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Chapter 4

Photodynamic Treatment of Actinic Keratosis Using Ameluz®: Recapitulation of Clinical Phase III Studies in the Light of Novel Preclinical Research

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Abstract

In photodynamic therapy (PDT) of neoplastic skin lesions, both ALA and MAL penetrate skin cells and serve as precursors in the synthesis of the photosensitizer, protoporphyrin IX (PpIX), in tumor cells. There has been a long debate on the differences between ALA and MAL regarding their clinical efficacy, adverse effects and molecular mechanisms underlying their cellular uptake and metabolism. Application site pain is one of the most disturbing side effects of PDT. Conflicting results were published regarding the severity of pain caused by ALA- and MAL-PDT. Also, some authors argued that MAL may display higher selectivity for tumor cells than ALA.

Here, we summarize a very recent multinational, multi-centre, prospective, placebo-controlled phase III study comparing an ALA (BF-200 ALA, marketed as Ameluz®) and a MAL preparation (Metvix®). We assess the clinical results in view of new preclinical research that explores the molecular mechanisms of ALA and MAL uptake and the action of PpIX in normal keratinocyte and keratinocyte tumor cell lines and nociceptive

neurons. Particularly relevant are new results on the interaction of keratinocytes and nerve cells during PDT.

The phase III study comparing Ameluz® and Metvix® demonstrated strongly superior efficacy of Ameluz® over Metvix®, in both the primary and secondary read-outs total patient and total lesion clearance. However, application site pain was not elevated along with the increased efficacy.

The better efficacy of Ameluz® may be based on the strongly improved skin penetration and PpIX formation that was demonstrated on normal pig skin. It may be argued that MAL forms less PpIX due to its supposedly higher tumor selectivity. However, the comparison of PpIX-formation in tumor and non-tumor keratinocyte cell lines loaded with ALA or MAL showed that both precursors produced up to 6-times higher PpIX levels in tumor cells than in non-tumor cells. In all cell types, PpIX synthesis was considerably faster with ALA than with MAL. Since both compounds are not sufficiently hydrophobic to diffuse through biological membranes, both must enter the cells via transporters, one of which is the GABA transporter (GAT)-3. The similar selectivity for tumor cells is reflected by the clinical results, where application site effects after PDT with Ameluz® are very similar to those after Metvix® PDT.

With respect to the burning pain experienced by many patients during and briefly after the PDT illumination, we recently demonstrated that ALA and MAL enter sensory neurons. A GAT-3 type transporter is involved in both ALA and MAL uptake into neurons. Further investigations of cellular events occurring during PDT and potentially causing sensory neuron activation revealed (i) a direct activation of neuronal voltage gated calcium and sodium channels by PDT-derived reactive oxygen, and (ii) an indirect activation of nociceptive neurons by acetylcholine secreted from keratinocyte tumor cells during PDT. Since only the ultimate nerve endings are exposed to ALA or MAL in the epidermis, this second mechanism seems most relevant for the generation of painful sensations during PDT. Since acetylcholine is increasingly produced in keratinocytes during their migration towards the upper layers of the epidermis, the enhanced penetration of Ameluz® may indeed lead to a better efficacy without increasing painful sensations, thus explaining the clinical results.

1. Three Challenges for Photodynamic Therapy in the Clinical Management of Actinic Keratosis

Photodynamic therapy (PDT) represents a clinically effective and minimally invasive treatment option for a variety of neoplastic and nonmalignant conditions (Agostinis *et al.*, 2011). As PDT relies on the application of a photosensitizing drug in combination with target area illumination with a light source, it has generated increasing interest in the field of dermatology (For reviews see Kennedy *et al.*, 1990; *Peng et al.*, 1997; Gold MH, 2007; McCormack MA, 2008). When treating cutaneous malignancies using PDT, drugs can mostly be applied topically. This allows rather specific spatial localization of the drug on the skin surface by local deposition of the photosensitizer drug. Also illumination can be performed unobstructed, as the skin surface is per se easily accessible for light. Still, photodynamic therapy in dermatology can face some hindrances. To overcome these or pass by them is the central challenge for PDT today.

