achieved.

These active concentrations may seem relatively high compared to other PS used at micromolar concentrations; indeed, for some other PS compounds, the application time is in minutes instead of hours [22,23]. However, the high selectivity of the compounds we have tested, as demonstrated in other medical domains, may provide the means to potentially eradicate bacterial infections without harming healthy surrounding tissues. Early clinical trials have demonstrated promising results from 5-ALA-mediated PDI of *H. pylori* [24], as well as the treatment of cutaneous mycosis [25] and acne [26]. In these contexts, not only is the ability to induce PAP formation important, but also the drug bioavailability. 5-ALA derivatives have

demonstrated some advantages as compared to the parent compound, including improved selectivity, homogeneity in tissue distribution, penetration, lack of systemic effects and less painful irradiation [10]. Furthermore, repeatability may be a major asset of PDI, as cumulative toxicity [27] and induced bacterial resistance upon repeated treatments [28] have not been reported to date.

## Conclusions

When optimal concentrations were used, 5-ALA and 5-ALA short-chained derivatives (5-ALA methylester and butylester) induced high PAP accumulation in both the gram-positive and gram-negative bacterial strains we tested, attaining the first prerequisite for a successful PDI. In contrast, long-chained 5-ALA derivatives (5-ALA pentylester, hexylester and octylester) demonstrated a high dark toxicity that increased with lipophilicity, and showed no significant PAP formation. As 5-ALA methylester efficiently induced PAP formation in bacteria and presented enhanced bioavailability in comparison to the parent compound, its capacities to treat local infections should be investigated in *in vivo* experiments.

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# CHAPTER VI

## 5-ALA derivatives-mediated photoinactivation of *Propionibacterium acnes*

Nicolas Fotinos[1], Juro Mikulic[1], Maruska Convert[2], Marino Andres Campo[1], Jean-Claude Piffaretti[2], Robert Gurny[1] and Norbert Lange[1]

[1] Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest Ansermet, 1211Geneva 4, Switzerland

[2] Cantonale institute of Microbiology, Via Mirasole 22A, 6500 Bellinzona, Switzerland

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## Abstract

Recent studies have shown that 5-aminolevulinic acid (5-ALA)-mediated photodynamic therapy (PDT) is applicable for the treatment of moderate to severe cases of acne vulgaris. However, due to sub-optimal pharmacokinetic properties of this molecule, more lipophilic derivatives have been developed and are currently in use for several clinical indications. The objective of the present study was the evaluation of different 5-ALA derivatives with respect to the generation of photoactive porphyrins (PAP) in *Propionibacterium acnes* and subsequent photodynamic inactivation (PDI).

For this purpose, suspensions of *P. acnes* were incubated with 5-ALA or 5-ALA derivatives as PAP precursors during 4 hours and then irradiated with white light. Photoinactivation was evaluated by counting the colony forming units before and after PDI treatment, while PAP formation was analyzed by extracting and measuring the porphyrins produced.

The different *P. acnes* strains were efficiently photoinactivated by 5-ALA, 5-ALA benzylester (BzAL), and 5-ALA-O-diethylenglycol-monomethylester (5-ALA DGME), while 5-ALA hexylester did not induce any PAP under the tested conditions. With regards to their promising *in vitro* results, the use of the moderately lipophilic 5-ALA derivatives BzAL and 5-ALA DGME should be considered to optimize PDT treatment of acne vulgaris, depending on the ability to increase drug bioavailability and selectivity, and to reduce side effects such as erythema, pustular eruptions, and epithelial exfoliation.

## Introduction

Although acne vulgaris is not a life-threatening pathology, severe forms of this disease may have a particularly negative influence on self-esteem. Acne vulgaris affects up to 85% of young people and can continue into adulthood. Treatment of severe cases frequently involves a combination of different topically or systemically applied agents, including antibiotics, retinoids, or hormonal agents [1]. This broad therapeutic mix, with potentially severe side effects, is required by the multifactorial aetiology of this complex and not yet fully understood pathology. Abnormal keratinisation, sebaceous gland hyperplasia, *Propionibacterium acnes* colonization, and inflammation processes, however, are broadly accepted as key factors associated with the disease.

Despite the well-documented colonization of pilo-sebaceous glands by *P. acnes*, its exact role and influence on the development, severity, and recurrence of acne is still unknown. However, the increased drug resistance of *P. acnes* after long-term oral antibiotic treatment and the emergence of serious side effects, including upper tract infections [2] and hepatitis [3], have raised serious doubts about the legitimacy of this antibacterial approach [4]. Thus, photodynamic therapy (PDT) may represent an alternative treatment. PDT is a relatively recent treatment modality that combines 3 individually non-toxic components: (i) a photosensitizing molecule (PS), (ii) light, and (iii) oxygen. In the PDT process, the photosensitizer accumulates at the target site and is activated to a higher energetic level upon local irradiation by light. The activated PS then transmits energy to the oxygen, resulting in the formation of short-living highly toxic reactive oxygen species, inducing selective target destruction without harming surrounding healthy tissues. Clinically, PDT is currently used for the treatment of various dermatological and non-dermatological pathologies [5].

Photodynamic inactivation (PDI) of microorganisms using different photosensitizing agents *in vitro* has led to much hope in the antimicrobial domain [6]. Nevertheless, only a few molecules have been evaluated clinically (*e.g.*, methylene blue, 5-aminolevulinic acid). One of the most promising approaches may come from the endogenous accumulation of photoactive porphyrins (PAP) in *P. acnes* [7], which can partially explain the significant improvements in patients suffering from acne vulgaris after light-based therapy [8,9]. To further enhance treatment efficacy, exogenous administration of 5-aminolevulinic acid (5-ALA), an endogenous porphyrin precursor, can selectively boost porphyrin accumulation within organisms having high metabolic rates [10], such as *P. acnes*. However, due to the unfavourable topical bioavailability of 5-ALA, more lipophilic derivatives have been developed, two of which have received marketed authorization [11]. The goal of this study is to evaluate the capacity of different 5-ALA derivatives, adapted for topical application, to photoinactivate *P. acnes*.

## Materials and methods

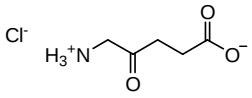
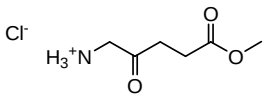
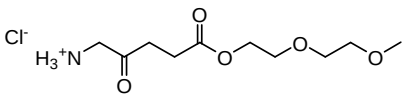
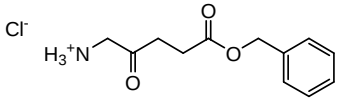
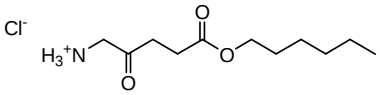
**Chemicals**: 5-ALA was obtained from Fluka (Buchs, Switzerland). Its methylester (MAL), hexylester (HAL), and benzylester (BzAL) were purchased from Organix (Colchester, UK). The O-diethyleneglycol monomethyl-ether ester of 5-ALA (5-ALA-DGME) was synthesized according to Berger *et al*. [12]. The structure of 5-ALA and its derivatives can be seen in Table 1. The porphyrin standards were purchased from Frontier Scientific (Logan, Utah, US). All solvents and other chemicals were of analytical grade and used without further purifications.

**Bacterial strains**: The laboratory strain *Propionibacterium acnes* CIP 53.117 was obtained from the Institut Pasteur in Paris (France). *P. acnes* E5947 and Va2376 were isolated from patients at the Istituto Cantonale di Microbiologia in Bellinzona, from blood and pleural puncture, respectively.

**Bacterial Growth:** All three *P. acnes* strains were grown for 72 hours on Columbia plates (Columbia Agar Base, defibrinated sheep blood 5% (v/v); Oxoid, Basel Switzerland) under anaerobic conditions (10% $CO_2$, 10% $H_2$, 80% $N_2$) at 37 °C. Initial bacterial suspensions were prepared by seeding approximately 3 loops of colonies in thioglycolate broth (TG, Beckton Dickinson, USA). After overnight incubation, the culture was diluted with TG to an optical density ($OD_{660}$) of 0.28 at 660 nm and incubated for approximately 90 minutes until an $OD_{660}$ of 0.30 was obtained. Before incubation with the substrates, the bacterial suspension was

washed twice with phosphate buffer saline (PBS, 0.1 N, pH 6.5, centrifugation 10 minutes at 9000 × *g*).

**Table 1**: Structure and lipophilicity of 5-aminolevulinic acid (5-ALA) and the different derivatives tested.

| Name | Abbreviation | Structure | Lipophilicity* |
|---|---|---|---|
| 5-aminolevulinic acid | 5-ALA |  | -2.1 |
| 5-ALA methylester | MAL |  | -1.2 |
| 5-ALA-O-diethylenglycol-monomethylether ester | 5-ALA DGME |  | -1.2 |
| 5-ALA benzylester | BzAL |  | 0.48 |
| 5-ALA hexylester | HAL |  | 1.26 |

* Lipophilicity is represented by the xlog *P* value calculated with ALOGPS 2.1 (VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org, 2005).

**Photosensitization**: Substrate solutions of 5-ALA or 5-ALA derivatives (MAL, BzAL, HAL and 5-ALA DGME) were freshly prepared by dissolving the different compounds in PBS (0.1 N, pH 6.5) and kept in the dark after proper dilution. The bacteria suspensions were centrifuged for 10 minutes at 9000 × *g*, and then the pellets were resuspended in the substrate solutions and incubated for 4 hours at 37 ℃ in the dark with constant agitation. One fraction of the suspension was used for the PDI procedure, while the other was collected for PAP analysis.

