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RESEARCH PAPER

Enhanced Topical and Transdermal Delivery of Antineoplastic and Antiviral Acyclic Nucleoside Phosphonate cPr-PMEDAP

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ABSTRACT

Purpose Acyclic nucleoside phosphonates possess unique antiviral and antineoplastic activities; however, their polar phosphonate moiety is associated with low ability to cross biological membranes. We explored the potential of transdermal and topical delivery of 2,6-diaminopurine derivative cPr-PMEDAP.

Methods *In vitro* diffusion of cPr-PMEDAP was investigated using formulations at different pH and concentration and with permeation enhancer through porcine and human skin.

Results Ability of 0.1–5% cPr-PMEDAP to cross human skin barrier was very low with flux values ~40 ng/cm^2/h, the majority of compound found in the stratum corneum. The highest permeation rates were found at pH 6; increased donor concentration had no influence. The permeation enhancer dodecyl 6-dimethylaminohexanoate (DDAK, 1%) increased flux of cPr-PMEDAP (up to 61 times) and its concentration in nucleated epidermis (up to ~0.5 mg of cPr-PMEDAP/g of the tissue). No deamination of cPr-PMEDAP into PMEG occurred during permeation studies, but *N*-dealkylation into PMEDAP mediated by skin microflora was observed.

Conclusions Transdermal or topical application of cPr-PMEDAP enabled by the permeation enhancer DDAK may provide an attractive alternative route of administration of this potent antitumor and antiviral compound.

KEY WORDS acyclic nucleoside phosphonates · antineoplastics · antivirals · permeation enhancer · topical skin application · transdermal delivery

ABBREVIATIONS

cPr-PMEDAP	N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine
DDAK	dodecyl 6-dimethylaminohexanoate
HBSS	Hanks balanced salt solution
PBS	phosphate-buffered saline
PMEDAP	2,6-diamino-9-[2-(phosphonomethoxy) ethyl]purine
PMEG	9-[2-(phosphonomethoxy)ethyl]guanine

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INTRODUCTION

Acyclic nucleoside phosphonates are analogues of nucleotides exhibiting broad-spectrum antiviral effects; the prototype compounds include cidofovir, adefovir, and tenofovir, which are approved for therapeutic use in human medicine aimed at hepatitis B, AIDS and various diseases caused by DNA viruses (1). Another member of this group, 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG, Fig. 1), is a potent antiproliferative agent (2,3). Its antitumor activity was demonstrated in mice engrafted with murine leukemia P388 cells or murine melanoma B16 cells (4). PMEG was further explored for its inhibitory effects on papillomavirus infections (5). However, its utility is limited by its poor cellular permeability and marked toxicity (4,5). These disadvantages were partially overcome by N^6-cyclopropyl-2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine (cPr-PMEDAP, Fig. 1), which has similar antiproliferative activity but reduced toxicity (6–8). For example, cPr-PMEDAP was highly efficacious in inhibiting choriocarcinoma development in rats (9) and proliferation of human papilloma virus-harboring cell lines (10). Although cPr-PMEDAP was structurally derived from 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine (PMEDAP), it is a prodrug of the guanine derivative PMEG. cPr-PMEDAP was found to be deaminated in cancer cells by N^6-methyl-AMP amino hydrolase (6,11).

In drugs with narrow therapeutic windows, maintaining stable blood levels is vital. One of the possibilities to overcome the peaks and troughs in plasma concentrations is the administration of the drug through the skin by a controlled release patch, i.e., transdermal delivery. Furthermore, in case of skin diseases, another opportunity to take advantage of the drug's potency without the risk of systemic toxicity is via its topical application. However, the uppermost skin layer, the stratum corneum, provides a formidable barrier to penetration of most compounds. In particular, cPr-PMEDAP is negatively charged at physiological pH; thus, its ability to cross lipophilic barriers including the skin is low. To improve its permeability through the skin barrier, a double prodrug GS-9191 for a possible treatment of papillomavirus lesions was studied (12).

Another possibility of overcoming the poor permeability of the stratum corneum is the use of permeation enhancers, i.e., compounds that temporarily decrease the skin barrier resistance (13). In our previous studies with adefovir, i.e. another acyclic nucleoside phosphonate, dodecyl 6-dimethylaminohexanoate (DDAK) displayed excellent permeation-enhancing activity (14,15). DDAK was designed by combining the structural features of two well-known enhancers—an ionizable dimethylamino polar head from dodecyl 2-dimethylaminopropanoate (DDAIP) and the 5-carbon linking chain between the nitrogen and ester carbonyl from Transkarbam 12 (16–18). This enhancer was found to possess highly favorable properties: broad-spectrum activity, negligible toxicity, and biodegradability by esterases. Moreover, its effect on the skin barrier was reversible (19).

The general purpose of this study was to investigate the transport of cPr-PMEDAP through and into the skin to assess its potential for transdermal and topical delivery. We hypothesized that the permeation enhancer DDAK may be effective in increasing the diffusion of this compound through the lipophilic skin barrier and aimed at studying its enhancing activity in relation to pH and cPr-PMEDAP donor concentration. In addition, *N*-dealkylation of cPr-PMEDAP was identified and studied during the permeation experiments.

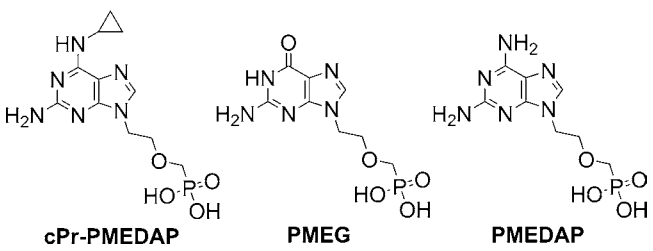

Fig. 1 Structures of acyclic nucleoside phosphonates cPr-PMEDAP, PMEG and PMEDAP.