A particularly well studied type of cutaneous lesion treated using PDT is actinic keratosis (AK) (Babilas *et al.*, 2010). These epidermal lesions, also classified as “carcinoma-in-situ”, are generally induced by extensive expose of skin to sunlight (Ko, CJ 2010). Clinically, these

lesions are described as discrete, premalignant, and intraepidermally localized and commonly occur on rather sun-exposed skin, as in the face or on the hands. While AKs are already endowed with true neoplastic characteristics (Ko, CJ 2010), they bear the risk to progress to invasive neoplastic lesions, namely squamous cell carcinomas (Braathen *et al.,* 2007). These tumour types are potentially metastatic and the initially localized lesion will spread into subepidermal layers and gather access to the bloodstream. In order to prevent the conversion from a rather benignant, locally treatable AK to a malignant systemic type of cancer, current international dermatological guidelines highly recommend the early attendance to AK. Photodynamic therapy has recently been ranked as a first-line treatment option for this particular purpose (Braathen *et al.,* 2007). While some drugs have already been approved for this, all have to face the above mentioned challenges. These challenges generally encompass: (a) selectivity, (b) drug or prodrug delivery and (c) an advantageous ratio of clinical efficacy versus side-effects.

PDT drugs for AK management often utilize protoporphyrin IX (PpIX) as the final photosensitizing compound, but rely on its synthesis from prodrugs inside AK cells (Ericson *et al.,* 2008). This approach bears a great advantage when addressing the selectivity problem. When using prodrugs of the endogenously occurring heme precursor PpIX, such as 5-aminolevulinic acid (ALA) and its methyl-ester (MAL), selectivity for neoplastic cells relies on two factors. One is the enhanced uptake of these prodrugs into neoplastic cells, the other is an enzymatic imbalance in tumor cells acting in favor of PpIX synthesis and hampering heme conversion (Van Hillegersberg *et al.,* 1992). Some studies have addressed this issue by comparing ALA und MAL efficiency in PpIX formation in cell cultures. Here, many of them could demonstrate superiority for ALA in PpIX production efficacy (e.g. Washbrook and Riley 1997, Uehlinger *et al.,* 2000; Gaullier *et al.,* 1997; Tunstall *et al.,* 2002; Rodriguez *et al.,* 2006 and Lee *et al.,* 2008). Additionally, cellular uptake routes seem to vary in different tumor cell-types and some authors have suggested divergent transport pathways for the structurally rather similar molecules ALA and MAL (Rud *et al.,* 2000; Rodriguez *et al.,* 2006). In order to shed light on this issue explicitly in the field of dermatology, our lab has conducted studies comparing healthy and malignant skin cells in their potential of PpIX synthesis from ALA and MAL. The results are presented below and may enhance the understanding of the different clinical characteristics experienced with ALA or MAL PDT.

A further challenge is the delivery of the rather hydrophilic molecules ALA and MAL through the *stratum corneum* in order to reach the neoplastic cells. Considerations in this field require different mechanistical approaches then those concerning selectivity and efficacy on a cellular level. As for MAL, it has been proposed that the addition of a single methyl-group to the acid residue of ALA might enhance lipophilicity sufficiently to allow passive membrane diffusion (Gederaas *et al.,* 2001). This may possibly mean a loss of selectivity towards neoplastic cells. Nevertheless, one advantage of MAL utilization is its increased stability in aqueous solution at physiological pH (Kaliszewski *et al.,* 2007). This feature seems to be gained at the expense of PpIX forming capacity.

Ideally, one would include the superior PpIX-producing characteristics of ALA into a drug delivery system circumventing instability problems and enhancing the ability to overcome the *stratum corneum*. A recent report introduced the ALA-containing nanoscale lipid vesicle formulation BF-200 ALA, now marketed as Ameluz®, as such a drug (Maisch *et al.,* 2010).

A combination of an active ingredient with a delivery system like this may at first sight seem a "perfect match" for photodynamic therapy. But it still has to master the final challenge: Ensuring high efficacies while not producing elevated side-effects in the clinic. One of the most common side effects reported by clinicians is pain at the application site during illumination. As illustrated below, pain depends on various parameters. One might generally assume a straight correlation between efficacy and pain, thus, recent clinical and preclinical studies hold proof that additional factors influence this equation and show how improved penetration characteristics keep pain at bay, while ALA as the PpIX precursor drives efficacy to novel heights.

2. Two Sides of the Same Medal: Combining Clinical Experience with Preclinical Understanding

With the previous points in mind, it is worthwhile pondering how a novel medication for PDT fulfilling those challenges could be conceived, created and clinically put to test. This achievement could only stem from a combination of a biological understanding of the addressed matter and its transition into the clinical setting with respect to the urges and needs of patients and health care professionals. Thus, the novel clinical data describing Ameluz® as a valuable drug for PDT in dermatological practice stand on solid scientific ground formed by intense preclinical and basic research.