**Illumination method:** Samples of 2.5 ml of bacterial suspension, containing approximately $4 \cdot 10^8$ bacteria, were introduced into sterile 35 mm diameter Petri dishes and illuminated by white light for 40 minutes, corresponding to a light dose of 120 $J \cdot cm^{-2}$. The light source was a 400 W halogen lamp, and the illumination surface was controlled for a homogenous and constant light intensity.

**Bacterial cell survival assay**: Colony forming units (CFU) were determined after appropriate dilutions of the bacterial suspensions (from $10^{-1}$ to $10^{-5}$) that were plated and cultured on Columbia agar. The survival fraction was calculated as $N_{PDI}/N_0$, where $N_{PDI}$ is the number of CFU per ml after photodynamic inactivation and $N_0$ is the number of CFU per ml in the initial sample. The dark toxicity of the substrates, defined as the intrinsic toxicity of the compounds in the absence of light, was monitored by evaluating the survival fraction of incubated but non-illuminated bacterial samples and calculated as $N_{DARK}/N_0$, where $N_{DARK}$ is the number of CFU per ml of the non-illuminated samples.

**HPLC analysis**. 10 ml of the incubated bacterial suspensions were centrifuged for 10 minutes at 9000 **×** *g*. The supernatant was discarded, and the bacteria were resuspended in 1.0 ml of extraction solvent (ethanol/DMSO/acetic acid; 80:20:1; v/v/v) and then stored at -80 °C until analysis. For the analysis, the bacterial wall was disrupted by 5 sonication cycles of 5 seconds at 0 °C using a sonicator probe (Branson digital sonifier, amplitude 30%, Danbury, USA) to extract the porphyrins. After centrifugation (4 minutes at 13,500 **×** *g*), the supernatant was collected and injected into the HPLC instrument.

Porphyrins were separated by reverse phase chromatography with a 125/4 Nucleodur C18 gravity 3 µm column (Macherey-Nagel, Oensingen, Switzerland) protected with the corresponding pre-column, using a gradient elution with solvent A (acetate buffer (pH 5.1 0.5M)/acetonitrile; 90:10) and solvent B (methanol/acetonitrile; 90:10). Detection was performed by a fluorescence detector (Merck-Hitachi, La Chrom L-7480) with an excitation wavelength of 407 nm and emission wavelength of 620 nm.

## Results

***P. acnes* CIP 53.117**: *P. acnes* naturally accumulates high amounts of porphyrins, and irradiation of untreated bacteria under our conditions photoinactivated approximately 45% of the microorganisms (data not shown). However, it was possible to significantly increase photoactive porphyrin (PAP) accumulation and, thus photoinactivation, after incubation with 5-ALA or some 5-ALA derivatives at the appropriate concentrations.

Different porphyrins can be formed within the heme biosynthesis cycle. The earliest porphyrin to be produced is the hydrophilic uroporphyrin (UP I & III), which presents 8 carboxylic functions that will undergo a series of enzymatic decarboxylations, leading to the formation of more lipophilic molecules, [*i.e*. coproporphyrin (CP I & III, 4 carboxylic groups)

and protoporphyrin IX (PpIX, 2 carboxylic groups)]. All these porphyrins are known to be photoactive [13] but may localize or interact differently due to different lipophilicities [14]. In the case of *P. acnes*, the major porphyrin types detected were uroporphyrins and coproporphyrins in varying proportions, but no protoporphyrin, which is the primary detected product in human cells. Depending on the substrate, slight variations in PAP-induced type distribution were observed (Table 2).

**Table 2**: Porphyrin type distribution in *P. acnes* CIP 53.117 after incubation with different compounds (mean and standard deviation). Uroporphyrin and coproporphyrin were the main PAP produced, while no protoporphyrin IX, the main product in mammalian cells, was detected.

| | Porphyrin distribution [%] | | | |
|---|---|---|---|---|
| Compound | uroporphyrin | coproporphyrin | protoporphyrin | other |
| 5-ALA | 45 ± 14 | 36 ± 11 | 0 | 19 ± 6 |
| MAL | 38 ± 27 | 62 ± 27 | 0 | 0 |
| DGME | 10 ± 19 | 88 ± 26 | 0 | 2 ± 7 |
| BzAL | 64 ± 10 | 28 ± 13 | 0 | 8 ± 6 |
| HAL | 62 ± 10 | 28 ± 14 | 0 | 10 ± 4 |

Figure 1 shows the total amount of PAP accumulated by *P. acnes* CIP 53.117 for 5-ALA and its derivatives and the survival fraction with and without illumination as a function of substrate concentration. It can be clearly seen that PAP accumulation and photoinactivation efficiency was strongly dependent on substrate concentration for each tested compound. In the case of 5-ALA, the highest PAP formation was observed after incubating *P. acnes* CIP 53.117 with an optimal substrate concentration of around 10 mM, while further increases in 5-ALA concentration resulted in a lower PAP accumulation. This specific PAP production profile as a function of substrate concentration is well-known in the 5-ALA-mediated PDT domain as a “bell-shaped curve”, and can also be observed for the PDI profile (Fig. 1A).

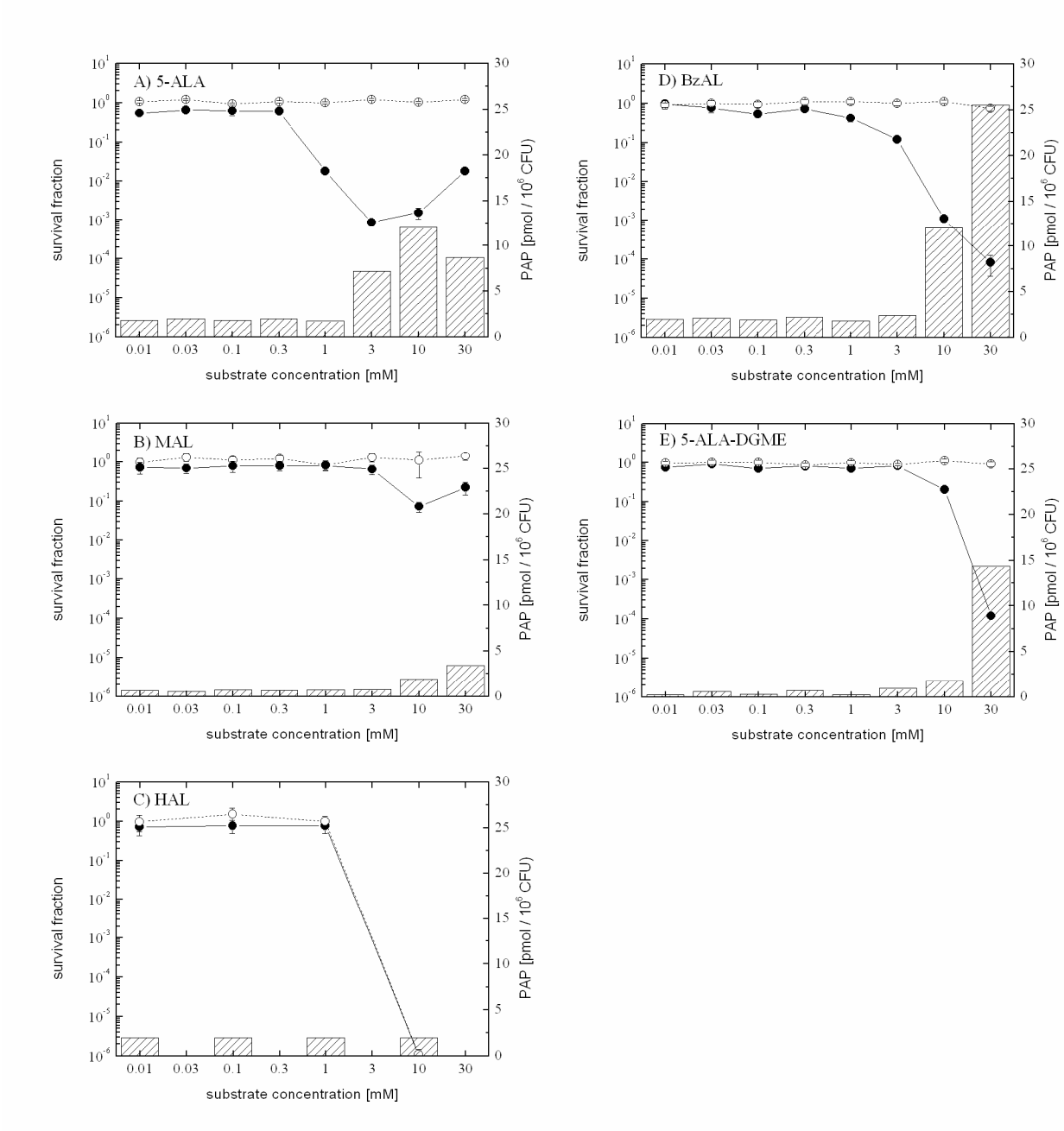



**Figure 1**: Photodynamic inactivation of *P. acnes* CIP 53.117. Survival fraction before (-○-) and after (-●-) irradiation, and PAP extracted from bacteria (bars) as a function of substrate concentration. A) 5-ALA, B) MAL, C) HAL, D) BzAL and E) 5-ALA-DGME.

In this case, a 4 hour incubation with 5-ALA at this optimal concentration drastically improved PDI efficiency, with a photoinactivated fraction of > 99.9%. Furthermore, no dark toxicity was observed after 5-ALA incubation with doses up to 30 mM.

