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RESEARCH PAPER

Transdermal Delivery and Cutaneous Targeting of Antivirals using a Penetration Enhancer and Lysolipid Prodrugs

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ABSTRACT

Purpose In this work, we investigate prodrug and enhancer approaches for transdermal and topical delivery of antiviral drugs belonging to the 2,6-diaminopurine acyclic nucleoside phosphonate (ANP) group. Our question was whether we can differentiate between transdermal and topical delivery, *i.e.*, to control the delivery of a given drug towards either systemic absorption or retention in the skin.

Methods The *in vitro* transdermal delivery and skin concentrations of seven antivirals, including (*R*)- and (*S*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (PMPDAP), (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine ((*S*)-HPMPDAP), its 8-aza analog, and their cyclic and hexadecyloxypropyl (HDP) prodrugs, was investigated with and without the penetration enhancer dodecyl-6-(dimethylamino)hexanoate (DDAK) using human skin.

Results The ability of ANPs to cross the human skin barrier was very low (0.5–1.4 nmol/cm^2/h), and the majority of the compounds were found in the stratum corneum, the uppermost skin layer. The combination of antivirals and the penetration enhancer DDAK proved to be a viable approach for transdermal delivery, especially in case of (*R*)-PMPDAP, an anti-HIV effective drug (30.2 ± 2.3 nmol/cm^2/h). On the other hand, lysophospholipid-like HDP prodrugs, *e.g.*, HDP-(*S*)-HPMPDAP, reached high concentrations in viable epidermis without significant systemic absorption.

Conclusions By using penetration enhancers or lysolipid prodrugs, it is possible to effectively target systemic diseases by the transdermal route or to target cutaneous pathologies by topical delivery.

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KEY WORDS acyclic nucleoside phosphonate antivirals · lysolipid prodrug · penetration enhancer · skin absorption · transdermal drug delivery

ABBREVIATIONS

ANP	acyclic nucleoside phosphonate
(*S*)-8-azaHPMPDAP	(*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diamino-8-azapurine
(*S*)-cHPMPDAP	cyclic (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine
CMV	cytomegalovirus
DDAK	dodecyl ester of 6-(dimethylamino) hexanoic acid
EBV	Epstein-Barr virus
HILIC	hydrophilic interaction liquid chromatography
HIV	human immunodeficiency virus
HDP	hexadecyloxypropyl ester
HDP-(*S*)-HPMPDAP	hexadecyloxypropyl ester of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy) propyl]-2,6-diaminopurine
HDP-(*R*)-PMPDAP	hexadecyloxypropyl ester of (*R*)-9-[2-(phosphonomethoxy) propyl]-2,6-diaminopurine
HHV	human herpesviruses
HPLC	high-performance liquid chromatography
(*S*)-HPMPDAP	(*S*)-9-[3-hydroxy-2-(phosphonomethoxy) propyl]-2,6-diaminopurine
HSV	herpes simplex virus
PBS	phosphate-buffered saline
PG	propylene glycol
(*R*)-PMPDAP	(*R*)-9-[2-(phosphonomethoxy) propyl]-2,6-diaminopurine

(*S*)-PMPDAP	(*S*)-9-[2-(phosphonomethoxy) propyl]-2,6-diaminopurine
SC	stratum corneum
VZV	varicella-zoster virus

INTRODUCTION

The skin is the largest organ in the human body. Due to its easy accessibility, the skin has been an attractive site for local (topical or cutaneous) or systemic (transdermal) drug administration. Topical drug delivery can be highly beneficial for the treatment of skin diseases, including skin cancer and viral infections, because it offers targeted therapy with limited side effects. However, this requires that the drug accumulates in the skin with minimal systemic absorption, especially in the case of highly potent drugs such as antineoplastics or antivirals. Transdermal delivery requires systemic drug absorption at a rate sufficient to reach therapeutically active plasma levels. Since the introduction of the first transdermal scopolamine patch in 1979, transdermal (percutaneous) delivery has become an established route of drug administration with numerous advantages over the oral or intravenous routes (1).

However, the limitation of both cutaneous and transdermal therapy is the skin itself. Skin provides a formidable barrier and has evolved to protect the human body from the loss of water and the penetration of harmful compounds from the environment. To overcome this barrier, numerous strategies have been developed including chemical penetration enhancers (2–4) and prodrugs (5,6). However, whether these penetration enhancing methods have the potential of controlling the drug delivery towards either transdermal absorption or cutaneous targeting still remains a question.