3. Clinical Impressions: Efficacy and Safety

Two clinical phase III studies were conducted with Ameluz® (then under its development code name BF-200 ALA), in order to test it for clinical efficacy and safety. The studies comprised 122 (Szeimies *et al.*, 2010) and 571 (Dirschka *et al.*, 2012) patients. The second study is to our knowledge the biggest prospective, randomized, multicentric and placebo-controlled study on PDT for Actinic Keratosis to date.

Patients included in these two phase III trials suffered from 4 to 8 mild to moderate AK lesions on face or scalp. In these studies, treatment was performed by treating the prepared lesions with Ameluz® or placebo (Szeimies *et al.*, 2010) or Ameluz®, Metvix® (a commercially available MAL-cream, Galderma) or placebo (Dirschka *et al.*, 2012) for 3 h followed by an illumination using either broad-spectrum or LED light sources. With broad spectrum light sources the total light dose was 75 – 200 J/cm², while it was 37 J/cm² using narrow spectrum LED devices. Lesions were assessed 12 weeks after the first PDT and remaining lesions, if present, were retreated. Finally, 12 weeks after the final PDT the number of patients with all lesions cleared and the total number of cleared lesions were determined.

As the first clinical trial, a randomized, double-blind, prospective, placebo-controlled phase III trial with 122 patients compared Ameluz® to placebo (Szeimies et al., 2010). The light sources used were either narrow-spectrum or broad-spectrum lamps. The patient complete clearance rates were significantly higher for Ameluz® than for placebo (66.3% versus 12.5%, respectively), and the lesion complete clearance rates were 81.1% versus 20.9%, respectively. The use of narrow-spectrum lamps emitting light at 630 +/- 9 nm

resulted in higher efficacy than that of broad-spectrum lamps (96% versus 53%, respectively, for total patient clearance, and 99% versus 70%, respectively, for total lesion clearance). Thus, an almost complete clearance could be achieved when the combination of Ameluz® and narrow-band PDT lamps was applied. However, the use of narrow-band lamps also resulted in stronger side effects. While in an overall counting, 59.3% of the patients reported pain during PDT using Ameluz® during the first PDT session (Placebo: 6.7%), this percentage was reduced to 24% during the second PDT session. When using broad spectrum lamps, 37.5% of the patients reported pain during illumination in the first PDT session, this value sunk to 19% during the second session (with placebo values of 11.8 and 6.3% respectively). Using narrow spectrum lamps, now proven to be more efficient in therapeutic outcome, the percentage of patients reporting pain was highest, reaching 85.1% and 50% during the first or the second PDT respectively.

In a second pivotal phase III trial (Dirschka et al., 2011) Ameluz® was compared with Metvix® and placebo in an investigator-blind setting. 571 patients were treated with photodynamic therapy using Ameluz®, comparator Metvix®, or placebo at a ratio of 3:3:1. Again, narrow-band and broad-band light sources were used.

The primary medical endpoint was the complete recovery of all of a patient's lesions 12 weeks after the last PDT. On average with all lamps, Ameluz® (78.2%) was significantly more efficient than MAL (64.2%) and placebo (17.1%). In addition, the total lesion clearance rates were higher for Ameluz® (90.4%) than for Metvix® (83.2%) and placebo (37.1%). This second study confirmed that the use of narrow-spectrum lamps resulted in higher efficacy, accompanied by stronger side effects. Using this type of lamps, the total patient clearance with Ameluz® was 84.8 %, with Metvix® 67.5 % and with Placebo 12.8 %. The total lesion clearance rates were 93.6 % and 89.3 % for Ameluz® and Metvix® respectively. Despite the significantly increased efficacy of Ameluz®, the frequency of pain was not different between Ameluz® (69.4%) and Metvix® (72.8%).

Pain intensity measured on an 11-point numeric rating scale (VAS score) was not significantly different between Ameluz® and Metvix®. Ameluz® PDT (in the first session for an AK in the face or forehead) resulted in an overall VAS score of 4.1 ± 3.42, while a Metvix® PDT for the same indication caused a VAS score of 4.3 ± 3.43.

The clinical studies asses a very good therapeutic profile for the novel PDT drug Ameluz®. While it proved to be superior to its competitor Metvix® at the primary study endpoint, it nevertheless did not present an augmented pain profile.

One might generally speculate that clinical efficacy and side-effect frequency would be somewhat proportional, i.e. a more efficient therapeutic approach would cause more ROS in the target area, elicit more cell death and thus cause more side effects in line with these events. This correlation can clearly be seen when the clinical data for broad and narrow spectrum lamps are analyzed. However, Ameluz® features an increased efficacy towards Metvix® without the parallel increase in pain during illumination as most disturbing side effect. This suggests that other factors than just the enhanced PpIX formation may be causative of this complex phenomenon. The preclinical investigations presented in the following sections may aid to solve this puzzle.

