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European Journal of Pharmaceutics and Biopharmaceutics 60 (2005) 439–444
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Topical glycerol monooleate/propylene glycol formulations enhance 5-aminolevulinic acid in vitro skin delivery and in vivo protophorphyrin IX accumulation in hairless mouse skin

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Received 8 March 2004; accepted in revised form 3 January 2005
Available online 7 April 2005

Abstract

Photodynamic therapy (PDT), a potential therapy for cancer treatment, utilizes exogenously applied or endogenously formed photosensitizers, further activated by light in an appropriate wavelength and dose to induce cell death through free radical formation. 5-Aminolevulinic acid (5-ALA) is a pro-drug which can be converted to the effective photosensitizer, protoporphyrin IX (PpIX). However, the use of 5-ALA in PDT is limited by the low penetration capacity of this highly hydrophilic molecule into appropriate skin layers. In the present study, we propose to increase 5-ALA penetration by using formulations containing glycerol monooleate (GMO), an interesting and useful component of pharmaceutical formulations. Propylene glycol solutions containing different concentrations of GMO significantly increased the in vitro skin permeation/retention of 5-ALA in comparison to control solutions. In vivo studies also showed increased PpIX accumulation in mouse hairless skin, after the use of topical 5-ALA formulations containing GMO in a concentration-dependent manner. The results show that skin 5-ALA penetration and PpIX accumulation, important factors for the success of topical 5-ALA-PDT in skin cancer, are optimized by GMO/propylene glycol formulations.
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Keywords: 5-Aminolevulinic acid; Monoolein; Photodynamic therapy; Protoporphyrin IX; Skin cancer

1. Introduction

Photodynamic therapy (PDT) is a relatively new approach for the treatment of some kinds of cancer and non-malignant conditions [1,2]. The therapy involves topical or systemic administration of photosensitizers, followed by local application of adequate doses of light of appropriate wavelength in the presence of oxygen to induce tissue or cell destruction, presumably via formation of reactive oxygen species [3–5]. Conventional photosensitizers (hematoporphyrin derivatives) have a prolonged photosensitive effect after the treatment, due to the relatively slow clearance rate of these compounds from skin and certain other normal tissues. Thus, patients receiving standard dosages of these compounds must avoid exposure to sunlight for at least 2 weeks following administration. The inconvenience could be alleviated by the search for alternative compounds to be used in PDT [1].

Endogenous induction of photosensitizers is a recent development in tumor destruction through the topical or systemic application of 5-aminolevulinic acid (5-ALA) [1] a metabolic precursor of protoporphyrin IX (PpIX) in the biosynthetic pathway of heme [6]. The photodynamically active PplX can act as an endogenous photosensitizer [7] and it is almost completely cleared from the body within 24 h, reducing the risks of a general photosensitization for longer periods of time [4]. Systemically administered photosensitizers like hematoprophyrin or its more purified form

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doi:10.1016/j.ejpb.2005.01.011

Photofrin®-II, when used in clinical trials caused a generalized skin photosensitivity persisting for up to 8–10 weeks after treatment due to its relatively slow skin clearance rates [8,9]. Such serious side effects have intensified dermatologic research on topical application of sensitizers over the last 10 years. Studies in human volunteers and experimental animals have shown that 5-ALA-induced PpIX is almost completely cleared from the body within 24 h. Such rapid clearance lowers the risk of PpIX accumulation leading to prolonged photosensitivity, even when PDT treatment is repeated as often as every other day [10].

Due to the efficient photosensitizing effect of PpIX, 5-ALA has been experimentally used in clinical PDT for the treatment of some cancers like squamous cell carcinoma, actinic keratosis, basal cell carcinoma, Bowen's disease, as well as in diagnostic evaluations of skin, bladder, gastrointestinal tract and lung tumors [11,12].

Hydrophilic 5-ALA is a zwitterion at physiological pH which poorly crosses biological lipophilic barriers such as cell membranes. Crossing biological barriers is a vital condition for the conversion of therapeutically active 5-ALA to PpIX [13] and its improved skin penetration by pharmaceutical vehicles may play an important role in the success of PDT [4].