Acyclic nucleoside phosphonates (ANPs) are potent broad-spectrum antiviral drugs. These nucleotide analogues include clinically used drugs such as tenofovir (against human immunodeficiency virus (HIV) and hepatitis B), adefovir (hepatitis B), and cidofovir (cytomegalovirus (CMV) retinitis). In addition, ANPs are highly active against viruses that cause cutaneous pathologies, including herpes simplex virus (HSV), varicella-zoster virus (VZV), CMV, Epstein-Barr virus (EBV), human herpesviruses 6 and 7 (HHV-6 and HHV-7), HHV-8 causing Kaposi sarcoma, human papillomavirus, and molluscum contagiosum virus (7,8).

One advantage of phosphonates is that they do not require kinases for phosphorylation and thus overcome one of the most common causes of viral resistance. Compared to phosphates, phosphonates do not suffer from a rapid degradation (9,10). On the other hand, the highly polar phosphonate moiety bears two negative charges at physiological pH; thus, the transport of ANPs through biological membranes is limited. Therefore, these highly active antivirals would benefit from improving their topical or transdermal delivery.

One approach to increase the absorption of ANP through the biological barriers is masking the phosphonate by prodrug formation (11,12). Another possibility, particularly for drug delivery through the skin, is the use of compounds known as permeation/penetration enhancers (2–4). We have recently shown that certain enhancers can increase the absorption of ANPs by two orders of magnitude; one such enhancer is the amino acid derivative DDAK (dodecyl ester of 6-(dimethylamino)hexanoic acid, Fig. 1) (13,14). This amino acid-based penetration enhancer increased skin flux of the structurally related ANPs adefovir by 179-fold (14,15) and cPr-PMEDAP by 61-fold (13). Furthermore, DDAK increased skin flux of theophylline by 17-fold, hydrocortisone by 43-fold, and indomethacin by 9-fold (16). DDAK also displayed low toxicity in keratinocytes and fibroblasts (17), and no toxicity after oral administration of 2 g/kg to mice and rats (16). In addition, it was biodegradable by esterases, and electrical impedance of a DDAK-treated skin barrier rapidly recovered, demonstrating at least partial reversibility of its action in the skin barrier (16).

In this work, we investigate the prodrug and enhancer approaches for transdermal and topical delivery of ANPs from the group of 2,6-diaminopurine derivatives. Our major question was whether we can differentiate between transdermal and topical delivery, *i.e.*, to control the delivery of a given drug towards systemic absorption or retention in the skin.

The studied ANPs (Fig. 1 and Table I) include (*R*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine ((*R*)-PMPDAP), which has excellent anti-HIV activity (18–21); its (*S*)-isomer ((*S*)-PMPDAP) (18); and its lysolipid-like prodrug, the hexadecyloxypropyl (HDP) ester of (*R*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HDP-(*R*)-PMPDAP) (22). The second series of compounds contains an additional hydroxyl: (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine ((*S*)-HPMPDAP), which has good activity against herpes viruses and poxviruses (19,23–25); its 8-aza analogue (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diamino-8-azapurine ((*S*)-8-azaHPMPDAP) (20,26); and its two prodrugs: cyclic ((*S*)-cHPMPDAP) (23) and HDP ester (HDP-(*S*)-HPMPDAP) (23,27).

MATERIALS AND METHODS

Chemicals

Acyclic nucleoside phosphonates (*R*)-PMPDAP, (*S*)-PMPDAP, (*S*)-HPMPDAP, (*S*)-cHPMPDAP, and (*S*)-8-azaHPMPDAP, and their lysolipid prodrugs HDP-(*R*)-PMPDAP and HDP-(*S*)-HPMPDAP were prepared as described previously (22,23). The penetration enhancer DDAK