4. Preclinical Explanations: Molecular and Cellular Mechanisms

Photodynamic therapy using ALA and MAL takes advantage of an evolutionary old and well conserved biochemical production chain – the heme pathway, giving rise to heme as the prosthetic group in hemoglobin and to cytochromes, present in the respiratory chains of all mitochondria. Therapeutic exploitation of this pathway usually starts by circumventing the rate limiting step of this process, ALA-synthase, the enzyme that creates ALA from glycin and succinyl-CoA. When adding extra ALA to a cell's metabolism, the end product feedback inhibition (heme to ALA-synthase) is passed by, and heme s consequently synthesized according to the potential of the subsequent enzymatic cascade (Peng *et al.*, 1997; McCormack MA, 2008). Thus, the production of PpIX - the actual photosensitizer - derived from ALA and other ALA-based PDT prodrugs, is possible in every somatic cell. This raises the questions how selectivity towards neoplastic cells is achieved in order to spare the surrounding healthy tissue?

Several studies have addressed the differences in prodrug uptake in healthy versus neoplastic cells. This *in vitro* approach allows focusing on cellular membrane uptake processes without having to consider additional parameters like tissue permeability. Thus, such studies greatly aid our understanding of the most basic processes. It has repeatedly been shown, that ALA and its methyl esters trigger higher PpIX formation in cells of neoplastic origin *in vitro* and *in vivo*. Two explanations for this phenomenon have been found. One is an increased activity of one enzyme in the heme synthesis pathway, namely porphobilinogen deaminase (PBG-D). This enzyme catalyses the deamination step from porphobilinogen to uroporphyrin III. The increased activity found in neoplastic cells will lead to an overall increase of PpIX formation (Kondo *et al.*, 1993; Leibovici *et al.*, 1988; Schoenfeld *et al.*, 1988). On the other hand, accumulation of PpIX is anything but the physiologically intended outcome of the heme pathway. Thus, after PpIX is built up, it is normally metabolized to heme through the addition of iron. Ferrochelatase is the responsible enzyme. This enzyme in particular was also shown to be functionally altered in neoplasms (el Sharabasy *et al.*, 1992; Peng *et al.*, 1997; van Hillegersberg *et al.*,, 1992). Its function is decreased, combining an already augmented anabolism of PpIX with decreased catabolic mechanisms. This finally renders the selectively increased build up in cancerous and pre-cancerous cells possible. An attempt to explain this is that cells with generally increased metabolism – such as neoplastic or tumor cells – may form increased amounts of PpIX due to a higher mitochondrial activity (Calvazara-Pinton *et al.*, 2007).

Still, this is just part of the truth. Before ALA or one of its derivatives may enter the pathways described above, they have to be taken up by the cells, and uptake routes seem to vary greatly between cells of different origin. Additionally, divergent pathways were described for ALA and methyl-ALA. While longer chain ALA-esters reach sufficient lipophilicity to overcome cellular membranes via diffusion, this is not true for ALA and MAL, as they are both markedly hydrophilic (Uehlinger *et al.*, 2000). The use of a more hydrophilic molecule poses the problem of getting it transported through membranes actively, but it holds a big advantage: Membrane transition has to be regarded as a selectivity gate with the potential of distinguishing neoplastic and healthy cells. Thus, prodrugs that do not unselectively pass any membrane in reach, may be advantageous since they gather selectivity

via transport processes. Various transporters have been described to carry ALA and MAL (Doering *et al.,* 1998; Rud *et al.,* 2000; Gederaas *et al.,* 2001; Rodriguez *et al.,* 2006; Frølund *et al.,* 2010). But as stated above, it is important to analyze uptake in cell types typical for the addressed organ, in this case epidermal keratinocytes, to fully understand the therapeutic potential (Casas *et al.,* 2002). Thus, it was needful to conduct *in vitro* studies comparing healthy and tumorous keratinocytes in terms of uptake and PpIX formation. The results described here were collected in a very practically orientated *in vitro* system for comparing ALA and MAL as prodrugs in epidermal neoplasms.