Several methodological or chemical proposals addressed this problem, like the use of iontophoresis [14,15], phonophoresis [16], conventional topical dosage forms [17], penetration enhancers [18,19], delivery systems [20–22] and 5-ALA derivatives [23,24], but there is still a gap in the provision of pharmaceutical preparations and methods to dermatologists for effective application in the topical PDT treatment of skin cancer [25].

One interesting strategy to be explored in topical PDT is the use of penetration enhancers [26], which reversibly reduce the barrier resistance of the stratum corneum (SC) and present low skin toxicity.

Monoolein is a mixture of glycerides and other fatty acids, mainly glycerol monooleate (GMO). It is biodegradable, nontoxic, biocompatible, generally recognized as safe [27,28], and has attracted great interest in the pharmaceutical area as a penetration enhancer in controlled drug delivery, bioadhesive systems and others [29]. It has been shown that GMO/solvent systems can be effective penetration enhancers for lipophilic drugs and highly polar compounds [30], probably acting through a temporary and reversible disruption of the ordered lamellar structure of SC bilayers. The SC intercellular lipid media is further increasingly fluidized by the removal of ceramides [31].

Structurally similar to oleic acid, a well-known skin penetration enhancer for several drugs [32,33], GMO enhances the skin penetration of several compounds such as urea, indomethacin, and steroids [30,34]. In addition, delivery systems for 5-ALA and photosensitizers based on GMO/water cubic phase gels showed adequate behavior in loading and preserving photochemical stability for these drugs [35].

The optimization of 5-ALA in vitro skin delivery and in vivo PpIX accumulation in hairless mouse skin promoted by the GMO enhancer were considered in this study, as decisive factors for the success of PDT in the treatment of skin cancer.

2. Materials and methods

2.1. Chemicals

5-Aminolevulinic acid hydrochloride was from Sigma Chemical Co. (St Louis, MO, USA); Monoolein–Myverol 18-99, containing distilled glycerol monooleate was from Quest International (Norwich, NY, USA). All other chemicals and solvents were of analytical grade.

2.2. Animals

The in vitro, in vivo and partition coefficient studies were carried out in male hairless mice, 6–8 weeks old (strain HRS/J Jackson Laboratories, Bar Harbor, ME, USA). The animals were housed at 24–26 °C, exposed to daily 12:12-h light: dark cycles (lights on at 6 a.m.), and had free access to standard mouse chow and tap water. To reduce the stress associated with the experimental procedure, mice were handled daily for 1 week before experimentation. They were euthanized by carbon dioxide vapor. The protocols were in accordance with the guidelines of the University of Sao Paulo Animal Care and Use Committee (Authorization number: 04.1.995.53.9).

2.3. Fluorometric assay

5-ALA was determined after conversion to its fluorescent derivative by reacting with acetylacetone and formaldehyde. The resulting derivative was assayed spectrofluorometrically using a HITACHI-F4500 spectrofluorometer (Tokyo, Japan), with excitation at 378 nm and emission at 464 nm [19,36].

2.4. Partition coefficient of 5-ALA between isopropyl myristate/water ($K_{IPM/water}$)

5-ALA partition coefficients between isopropyl myristate (IPM) and water were determined using the shake-flask method. Aqueous solutions of 5-ALA (60 μg/ml) were shaken with an equal volume of IPM for 30 min, the optimal time found for partition equilibrium of the drug between IPM and water phases. After standing for 5 min, the supernatant was removed and the residue centrifuged for 10 min at 2000*g* [19]. The content of the aqueous phase was determined by the spectrofluorometric assay described above at time zero (C_0) and after shaking to ensure partition (C). $K_{IPM/water}$ was calculated as $(C_0 - C)/C$.

2.5. Partition coefficient of 5-ALA between stratum corneum (SC)/vehicle ($K_{SC/V}$)

SC samples were obtained by floating dorsal full thickness hairless mouse skin for 14 h on a solution of 0.1% w/v trypsin and 0.5% w/v sodium bicarbonate at room temperature followed by rinsing of the SC sheets with distilled water. SC samples were spread on filter paper and dried by storage in a desiccator over silica-gel, for a maximum of 1 week prior to use [19]. $K_{SC/V}$ was determined by agitating treated SC samples in propylene glycol containing 5-ALA (60 μg/ml) and GMO at 10 or 20% (w/w) for 6 min. 5-ALA concentrations prior (C_0) and after partition (C) were determined by spectrofluorometric assay and $K_{SC/V}$ calculated as described above.