MAL-induced PAP formation in *P. acnes* CIP 53.117 (Fig. 1B) appeared to be less efficient than with 5-ALA at the concentrations tested on this bacterial strain. Furthermore, within our experimental conditions, PDI resulted in a CFU reduction of less than 99%. Similarly to 5-ALA, no dark toxicity was observed with this hydrophilic derivative of 5-ALA. On the other hand, the lipophilic HAL induced no PAP accumulation at all and revealed a high dark toxicity, with a viability reduction of more than 5 orders of magnitude when using a concentration of 10 mM (Fig. 1C).

The two other 5-ALA derivatives, 5-ALA benzylester (Fig 1D) and 5-ALA DGME (Fig. 1E), which are moderately lipophilic, both demonstrated the capacity to (i) induce high PAP accumulation when using doses up to 30 mM, (ii) efficiently photoinactivate high bacterial fractions, up to 99.99% , and finally (iii) show no dark toxicity at doses up to 30 mM.

***P. acnes* E5947 and VA2376:** The results obtained with *P. acnes* E5947 and VA2376, two clinical strains, were very similar to the laboratory strain *P. acnes* CIP 53.117 at the tested concentrations. Table 3 shows the minimal substrate concentration necessary to inactivate >99% CFU for all three bacterial strains. In *P. acnes* E5947, 5-ALA, BzAL, and 5-ALA-DGME induced high PAP formation at a concentration of 10 mM. As for strain CIP 53.117, MAL exerted limited PDI, while HAL again presented a high dark toxicity ($10^{-6}$ survival fraction) and failed to induce any PDI effect. In the case of *P. acnes* VA2376, 5-ALA induced PDI at 1 mM, while 10 mM was needed for DGME and BzAL. The high dark toxicity of HAL and low PDI efficiency of MAL were also confirmed with this strain.

**Table 3**: Minimal substrate concentrations necessary to induce a PDI of 99% of CFU for *P. acnes* CIP 53.117, E5947, and VA2376.

| | CIP 53.117 | E5947 | VA2376 |
|---|---|---|---|
| 5-ALA | 1 mM | 10 mM | 1 mM |
| MAL | N/A | d.t. | N/A |
| DGME | 30 mM | 10 mM | 10 mM |
| BzAL | 10 mM | 10 mM | 10 mM |
| HAL | d.t. | d.t. | d.t. |

d.t. represents dark toxicity when a reduction of > 99% of CFU is observed in substrate-incubated but non_irradiated samples; .N/A. indicates that a PDI of 99% is not achieved

## Discussion

While the efficacy of 5-ALA-mediated PDT is widely accepted, the use of optimized derivatives allows significant improvements in terms of selectivity, bioavailability, and reduction of adverse effects [11]. However, n-alkyl derivatives of 5-ALA that were successful in neoplastic cell photoinactivation gave surprising results when tested on different bacteria [15]. Lipophilic, long-chained esters, such as HAL, induced similar PpIX production as did 5-ALA in human cancer cells but at 100 times lower doses [16]. Nevertheless, these derivatives did not induce a detectable porphyrin production in the tested bacterial strains. In contrast, more hydrophilic short chained derivatives like MAL induced an increased porphyrin accumulation and efficient bacterial photoinactivation in a dose-dependant manner. The results obtained in this study with MAL- and HAL-mediated photoinactivation of *P. acnes* were concordant with this tendency. HAL failed to induced PAP expression and appeared to exert a high bactericidal effect at 10 mM, presumably due to its highly amphiphilic properties that act on membrane integrity. Therefore, HAL may be inappropriate for *P. acnes* photoinactivation. In contrast, MAL induced PAP over-expression and photoinactivation in *P. acnes* CIP 53.117.

Prone to their authorization for the treatment of diverse dermatological conditions, 5-ALA [17,18,19] and MAL-mediated PDT [20,21] of acne vulgaris have already been investigated in various clinical studies. Positive therapeutic outcomes were reported with respect to the number of inflammatory lesions and decrease in the severity grading of the pathology. A recent comparative study by Wiegell *et al*. that compared 5-ALA and MAL applied to each side of the foreheads of patients suffering from acne vulgaris [20] revealed no significant response rate differences for the compounds. Nevertheless, 5-ALA PDT resulted in more prolonged and severe side effects, such as erythema, pustular eruptions, and epithelial exfoliation. These results were not surprising, as it was previously demonstrated that 5-ALA-mediated PDT was more painful than MAL PDT, presumably due to the lower selectivity of 5-ALA, which induces higher PAP formation in healthy tissue than MAL, or by the activation of the GABA receptor by 5-ALA but not 5-ALA derivatives due to structural similarities [22].

To the best of our knowledge, the PDI ability of the non-n-alkyl derivatives BzAL and 5-ALA DGME have not been tested until now. Their intermediate lipophilic properties, apparently favourable for bacterial photoinactivation, should avoid an accumulation of these compounds in the bacterial membrane. In fact, their ability to induce PAP formation and to photoinactivate *P. acnes* appeared to be high, while no dark toxicity was observed up to a concentration of 30 mM. Furthermore, the covalent linking of the absorption promoter diethyleneglycol monomethylether to 5-ALA might be of potential benefit for the treatment of acne. The fact that this molecule, which was especially designed for an optimal topical

delivery, can also induce efficient PDI of *P. acnes* is very promising and needs further investigation.

5-ALA PDT treatment of acne may result in a combination of the following beneficial effects: (i) inactivation of sebaceous glands and inhibition of sebum production; (ii) photo-inactivation of *P. acnes*, thus reducing inflammation; and (iii) a decrease in hyperkeratinisation by PDT-induced peeling, reducing follicular obstruction The exact therapeutic mechanism of PDT, however, is still controversial. For instance, Hongcharu *et al*. [18] have shown reductions in sebum production and *P. acnes* fluorescence; in contrast, Pollock *et al.* [19] observed no significant reduction in sebum excretion and *P. acnes* population. Differences in PDT regimens may be responsible for these variations and underline the complexity of the procedure and the need for an optimized protocol.

Apart from drug selection, other parameters have to be optimized in order to obtain optimal treatment and reduced adverse effects. The formulation of the drug is another key parameter [11,23] that is often underestimated in the development process. For example, Han *et al*. [24] have developed liposomal formulations of 5-ALA that were able to induce higher PAP formation in sebaceous glands than standard solutions. In this context, we have shown that 5-ALA DGME was liberated *in vitro* much faster when incorporated into a hydrogel of hydroxyethylcellulose than into a lipophilic cream (vaseline/paraffin 95:5) (results not shown). The relevance of these results has to be confirmed *in vivo* in order to evaluate the effect of formulation on the local bioavailability and also pilosebaceous selectivity.

As reviewed by Ross *et al*. [25], it seems evident that the use of 5-ALA will expand into acne treatment, but the PDT regimen has to be optimized in terms of application time, vehicles, drug dose, irradiation conditions, and treatment repetition. In this context, the use of better adapted 5-ALA derivatives for topical administration has to be considered.

## Conclusions

It is already known that a sufficiently high light dose photoinactivates *P. acnes* due to the innate bacterial accumulation of PAPs and that pre-treatment with 5-ALA drastically increases porphyrin accumulation and, therefore, photoinactivation. Our investigations with 5-ALA derivatives have revealed significant differences between simple n-alkyl versus non-n-alkyl ester derivatives. Despite their similar lipophilicity, MAL and 5-ALA DGME showed different abilities to induce PAPs and PDI of bacteria, with the latter compound being more

effective. The more lipophilic n-alkylester HAL failed to induce PAPs and consequently PDI. In our study, BzAL, as well as 5-ALA DGME, induced high PAP accumulation and efficient photoinactivation of *P. acnes*. As MAL, BzAL and 5-ALA-DGME appeared to be promising candidates, their capacity to target sebaceous glands, induce less pain during illumination, and display a good local bioavailability should be investigated for an optimized PDT treatment of acne vulgaris as an alternative to 5-ALA.

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# CHAPTER VII

# Conclusions

Photodynamic therapy in general, but especially 5-aminolevulinic acid (5-ALA) and 5-ALA derivative-mediated PDT, has been shown to be amongst the most selective treatments for neoplastic, but also non-neoplastic diseases. To understand the reason of this success, but also the direction that future development should follow, one has to focus on some physico-chemical and biological properties of 5-ALA.

The fact that 5-ALA is not a photosensitizer by itself, but only a precursor of the photoactive porphyrins, is of particular interest, with respect to the different advantages reported, *i.e.* the high selectivity for pathological tissues, and limited skin photosensitization. Furthermore, the fact that 5-ALA is an endogenous molecule is favourable regarding questions of security, or tolerance. However, the expanded use of 5-ALA was drastically hindered by a poor bioavailability. Therefore, amongst different more or less successful strategies applied to overcome this drawback, the development of 5-ALA derivatives has shown to significantly improve 5-ALA-mediated PDT.

A rich literature review compiles the different aspects of the development 5-ALA derivatives, and provides an exhaustive view on the different derivatization strategies that have been considered. It attempts to explain why only few of the numerous different derivatives were assessed in preclinical studies, through some predicable limitations, or promising approaches. Furthermore, as two 5-ALA derivatives have entered clinical studies, an overview of the clinical experience is presented, from early trials to long term studies, and the reasons they gained market authorization.

Besides the pre-cited successful developments, research using 5-ALA derivatives for systemic administration remains somehow disappointing. For more pertinent investigations in this domain, we looked to improve the limited test models available. In this context, the chick embryo model was used for the *in vivo* evaluation of newly designed 5-ALA derivatives. It allowed us not only to select some promising molecules that would have been

withdrawn if only *in vitro* cell culture was available, but also to evaluate rapidly the acute toxicity exerted.