Side-by-side studies in this system, comparing ALA and MAL uptake into healthy (CCD 1106 KERTr cells) and neoplastic keratinocytes (A431 cells from a squamous cell carcinoma) could reveal, that - regardless of the prodrug used - PpIX production is more pronounced in neoplastic keratinocytes by a factor of at least 4. With ALA even a factor of 5 is observed. Such differences in uptake rates and amounts of PpIX formation, indicating superiority of ALA in these parameters, have also been described in various other cells lines (e.g. Washbrook and Riley 1997; Uehlinger *et al.,* 2000; Gaullier *et al.,* 1997; Tunstall *et al.,* 2002; Rodriguez *et al.,* 2006 and Lee *et al.,* 2008).

Our studies also revealed that the accumulation of PpIX in A431 cells peaks faster and at lower prodrug concentrations with ALA than with MAL. Still, when prolonging MAL incubation times or increasing prodrug concentration, total fluorescence maxima do not differ greatly anymore (Schulten *et al.,* 2012). Thus, any clinical study in patients evaluating ALA versus MAL medication efficacy should be carefully interpreted and checked for differences in incubation times and concentration of the applied drugs. Both, selectivity for tumour cells and amount of PpIX formation can be influenced by the time of incubation. Tumour selectivity is higher with shorter incubation times, at the expense of lower overall PpIX levels in the tumour cells. Additionally, 5-ALA shows higher selectivity at lower concentrations (Schulten *et al.,* 2012). This may explain better tumour selectivity described for MAL over ALA, where the incubation time and prodrug concentrations were kept constant (Fritsch *et al.,* 1998).

In order to identify the relevant pathways for ALA and MAL uptake, experiments were performed with inhibitors and competitors of the uptake. For ALA, the most significant uptake pathway is the GABA transporter 3 (GAT-3), as the highly selective GAT-3 blocker (S)-SNAP-5114 is capable of blocking most PpIX formation in ALA incubated cell types, both normal and neoplastic. A GABA-associated uptake route had already been published for human and murine adenocarcinoma cells (Rud et al., 2000; Rodriguez et al., 2006). Additional transporters involved in ALA uptake may be amino acid transporters. MAL is also taken up via these transporters but their relative importance seems to be different from ALA (Gederaas et al., 2001; Schulten *et al.,* 2012). While in many cell types the GABA 3 transporter seems less relevant for MAL uptake, this is different in neurons where both ALA and MAL are taken up through this transporter (Novak et al., 2011).

Penetration depth into the epidermis is a fundamental parameter for PDT efficacy. The delivery of the rather hydrophilic molecules ALA and MAL over the *stratum corneum* into the vital epidermal layers represents a challenge for dermatological PDT medications. Regarding Ameluz® this challenge has been met combining ALA with a nanoscale lipid vesicle formulation. This formulation not only chemically stabilizes ALA, a molecule normally rather unstable in aqueous solutions at neutral pH (Kaliszewski *et al.,* 2007), but also greatly improves ALA transport into deep epidermal regions. While the detailed

biochemical mode of action is in this case still subject to detailed investigations, the enhanced penetration efficacy has been shown in a preclinical study by Maisch and co-workers in 2010. In this study, the penetration depth and speed of BF-200 ALA and a commercially available MAL cream (Metvix®) were compared. This study used PpIX formation in slices of the *ex-vivo* porcine skin samples as readout. Fluorescence was measured at different time points (3, 5, 8 and 12 h) after drug application. The collected data showed that BF-200 ALA leads to an appearance of PpIX fluorescence in much deeper layers of the epidermis. After the longest evaluated time point (12 h) BF-200 ALA induced PpIX fluorescence throughout the epidermis down to the basal membrane. This could not be seen after any time point using the MAL-containing cream which caused more superficial PpIX synthesis at all time points evaluated. No evidence for PpIX formation in the dermis were detected with either the ALA or the MAL formulations (Maisch *et al.,* 2010).

These preclinical findings are in good agreement with the phenomena observed in the clinic, namely the data from Dirschka *et al.,* 2012, which reported higher efficacy for Ameluz® in the clearance of actinic keratosis than with the MAL cream Metvix®. An increased penetration depth will in all likelihood lead to a more profound photosensitization of the AK lesions and therefore result in an increased clearance. Neoplastic cells can only endure and form lesions if they are born in the lower epidermal layers where proliferation takes place. Thus, successful drug treatment has to reach the *stratum basale*.

Yet, the side effect profile of Ameluz® appeared unaltered towards the one of Metvix® (Dirschka *et al.,* 2012). Side effects of ALA or MAL PDT are transient and mostly restricted to the application site. The most disturbing side effect in the clinics is application site pain during the illumination. Various theories have emerged on the source and molecular nature of pain during dermatological PDT treatments. Still, most of these theories lacked experimental proof so far. Combining clinical findings with recent results from basic research now allows a better understanding of the origin of pain during PDT illumination.