MATERIALS AND METHODS

Chemicals

Acyclic nucleoside phosphonates cPr-PMEDAP, PMEG, and PMEDAP (20) and the permeation enhancer DDAK (19) were synthesized as described previously. All other chemicals were from Sigma-Aldrich (Schnelldorf, Germany). Ultrapure water was prepared using a Milli-Q Water Filtration System (Millipore, Bedford, MA).

Porcine and Human Skin

Porcine ears were obtained from a local slaughterhouse. To ensure integrity of the skin barrier, ears were removed post-sacrifice before the carcass was exposed to the high-temperature cleaning procedure. Full-thickness dorsal skin was excised by blunt dissection, and hairs were carefully trimmed. The skin fragments were immersed in 0.05% sodium azide solution in saline for 5 min for preservation and were stored at −20°C.

Human skin from Caucasian female patients who had undergone abdominal plastic surgery was used. The

procedure was approved by the Ethics Committee of the University Hospital Hradec Králové, Czech Republic (No. 200609 S09P) and conducted according to the principles of the Declaration of Helsinki. The skin fragments were used immediately after excision.

Donor Samples

Donor samples were prepared by stirring an appropriate amount of cPr-PMEDAP (1, 10, 30, and 50 mg, respectively) in 1 ml of 100 mM phosphate buffer either with or without 10 mg of DDAK. The sample pH was adjusted by H_3PO_4 and NaOH, respectively, using a microelectrode HC153 (Fisher Scientific, Pardubice, Czech Republic). The samples were allowed to equilibrate at 37 C for 48 h before application to the skin.

Permeation of cPr-PMEDAP Through the Skin

Porcine Skin

The skin permeation of cPr-PMEDAP was evaluated using Franz diffusion cells with an available diffusion area of 1 cm^2 and an acceptor volume of approximately 17 ml. The skin fragments were slowly thawed immediately before use and carefully inspected for any visual damage. They were cut into squares ca. 2×2 cm, mounted into the diffusion cells dermal side down and sealed with silicone grease. The acceptor compartment of the cell was filled with phosphate-buffered saline (PBS, containing 10 mM phosphate buffer, 137 mM NaCl and 2.7 mM KCl) at pH 7.4 with 1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml. The precise volume of the acceptor liquid was measured for each cell and included in the calculation. The Franz diffusion cells with mounted skin samples were placed in a water bath with a constant temperature of 32°C equipped with a magnetic stirrer. After an equilibration period of 1 h, the skin integrity was checked by measuring the electrical impedance (see later). The donor sample (150 μl) was applied to the skin surface and covered with a glass slide. The acceptor phase was stirred at 600 rpm at 32°C throughout the experiment. Samples of the acceptor phase (0.6 ml) were withdrawn at predetermined time intervals over 48 h and replaced with fresh PBS.

Human Skin

The experiment with human skin was performed as above, except for the acceptor phase, which was 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Hanks balanced salt solution (HBSS) at pH 7.4 without phenol red containing 1,000 units penicillin, 1 mg streptomycin and 2.5 μg amphotericin B per ml. This acceptor phase was previously shown to maintain skin viability and metabolic activity during storage and permeation experiment (21). Samples of the acceptor phase were collected over 72 h to achieve steady state flux.

Concentration of cPr-PMEDAP in the Skin

Validation of the Extraction Procedure

A known amount of 100 μg/ml solution of cPr-PMEDAP (1, 5, and 20 μg) was applied to a 1-cm^2 skin fragment in a glass vial and was allowed to penetrate into the skin at 32°C for 48 h. Then, 5 ml of a methanol/PBS mixture with 1,000 units of penicillin, 1 mg of streptomycin, and 2.5 μg of amphotericin B per ml were added and allowed to stir at 32°C for 24 h or 48 h. The solvent was filtered and analyzed by HPLC. Each experiment was performed in triplicate.

Porcine Skin

At the end of the permeation experiment (48 h), the diffusion cells were dismounted and the skin surface washed three times with 0.5 ml of PBS. The exposed area of 1 cm^2 was punched out, blotted dry, and weighed. The skin sample was then extracted with 5 ml of 20% methanol in PBS containing 1,000 units of penicillin, 1 mg of streptomycin, and 2.5 μg of amphotericin B per ml for 24 h, and the concentration of the model drugs was determined by HPLC.

Human Skin

At the end of the permeation experiment (72 h), the diffusion cells were dismounted and the skin washed as described above. The stratum corneum was collected by tape stripping with an UrgoFilm tape (Laboratories Urgo, Chenove, France). The tape was applied using a homogenous pressure (1.5 kg per 1 cm^2) for 5 s and then removed with forceps. The strips were collected until the skin surface was glistening. To collect only the stratum corneum from the skin area that had been exposed to the donor compartment, a "mask" made from a plastic foil with a 1 cm^2 opening was attached to the skin fragment. Due to the high variability in the stratum corneum removed in one tape strip due to an excessive hydration of the skin during the 72 h experiment, the data were pooled and are presented as the overall cPr-PMEDAP amount in the stratum corneum. Then, 1 cm^2 of the remaining tissue was punched out, wrapped in an aluminum foil and heated to 60°C for 1 min. The epidermis was then carefully peeled off from the dermis. Both epidermis and dermis were

precisely weighed and placed separately into glass vials. The tape strips, epidermis and dermis were extracted with 1 ml, 1 ml, and 5 ml of 20% methanol in PBS containing 1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml, respectively, for 24 h. The extract was filtered and analyzed by HPLC.