Furthermore, the promising *in vivo* results obtained with 5-ALA diacylglycerides derivatives, supposed to be ineffective due to a branched alkyl group in alpha position of the carboxylic group, reopened some questions about the exact befall of 5-ALA derivatives once internalized into the cell. From a purely mechanistic point of view, the carboxylic function of 5-ALA is not involved in the enzymatic condensation forming porphobilinogen. It was reported that only the amino moiety was concerned in the reaction. This raises the question if 5-ALA carboxy-esters may be direct substrates for the heme biosynthesis. In the latter case, sterical hindrance should be considered as one of the main limitations. As a matter of fact, the specially designed AOMM, becoming porphyrin substrate only after enzymatic activation, has shown that the cleavage of esters within the cell was not mandatory. We were not able to give a clear answer to the exact fate of 5-ALA derivatives following their administration, but considered necessary to mention this issue, and input some more knowledge in the understanding of the process.

Although we were initially interested into anti-cancer applications, we noticed that the use of 5-ALA derivatives was unexplored in some areas, *i.e.* microbial photo-inactivation (PDI). PDI is an expending branch of PDT, and successful achievements in this domain may potentially lead to effective treatments for different pathologies, *e.g.* resistant wound infections, dental colonization, gastric ulcer, or other dermatological illness. Nevertheless, 5-ALA-mediated PDI is somewhat neglected, although literature shows that it was possible to induce porphyrin accumulation in micro-organisms after incubation with 5-ALA. However, photo-inactivation efficacy was not constant, depending on different factors related to treatment conditions and the nature of target organism. While some studies concluded that 5-ALA mediated PDI was efficient only against Gram-positive bacteria, other contradictory results mentioned the efficient 5-ALA-mediated photo-inactivation of Gram-negative strains. As the limitation was postulated to be related to the efficient Gram negative bacterial barrier, we wanted to test whether lipophilic 5-ALA derivative could bring similar improvements, as demonstrated in cancer-related domains.

We evaluated 5-ALA's capacity to induce PAP accumulation, and efficient PDI in different bacterial strains, compared to 5-ALA n-alkylester with increasing chain length. We found that PAP formation was strongly dependant on the substrate concentration. Furthermore, optimal concentrations varied strongly between both different substrates and bacterial strains. This work underlined the importance of this parameter when studying porphyrin induced PDI. Our results were significantly different from those observed in

mammalian cells lines. The long chained lipophilic 5-ALA derivatives exerted a high intrinsic toxicity and were unable to induce any PAP formation in all tested bacteria strains. In contrast, short chained derivative induced PAP accumulation and enhanced PDI, indicating the possibility for target-specific optimization.

Furthermore, we investigated the potential use of this PDI in a specific therapeutic application. One opportunity came from the investigation undergone with 5-ALA for the PDT treatment of severe cases of acne vulgaris. Although follicles colonization by *Propionibacterium acnes* is not the only element involved in this complex multi-causal pathology, the optimization of *P. acnes* photo-inactivation may improve treatment outcome. We outlined two potential candidates, 5-ALA benzylester and diethylengylcol-monomethylether ester, that surpassed 5-ALA and MAL ability to photo-inactivate *P. acnes*. Considering that the latter have shown promising results in the clinical treatment of this disease, we hypothesize that the selected 5-ALA derivatives will lead to significant improvements.

The utilization of 5-ALA-induced PpIX in photomedicine can be considered as a milestone in the development of photodynamic therapy and fluorescence detection, due to an innovative approach of “on site” photosensitizer production. Significant advantages towards first generation photosensitizers, in terms of ease of utilization and reduced skin photosensitivity are unanimously admitted. However, regarding 5-ALA’s low bioavailability, there is no doubt that it will be supplanted by specifically designed derivatives. This is illustrated by the tremendous success of 5-ALA methylester and 5-ALA hexylester in clinics. Therefore, we can assume that the future expansion of 5-ALA derivatives-related photomedicine will depend mainly on the capacity of researchers to develop new 5-ALA derivatives for specific indications, and optimized formulations.

# CHAPTER VIII

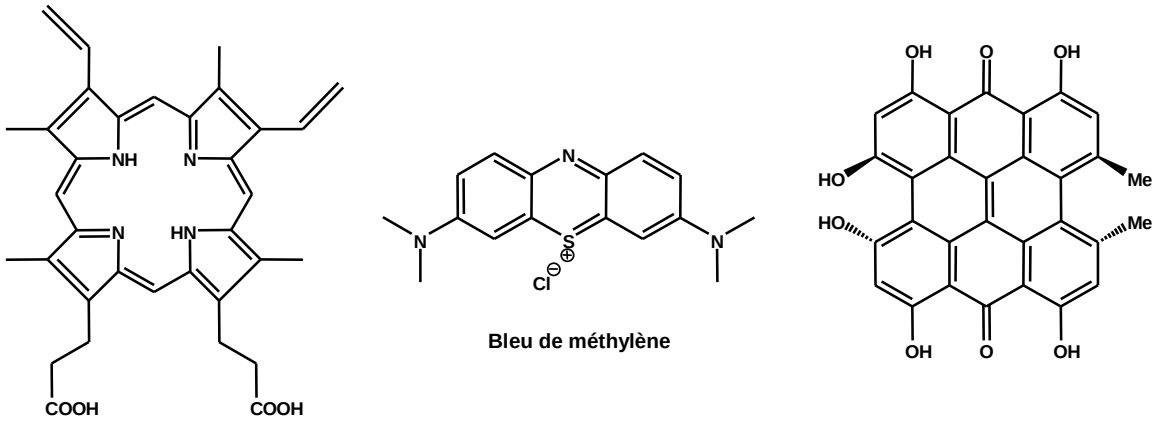
# RÉSUMÉ

## Introduction

La thérapie photodynamique (PDT) et la détection par fluorescence (FD) sont des techniques encore peu connues car relativement récentes. Elles ont été développées grâce aux travaux d'Oscar Raab et de Hermann Von Tappeiner au début du XX$^{ème}$ siècle, en Allemagne. Ces deux chercheurs ont découvert que la présence de lumière avait un effet prépondérant sur l'efficacité antiparasitaire de colorants qu'ils étudiaient. Alors que le concept de base de la PDT était posé, il a toutefois fallu attendre la fin de la seconde guerre mondiale pour que la PDT et la FD connaissent un véritable essor.

Dans la thérapie photodynamique, le terme *photos* indique que la lumière est une composante principale de cette méthode thérapeutique, mais celle-ci diffère de la simple photothérapie dans le sens où deux autres éléments, un agent photosensibilisant (PS) ainsi que l'oxygène, sont nécessaires au déroulement du processus thérapeutique.

Le PS est une molécule, généralement de structure polycyclique aromatique (Fig. 1), capable d'absorber la lumière afin de se retrouver en un état d'énergie élevé.




**Protoporphyrine IX** **Bleu de méthylène** **Hypéricine**

**Figure 1** : Les photosensibilisants sont généralement des molécules de type polycyclique aromatique capables d'absorber l'énergie lumineuse afin d'atteindre un état énergétique élevé. La plupart des photosensibilisants utilisés ont une structure de type porphyrine (telle la protoporphyrine IX) ou dérivé. On trouve également d'autres molécules, de type phénothiazine, (premiers photosensibilisants utilisés, tels que le bleu de méthylène), ou encore l'hypéricine, molécule naturelle extraite du Millepertuis (*Hypericum perforatum*).

Le PS, une fois activé, va retrouver son état d'énergie initial selon différents processus, soit en émettant de la lumière (fluorescence) dans le cas de la FD, soit en utilisant ce surplus d'énergie pour produire des éléments toxiques dans le cas de la PDT.

Le terme « dynamique » quant à lui, s'est imposé suite à une proposition de Von Tappeiner pour indiquer que de l'oxygène était impliqué dans le processus. Le PS activé par la lumière va transmettre l'énergie à l'oxygène, induisant la formation d'espèces d'oxygène réactives (ROS), telles que l'oxygène singulet ou le radical hydroxyle. Une fois formés, ces ROS hautement toxiques vont réagir avec les différents constituants des cellules (protéines, membranes, organelles) et entraîner une destruction sélective des cellules et tissus cibles (Fig. 2).

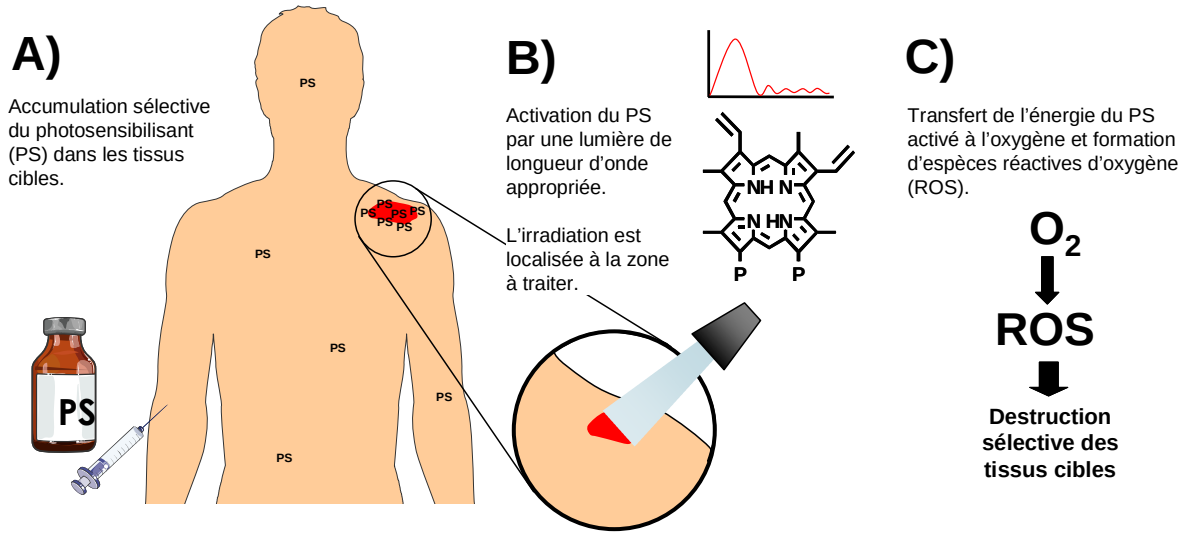



**Figure 2** : Principe de base de la PDT. Le photosensibilisant s'accumule préférentiellement dans les tissus cibles (A), puis est activé par la lumière (B). Un transfert d'énergie du photosensibilisant activé à l'oxygène induit la formation d'espèces d'oxygène réactives qui vont entraîner la destruction sélective des cellules ciblées (C).