5. The Source, the Depth and the Transmission: Novel Insights into Pain during PDT

Many authors have described pain during PDT as a common side effect of differing intensity (Wiegell *et al.*, 2003; Kasche *et al.,* 2006; Moloney and Collins 2007; Gholam *et al.,* 2011). Both clinical studies reviewed above (Szeimies *et al.,* 2010; Dirschka *et al.,* 2012) found that the PDT light source correlates not just with treatment efficacy but also with the severity of PDT pain. A direct link between these two parameters may be assumed, such that a more efficient treatment may give rise to more singlet oxygen in the epidermis, causing elevated decay of neoplastic cells and a higher intensity of PDT pain. While this observation is rather intuitive, as it describes the proportional relationship of more light energy with stronger effects – both desired and unwanted - the results of the clinical study comparing the efficacy of Ameluz® and Metvix® did not hint augmented pain during treatment with Ameluz® (Dirschka *et al.*, 2012). This apparent discrepancy can be explained by studying the mechanism of PDT pain, in particular since the observation is contrary to previous publications where a lower pain profile was described for MAL than for ALA PDT (Kasche *et al.*, 2006).

Any treatment related pain arising during PDT will manifest itself in the primary sensory neurons innervating the epidermis. Epidermal nerve endings stem form pseudounipolar neurons in the dorsal root or trigeminal ganglia that send processes into the periphery and propagate excitations to the dorsal horn in the spinal cord or the trigeminal nucleus caudalis (Lumpkin and Caterina, 2007). Some of these neurons, mainly those giving rise to Aδ and C fibers are specialized nociceptors, i.e. detectors for noxious stimuli, capable of perceiving various damage associated events (Julius D., 2001). During PDT these neurons must somehow be activated - a necessary prerequisite for acute pain.

From a theoretical point of view, two pathways are conceivable for neuronal excitation through PDT. Singlet oxygen is formed in the target cells during PDT. Thus, if neuronal processes in the epidermis could also be considered as cellular targets, one explanation could be a direct neuronal activation during PDT. This theory has been formulated previously by authors attempting to speculate on molecular explanations for their findings that ALA PDT appeared clinically more painful than MAL PDT. The assumption was often that ALA, but not MAL, would enter sensory nerve endings, induce local PpIX production and illumination would then be the reason for neuronal excitation. These assumptions were based on the finding that in an adenocarcinoma cell line ALA but not MAL was taken up via GABA transporters (Rud et al., 2000).

Although only sparse data was available on the expression of GABA transporters in primary sensory neurons, this particular theory reached some acceptance in the community. Very recent findings from our own laboratory now greatly question the coherency of this theory (Novak et al., 2011). Using a rat primary sensory neuron model *in vitro*, we showed that both, ALA and MAL are taken up into sensory neurons and are capable of forming PpIX there. More detailed observations on the responsible uptake pathway also showed that the GABA transporter 3 (GAT-3) presents the common uptake route for ALA and MAL in these cells. When discerning the various nociceptor cells types, our studies provided evidence that also C-fiber neurons, that reach deep into the epidermal layers *in vivo* (neurons positive for the markers CGRP and isolectin B4 (IB4); Stucky and Lewin 1999; Priestley *et al.*, 2002) are capable of PpIX production. While PpIX synthesis after ALA application was faster than after MAL application, the maximally observed synthesis amount was nearly the same (Novak et al., 2011). Thus, when comparing clinical data on pain during PDT, close attention has to be paid to the study parameters, since shorter incubation times might lead to reduced pain using MAL (Novak et al., 2011).

Following this direct path of pain formation during PDT, one might wonder how ROS production in nociceptive nerve endings might lead to neuronal excitation. To gather data on this issue, we have turned to calcium microflurimetry using Fura—2/AM in cultured rat sensory neurons loaded with PpIX by ALA incubation. While these neurons could still respond normally to physiological depolarization stimuli with a calcium influx, they also responded to 10 minute blue light (380 nm) illumination (*in vitro* PDT) with a massive rise in intracellular calcium. Control neurons that were not incubated with ALA showed no such reaction. The level of the calcium rise was within the range of calcium transients seen by depolarization.

To further analyze the mechanisms involved, we investigated the source of the calcium rise. By omitting extracellular calcium during *in vitro* PDT, we demonstrated that extracellular calcium, and not intracellular stores, was mainly responsible. By using various

channel blockers we furthermore confirmed that voltage gated calcium channels (VGCCs) were involved in this process.