Skin Electrical Impedance

The electrical impedance of the skin was recorded using an LCR meter 4080 (Conrad Electronic, Hirschau, Germany, measuring range 20 Ω–10 MΩ, error at kΩ values <0.5%), operated in a parallel mode with an alternating frequency of 120 Hz. The impedance of the skin was obtained by immersing the tip of the stainless-steel probes, one each into PBS in the donor and acceptor compartment of the Franz diffusion cell. The impedance was 5–30 kΩ×cm^2 and 15–45 kΩ×cm^2 in porcine and human skin, respectively.

Degradation of cPr-PMEDAP

After initial control experiments without the antibiotic/antimycotic mixture, significant decomposition of cPr-PMEDAP was found. To study this reaction further and to identify the major degradation product, cPr-PMEDAP at 25 μg/ml was added to porcine or human skin homogenate in HBSS at pH 7.4. After predetermined time intervals, 500 μl of acetonitrile was added to stop the reaction, and the sample was centrifuged at 6,700×g for 5 min. The supernatant was withdrawn, filtered through a 0.2 μm filter and analyzed by HPLC (n=12). As a negative control, the same cPr-PMEDAP sample in HBSS at the same pH without the skin was treated likewise (n=4). Further experiments were performed with a) skin "sections" (skin surface washings, tape-stripped stratum corneum, and the remaining epidermis + dermis), b) skin homogenates after addition of 1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml, and c) sonicated skin homogenates.

Solubility and Stratum Corneum Distribution Coefficients

For the determination of cPr-PMEDAP solubility in water, donor solvent at pH 6, and acceptor phases (PBS or HBSS), an excess of cPr-PMEDAP was added to the pertinent solvent either with or without 1% DDAK, and the suspension was allowed to equilibrate. All of the solvents were supplemented with 1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml. After 24 h, the suspensions were centrifuged at 6,700 × g for 5 min; the supernatant was withdrawn, diluted with the pertinent mobile phase and analyzed by HPLC. Three replicates were performed in each solvent.

The relative stratum corneum distribution coefficients of cPr-PMEDAP were determined as described previously (15,22) using the same solvents as above. Human stratum corneum sheets were prepared by trypsin treatment as described elsewhere (23) and dried in vacuo. Before the experiment, stratum corneum sheets of ca. 10 mg were precisely weighed and hydrated in 1 ml of saline with 1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml. After 24 h, stratum corneum was withdrawn and blotted dry on a filter paper. Ten μg/ml of cPr-PMEDAP solution either with or without DDAK (10 μg/ml) in the pertinent solvent was added to each stratum corneum sheet (1 ml of the solution per 10 mg of the stratum corneum) and allowed to equilibrate for 24 h. The sample was centrifuged at 6,700×g for 5 min, and the concentration of cPr-PMEDAP in the supernatant was determined by HPLC (c). The same solution without the stratum corneum was treated likewise (c_0). The distribution coefficient D was determined as follows: $D=(c_0-c)/c$. The concentration used in this experiment was different from those in the permeation study; however, irrespective of the drug concentration used, the ratio derived should always be the same because the partitioning (distribution) coefficient measurement is an equilibrium phenomenon (22).

HPLC Conditions

The samples from the permeation experiments were analyzed using a Shimadzu Prominence instrument (Shimadzu, Kyoto, Japan) consisting of LC-20AD pumps with DGU-20A3 degasser, SIL-20A HT autosampler, CTO-20AC column oven, SPD-M20A diode array detector, and CBM-20A communication module. Data were analyzed using the LCsolutions 1.22 software. Reverse phase separation of cPr-PMEDAP, PMEDAP, and PMEG was achieved on an AscentisTM RP-Amide 150×4.6 mm column, 5 μm (Supelco, Sigma Aldrich, Darmstadt, Germany) at 35°C using 5% methanol in 0.1% acetic acid (v/v) as the mobile phase at a flow rate of 1.5 ml/min. Twenty μl of the sample was injected on the column, and the effluent was measured at 292 nm for cPr-PMEDAP and PMEDAP, and at 254 nm for PMEG. The retention times of cPr-PMEDAP, PMEDAP, and PMEG were 7.5, 2.1, and 4.5 min, respectively.

HPLC-MS analysis using an LC 20A Prominence chromatographic system (Shimadzu, Kyoto, Japan) coupled with an LCQ Max advantage mass spectrometer (Thermo Finnigan, San Jose, U.S.A.) was used to identify PMEDAP as the N-dealkylation product of cPr-PMEDAP. The same column and a mobile phase consisting of 10% methanol in 0.05% acetic acid (v/v) at a flow rate of 0.3 ml/min were

used. The retention times of cPr-PMEDAP and PMEDAP were 28 min and 9 min, respectively. ESI in positive mode was employed in these experiments. The detector was set as follows: capillary voltage of 3.5 kV, capillary temperature of 200°C, sheet and auxiliary gas flows of 75 and 30 units, respectively. Full scan spectra were recorded from 100–600 m/z; MS^n experiments were done using a collision energy of 38–40%. The data were processed using Xcalibur software (Thermo Finnigan, San Jose, U.S.A.).

Data Treatment

The cumulative amount of cPr-PMEDAP having penetrated the skin, corrected for the acceptor sample replacement, was plotted against time. The steady state flux was calculated from the linear region of the plot and lag time by extrapolation of the linear part to the x-axis. Skin concentration, expressed as μg of cPr-PMEDAP per g of the tissue, was calculated by dividing cPr-PMEDAP amount by the respective skin weight. The enhancement ratio (ER) was calculated as a ratio of the permeation characteristics, either flux or skin concentration, with and without the enhancer. The data are presented as means ± SEM; *n* is given in the pertinent figures. Statistical significance was determined using t-test or Rank Sum Test, where appropriate.