La sélectivité est incontestablement un des principaux avantages de la PDT, raison pour laquelle elle s'est rapidement imposée dans un domaine où les traitements efficaces sont rarement sélectifs : l'oncologie. Pour traiter un cancer, les principales options disponibles actuelles sont premièrement la chirurgie, très invasive, et qui ne permet souvent pas d'éliminer toutes les cellules cancéreuses entraînant ainsi un risque de récidive, et deuxièmement les chimio- et radiothérapies, efficaces mais au prix de nombreux et

importants effets secondaires résultant du fait que toutes les cellules en division rapide sont touchées, et pas uniquement les cellules pathogènes. La sélectivité de la PDT pour les tumeurs est obtenue à plusieurs niveaux : (i) le PS s'accumule préférentiellement dans les tissus cibles en raison de différents mécanismes, tels que la moins bonne étanchéité de la neo-vascularisation tumorale qui permettant une extravasation du PS, ou les défauts dans le drainage lymphatique prévenant une élimination rapide du PS ; (ii) seule la zone à traiter est irradiée, évitant ainsi d'activer les PS qui se seraient accumulés dans des zones non désirées ; (iii) la faible durée de vie des ROS, de l'ordre de quelques millisecondes, empêchant la migration de ces éléments toxiques vers les tissus sains environnants, et entraînant ainsi une destruction tissulaire confinée à la zone traitée.

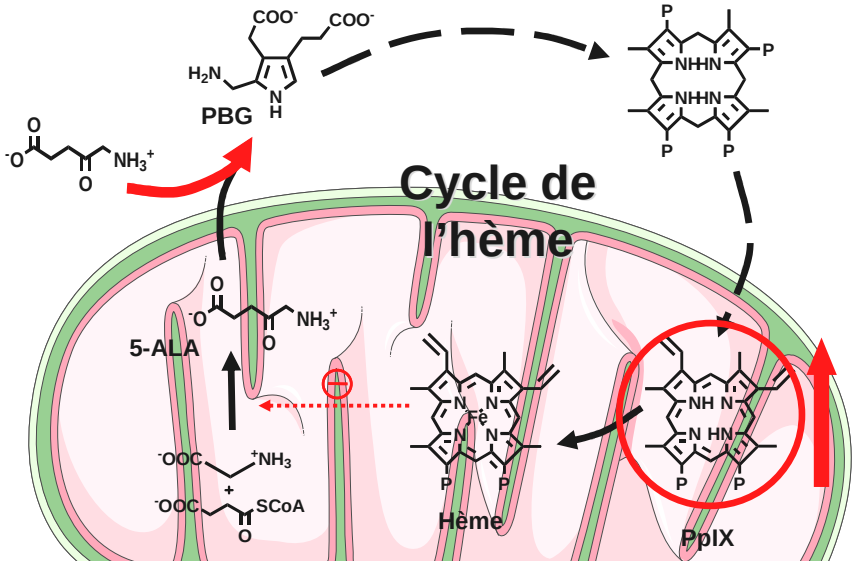



**Figure 3** : Biosynthèse de l'hème : cycle naturel quasi-ubiquitaire débutant par la formation d'acide 5-aminolévulinique (5-ALA) dans la mitochondrie, puis condensation enzymatique de deux molécules de 5-ALA en une molécule de porphobilinogène (PBG). Quatre PBG sont assemblés pour former un tétrapyrrole, qui sera transformé par une succession de décarboxylations enzymatiques en une porphyrine, la protoporphyrine IX (PpIX), molécule photoactive. La ferrochélatase va ensuite incorporer un atome de fer dans la PpIX pour former l'hème, biologiquement active, mais non photoactive. L'hème exerce un rétrocontrôle négatif sur la formation de 5-ALA, empêchant ainsi l'accumulation intracellulaire de porphyrines photoactives. Toutefois, une administration exogène de 5-ALA contourne ce rétrocontrôle négatif, entraînant une accumulation de PpIX, principalement dans les cellules très actives métaboliquement, par exemple les cellules cancéreuses.

Dans le domaine de la PDT et de la FD, de nombreux PS ont été étudiés, la majorité ayant une structure de type porphyrine ou dérivée. Ces PS ont par la suite été développés en modifiant leurs propriétés physicochimiques, dans le but d'améliorer leur efficacité et leur sélectivité, ou de diminuer leurs effets secondaires, et d'accélérer leur élimination. Néanmoins, une approche innovante et radicalement différente de l'utilisation de photosensibilisants classiques a convaincu une grande majorité des praticiens: l'induction de porphyrines photo-actives (PAP) endogènes par administration exogènes d'un précurseur, l'acide 5-aminolévulinique (5-ALA) (Fig. 3).

En effet, il est connu que les cellules tumorales se distinguent des cellules saines entre autres par un métabolisme « suractivé ». Ainsi, à la fin des années huitante, il a été envisagé d'utiliser cette particularité pour entraîner une surproduction de porphyrines endogènes par les cellules cancéreuses, après administration de 5-ALA. Les cellules saines accumulent également des porphyrines endogènes, mais dans une moindre mesure comparé aux cellules cancéreuses. Cette différence de production de PS endogène devient ainsi un vecteur de sélectivité supplémentaire pour la PDT. Par la suite, en fonction de la dose de lumière administrée, seules les cellules ayant accumulé une quantité suffisante de porphyrines seront détruites, alors que les autres ne subiront aucun dégât (effet de seuil).

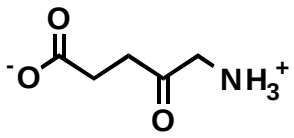
L'utilisation du 5-ALA en PDT n'est pas restée confinée au stade expérimental, car elle a été testée en clinique, notamment pour le traitement PDT de certains cancers ou désordres cutanés, et a démontré des capacités prometteuses. Toutefois, la nature zwitterionique de cette molécule (charge positive sur le groupement amine, et négative sur le groupe carboxylique) à pH physiologique a un effet dramatique sur sa biodisponibilité, et a rendu nécessaires l'élaboration de méthodes permettant de surmonter cette limitation. Dans cette optique, différentes techniques ont été évaluées, telles que la ionophorèse, l'ajout de promoteurs d'absorption, l'optimisation de la formulation, ou encore l'élaboration de dérivés de 5-ALA. C'est cette dernière méthode qui s'est montrée la plus prometteuse jusqu'à aujourd'hui, et qui est le sujet de ce travail de thèse.

## Dérivés de l'acide 5-aminolévulinique en photomédecine : caractéristiques, applications et perspectives

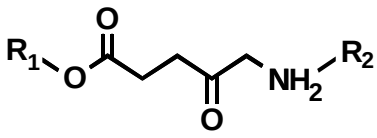
Comme mentionné précédemment, la principale limitation de l'utilisation du 5-ALA provient sa faible biodisponibilité, laquelle est due au fait que ce précurseur de PAP est un zwitterion en milieu physiologique et ne peut donc pas traverser librement les membranes

biologiques. Des dérivés plus lipophiles ont été développés afin de contourner cette limitation (Fig. 4). De nombreuses molécules ont été synthétisées puis testées *in vitro* en culture cellulaire ou *in vivo* au cours d'essais précliniques et cliniques. Leur développement, leur mécanisme d'action, ainsi que les principaux résultats obtenus sont résumés dans cette revue de littérature.

La première stratégie adoptée a été l'augmentation de la lipophilie par esterification du groupement carboxylique avec des chaînes alkyles, linéaires ou substituées. Les trois principales conclusions de cette étude sont les suivantes : (i) la lipophilie a une forte influence sur la concentration de substrat nécessaire à la production de porphyrines *in vitro*, le 5-ALA hexylester produisant autant de porphyrines que le 5-ALA mais à des concentrations jusqu'à 100 fois plus faibles ; (ii) les dérivés lipophiles pénètrent passivement les membranes biologiques au contraire du 5-ALA qui pénètre par transport actif ; (iii) les dérivés substitués en position alpha de la fonction carboxylique sont incapables d'induire la production de porphyrines. Différentes modifications ont par la suite été évaluées, en ajoutant par exemple des substituants halogénés, ou en remplaçant les chaînes aliphatiques par des groupements cycliques ou aromatiques.



5-ALA



Dérivé $R_1$ et/ou $R_2$ du 5-ALA

**Figure 4 :** Structure du 5-ALA présentant une double charge (zwitterion) en milieu physiologique. La dérivatisation est possible au niveau des groupes carboxyliques $R_1$ et amino $R_2$, mais ces derniers présentent une capacité réduite à induire la production de porphyrines.

