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Evidence That P-glycoprotein Inhibitor (Elacridar)-Loaded Nanocarriers Improve Epidermal Targeting of an Anticancer Drug via Absorptive Cutaneous Transporters Inhibition

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ABSTRACT

Because P-glycoprotein (P-gp) plays an absorptive role in the skin, its pharmacological inhibition represents a strategy to promote cutaneous localization of anticancer agents that serve as its substrates, improving local efficacy while reducing systemic exposure. Here, we evaluated the ability of a nanoemulsion (NE) coencapsulating a P-gp inhibitor (elacridar) with the antitumor drug paclitaxel to promote epidermal targeting. Loaded NE displayed a nanometric size (45.2 ± 4.0 nm) and negative zeta potential (−4.2 ± 0.8 mV). Elacridar improved NE ability to inhibit verapamil-induced ATPase activity of P-gp; unloaded NE-inhibited P-gp when used at a concentration of 1500 μM, while elacridar encapsulation decreased this concentration by 3-fold (p <0.05). Elacridar-loaded NE reduced paclitaxel penetration into the dermis of freshly excised mice skin and its percutaneous permeation by 1.5- and 1.7-fold (p <0.05), respectively at 6 h, whereas larger drug amounts (1.4-fold, p <0.05) were obtained in viable epidermis. Assessment of cutaneous distribution of a fluorescent paclitaxel derivative confirmed the smaller delivery into the dermis at elacridar presence. In conclusion, we have provided novel evidence that NE containing elacridar exhibited a clear potential for P-gp inhibition and enabled epidermal targeting of paclitaxel, which in turn, can potentially reduce adverse effects associated with systemic exposure to anticancer therapy.

Introduction

The overexpression of members of the ATP-binding cassette superfamily of efflux transporters has attracted significant attention due to their relationship to chemotherapeutic drug resistance, which hinders cancer treatment.[1] The most well-known examples of these transporters are P-glycoprotein (P-gp), multidrug resistance protein and breast cancer resistance protein (BCRP).[2] In addition to cancer cells, these transporters are expressed in various physiological barriers, including the skin, where they influence drug disposition.[3-5]

The P-gp is constitutively expressed in keratinocytes at the basal layer of the epidermis and in dermal components including endothelial cells, sweat ducts, nerve sheaths, and muscles of human skin.[6] In mice, it has been detected in basal layer keratinocytes and dermis (mainly).[7,8] BCRP has also been detected in mouse and human dermal endothelial cells.[3] Despite the fact that the transport mechanism remains complex, these carriers have been demonstrated to influence the cutaneous distribution and transdermal transport of compounds with varying physicochemical properties and pharmacological activity, including steroidal antiinflammatory drugs, for example, dexamethasone, the antifungal agent itraconazole and the multi-kinase inhibitor regorafenib.[3,7,9,10]

To our knowledge, Ito et al.[7] were the first to demonstrate the absorptive role of P-gp in the skin by showing that topical application of a P-gp substrate (rhodamine 123) resulted in a lower percutaneous permeation in mdr1a/1b−/− mice compared with wild-type animals, whereas transport of FD-4 (non-substrate for P-gp) was similar in mdr1a/1b−/−and wild-type mice. Moreover, skin distribution of rhodamine 123 after intravenous infusion was higher in mdr1a/1b/bcrp−/− than in the wild-type mice, which indicated P-gp and BCRP influence on cutaneous drug distribution and transdermal delivery.[3] Coadministration of a P-gp inhibitor (itraconazole) reduced rhodamine concentration in the dermis and plasma only in wild-type mice,[3] suggesting that absorptive transporters may be inhibited to reduce drug transdermal delivery, and consequently, to limit systemic exposure.

Based on these studies, we hypothesized that coencapsulation of the antitumor agent and P-gp/BCRP substrate paclitaxel with a

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P-gp inhibitor (elacridar) in nanocarriers may be an effective strategy to promote epidermal paclitaxel targeting while limiting its permeation across the skin, which should provide local efficacy and reduce systemic adverse effects that hinder its use for treatment of skin cancers.[11-13] Because paclitaxel transport across the stratum corneum (the main barrier for penetration) is stalled by its size and lipophilicity,[14-16] we employed a nanoemulsion previously demonstrated to improve drug penetration across the stratum corneum as a cutaneous delivery system. Even though nanocarriers have been studied for the improvement of paclitaxel localization in the skin,[14,15] to the best of our knowledge, the present study is the first to address the pharmacological inhibition of cutaneous absorptive transporters to improve paclitaxel localization in the epidermis, where tumor lesions develop. We have provided clear evidence that elacridar affected the nanoemulsion (NE) ability to inhibit P-gp ATPase activity and penetrates into viable skin layers for the inhibition of transporters within 4 h. Furthermore, elacridar interfered with paclitaxel cutaneous distribution and percutaneous permeation.

Material and Methods

Material

Tween 80 (polysorbate 80), tributyrin, elacridar, dimethyl sulfoxide (DMSO), oleic acid, poloxamer 407, and propylene glycol were obtained from Sigma (St. Louis, MO), whereas tricaprylin was kindly supplied by Abitec Corporation (Janesville, WI). Acetonitrile, ethanol, and methanol were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Plain paclitaxel was obtained from Polymed Therapeutics (Houston, TX), whereas a fluorescent derivative of paclitaxel (Oregon Green 488 conjugate) was obtained from Invitrogen (Carlsbad, CA).