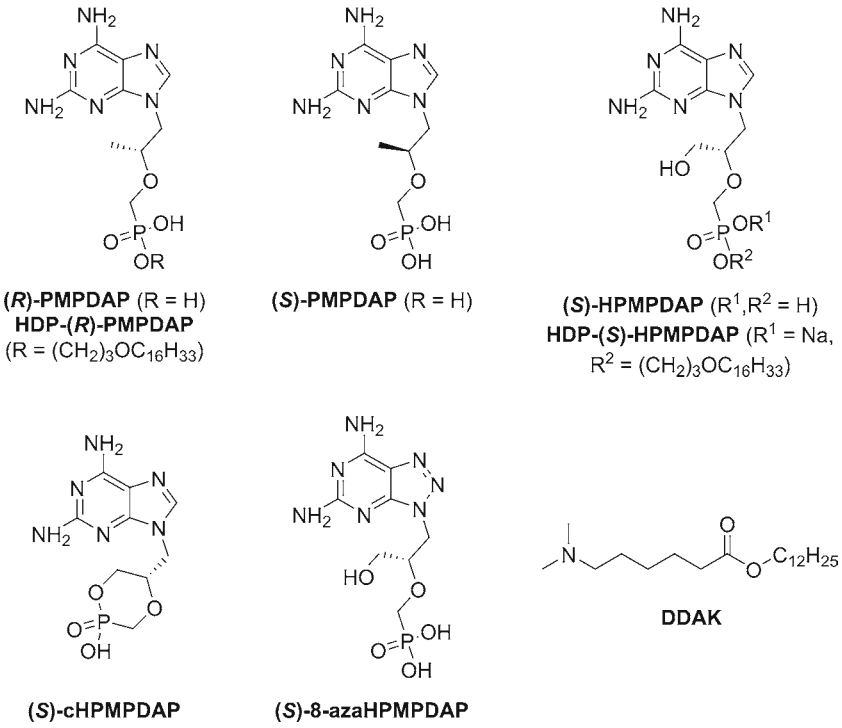

Fig. 1 Structures of the studied 2, 6-diaminopurine antivirals, their lysolipid prodrugs (HDP esters), and the penetration enhancer DDAK.

was synthesized according to Novotný *et al.* (16). All other chemicals were from Sigma-Aldrich (Schnelldorf, Germany). Ultrapure water was prepared using a Milli-Q Water Filtration System (Millipore, Bedford, MA).

Skin

We used human skin from Caucasian female patients who had undergone abdominal plastic surgery. The procedure for this work 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 subcutaneous fat was carefully removed and the remaining skin fragments were washed with saline, blotted dry, and stored at –20°C.

Donor Samples and Solubility of the Drugs

Saturated drug donor samples for the permeation and penetration studies we prepared by stirring an excess (60 μmol) of each ANP, either without or with 10 mg of DDAK, into 1 ml of the donor solvent, which is composed of 60% propylene glycol (PG) in 10 mM phosphate buffer at pH 6. The samples were allowed to equilibrate at 37°C for 24 h before being applied to the skin. All donor samples were applied as suspensions, *i.e.,* at equal (maximum) thermodynamic activity.

To determine the solubility of a drug in the donor solvent, the samples were prepared as above and centrifuged at 6, 700×g for 5 min; the supernatant was withdrawn, diluted with phosphate-buffered saline at pH 7.4 (PBS, containing 10 mM phosphate buffer, 137 mM NaCl and 2.7 mM KCl) and analyzed by HPLC. The solubility of each ANP in the acceptor media was determined by stirring an excess drug in PBS and then treating the sample likewise.

Transdermal Permeation Experiments

The skin permeation of the studied drugs was evaluated using Franz diffusion cells with an available diffusion area of 1 cm^2. The skin fragments were slowly thawed immediately before use, inspected for any visual damage, and cut into squares approximately 2 cm×2 cm. The cut squares were mounted into the diffusion cells with the dermal side down and the edges were sealed with silicone grease. The acceptor compartment of the cell (approximate volume of 6.5 ml) was filled with PBS at pH 7.4 with 50 mg/l gentamicin as a preservative. The precise volume of the acceptor liquid was measured for each cell and included in the calculation of cumulative amount and flux. The Franz diffusion cells were stirred at 600 rpm in a water bath at 32°C. After an 1 h equilibration period, the skin integrity was checked by measuring the electrical impedance using an LCR meter 4080 (Conrad Electronic, Hirschau, Germany) as described previously (13,17). The acceptable Impedance limit was set to 12 kΩ×cm^2.