Dans une optique différente, un groupe de recherche a synthétisé des dérivés N-peptidiques dans le but de cibler spécifiquement certaines enzymes, les N-aminopeptidases, qui sont surexprimées dans les tissus tumoraux. Les résultats n'ont toutefois pas été concluants, et il a été observé que les dérivés du 5-ALA sur la fonction amine n'étaient pas de bons substrats pour induire la formation de porphyrines. Mis à part les n-alkylesters du 5-ALA, d'autres molécules en développement se sont montrées

prometteuses ; il s'agit du benzylester du 5-ALA, de ses dérivés éthylèneglycols, ou encore de dendrimères de 5-ALA.

En clinique, deux dérivés du 5-ALA ont prouvé leur efficacité ainsi que leur supériorité par rapport à la molécule mère, et ont par conséquent obtenu une autorisation de mise sur le marché. Le méthylester du 5-ALA, sous le nom de Metvix®, a reçu une autorisation pour le traitement PDT de certaines pathologies cutanées, plus précisément la kératose actinique (AK) et certains cancers des cellules basales (BCC). Les différents essais cliniques comparatifs ont montré que le traitement par Metvix®-PDT était aussi efficace que les méthodes standards (AK :cryothérapie ; BCC : chirurgie), mais présentait deux avantages majeurs . Premièrement, le traitement PDT étant non invasif, il peut être répété, avantage non négligeable notamment en cas de récidive. Deuxièmement, le résultat cosmétique obtenu est bien meilleur que celui de la chirurgie. Ce dernier point peut paraître secondaire à première vue, mais il faut souligner que ce type de pathologie affecte majoritairement les zones du corps exposées au soleil, dont le visage et la nuque, un traitement invasif risquant de défigurer gravement le patient.

Le second dérivé du 5-ALA utilisé couramment en clinique est l'hexylester du 5-ALA, commercialisé sous le nom de Hexvix® pour la détection améliorée du cancer de la vessie. Ce type de cancer est fréquent en Europe (5$^{ème}$ cancer par incidence), mais sa mortalité n'est pas parmi les plus élevées. Toutefois, le risque de récidive est relativement élevé. Actuellement, le traitement de choix du cancer de la vessie est la chirurgie, mais les difficultés rencontrées par le chirurgien pour détecter un type particulier de lésion plate, les carcinomes *in situ* (CIS), sont une des causes majeures des échecs thérapeutiques. C'est précisément à ce niveau que la détection par fluorescence avec Hexvix® est avantageuse. En effet, après l'instillation intra-vésicale de 5-ALA hexylester, les cellules cancéreuses accumulent de grandes quantités de porphyrines fluorescentes, au contraire des cellules saines de l'urothélium, et se distinguent par une forte émission de fluorescence rouge lorsque la vessie est irradiée par lumière bleue (Fig. 5). Différentes études cliniques ont souligné que la cystoscopie par fluorescence permettait d'améliorer significativement la détection des CIS, sans entraîner d'effets secondaires ou d'inconfort majeurs pour le patient. Cette méthode est en train de devenir le « gold-standard » dans le domaine.

Le 5-ALA hexylester est autorisé uniquement pour la détection du cancer de la vessie, toutefois des essais en vue d'un traitement PDT de cette pathologie sont actuellement à l'étude.

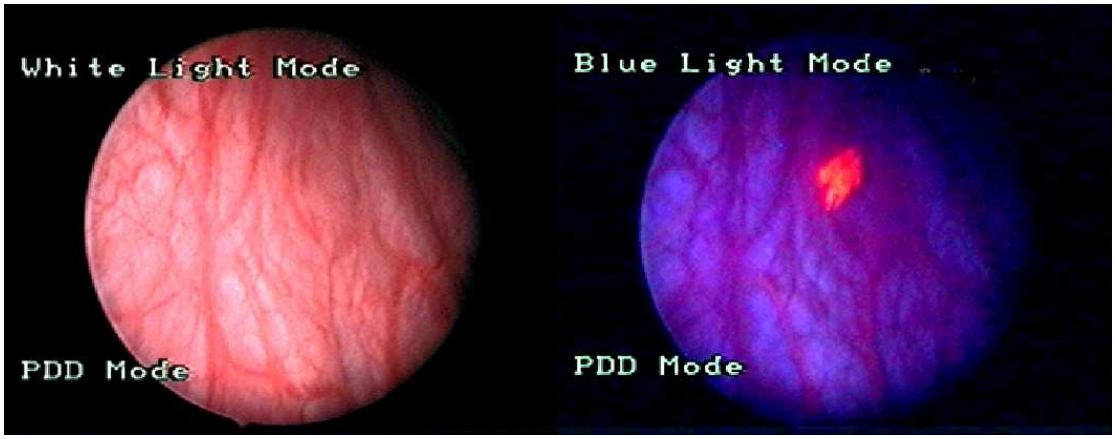



**Figure 5 :** Cystoscopie d'un patient diagnostiqué pour un cancer de la vessie ; à gauche, image prise en cystoscopie par lumière blanche ne présentant pas de lésion détectable ; à droite, la même zone irradiée en lumière bleue après instillation de Hexvix® permet de mettre en évidence carcinome *in situ* grâce à la forte fluorescence rouge émise par les cellules cancéreuses.

D'autres maladies seront peut-être traitées un jour par les dérivés du 5-ALA, des recherches étant en cours dans différents domaines, par exemple en dermatologie pour le traitement du psoriasis, de la leishmaniose, de l'acné, des verrues, ou en oncologie pour le traitement du cancer de l'utérus ou de l'œsophage de Barrett. D'autres pathologies, telles que les cancers de la prostate, du sein ou du cerveau pourraient être potentiellement traités par cette méthode, mais nécessiteraient l'élaboration de dérivés du 5-ALA administrables par voie systémique.

## Du métabolisme des dérivés de l'acide 5-aminolévulinique

Malgré le développement clinique de l'utilisation des dérivés du 5-ALA en photomédecine, il subsiste des interrogations au niveau de quelques mécanismes fondamentaux. Par exemple, il a été observé que, tandis que les molécules de 5-ALA doivent être transporté par des mécanismes actifs, les dérivés lipophiles du 5-ALA traversent les membranes biologiques par voie passive. Il a été supposé que, par la suite, les esters du 5-ALA doivent être clivés en 5-ALA et son alcool correspondant, par des estérases cytosoliques non spécifiques, pour entrer dans la biosynthèse de l'hème. Toutefois, la formation de porphyrines ne peut être considérée comme une preuve suffisante de l'hydrolyse des dérivés du 5-ALA. En considérant les dernières découvertes dans le domaine

de la biosynthèse de l'hème, il apparaît que le mécanisme d'action de la porphobilinogène synthase (PBGS), l'enzyme catalysant la condensation de deux molécules de 5-ALA en une molécule de porphobilinogène (PBG), n'implique a aucun moment les groupements carboxyliques du 5-ALA concernés par la dérivatisation. L'hydrolyse des dérivés du 5-ALA en 5-ALA ne serait donc pas un prérequis pour devenir substrat de la biosynthèse de l'hème, pour autant que la dérivatisation soit effectuée sur le groupement carboxylique et que l'encombrement stérique ne gène pas l'entrée de la molécule dans les poches fonctionnelles de l'enzyme (Fig. 6).

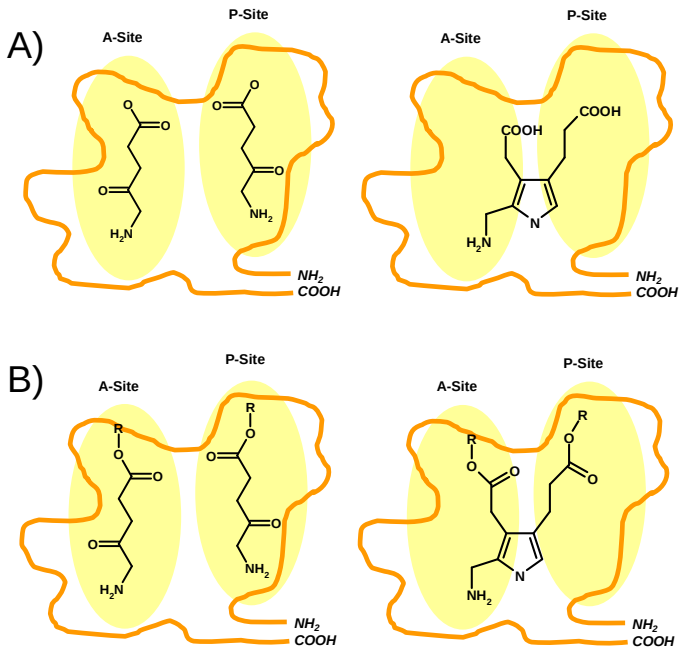



**Figure 6** : Représentation schématique de la porphobilinogène synthase (PBGS). A) Deux molécules de 5-ALA prennent place dans les poches de l'enzyme qui catalyse une réaction de condensation. B) Les groupements carboxyliques n'étant pas impliqué dans le processus enzymatique, il est mécanistiquement possible que les dérivés du 5-ALA soient directement substrat de l'enzyme, résultant en la formation de dérivés de porphobilinogène.