Methods

Nanoemulsion Preparation and Characterization

NEs were obtained by vortex mixing the oil phase (tributyrin:oleic acid:tricaprylin, 2:1:1, w/w/w) and polysorbate 80:DMSO (surfactant:cosurfactant, 2:1, w/w) at 1:1 (w/w), followed by the addition of the aqueous phase (65%, comprised of a solution of poloxamer 407 at 2%, w/w) and sonication for 20 min in pulses (58 s on and 30 s off) in an ice bath using 40% maximum amplitude (VCX500, Sonics, Newtown, CT) as previously described.[17] Paclitaxel was incorporated in the mixture of surfactant and oil phase of the nanocarriers to obtain a final concentration of 0.50% (w/w). Elacridar was dissolved in DMSO before the preparation of the surfactant-co-surfactant blend to obtain a final elacridar concentration of 0.07% (w/w). Three-fold lower amounts of elacridar were encapsulated when it was added directly into the oil phase-surfactant blend (as performed for paclitaxel), which justifies its prior solubilization in DMSO. Size and zeta potential of the unloaded NE or the nanocarrier coencapsulating elacridar and paclitaxel were determined using Zetasizer NanoZS equipment (Malvern, UK) after NE dilution with water at 1:10 (w/w).

P-glycoprotein ATPase Activity in the Presence of the Nanoemulsion With and Without Elacridar

Elacridar has been described to inhibit ATP hydrolysis, modulating the transporter ATPase activity.[18] Thus, in this experiment, we assessed elacridar influence on the NE ability to inhibit the ATPase activity of P-gp using the Pro-glo assay (Promega, Madison, WI) and recombinant P-gp in membrane fractions according to the manufacturer's instructions.[16] ATP consumption was quantified based on the luminescence resulting from a second reaction with a recombinant firefly luciferase. NE with and without elacridar were prepared and diluted with the manufacturer's assay buffer as directed, so that final NE concentration (which was expressed as polysorbate concentration) in the assay wells would be 0, 100, 500, 1000, and 1500 μM.

The P-gp enriched membranes were treated with the unloaded or elacridar-loaded NE in the presence of the L-type calcium channel blocker verapamil (50 μM) for 1 h; untreated and Na_3VO_4-treated (100 μM) membranes were used as controls. Verapamil is a substrate and activates P-gp, and ATP consumption is higher in its presence. On the other hand, Na_3VO_4 is a selective inhibitor of P-gp, and ATP consumption is negligible in its presence. Raw data were collected as relative light units and converted to ATP concentration with a calibration curve obtained using ATP standards at 0.375-3 mM.

Cutaneous Availability and Percutaneous Permeation

Skin penetration of paclitaxel and elacridar was studied using mice (Balb/c) skin as model tissue mounted in Franz diffusion cells (diffusion area of 1 cm^2; Hanson, Chatsworth, CA). Animals were housed in the Department of Pharmacology animal facility with free access to food and water. The animal room was kept under a 12:12 h light-dark cycle (lights on at 7:00 AM), and temperature was maintained between 22°C and 23°C. The protocol was conducted in accordance with the guidelines from the Brazilian Council for Control of Animal Experimentation and approved by the Animal Care and Use Committee at the Institute of Biomedical Sciences of the University of São Paulo (São Paulo, Brazil, protocol#100, 2013).

Briefly, mice were anaesthetized with isoflurane (Cristalia, Itapira, Brazil) and their dorsal skin was shaved using a clipper (model ER389, Panasonic, Kadoma, Japan). Immediately following euthanasia, the dorsal skin was removed and cleaned from the subcutaneous tissue and mounted in Franz diffusion cells for the penetration experiments with the dermis facing the receptor compartment.[19] The receptor phase consisted of 100-mM phosphate buffer (pH 7.4) maintained at 37°C under constant stirring (350 rpm). The skin was treated with the undiluted NE loaded with elacridar, paclitaxel or both compounds; in other words, we compared paclitaxel delivery from NE containing elacridar and NE without elacridar to evaluate the influence of elacridar encapsulation on paclitaxel cutaneous transport.

The formulations were placed in the donor compartment of diffusion cells (volume equivalent to 100 mg of each formulation) for 2-6 h. Six hours was selected as the latest time point to ensure tissue viability. Freshly excised human and animal skin mounted in Franz diffusion cells have been employed in studies to assess cutaneous metabolism of xenobiotics, indicating that protein activity and viability of cutaneous tissues are maintained during permeation experiments.[20-22] However, viability and protein activity depended on time and type of receptor phase used. Tissue ability to metabolize glucose during *in vitro* permeation experiments decreased after 16-24 h postharvesting when Eagles minimum essential medium and Dulbecco-modified phosphate-buffered saline were used as receptor phases.[23,24] On the other hand, using PBS as receptor phase eliminated aerobic and anaerobic glucose use in 12 h,[24] indicating that viability is compromised faster. Because paclitaxel could not be quantified in tissue culture medium using HPLC (due to interference of other components), we opted to use PBS as receptor phase and assessed cutaneous delivery and percutaneous permeation up to 6 h to avoid viability loss, which would compromise the transporter activity and interpretation of results. Previous *in vitro* studies that assessed P-gp influence on skin transport of substrates using excised mice skin and buffer as receptor phase were carried out with a similar time window to preserve skin viability.[7]