Next, donor samples (200 μl) were applied to the skin surface and covered with glass slides. Sink conditions were maintained for all drug. Samples from the acceptor phase (0.3 ml) were withdrawn at predetermined time intervals over

Table I Characteristics of the Studied Antivirals and Their Selected Antiviral Activities

Drug	(*R*)-PMPDAP	(*S*)-PMPDAP	(*S*)-HPMPDAP	(*S*)-c-HPMPDAP	(*S*)-8-aza-HPMPDAP	HDP-(*R*)-PMPDAP	HDP-(*S*)- HPMPDAP
MW (g/mol)	302.2	302.2	318.2	300.2	319.2	584.7	622.7
Solubility (mM)							
donor	19.8 ± 0.9	19.7 ± 1.6	16.6 ± 1.5	47.7 ± 0.8	57.6 ± 1.7	0.25 ± 0.05	0.11 ± 0.01
donor + DDAK	21.6 ± 1.2	22.7 ± 0.7	14.4 ± 0.3	43.5 ± 0.9*	47.5 ± 1.2*	5.7 ± 0.3*	3.7 ± 0.1*
acceptor	12.1 ± 0.9	12.2 ± 0.4	19.4 ± 0.3	20.5 ± 0.3	22.6 ± 0.4	0.16 ± 0.02	0.11 ± 0.03
Antiviral activity (EC_{50}, μM)							
HIV-1	0.01 − 2.8 (18)[a] 0.17 ± 0.08(19)	7.4 ± 1.8 (19)	2.3 (25) ≥62 (19)		>313 (26)	0.0022 (22)	0.04 ± 0.02 (27)
HIV-2	0.18 ± 0.04(19)	7.9 ± 1.8 (19)	≥62 (19)		>313 (26)		
FIV	0.07 − 0.1 (21)					0.0006(22)	
HSV-1 (HHV1)	496 (19)	993 (19)	0.2 ± 0.2 (19) 1.3 − 2.9 (23)	3.6 ± 1.8 (23)	63 (26)		0.0027 − 0.0045(23)
HSV-2 (HHV2)	232 (19)	232 (19)	1.0 ± 0.6 (19) 2.8 ± 1.4 (23)	4.3 ± 4.0 (23)	63 (26)		0.007 ± 0.011(23)
VZV (HHV3)	>30 (19)		2.0 ± 1.1 (19) 0.01 − 0.2(23)	0.4 − 0.8 (23)	19 − 28 (26)	0.1 (22)	0.0021 − 0.0027(23)
EBV (HHV4)			2.0 (24)				
CMV (HHV5)	>30 (19)		7.8 ± 4.7 (19) 6 − 11(23)	10 − 15 (23)	>313 (26)		0.004 − 0.02(23)
HHV-6	>300 (20)		17.6 ± 3.7(23)		>300 (20)		
VV (VACV)	>1,200 (19)		12.6 ± 6.2(19) 0.5 ± 0.2 (23)	0.5 ± 0.2(23)	63 (26)		0.0007 ± 0.0006(23)

* indicates a statistically significant difference between the samples with and without DDAK at $p < 0.05$ ($n \geq 3$)

[a] references in brackets

72 h and replaced with fresh PBS. The drug content of the acceptor phase was determined by HPLC.

Topical Skin Absorption Experiments

First, the extraction efficiency from the skin (recovery) for each drug was determined. A known amount of each drug (1, 10, and 50 nmol) 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 methanol (for HDP ester prodrugs) or a 1:1 (v/v) mix of methanol and PBS, pH 7.4 (for all other compounds) was added and allowed to stir at 32°C for 24 h. The solvent was filtered and analyzed by HPLC. Each concentration level was tested in triplicate for each drug.

To determine the topical/cutaneous drug absorption, the skin was mounted into Franz diffusion cells with the diffusion area of 2 cm^2 and the donor sample (25 μl/cm^2) was applied to the skin surface for 8 h. All other conditions were the same as those in the transdermal permeation studies. At the end of the penetration experiment (8 h), the diffusion cells were dismounted and the skin washed three times with 0.5 ml of PBS (or 0.5 ml of methanol in case of the HDP prodrugs). The stratum corneum (SC) was collected by tape stripping with an UrgoFilm tape (Laboratories Urgo, Chenove, France) as described previously (13). The remaining tissue that had been exposed to the donor sample was then punched out, wrapped in an aluminum foil, and heated to 60°C for 3 min in an oven. The epidermis was then carefully peeled off from the dermis. The tape strips, epidermis and dermis were extracted with 1 ml, 1 ml, and 5 ml of the extraction solvent, respectively, for 24 h. The extract was filtered and analyzed by HPLC.