Afin d'étudier le métabolisme des dérivés du 5-ALA ainsi qu'une possibilité d'améliorer la stabilité de ces molécules, nous avons synthétisé le 2-amino-3-oxohenadioic-1,6-diméthyl ester (AOMM). Cette molécule se base sur le 5-ALA méthylester (MAL) auquel a été ajouté un groupement methoxycarbonyl à proximité de la fonction amine. Cette

molécule présente des propriétés de taille, de charge, et de lipophilie proches de la molécule parente, mais doit impérativement subir un clivage par une estérase pour devenir active. En effet, le groupement supplémentaire bloque la fonction amine, ce qui empêche à la fois la dégradation de la molécule par dimérisation, et sa transformation enzymatique en PBG.

En cas de présence d'activité estérasique, AOMM devrait être transformé en 5-ALA méthlyester et en 5-ALA, et devenir substrats du cycle de biosynthèse de l'hème. Des essais enzymatiques *in vitro* ont montré qu'il y avait bien transformation rapide des cette molécule « témoin » préférentiellement en MAL, puis en 5-ALA, en présence d'estérases de foie de porc. Néanmoins, les cellules cancéreuses incubées avec AOMM n'ont pas accumulées de porphyrines fluorescentes, au contraire de celles incubées avec 5-ALA ou MAL, indiquant une incapacité des cellules à hydrolyser ce substrat, et remet en question la présence et/ou l'importance de cette activité estérasique présumée. De plus, l'injection d'AOMM dans le modèle le l'embryon de poulet a entraîné l'accumulation de porphyrines fluorescentes dans les différents organes analysés.

Ceci confirme la nécessité de poursuivre les recherches fondamentales sur le métabolisme des dérivés du 5-ALA, afin de pouvoir adapter au mieux les futures stratégies de ciblage des dérivés du 5-ALA.

## Nouvelle méthode d'évaluation de dérivés de l'acide 5-aminolévulinique pour de nouvelles indications

Il a été montré précédemment que la biodisponibilité du 5-ALA était grandement améliorée par la dérivatisation de cette molécule en n-alkylester plus lipophiles, tels le méthylester et l'hexylester du 5-ALA. Toutefois, ces dérivés ont été développés exclusivement pour un usage topique. Leur utilisation par voie systémique n'étant pas possible pour des raisons de toxicité, certaines pathologies telles que les cancers du sein, de la prostate ou du cerveau, ne bénéficient pas de ces évolutions. Pourtant, le 5-ALA a déjà été utilisé pour la FD ou le traitement PDT de glioblastomes, et a montré un potentiel certain. Néanmoins la faible capacité du 5-ALA à traverser la barrière hémato-encéphalique pénalise et rend nécessaire le développement de dérivés du 5-ALA conçus spécifiquement pour une administration systémique et présentant une capacité améliorée à atteindre le système nerveux centrale.

Dans cette optique, différents modèles expérimentaux sont disponibles pour l'évaluation de ces nouvelles molécules, chacun de ces modèles présentant des avantages

et inconvénients qui leurs sont propres. La culture cellulaire, par exemple, est facilement accessible, bon marché, mais reste un model basique peu représentatif de la situation complexe d'un organisme vivant. D'un autre côté, les essais *in vivo* sur des animaux donnent une idée beaucoup plus proche du devenir des molécules actives chez l'homme, mais pour un coût très élevé, et en présentant de plus des limitations éthiques et légales qui les rendent inadaptés en phase de criblage.

Pour ces raisons, nous avons envisagé l'utilisation du modèle de l'embryon de poulet pour évaluer les nouveaux dérivés du 5-ALA. L'embryon de poulet est utilisé depuis de nombreuses années dans le domaine de la PDT pour sa membrane chorio-allantoïque (CAM), fortement vascularisée et accessible, qui permet l'évaluation des propriétés vaso-occlusives de différents régimes PDT. La procédure appliquée a été la suivante. Des œufs fécondés ont été préparés et incubés jusqu'au douzième jour de développement embryonnaire. Les molécules à tester ont été dissoutes dans un certain volume de solvant, puis injectées dans une large veine du CAM. Après un certain temps d'incubation, l'embryon a été congelé à -80°C. Après dissection et prélèvement des différents organes, les porphyrines produites ont été extraites des échantillons, puis dosées par HPLC-FD. Des essais effectués avec le 5-ALA ont montrés que la production de porphyrines dans les différents organes était dépendante de la concentration de 5-ALA administrée. De plus, les valeurs obtenues étaient comparables aux données de la littérature concernant la biodistribution des porphyrines après administration du 5-ALA à des souris. De même, une injection d'hexylester du 5-ALA a montré une forte toxicité aiguë, telle que celle observée après administration systémique chez la souris.

Des essais ont été effectués avec des dérivés diacylglycérides du 5-ALA (Fig. 7) spécialement synthétisés pour cibler le cancer du cerveau. Ces molécules doivent être adaptées à une administration par voie systémique, présenter une lipophilie accrue afin de traverser aisément la barrière hémato-encéphalique tout en présentant une faible toxicité. Après avoir été injectées dans le model de l'embryon de poulet, ces dérivés ont montré la capacité à induire la production de porphyrines dans les différents organes, tandis qu'aucune toxicité aiguë n'a été observée.

Ce modèle peut nous permettre de déterminer les candidats les plus prometteurs en terme de capacité à induire une accumulation de porphyrines dans les tissues cibles, ainsi que d'absence de toxicité aiguë, et pourrait devenir une alternative intéressante aux études précliniques de biodistribution et de toxicité.

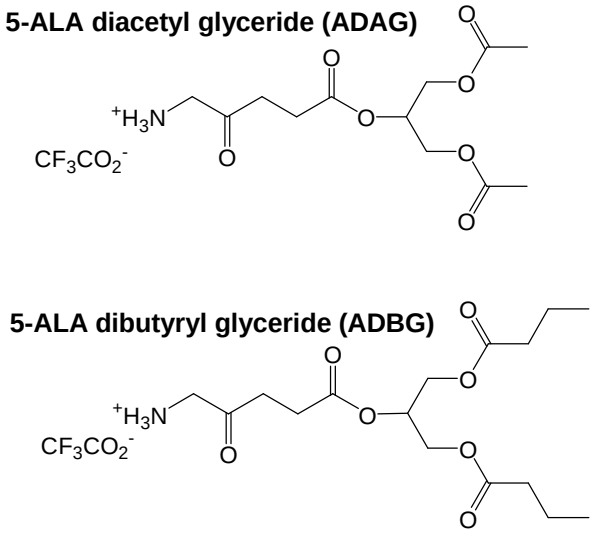



**Figure 7 :** Structures de deux dérivés diacylglycérides du 5-ALA conçus pour une utilisation systémique dans le ciblage du cancer du cerveau.

L'avantage de ce modèle est qu'il permet d'étudier les dérivés du 5-ALA quant à leur capacité à induire la production de PAP, la biodistribution de ces porphyrines dans les différents organes, ainsi que leur toxicité systémique aiguë suite à l'injection du produit. De plus, contrairement à la culture cellulaire, les molécules faiblement hydrosolubles peuvent être facilement testées puisque l'injection de faibles volumes de solvant, ici un mélange de polyéthylène glycol, d'éthanol et d'eau, est bien tolérée. Ce modèle présente une grande flexibilité puisque différentes voies d'administration peuvent être testées (intraveineuse, intraperitonéale, topique). De plus, il est possible d'implanter des tumeurs directement sur la CAM.

## Effets de l'acide 5-aminolévulinique et des dérivés de l'acide 5-aminolévulinique sur des bactéries gram-négatives et gram-positives

Alors que la découverte des antibiotiques par Flemming et les progrès réalisés dans le domaine durant la décennie qui suivit avaient laissé espérer une éradication totale des infections bactériennes, nous sommes toutefois obligés de constater la relative impuissance de la recherche pharmaceutique face au pouvoir d'adaptation des bactéries. La capacité qu'ont les bactéries à devenir résistantes aux nouveaux traitements et à propager cette

résistance à leurs congénères est en train de surpasser celle de la médecine à développer de nouvelles molécules antibactériennes. Une des raisons pour expliquer ces rapides adaptations aux antibiotiques est l'usage injustifié et immodéré de grandes quantités d'antibiotiques, non seulement dans les domaines de la santé, mais également de l'agroalimentaire. Dans ce contexte, des alternatives thérapeutiques aux antibiotiques doivent être développées.

Une de ces alternatives pourrait être la PDT. En effet, cette technique actuellement utilisée pour le traitement de certains cancers ou de la dégénérescence maculaire liée à l'âge a pour la première fois été observée pour ses propriétés antimicrobiennes. De nombreux essais dans ce domaine ont été effectués et ont montré une efficacité de la PDT dans l'élimination *in vitro* de bactéries, dans la désinfection de l'eau ou du plasma, ainsi que pour le traitement *in vivo* de certaines infections. Dans ce dernier cas, le choix du PS reste crucial, et des propriétés telles qu'une faible toxicité, une sélectivité accrue pour les tissus cibles ainsi qu'une absence de photosensibilité cutanée résiduelle sont recherchées. Différentes études ont montré que le 5-ALA induisait une production plus ou moins grande de porphyrines selon le type de bactérie, la phase de croissance ou les conditions d'incubations.