RESULTS

Identification of *N*-dealkylation of cPr-PMEDAP

First, we aimed at developing an HPLC method for the determination of cPr-PMEDAP in the Franz cell acceptor phase and skin samples. This method was adjusted to simultaneously follow PMEG to be sure that no deamination into this highly toxic guanine derivative occurred in normal skin cells. Initially, we modified our previous method used for adefovir determination (24); RP18 column and a mobile phase consisting of 10 mM KH_2PO_4 and 2 mM Bu_4NHSO_4 at pH 6 with 12 % acetonitrile seemed to be sufficient for the separation of both cPr-PMEDAP (retention time of 7.4 min) and PMEG (2.5 min) from the endogenous components extracted from the skin.

During the preliminary permeation studies and method validation, a substantial degradation of cPr-PMEDAP in the acceptor phase was observed. The peak of this degradation product displayed very similar retention to PMEG, but its UV spectrum was different, with maxima at 286, 251 and 226 nm, while that of PMEG had only one maximum at 253 nm. Subsequently, the original HPLC method was modified to allow the simultaneous analysis of unknown degradation product, cPr-PMEDAP and PMEG. The desired separation was achieved on RP-Amide column, which is less hydrophobic than common C18 phase, possesses unique selectivity, and is compatible with highly aqueous mobile phases. Moreover, no ion-pairing additive was needed for acceptable retention of the polar acyclic nucleoside phosphonates, and the column life was much longer compared to the previous setup. The best separation with acceptable analysis time (9 min) was achieved using 5% methanol in 0.1% acetic acid. The retention times of cPr-PMEDAP, PMEG, and the degradation product were 7.5, 4.5, and 2.1 min, respectively.

This method was then used to study the observed cPr-PMEDAP decomposition in acceptor phase and skin samples. The UV spectrum of the degradation product as well as its chromatographic behavior suggested that it could be an *N*-dealkylation product PMEDAP. This was confirmed by the retention of an authentic standard (Fig. 2a) under various chromatographic conditions and matching UV spectra. Thereafter, the HPLC method was modified for MS detection, and the degradation product was found to have *m/z* 289, which is fully consistent with PMEDAP $[M + H]^+$. In addition, the same fragmentation pattern of both cPr-PMEDAP and PMEDAP was found in MS^n experiments. First, the neutral loss of water from the protonated phosphonate was observed in MS^2 spectra. The MS^3 experiments of these fragment ions yielded a neutral loss of water (−18 Da), HPO_2 (−64 Da), and formaldehyde (−30 Da) via a rearrangement process (Fig. 2c). The proposed fragmentation of cPr-PMEDAP and PMEDAP is in accordance with that described previously for adefovir (25). Two minor peaks were also found in the chromatograms, but no deamination to PMEG was observed (Fig. 2b).

The cPr-PMEDAP *N*-dealkylation was further studied to identify the source of this reaction. cPr-PMEDAP was generally stable in various buffers and mobile phases, excluding the possibility of simple chemical decomposition. Skin metabolism was also rejected, because the dealkylation was found in both viable (Fig. 2d) and frozen-and-thawed skin. Moreover, no reaction was observed in tape-stripped skin (i.e., without the stratum corneum), sonicated skin homogenate samples, and those with an antibiotic/antimycotic mixture (1,000 units of penicillin, 1 mg of streptomycin and 2.5 μg of amphotericin B per ml). Thus, the dealkylation was attributed to skin microflora. Finally, after addition of this antibiotic/antimycotic mixture, no further decomposition was observed during the permeation studies.

Therefore, this acceptor phase was then used for the validation of the HPLC method and for studying the penetration of cPr-PMEDAP through and into the skin. Typical chromatograms of the blank acceptor phase and that spiked with all three analytes at 6 μg/ml are shown in

