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Comparison of ALA- and ALA hexyl-ester-induced PpIX depth distribution in human skin carcinoma

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ABSTRACT

Photodynamic therapy (PDT) based on the use of photoactivable porphyrins, such as protoporphyrin IX (PpIX), induced by the topical application of amino-levulinic acid (ALA) or its derivatives, ALA methyl-ester (m-ALA), is a treatment for superficial basal cell carcinoma (BCC), with complete response rates of over 80%. However, in the case of deep, nodular-ulcerative lesions, the complete response rates are lower, possibly related to a lower bioavailability of PpIX. Previous in vitro skin permeation studies demonstrated an increased penetration of amino-levulinic acid hexyl-ester (h-ALA) over ALA. In this study, we tested the validity of this approach in vivo on human BCCs. An emulsion containing 20% ALA (w/w) and preparations of h-ALA at different concentrations were applied topically to the normal skin of Caucasian volunteers to compare the PpIX fluorescence intensities with an optical fiber-based spectrofluorometer. In addition, the PpIX depth distribution and fluorescence intensity in 26 BCCs were investigated by fluorescence microscopy following topical application of 20% ALA and 1% h-ALA. We found that, for application times up to 24 h, h-ALA is identical to ALA as a PpIX precursor with respect to PpIX fluorescence intensity, depth of penetration, and distribution in basal cell carcinoma, but has the added advantage that much smaller h-ALA concentrations can be used (up to a factor 13). We observed a non-homogenous distribution in BCCs with both precursors, independent of the histological type and depth of invasion in the dermis.

1. Introduction

Photodynamic therapy (PDT) has been investigated clinically as a treatment of malignant and non-malignant skin diseases over the last years [1–6]. In particular, the use of 5-aminolevulinic acid (ALA) and some of its ester derivatives, mainly 5-aminolevulinic acid methyl-ester (m-ALA), for PDT of actinic keratosis (AK) and basal cell carcinoma (BCC) has been approved by the European Union (m-ALA–PDT for BCC and AK in 2001) and the US Food and Drug Administration (ALA–PDT for AK in 1999) [1,2,7], and is now a well-established therapeutic option [5].

This treatment modality involves the topical application of a precursor (ALA or one of its derivatives) onto the diseased area for several hours, which results in the generation of photoactivable porphyrins such as protoporphyrin IX (PpIX). The diseased area is then irradiated with light from a light emitting diode (LED) matrix, a laser or a filtered lamp, illuminating the tissue in the violet or in the red, mainly at a wavelength of 635 nm. The later wavelength penetrates up to several millimeters into soft biological tissues. This wavelength is also absorbed by PpIX, which, upon photoactivation, generates cytotoxic singlet oxygen locally. This causes cell death in the irradiated areas [3,8].

PDT following the topical application of ALA or m-ALA is an effective treatment for superficial basal cell carcinoma (BCC), superficial squamous cell carcinoma and actinic keratosis [1,2,9–14], with over 80% success rates reported [1,15], despite a significant decrease in the cure rate after long-term (>12 months) follow-up [4,5,12]. A much lower complete response (CR) rate is reported with ALA–PDT or m-ALA PDT for deep, nodular-ulcerative BCC lesions [1,8,16–24]. This may be explained by the fact that hydrophilic ALA, a zwitterion under physiological conditions, passes only poorly across biological barriers with lipophilic characteristics [19,25]. More generally, the percutaneous drug absorption can be described by Fick's first law of diffusion [26]. In order to enhance the drug delivery, the manipulation of any of its parameters (partition coefficient of the precursor, its diffusion coefficient, ...) may influence the flux of the drug through the stratum corneum.

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It should be borne in mind that the alteration of one component might influence another factor of Fick's Law. For example, increasing the partition coefficient of the precursor can also increase its affinity for the administration vehicle. The overall effect in such a case might be balanced or even negative. It should also be noted that the PpIX production is a complex process involving several steps. In particular, the differential metabolic rate of BCC cells is an element that is likely to have an impact on the overall production of PpIX, as illustrated by our study.

Several approaches have been explored to increase the local bioavailability and penetration depth of ALA, such as, for instance, the concomitant use of dimethylsulfoxid (DMSO) or DMSO combined with ethylene-diamine-tetraacetic acid (EDTA) [27–30]. Alternatively, the stratum corneum (SC) and/or the epidermis can be removed mechanically prior to ALA application [31]. Another approach focuses on the use of more lipophilic derivatives of ALA, such as ALA alkyl esters, which penetrate tissue better than ALA [1,19,32]. In particular, m-ALA–PDT has been shown to be an effective treatment modality for thin nodular BCC less than 2 mm in depth [1,13,18,20,23,33], and h-ALA has been successfully used to improve the detection of superficial bladder cancer [34,35]. Furthermore, h-ALA has been envisioned as a potentially interesting option to optimize PpIX PDT of skin cancers following the topical application of precursors in vitro [36,37], and on animal models [38,39]. Indeed, it has been shown that the uptake mechanisms of ALA alkyl esters haveare uptake mechanisms different from than those of ALA [26]. While ALA uptake depends on carriers like the di- and tri-peptide transporters PEPT1 and PEPT2 [40,41] or BETA transporters [42,43], m-ALA is actively taken up using transporters of nonpolar amino acids [44]. In contrast to this, longer chain aliphatic ALA-esters, including h-ALA, are only partially taken up by transporters, but rather by simple diffusion [43,45,46]. However, there is some evidence that h-ALA may be taken up by PEPT2 in some epithelial cell types [43].