2.6. In vitro permeation studies

In vitro permeation studies were carried out using full-thickness mouse skin excised from the dorsal surface and mounted in a diffusion cell (1.13 cm^2 diffusion surface area) maintained at 37 °C by a circulating water bath. Isotonic phosphate buffer (7 ml) pH 5.0, was used as the acceptor medium [25], and samples were removed at regular intervals up to 12 h. Donor media were 250 mg aliquots of formulations containing 5% (w/w) 5-ALA and GMO at 0, 1, 5, 10, 20 or 30% (w/w) in propylene glycol. Permeation profiles were constructed by plotting the total amount of spectrofluorometrically determined 5-ALA transported across the hairless mouse skin (μg/cm^2) against time (h). Drug flux across the membrane (J) was calculated from the slope of the curve at steady state and expressed as μg/cm^2/h.

2.7. 5-ALA retention and tape stripping

The initial phase of these experiments was similar to the in vitro permeation studies and the full method adapted from our previous work [37]. After 8 h, the permeation procedure was stopped, the skins removed and carefully washed with distilled water to discard residual formulation. Excess water was absorbed with tissue paper, and the SC was removed from the diffusion surface by ten tape strippings (Scotch Book Tape no. 845, 3 M, St Paul, MN, USA). The tape-strips were placed in 10 ml of methanol in a glass tube, stored overnight and then stirred for 1 min before filtration. The remaining skin [epidermis + dermis] was weighed and cut in small pieces, added to 5 ml isotonic pH 5.0-phosphate buffer in a tissue homogenizer, sonicated for 20 min and then filtered through a 0.45 μm policarbonate membrane. 5-ALA amounts from tape-strips and [epidermis + dermis] were assayed as described before [24].

2.8. In vivo studies of PpIX skin accumulation

The formulations tested had 5% (w/w) 5-ALA and MO at concentrations of 5, 10 or 20% (w/w) in propylene glycol. The controls were: untreated skin; propylene glycol treated skin; GMO treated skin; skin treated with 20% (w/w) GMO in propylene glycol and 5% (w/w) 5-ALA in propylene glycol. Experimental formulations were applied on the dorsal region of hairless mice for 4 h. After sacrifice of the animals the removed treated skin areas were submitted to PpIX extraction as described before [19]. All procedures were performed under subdued light. Briefly, treated hairless mouse skin areas weighing approximately 0.2 g were homogenized in 25 ml of a methanol/water mixture (9:1 v/v) and sonicated for 15 min. This procedure was repeated once more to complete the extraction of PpIX. The supernatant fluorescence was measured spectrofluorometrically at λ excitation/emission 400/632 nm and the assay was linear between 0.5 and 250.0 μg/ml ($r = 0.998$) (bandwidth 10.0). Skin and formulation components did not interfere with the assay. The results were expressed as ng PpIX/g skin.

2.9. Statistics

All values are expressed as means ± SD. Comparisons were made by the Kruskal-Wallis nonparametric ANOVA test and post-test Dunn's multiple comparisons. Differences considered significant had $P < 0.05$ values.

3. Results and discussion

GMO, an effective penetration enhancer belongs to a class of water insoluble polar lipids, which swell in water forming mesophases dependent on the water content, temperature and the presence of additional substances in the system [28,29]. Propylene glycol, the chosen vehicle is freely miscible with monoolein (GMO) [38] and a good 5-ALA solvent [39]. Like several other solvents, it may also enter into the SC, increasing drug partition into skin layers and, thus improving skin drug permeation.

$K_{IfPM/water}$ is generally believed to be a good characterization parameter of drug partitioning between SC and the underlying hydrophilic viable epidermis. Although several other solvent systems have been used to relate partition coefficients to percutaneous absorption, IPM is a suitable one, since its polar and non-polar nature mimics the skin is complex nature [40].