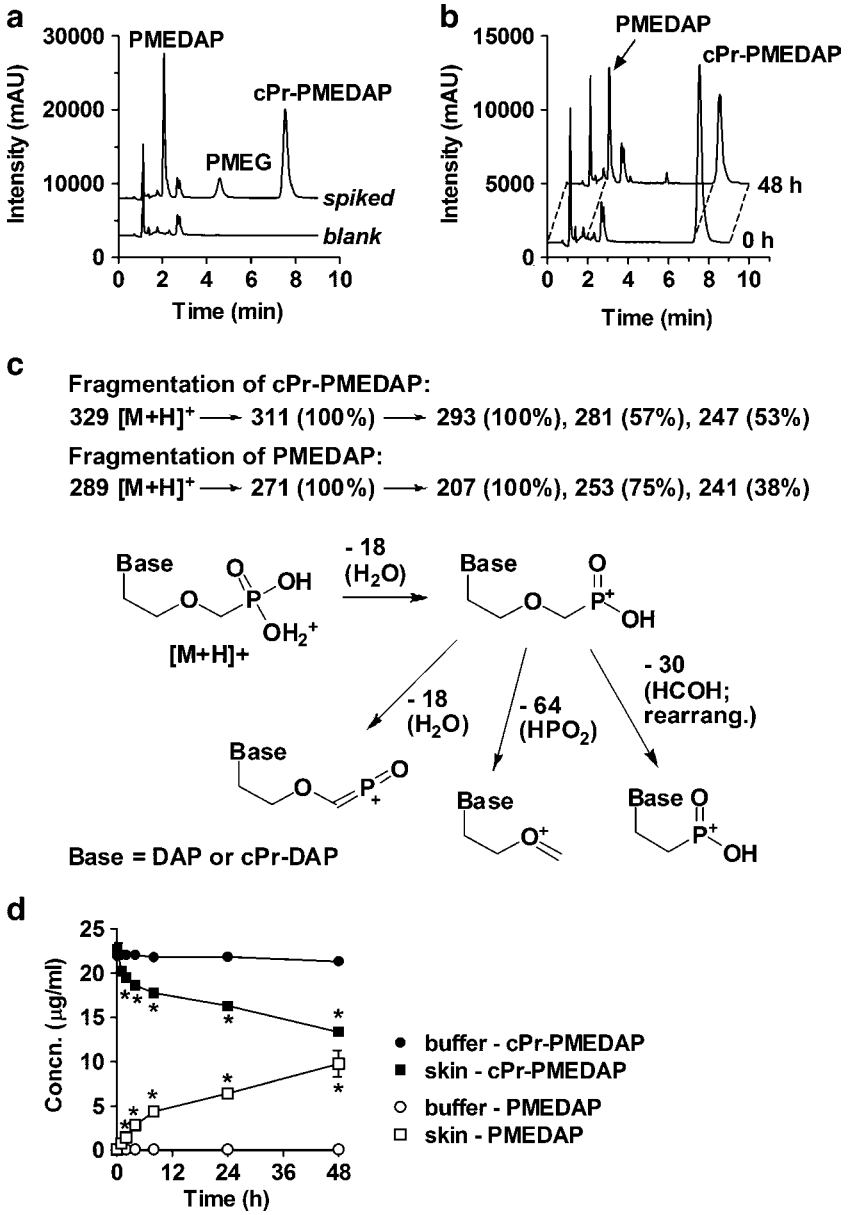

Fig. 2 HPLC method and *N*-dealkylation of cPr-PMEDAP. (**a**) Chromatograms of the blank acceptor phase and that spiked with 6 μg/ml cPr-PMEDAP (7.5 min), PMEDAP (2.1 min), and PMEG (4.5 min) on an AscentisTM RP-Amide column using 5% methanol in 0.1% acetic acid (v/v) at a flow rate of 1.5 ml/min, detected at 292 nm. (**b**) Chromatogram of cPr-PMEDAP in porcine skin homogenate in HBSS (freshly prepared = 0 h) and evidence of *N*-dealkylation of cPr-PMEDAP into PMEDAP (48 h). (**c**) MS fragmentation of cPr-PMEDAP and its *N*-dealkylation product PMEDAP (*m/z* with relative abundance in parentheses) and the proposed fragmentation mechanism for both compounds. (**d**) *N*-Dealkylation of cPr-PMEDAP (25 μg/ml, filled squares) into PMEDAP (open squares) in porcine skin homogenate in HBSS at pH 7.4 in time (mean ± SEM, $n = 12$; * indicates significant difference against time 0). As a negative control, a cPr-PMEDAP sample at the same concentration in HBSS at pH 7.4 without the skin was followed for the same time (filled circles, mean ± SEM, $n = 4$).

Fig. 2a. The calibration curves were linear in the range of 0.2–100 μg/ml ($r^2 = 0.999$, $p < 0.001$ for all three compounds). The precision and accuracy were within the limits of the FDA guideline for bioanalytical method validation (2001). The efficiency of the cPr-PMEDAP extraction from the skin was 91 ± 8% and 93 ± 5% after 24 h and 48 h, respectively.

The solubility of cPr-PMEDAP in water was 10.3 ± 1.5 mg/ml and was not significantly influenced by 1% DDAK (11.5 ± 0.8 mg/ml). Similar values (~11 mg/ml) were found for cPr-PMEDAP solubility in both acceptor phases, i.e., PBS and HBSS at pH 7.4 with the antibiotic/antimycotic mixture, either with or without DDAK. Thus, sink conditions were maintained throughout the permeation studies because even the highest donor concentration without any skin barrier would lead only to ~0.4 mg/ml of cPr-PMEDAP in the acceptor phase. It should be noted that at such high concentrations of cPr-PMEDAP, the buffering capacity is not sufficient and the pH gradually decreases. In the donor samples adjusted at pH 6, the solubility of cPr-PMEDAP was over 150 mg/ml.

The partitioning and distribution coefficients between the isolated human stratum corneum and various aqueous solvents confirmed the hydrophilic nature of cPr-PMEDAP. The logP/logD values varied between −0.9 and −1.6 and were not significantly affected by added enhancer DDAK.

Delivery of cPr-PMEDAP Through and into Porcine Skin and Effects of pH, Concentration, and Enhancer DDAK

The behavior of cPr-PMEDAP was first studied using porcine skin, which is an easily available, relevant model for human skin. The effect of pH of the donor sample and cPr-PMEDAP concentration on its transdermal flux and skin concentration is shown in Fig. 3. Without an enhancer, the flux of 1% cPr-PMEDAP through full-thickness porcine skin was low, with values from 0.3±0.1 μg/cm^2/h at pH 4 to 1.6±0.8 μg/cm^2/h at pH 8 (Fig. 3a). This increase of flux in a slightly alkaline environment was accompanied by a decrease of skin impedance (data shown elsewhere (26)). Thus, it can be attributed to a negative effect of the higher pH itself, not to a different ionization of cPr-PMEDAP. The concentration of cPr-PMEDAP retained in the skin ranged from 180±39 μg per g of the tissue at pH 3 to 346±64 μg/g at pH 7. With further increase to pH 8, the skin concentration of cPr-PMEDAP decreased, but these differences were not statistically significant (Fig. 3c). Increasing the cPr-PMEDAP concentration in the donor sample at pH 6 from 1% to 5% had no positive impact on either flux or skin concentration of this drug (Fig. 3b and d).