Blocking L-type, T-type and P/Q-type Ca2+ channels reduces the calcium influx caused by *in vitro* PDT to 25% of the value without VGCC blocking. Further investigations shed light on the question whether VGCC opening is directly caused by PpIX derived ROS or whether a membrane depolarization might be the driving force. Omitting extracellular sodium, physiologically responsible for action potential generation via voltage gated sodium channels (VGSCs), led to a 50% reduction of neuronal calcium responses to *in vitro* PDT. This argues in favor of a depolarization associated process underlying the neuronal activation to PDT derived ROS (Novak *et al.*, unpublished data). Cytosolic calcium alterations had already earlier been found as a consequence of photodynamic action in a variety of other cell types (for a review see Almeida *et al.*, 2004). While some studies attributed this to a depletion of intracellular stores (Cui *et al.*, 1997; Ricchelli *et al.*, 1999; Granville *et al.*, 2001), others described calcium influx over the plasma membrane (Specht and Rodgers 1991; Joshi *et al.*, 1994; Gederaas *et al.*, 1996; Tajiri *et al.*, 1998). Although few researchers had so far focused on the molecular pathway of this phenomenon, there is some additional evidence that cell membrane calcium channels are a potential influx route (Joshi *et al.*, 1994; Hill and Schaefer 2009). Also, evidence exists that neuronal calcium channels undergo modifications when exposed to ROS (Todorovic *et al.*, 2001; Annunziato *et al.*, 2002). Neurons depend greatly on a tight regulation of cytosolic calcium.

Any disturbance leads to altered neuronal activity, transmitter release, energy balance, excitability and ultimately death (Bergmann and Keller 2003; Berridge MJ, 1998; Svichar *et al.*, 1998; Thayer *et al.*, 2002; Vanden Berghe *et al.*, 2002). Studies on invertebrate neurons (Uzdensky et al., 2001; Uzdenskii *et al.*, 2008) as well as an early study on PpIX neurotoxicity in sensory neurons of chicken (Riopelle and Kennedy 1982) support our data as well as a report on the neurotoxic effect of the photosensitizer mTHPC in rat sensory neurons (Wright *et al.*, 2009). Thus, these new experiments are in good agreement with other published observations and add some functional knowledge on ROS biology in the peripheral nervous system in general.

These results may aid in understanding direct mechanisms leading to nociceptor activation during PDT and rule out the assumption that ALA is more pain inducing due to exclusive uptake into sensory nerve endings.

However, only the very final tips of the nerve fibers innervate the vital layers of the epidermis and represent a very limited area for PpIX synthesis directly inside these cells. Therefore, potential interactions between neurons and keratinocytes should also be given attention.

The interaction of nerve endings and keratinocytes in nociception is an important new aspect in cutaneous sensory biology (Chateau and Misery 2004). At least two types of C-fiber nociceptors project processes into the epidermis (Zylka *et al.*, 2005; Lumpkin and Caterina 2007), which terminate directly adjacent to the keratinocytes' membranes. Keratinocytes themselves also express nociception-associated receptors such as the ATP-sensitive $P2X_3$ channel or the TRPV1 receptor (Denda *et al.*, 2006). This enables keratinocytes to sense pain-related stimuli and communicate them via calcium waves through gap junctions and paracrine signaling via transmitter release to other keratinocytes or to the nerve endings (Koizumi *et al.*, 2004; Denda and Denda 2007; Tsutsumi *et al.*, 2009). One important messenger in neuronal activation is ATP (Koizumi *et al.*, 2004; Denda and Denda 2007). ATP is secreted in response

to mechanical, thermal and chemiosmotic stress, while it can be detected by the sensory neuronal ATP receptors $P2X_3$ and $P2X_{2/3}$ (Burnstock, G. 2000). Another promising candidate for keratinocyte-neuron communication is acetylcholine. Keratinocytes produce and release this transmitter (Grando *et al.,* 1993), which can be triggered for example by UV-light exposure and is involved in sunburns (Kurzen et al. 2007). Furthermore nicotinic acetylcholine receptor subunits as well as muscarinic acetylcholine receptors have been detected in dorsal root ganglion neurons (Shelukhina *et al.,* 2009; Cao *et al.,* 2011). Subunits of the nicotinic receptor were also documented in isolectin B4 (IB4) and Mrgprd positive non-peptidergic nociceptors (Khan *et al.,* 2003), a type of C-fiber neuron that deeply innervates epidermal layers (Dussor *et al.,* 2008; Dussor *et al.,* 2009).