The very lipophilic *stratum corneum*, the outermost horny layer of the epidermis is primarily a water-loss barrier and an inhibitor to the entry of microorganisms and molecules into the body. On the other hand, our studies show a low $K_{IPM/water}$ value, 0.0439, for the 5-ALA/water system indicating hydrophilic characteristics for 5-ALA (Table 1). Other authors have confirmed this property [19,41], obtaining low values of K even with other solvents like octanol and PBS buffer at pH 7.4 [41]. This basic incompatibility impairs 5-ALA transport across lipophilic biological barriers such as SC, and its conversion to

Table 1
5-ALA partition coefficients in different conditions

Partition coefficient-condition	K[a]
$K_{IPM/water}$	0.0439 (±0.0043)
$K_{SC/V}$[b]	
0% GMO	0.0931 (±0.0193)
10% GMO (w/w)	0.0769 (±0.0231)[b]
20% GMO (w/w)	0.1077 (±0.0283)[b]

[a] Mean values ± SD ($n=4$).
[b] Statistical test was not significant in comparison to 0% GMO ($P>0.05$).

Table 2
Effect of GMO on the in vitro skin permeation of 5-ALA

Formulations (w/w)	Flux J (μg/cm^2/h)[a]
5% 5-ALA (control)	6.29 (±4.53)
5% 5-ALA + 1% GMO	134.81 (±37.77)
5% 5-ALA + 5% GMO	169.27 (±47.52)
5% 5-ALA + 10% GMO	251.78 (±103.78)[b]
5% 5-ALA + 20% GMO	476.18 (±106.52)[b]
5% 5-ALA + 30% MO	318.21 (±92.84)[b]

[a] Means ± SD ($n\geq6$).
[b] Statistically significant ($P<0.05$) in comparison to control.

PpIX, the therapeutically active compound in viable skin layers [4]. To overcome this limitation, 5-ALA propylene glycol formulations containing different concentrations of GMO (10 and 20% w/w) were tested. The results presented in Table 1 show that these GMO concentrations did not significantly affect the $K_{SC/V}$ values ($P>0.05$) for 5-ALA.

Chemical properties of 5-ALA result in a low percutaneous absorption. Its permeation properties could be investigated with the proposed in vitro model. Several other methods in different models have been used to enhance 5-ALA penetration into skin, like ester derivatives of 5-ALA [24,25,42], use of penetration enhancers [15,16] and iontophoresis [15,37,43]. Fig. 1 shows that 5-ALA in vitro permeation through hairless mouse skin is increased in the presence of GMO in a concentration-dependent manner. The flux values listed in Table 2 were calculated by linear regression of the data up to 12 h. A significant difference ($P<0.05$) in these values was observed when comparing formulations containing GMO (10, 20 or 30% w/w) with a control formulation (without GMO).

For the determination of 5-ALA retention in SC and [epidermis + dermis], tape stripping of SC layers and tissue homogenization techniques were used. Fig. 2 shows that 5-ALA concentrations are significantly higher in [epidermis + dermis] than in SC ($P<0.05$) for formulations containing 5, 10 or 20% GMO (w/w), when compared to control formulations.

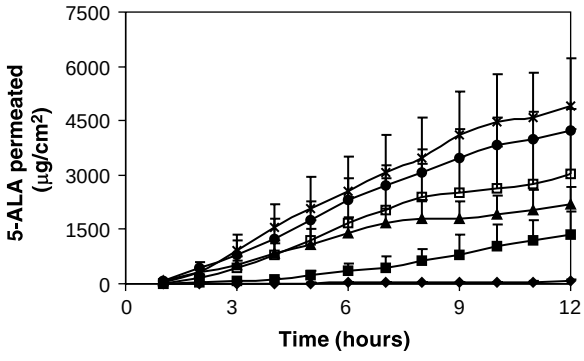

Fig. 1. In vitro 5-ALA permeation through hairless mouse skin. All formulations contained 5.0% 5-ALA (w/w) and GMO at: (♦) control; (■) 1% w/w; (▲) 5% w/w; (□) 10% w/w; (*) 20% w/w and (●) 30% w/w. Results presented are means ± SD of six or more experiments.

These results show that the presence of GMO improves the penetration of 5-ALA in skin layers, which can lead to an accumulation of PpIX in adequate amounts when the formulations are applied in vivo. Other authors [44] showed that this is the major factor limiting the efficacy of topical 5-ALA-PDT. The drug penetration depth and its in situ conversion to PpIX in sufficient and homogeneously distributed amounts in the tissue are a sine qua non condition for a successful therapeutic effect. In order to verify if GMO/propylene glycol formulations were also effective in PpIX accumulation, in vivo studies were also carried out in the animal model.