To promote the transport of cPr-PMEDAP through and into the skin, DDAK was selected as an enhancer, since this compound had proved to be highly potent in our previous study using another acyclic nucleoside phosphonate, adefovir (15). Indeed, addition of 1% DDAK significantly increased both the flux and skin concentration of cPr-PMEDAP. The highest flux was observed at pH 6, reaching a value of 8.4±1.0 μg/cm^2/h (Fig. 3a). This corresponds to the enhancement ratio (ER) of 23. Further increase of flux was reached with higher cPr-PMEDAP concentration, with flux values up to 23.8±5.1 μg/cm^2/h and ER of 48 (Fig. 3b). In addition, the concentrations of cPr-PMEDAP in the skin were 1.3–2.6 times higher upon addition of 1% DDAK (Fig. 3c). Better results were again achieved with 2–5% cPr-PMEDAP in the donor sample at pH 6, where 1% DDAK increased the overall skin concentration of this drug up to 6.5 times (Fig. 3d).

Delivery of cPr-PMEDAP Through and into Different Human Skin Layers

The behavior of cPr-PMEDAP was further investigated using freshly excised human skin, which is the best *in vitro* model for the real *in vivo* situation. The diffusion of this drug through human skin was very slow; thus, the experiment had to be prolonged to 72 h to reach steady state. The permeation profiles are shown in Fig. 4a. Without the enhancer, the flux values were 38±2, 39±5, and 44±8 ng/cm^2/h from the donor sample containing 0.1, 1, and 5% of cPr-PMEDAP at pH 6, respectively, i.e., approximately an order-of-magnitude lower than through the porcine skin (Fig. 4b). A similarly low flux (25±2 ng/cm^2/h) was observed also from the lipophilic isopropyl myristate donor solvent (not shown).

Nevertheless, the addition of 1% DDAK resulted in a significantly higher flux of cPr-PMEDAP through human

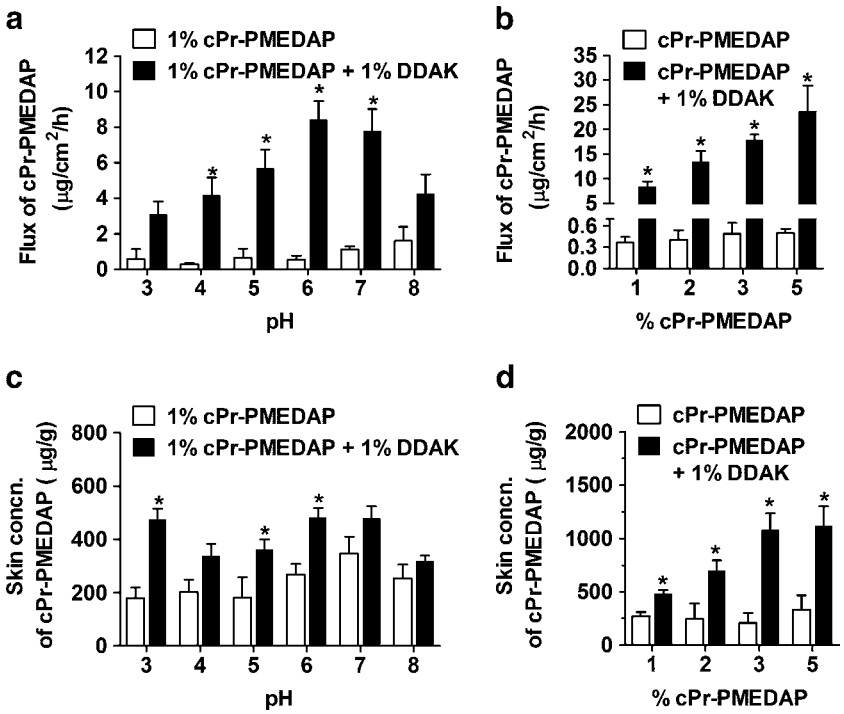

Fig. 3 Delivery of cPr-PMEDAP through and into porcine skin. (**a**) Flux of 1% cPr-PMEDAP formulated at different pH through porcine skin with and without 1% of DDAK. (**b**) Flux of cPr-PMEDAP (applied at 1–5% at pH 6) through porcine skin with and without 1% of DDAK. (**c**) Skin concentration of cPr-PMEDAP formulated at different pH with and without 1% of DDAK. (**d**) Skin concentration of cPr-PMEDAP (applied at 1–5% concentration at pH 6) with and without 1% of DDAK. Indicated values are means ± SEM from at least 3 experiments, and * indicates significant difference compared to the sample without DDAK at the same pH or concentration.

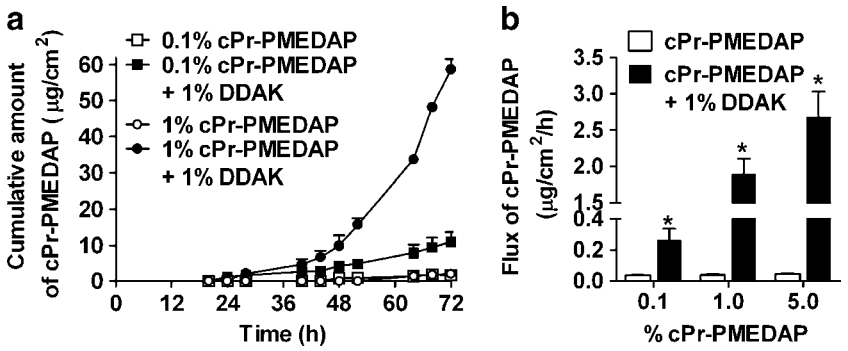

Fig. 4 Permeation of cPr-PMEDAP through human skin. (**a**) Permeation profiles of cPr-PMEDAP (0.1% and 1% at pH 6) through human skin with and without 1% of DDAK. (**b**) Flux of cPr-PMEDAP (0.1–5% at pH 6) through human skin with and without 1% of DDAK. Indicated values are means ± SEM from at least 3 experiments, and * indicates significant difference compared to the sample without DDAK at the same concentration.

skin; the values obtained from 0.1, 1, and 5% cPr-PMEDAP donor sample were 0.26±0.07, 1.90±0.22, and 2.69±0.34 μg/cm^2/h, corresponding to the ERs of 6.7, 53, and 61, respectively (Fig. 4b).