In this study, we compare two precursors of PpIX: ALA at 20% (w/w) concentration, and h-ALA at different concentrations. We determine the optimal concentration of h-ALA by means of in vivo fluorescence spectroscopy performed on the normal skin with an optical fiber-based spectrofluorometer. Then we assess the fluorescence intensity of PpIX in BCCs after topical application of the precursor in vivo, thus measuring the quantity of PpIX generated. Finally, we measure the depth of PpIX generation in BCCs induced by the application of h-ALA and ALA by fluorescence microscopy.

2. Methods and materials

2.1. Chemicals

PpIX fluorescence in normal skin and BCCs was induced by topical application of an amphiphilic emulsion (Decoderm Basiscreme, Hermal Kurt Herrmann, Reinbek, Germany) containing 5% (w/w) DMSO (Fluka, Neu-Ulm, Germany) and the PpIX precursor at the following concentrations: 20% (w/w) ALA (ALA hydrochloride, Merck, Darmstadt, Germany) or 0.25%, 0.5%, 1%, 1.5%, 2.5%, 5% h-ALA produced in our facilities according to the method described by Lange et al. [35]. In dermatology, an ALA concentration of 20% is frequently used for PDT because PpIX fluorescence generation is poorly dose-dependent for ALA applications between 10 and 30%. The emulsions were prepared less than 24 h before application and stored at 4 °C in the dark until use.

2.2. Patients

The protocol and procedures have been approved by the ethical committee of the Geneva University Hospital. All patients gave their agreement by written informed consent. Five Caucasian volunteers (two female, three male) participated in the fluorescence spectroscopy study on normal skin. Emulsions were applied to eight separate sites of their inner fore arms (one emulsion with each precursor concentration as described above, plus one without precursor) for up to 23 h on to a surface of 10×20 mm. The application site was then protected using a transparent dressing (Tegaderm, 3M, Switzerland) and opaque tissue to avoid unwanted light exposure. Prior to measurements, the emulsions were removed.

Fifteen Caucasian patients (six female, nine male) with a BCC necessitating treatment by surgical excision were admitted to the fluorescence microscopy study of PpIX distribution in skin lesions following topical application of ALA and h-ALA. Twenty-six tissue samples from 23 BCCs and three spinocellular carcinoma were taken from 14 lesions located on the head and neck, 11 on the thorax or shoulder, and one on the leg. A 1 mm thick layer of emulsion containing 1% h-ALA or 20% ALA was applied on 15 and 11 lesions, respectively. The area was then covered with transparent dressing (Tegaderm, 3M, Switzerland) and opaque tissue to avoid unwanted light exposure. The dressings were removed shortly before surgical excision of the lesions after an application time ranging from 140 min to 24 h. The excised tissue samples were immediately frozen and kept at −80 °C in the dark. Ten tissue cross sections of 5 μm thickness were cut from each tissue sample with a cryostat microtome while avoiding direct light exposure. The cross sections were stored at −20 °C in the dark until fluorescence microscopy was carried out. Usually three tissue cross sections per tissue sample were used for fluorescence microscopy.

2.3. In vivo fluorescence spectroscopy and ex vivo fluorescence microscopy

The pharmacokinetics of PpIX and the autofluorescence of normal skin were measured in vivo with an optical fiber-based spectrofluorometer described elsewhere [47]. This system is functionally equivalent to fiber-based fluorescence spectrometer developed by other groups [48,49]. Briefly, it consists of a Xenon lamp, an excitation monochromator, a spectrograph, an intensified diode array and an optical multi-channel analyzer. Excitation and detection wavelengths were separated by a 525 nm excitation short pass filter, a 500 nm dichroic mirror, and a 590 nm emission long pass filter. The PpIX fluorescence was excited at 510 nm (7 nm FWHM) with a power of 20 μW at the distal tip of the quartz fiber (core diameter: 600 μm, length: 5 m, NA: 0.4). The distal end of the fiber was brought into slight contact with the skin and placed on the skin perpendicularly prior to the recording of the fluorescence. Then, the fiber tip was cleaned with ethanol, and shifted to a site with a different PpIX precursor concentration. For each site, four fluorescence spectroscopic measurements were performed. Each spectrum was corrected for the system background and for day-to-day fluctuations of the excitation light energy or detection pathway alignment by using a Rhodamine B ethanol solution (10^{-6} mol/l) in a quartz cuvette as reference.

Fluorescence microscopy on excised tissue samples of human skin lesions was carried out with a quadro-ocular epi-fluorescence microscope (Labophot-2, Nikon, Japan) comprising a mercury vapor lamp HbO 100 W (Osram, Germany), a microscopy lens Plan Fluor 10/0.30 (Nikon, Japan), and a Nikon Cube BV2A (excitation filter 400–440 nm, dichroic mirror 455 nm, barrier filter 470 nm). PpIX fluorescence and autofluorescence were detected with a long pass filter 610 nm (RG610, Schott, Germany) and a band pass filter 550 ± 20 nm (550FS40-25, Andover, NH, USA), respectively.

Fluorescence microscopy images were recorded with a 12–16 bits ST 133 MicroMax controller (Princeton Instruments, NJ, USA) connected to a Peltier-cooled CCD camera (Kodak KAF1600, 1536×1024 pixels chip) operated in the 2×2 binning mode,