We have therefore tried to analyze the functional impact of molecules secreted from a squamous cell carcinoma cell line treated with *in vitro* ALA-PDT on cultured rat sensory neurons. It turned out that the supernatant of A431 cells elicited responses in cultured sensory neurons that were visualized by calcium imaging with Fura-2/AM. These responses were only present when the A431 cells were subjected to the combination of ALA incubation and illumination. Such calcium signals in peripheral neurons were also observed, when the supernatants of PDT-treated A431 cells were transferred to the neurons. These signals were completely inhibited by a mixture of antagonists for the nicotinic (mecamylamine) and the muscarinic (atropine) acetylcholine receptors. This result strongly supports the hypothesis that acetylcholine is secreted from neoplastic epidermal cells upon PDT, and that this transmitter excites primary sensory neurons.

In order to interprete this finding with the clinical observations that (a) Ameluz® (ALA) PDT is more effective than MAL PDT in clinical studies, (b) Ameluz® (ALA) and Metvix® (MAL) show nearly the same pain profile during treatment, (c) Ameluz® drives PpIX formation in by far deeper epidermal layers than Metvix®, one has to consider the expression and function of acetylcholine in the epidermis.

Acetylcholine is present in the skin, where it serves multiple physiological functions. It is involved in keratinocyte barrier formation, migration, proliferation and differentiation. It thereby greatly regulates skin homeostasis via autocrine and paracrine signaling pathways (for a review see Kurzen *et al.,* 2007). As described above, acetylcholine release from keratinocytes is also triggered by UV-light. Kurzen *et al.* summarized the role of acetylcoline as an important messenger in epidermal responses to UV light exposure: Suprabasal keratinocytes are exposed to UV light, release acetylcholine which in turn activates basal keratinocytes to release nitric oxide. Nitric oxide causes an increased blood flow in adjacent vessels, thereby causing erythema. Excess UV light exposure cause sustained acetylcholine release, which disturbs epidermal homeostasis and leads to a loss of cellular attachment, blistering and keratinocyte cell death (Kurzen *et al.,* 2007). Translated to the PDT scenario, the identical mechanism might elicit PDT side effects, but rather than UV irradiation it is red light in combination with a photosensitizer that is responsible for this. Local pain, erythema and blistering are the major PDT side effects.

To finally close the circle to the clinical observations, it is important to understand that acetylcholine is spatially unevenly distributed throughout the epidermis. The most apical vital layers (granular and spinous) contain higher amounts of acetylcholine than the basal layer. The latter is almost devoid of the messenger and instead expresses acetylcholine esterase, the principal catabolic enzyme for acetylcholine (Nguyen et al., 2001). These authors also showed that acetylcholine triggers the final secretion processes in keratinocytes prior to their

programmed cell death and transition to corneocytes of the stratum corneum. This acetylcholine gradient may now be the ultimate key to understand why a PDT drug inducing PpIX until the basement membrane of the epidermis, in spite of higher efficacy, is not more prone to PDT side effects – especially pain – than a drug that mostly photosensitizes the apical layers of the epidermis. PpIX related pain seems to a major part related to induction by acetylcholine which is mostly secreted from the more superficial epidermal layers. Deeper penetration of the photosensitizer may thus improve clinical efficacy without causing additional nociceptor excitation.

Conclusion

Clinical studies have identified a novel ALA-containing nanoscale vesicle formulation (Ameluz®) as a valuable and efficient medication for the photodynamic treatment of actinic keratosis. The combination of deep epidermal penetration and the highly effective protoporphyrin IX prodrug ALA leads to extremely good clearance rates of AK lesions, especially when LED-lamps are used. Side effects on the one hand increase along with higher efficacy but are not augmented compared to a MAL containing cream of lower efficacy.

While deep efficient penetration of the entire epidermis is a key to high efficacy rates, exacerbated pain was not clinically observed. In an adequate *in vitro* setup comparing normal and neoplastic skin cells, ALA showed similar or higher selectivity towards neoplastic cells than MAL.

Pain differences between ALA and MAL PDT cannot be attributed to differential uptake of these compounds into sensory nerve endings, as preclinical data clearly showed that both PpIX prodrugs are transported into primary sensory neurons via the same transporter protein. Further preclinical studies revealed that acetylcholine plays a pivotal role in pain formation during PDT. Acetylcholine is distributed in a gradient through the epidermis, such that the uppermost vital layers show the highest synthesis.

Hence, this may be the spatial zone responsible for nociceptive processes during PDT that involve keratinocyte-neuron communication. Increasing penetration depth therefore seems mostly beneficial in terms of therapeutic outcome while it does not necessarily worsen the side effect profile.

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