Apart from the permeation of cPr-PMEDAP through the skin into the receptor fluid, i.e., a model of the transdermal delivery into the systemic circulation, its concentration in the individual skin layers was investigated to assess its potential for the treatment of various skin diseases. Despite the relatively high overall skin concentration of cPr-PMEDAP, the analysis of the skin layers revealed that the majority of the compound was located in the stratum corneum (Fig. 5a). Thus, the stratum corneum acted as a highly effective barrier for the diffusion of this polar phosphonate-containing compound. Moreover, the transport seemed to be saturated already after the application of 0.1% cPr-PMEDAP; for example, the amounts of this compound penetrating through the stratum corneum into the living epidermis were 47±9 and 46±6 μg/g from the 0.1 and 1% cPr-PMEDAP donor sample, respectively (Fig. 5b). Similarly, no difference was found between the concentrations in the dermis after the

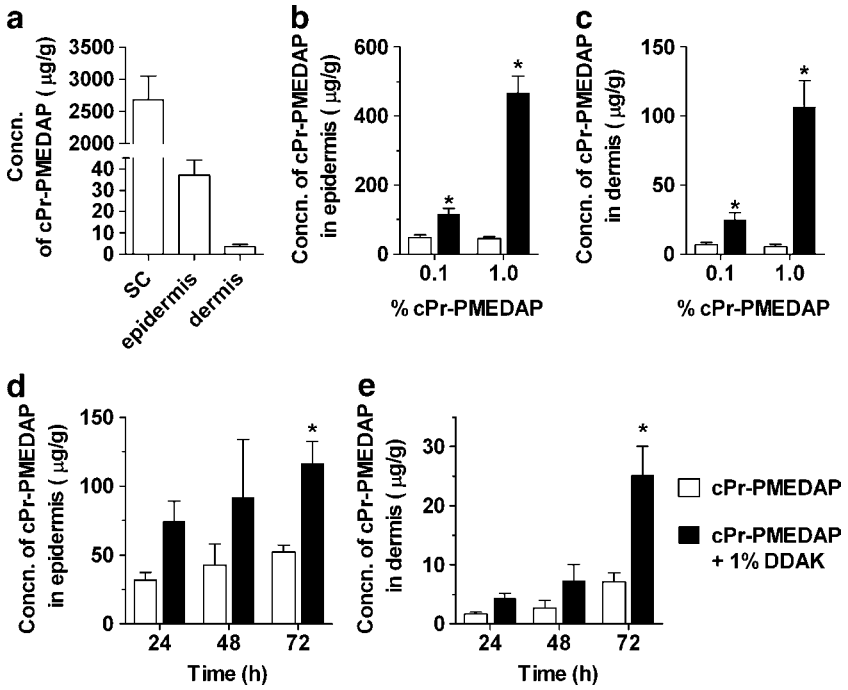

Fig. 5 Penetration of cPr-PMEDAP into human skin layers. (**a**) Concentrations of cPr-PMEDAP (1%, without the enhancer at pH 6) in the stratum corneum (SC), living epidermis, and dermis at 72 h. (**b**) Concentration of cPr-PMEDAP (applied at 0.1 and 1% at pH 6) in the living epidermis with and without 1% of DDAK at 72 h. (**c**) Concentration of cPr-PMEDAP (applied at 0.1 and 1% at pH 6) in the dermis with and without 1% of DDAK at 72 h. (**d**) Concentration of 0.1% cPr-PMEDAP in the living epidermis with and without 1% of DDAK at 24, 48, and 72 h. (**e**) Concentration of 0.1% cPr-PMEDAP in the dermis with and without 1% of DDAK at 24, 48, and 72 h. Indicated values are means ± SEM from at least 3 experiments, and * indicates significant difference compared to the sample without DDAK at the same concentration.

application of 0.1 and 1% cPr-PMEDAP; values of 7.0±1.5 and 5.5±1.5 μg/g, respectively, were reached (Fig. 5c).

Nevertheless, after the addition of 1% DDAK, significantly higher concentrations of cPr-PMEDAP were detected in the deeper skin layers. After the application of 0.1 and 1% cPr-PMEDAP with the enhancer, the living epidermis contained 116±16 and 468±48 μg of cPr-PMEDAP per g of the tissue (i.e. 2.5 and 10 times higher amounts than without the enhancer), and dermis 25±5 and 107±19 μg/g (i.e. 3.6 and 19 times higher amounts), respectively.

All the above skin concentrations are reported at 72 h, i.e. the end of the permeation experiments. To assess the time-course of the diffusion of 0.1% cPr-PMEDAP into the individual skin layers, some experiments were shortened to 24 and 48 h (Fig. 5d and e). In the epidermis, the cPr-PMEDAP concentrations were similar at all time intervals. On the other hand, the concentration of cPr-PMEDAP in the dermis increased slowly, in line with the concentration in the acceptor compartment.