Fig. 3 shows levels of PpIX extracted from hairless mouse skin after in vivo application of test formulations and controls. Skin accumulated PpIX concentrations from applied formulations containing 5-ALA and 10% GMO showed a statistically significant ($P<0.05$) increase when compared to controls. In the absence of 5-ALA, the smaller accumulation of PpIX compared to other formulations probably represented the basal PpIX skin cell production [5,6].

Other reports [17,18] show that penetration enhancers, like DMSO, in different formulations containing 5-ALA, increase superficial porphyrin synthesis and/or accumulation

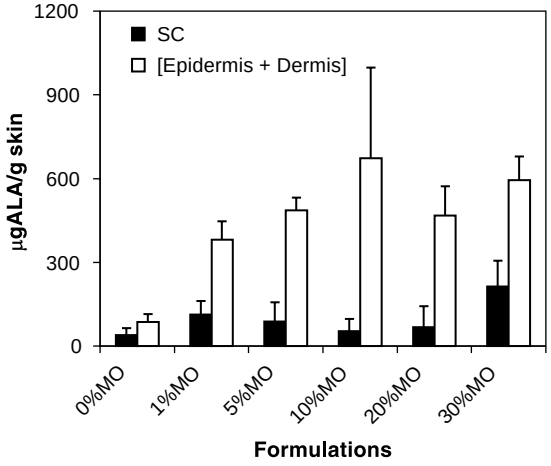

Fig. 2. In vitro retention of 5-ALA in hairless mouse skin following 8 h application. Results are means ± SD of five determinations.

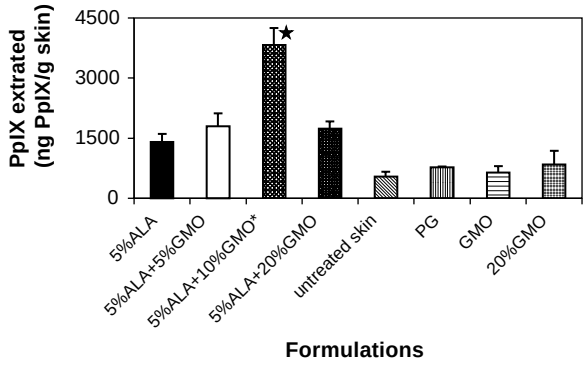

Fig. 3. In vivo 5-ALA induced PpIX accumulation in hairless mouse skin following 4 h of topical application of formulations containing 5.0% 5-ALA (w/w) and different concentrations of GMO and controls without 5-ALA. Results presented are means ± SD of five determinations. *Statistically significant ($P < 0.05$).

in the skin, when compared with formulations without penetration enhancers. These results are confirmed in the present work with still another enhancer and they emphasize the potential importance of these compounds when using 5-ALA in PDT for the treatment of skin cancer.

Our previous work [19] showed that DMSO (20% w/w) associated to EDTA (3% w/w) induced a similar in vivo PpIX accumulation in hairless mouse skin using a twofold 5-ALA concentration, what allow the obtainment of formulations with lower cost. The non-toxicity of GMO compared to DMSO, is also an important factor in considering that formulations containing 5-ALA and GMO as penetration enhancers can be effective therapeutic and life saving agents. However, some major questions have to be answered in careful studies before this formulation is introduced in clinical practice. These include determination of the best time period for application of the formulations and the minimal 5-ALA concentrations producing an effective in vivo PpIX accumulation, homogeneously distributed in tumor tissue.

4. Conclusion

GMO at 10% w/w in propylene glycol significantly improves 5-ALA skin delivery for PDT use. The increased in vivo PpIX accumulation in hairless mouse skin after applying this formulation highlights its potential. Further studies will be addressed to verify whether this formulation will be effective in the regression of skin tumors in topical PDT.

Acknowledgements

The authors would like to thank the Fundação de Amparo a Pesquisa do Estado de São Paulo—FAPESP and Conselho Nacional de Pesquisa—CNPq, Brazil for supporting this study.

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