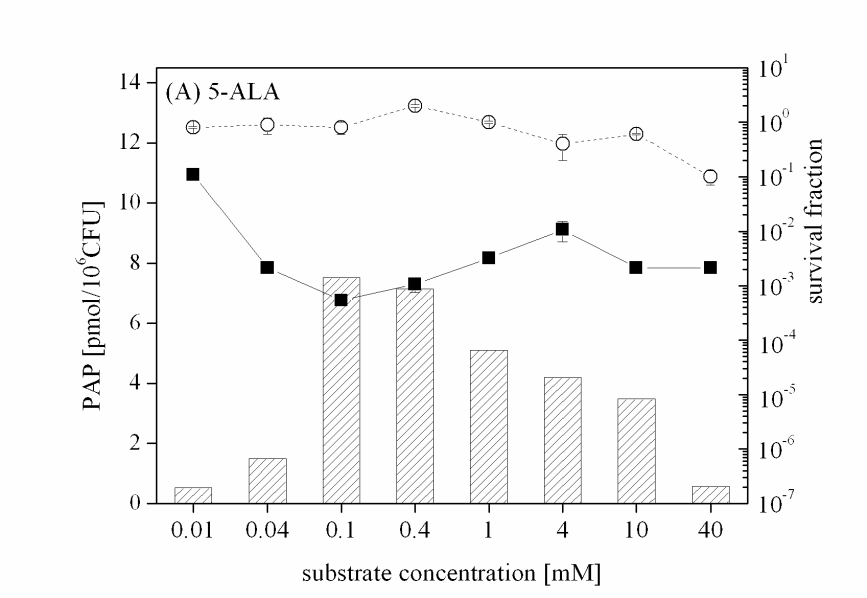



**Figure 8 :** Production de porphyrines (barres), et fraction survivante sans irradiation (○) et après irradiation (■) de *E. coli* K12, en fonction de la concentration de 5-ALA (temps d'incubation 3 heures ; irradiation 120 $J \cdot cm^{-2}$).

Le but de cette étude était de déterminer s'il était possible d'améliorer ou d'influencer la production de porphyrines dans différentes souches bactériennes en utilisant des dérivés lipophiles du 5-ALA. Pour cela, le 5-ALA et des n-alkylesters du 5-ALA de lipophilie croissante ont été incubés à différentes concentrations avec des souches d'*Escherichia coli* K12, *Escherichia coli* Ti05, *Pseudomonas aeruginosa* et *Staphylococcus aureus* MRSA. Leur survie avec et sans irradiation a ensuite été déterminée, de même que la quantité et le type de porphyrines produites.

Les premiers essais effectués sur *E. coli* K12, une souche de laboratoire non pathogène, ont montré que la production de porphyrines était fortement dépendante de la concentration en substrat. En effet, la production de porphyrines augmente avec celle de substrat jusqu'à un optimum d'environ 0.1 mM, puis diminue malgré l'augmentation de la concentration de 5-ALA (Fig 8). Cette « courbe en cloche » a déjà été observée par exemple en culture cellulaire, et l'hypothèse communément admise pour expliquer cette baisse est celle du blocage de certaines enzymes par un excès de 5-ALA. De plus, la production de porphyrines est bien corrélée à l'efficacité de la photoinactivation, puisque c'est à cette concentration optimale de production de PAP que la photoinactivation est la plus efficace, (> 99.9% d'inactivation des bactéries). Les porphyrines majoritairement détectées sont l'uroporphyrine et la coproporphyrine, tandis qu'on ne trouve que quelques traces de protoporphyrine IX.

Les esters du 5-ALA à courte chaîne (méthyl- et butylester) ont entraîné la production de grandes quantités de porphyrines après incubation à une concentration de l'ordre de 10 mM. Au contraire, les n-alkylesters à longue chaîne du 5-ALA (pentyl-, hexyl- et octylester) n'ont montré aucune capacité à induire la formation de porphyrines à quelque concentration que ce soit. De plus, ces molécules ont démontré une grande toxicité intrinsèque, augmentant avec la lipophilie, non liée à une action photochimique, et ne sont donc pas des candidats intéressants pour une photoinactivation antibactérienne.

Les essais avec les autres souches précitées ont montré des résultats similaires, le méthylester du 5-ALA induisant des grandes quantités de porphyrines et entraînant une photoinactivation efficace des bactéries. Cette molécule, déjà utilisée pour le traitement PDT de différents désordres cutanés, pourrait être un candidat potentiel pour le traitement PDT d'infections cutanées.

## Dérivés de l'acide 5-aminolévulinique pour une photoinactivation améliorée de *Propionibacterium acnes*

Actuellement, différents traitements monosubstances ou combinés sont utilisés dans le traitement de l'acné modéré ou sévère, mais leur efficacité doit être mise en relation avec de lourds effets secondaires, ainsi qu'un problème de résistance des bactéries.

Une solution envisagée pour remplacer ces traitements est la PDT. Elle permettrait de traiter à la fois le désordre glandulaire, l'hyperproduction sébacée et la prolifération bactérienne (*Propionibacterium acnes*) (Fig. 9), cette dernière étant responsable de l'inflammation. Des études cliniques ont montré la faisabilité de ce type de traitement de l'acné, avec notamment une amélioration de la maladie chez des patients ayant subi un traitement PDT avec 5-ALA. Une étude a même comparé directement l'efficacité d'un traitement PDT après application topique de 5-ALA ou de méthylester du 5-ALA (MAL). L'efficacité des deux traitements s'est montrée similaire, la différence venant du fait que l'irradiation était moins douloureuse après application du MAL que du 5-ALA.

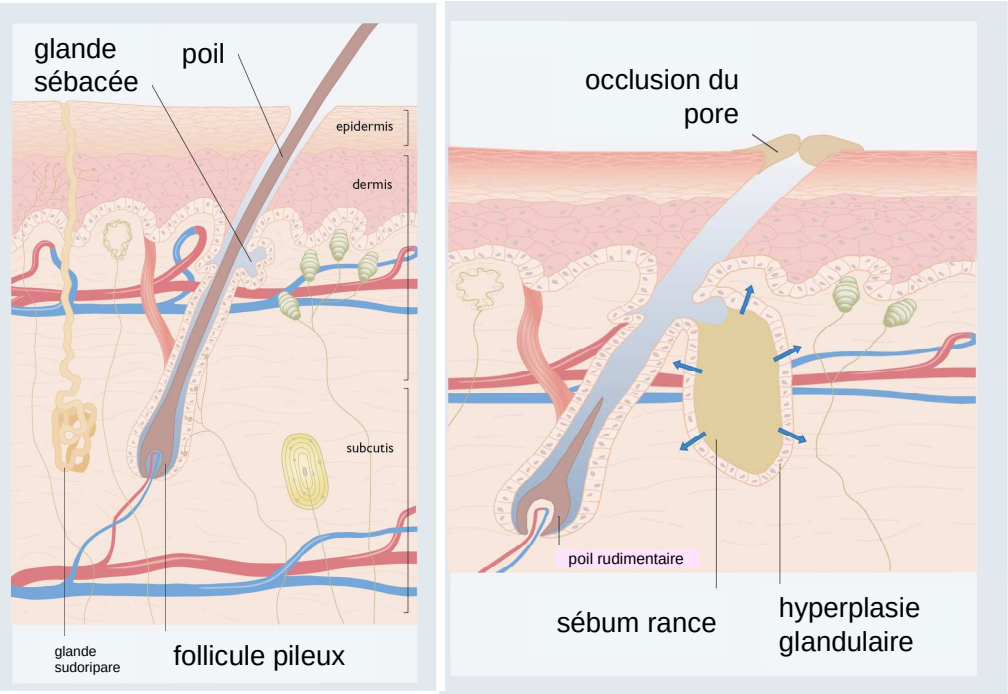



**Figure 9** : Schéma représentant un follicule pileux sain (gauche) et acnéique (droite) ; l'occlusion du pore, l'hyperplasie glandulaire et l'accumulation de sébum installent un environnement propice à la colonisation du follicule pilo-sébacé par *Propionibactrium acnes*, qui va induire ou aggraver le processus inflammatoire (tiré de « Integrated Pharmacology » 2ème édition).

Le but de cette étude était de déterminer quel dérivé du 5-ALA serait le plus efficace pour une photoinactivation des bactéries de type *P. acnes*. Pour cela, une souche de laboratoire de *P. acnes*, ainsi que deux souches pathogènes ont été incubées avec le 5-ALA et différents dérivés. Les candidats sélectionnés étaient le méthylester du 5-ALA, qui a montré une bonne efficacité contre les différentes souches testées dans une étude précédente, l'hexylester (HAL), le benzylester (BzAL) et le di-éthylène-glycol-monométhylether ester (DGME) du 5-ALA. Les résultats ont montré qu'il était possible d'induire une forte accumulation de porphyrines chez *P. acnes* generale après incubation avec le 5-ALA. La concentration optimale de 5-ALA se situe entre 3-10 mM, tandis que les types de porphyrines les plus représentées dans ces conditions expérimentales sont l'uroporphyrine et la coproporphyrine. MAL n'a que faiblement induit la production de porphyrines, tandis que HAL a démontré une grande toxicité intrinsèque à des doses supérieures à 1 mM.

Les deux molécules les plus prometteuses sont BzAL et DGME qui ont induit l'accumulation de grande quantités de porphyrines chez *P. acnes* generale, et une photoinactivation de >99.9 % des bactéries. Des résultats similaires ont été obtenus sur les souches pathogènes testées, BzAL et DGME induisant une photoinactivation plus importante que MAL.

Une production élevée de porphyrines *in vitro* chez *P. acnes* ne représente toutefois pas une garantie pour un traitement efficace de l'acné, et l'accumulation de ces molécules au niveau des follicules pilo-sébacés devra également être étudiée, de même qu'une optimisation de la formulation et des conditions d'irradiation. Des essais précliniques se déroulent en ce moment dans le but d'évaluer le réel apport de ces molécules, comparé au 5-ALA et au MAL, pour le traitement PDT de l'acné.