DISCUSSION

Acyclic nucleoside phosphonates possess unique antiviral and antineoplastic activities; however, their polar phosphonate moiety is associated with low ability to cross biological membranes. In this study, the potential of transdermal and topical skin delivery of cPr-PMEDAP, a potent antiproliferative and antiviral agent, was investigated. During the preliminary permeation studies and HPLC method development, *N*-dealkylation of cPr-PMEDAP into PMEDAP was found. At first, this was rather surprising, because only the deamination process into PMEG was known previously (6,9,11). Nevertheless, shortly after this finding, a paper describing this dealkylation into PMEDAP in the liver of dogs with spontaneous non-Hodgkin's lymphoma treated with the cPr-PMEDAP prodrug GS-9219 appeared (27). Thus, this transformation into PMEDAP should be considered in the further development of cPr-PMEDAP. PMEDAP is a broad-spectrum antiviral agent against DNA viruses; it inhibits replication of human immunodeficiency virus in human T-lymphocyte MT4 cells and suppresses tumor formation and mortality in newborn mice inoculated with Moloney murine sarcoma virus (28,29). Moreover, PMEDAP showed an antitumor effect on T-cell lymphoma in an *in vivo* model and showed synergy with docetaxel (30–32). For a review on PMEDAP, see Ref. (1). In our study, this reaction was attributed to skin microflora; however, the extent of this reaction *in vivo* and its practical implications certainly warrant further investigation.

An even more important finding was that no deamination to PMEG occurred during the permeation studies using viable human skin. This confirms the previous observations that PMEG is not released from cPr-PMEDAP in untransformed cells. This is particularly important because PMEG is relatively toxic; it caused significant tissue necrosis already at 0.1% concentration applied topically to rabbit papillomas twice-daily 5 days a week for 8 weeks (33). Moreover, PMEG caused lymphotoxicity and nephrotoxicity in rats at doses higher than 1 mg/kg. At 2.5 mg/kg, severe renal impairment was apparent (9). However, our results showed that there would be no risk of PMEG-associated toxicity to untransformed skin cells during transdermal or topical administration of cPr-PMEDAP.

The initial permeation and penetration studies were performed in porcine skin. We started with 1% cPr-PMEDAP because this concentration applied topically caused moderate anti-papillomavirus activity in rabbits *in vivo* (33). The flux of cPr-PMEDAP through the skin was relatively low, and both the penetration and permeation characteristics were roughly comparable to adefovir (14,15). An increase in the donor concentration from 1 to 5% improved neither the flux nor skin concentration, which explains the cPr-PMEDAP anti-papillomavirus activity in rabbits, where the 5% sample produced similar results as the 1% one (33). On the other hand, the addition of 1% DDAK, a permeation enhancer, resulted in significantly higher transport of cPr-PMEDAP through and into the skin. The best values were achieved at pH 6, which is the same as in adefovir; possible causes were studied and discussed in our previous work (15). This optimized pH was then used in the human skin experiments.

In human skin, the permeation of cPr-PMEDAP into the acceptor compartment was even lower. Apparently, the human stratum corneum acted as a more effective barrier against the penetration of this hydrophilic phosphonate that the porcine one, which is in good agreement with previous results (15). Without the enhancer, the flux values were 38–44 ng/cm^2/h, which means only up to 32 μg of cPr-PMEDAP would be delivered from a 30 cm^2 patch during 24 h. In comparison, complete inhibition of choriocarcinoma tumor development in rats was achieved upon daily intraperitoneal treatment with 10 mg/kg of cPr-PMEDAP, and the dose producing a minimal yet meaningful effect was 1 mg/kg (9). Another relevant example is an administration of GS-9219, a cPr-PMEDAP prodrug, in a pet dog model of non-Hodgkin's lymphoma, where daily administration of 0.20 mg/kg GS-9219 produced very good antitumor response (27). This dose equals 0.12 mg/kg of cPr-PMEDAP. With the aid of 1% DDAK, the flux of cPr-PMEDAP through human skin increased up to 61 times and reached values equal to almost 2 mg of cPr-PMEDAP delivered from a 30 cm^2 patch during 24 h. This is still not comparable to the single doses mentioned above,

but the possibility to produce more prolonged drug exposure via the transdermal route may be attractive in the treatment of various tumors.

The topical administration of cPr-PMEDAP revealed that even after prolonged administration, the majority of this compound stays in the uppermost skin layer, the stratum corneum, and the resultant concentrations in the epidermis and dermis are low. This explains the relatively weak effect of this compound on human papillomas after topical administration (33) compared to its excellent *in vitro* activities. Again, the cPr-PMEDAP concentration in the nucleated epidermis was approximately an order-of-magnitude higher when applied with 1% DDAK than without the enhancer, reaching values up to 468 μg/g of cPr-PMEDAP. Thus, the use of DDAK proved to be a promising means of increasing the penetration of cPr-PMEDAP into the deeper skin layers, the potential sites of an infection or tumor.

CONCLUSION

In this study, we explored the potential of both transdermal and topical delivery of cPr-PMEDAP, a potent antiproliferative agent. The *in vitro* diffusion experiments revealed its very low ability to cross the skin barrier, with the majority of the compound found in the uppermost skin layer, the stratum corneum. Nevertheless, the permeation enhancer DDAK increased both the flux of cPr-PMEDAP and its concentration in nucleated epidermis. No deamination of cPr-PMEDAP into PMEG occurred during the permeation studies, but *N*-dealkylation into PMEDAP mediated by skin microflora was observed. In conclusion, transdermal or topical application of cPr-PMEDAP enabled by the permeation enhancer DDAK may provide an attractive alternative route for administration of this compound.

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