HPLC Conditions

The samples were analyzed using a Shimadzu Prominence instrument (Shimadzu, Kyoto, Japan) consisting of LC-20AD pumps with a DGU-20A3 degasser, an SIL-20A HT autosampler, a CTO-20AC column oven, an SPD-M20A diode array detector, an RF10AXL fluorescence detector, and a CBM-20A communication module. The data were analyzed using LCsolutions 1.22 software.

(*R*)-PMPDAP, (*S*)-PMPDAP, (*S*)-HPMPDAP, (*S*)-cHPMPDAP, and (*S*)-8-azaHPMPDAP were analyzed in HILIC mode using a SeQuant™ ZIC®-HILIC 150−4.6 mm column with 5-μm particles with 200 Å porosity (Merck, Darmstadt, Germany) at 30°C. The mobile phase, composed of 50 mM ammonium acetate/acetonitrile 1:3 (v/v) at a flow rate of 2 ml/min, was used. The samples were monitored at 280 nm; for (*S*)-8-azaHPMPDAP, fluorescent detection was carried out, with an excitation wavelength of 280 nm and an emission wavelength of 350 nm. The retention times of (*R*)-PMPDAP, (*S*)-PMPDAP, (*S*)-HPMPDAP, (*S*)-cHPMPDAP, and (*S*)-8-azaHPMPDAP were 3.5, 3.5, 4.3, 2.1, and 3.3 min, respectively. The calibration curves were linear in the range of 0.01 – 200 μM for (*S*)-8-azaHPMPDAP and in the range of 0.1 – 200 μM for the other compounds ($r^2 \geq 0.999$, $p < 0.001$).

The lysolipid prodrugs were analyzed by reverse-phase chromatography using a LiChroCART 125-4 column (Purospher STAR, RP-8e, 5 μm, Merck, Dartmstadt, Germany) at 30°C using 50 mM ammonium acetate/acetonitrile 2:3 (v/v) as the mobile phase with a flow rate of 1 ml/min and UV detection at 280 nm. The retention times of HDP-(*R*)-PMPDAP and HDP-(*S*)-HPMPDAP were 3.3 and 3.0 min, respectively. The calibration curves were linear in the range of 0.1 – 20 μM ($r^2 \geq 0.999$, $p < 0.001$). The precision and accuracy of these methods were within the limits of the FDA guidelines for bioanalytical method validation (2001).

Data Treatment

The cumulative amount of each drug that penetrated the skin, corrected for the acceptor sample replacement and the exact acceptor volume, was plotted against time. The steady state flux was calculated from the linear region of the plot. The skin drug concentration was calculated by dividing the amount of each drug by the respective skin layer weight. The enhancement ratio (ER) was calculated as a ratio of the permeation characteristics, either the flux or the skin concentration, with and without the enhancer.

The data are presented as the means ± SEM, and the number of replicates (n) is given in the pertinent figures. A two-tailed Student's t-test was used when comparing two different conditions. When comparing three or more conditions, a one-way analysis of variance (ANOVA) with a Dunnett's post-hoc test was performed. In all cases, $p < 0.05$ was considered significant.

RESULTS

Chromatographic Conditions

First, we needed an analytical method to determine the studied ANPs in the acceptor phase samples and skin extracts. The retention of these hydrophilic compounds in reverse-phase chromatography is generally unsatisfactory. Although their retention can be improved by ion-pairing reagents (14,15,28), this approach requires highly aqueous mobile phases and leads to short column life. Another option that has been used previously for the determination of N^6-cPrPMEDAP, PMEDAP and PMEG (13) is a reversed phase containing an amide linker, which is less hydrophobic than common C18 phase, possesses unique selectivity, and is compatible with highly aqueous mobile phases. However, the separation of the studied 2,6-diaminopurine derivatives was

not fully successful on an RP-amide column; particularly problematic was highly hydrophilic (*S*)-HPMPDAP.

Therefore, we changed the chromatographic mode to a HILIC column with zwitterionic groups. This enabled good separation of the free phosphonates and (*S*)-cHPMPDAP from the skin components. Only (*S*)-8-azaHPMPDAP displayed strong fluorescence, yielding a 10 nM limit of quantification; all other compounds were quantified at 280 nm with the quantification limit of 0.1 μM. The HDP prodrugs were separated using a C8-reversed phase column; these samples were also checked for the presence of (*R*)-PMPDAP and (*S*)-HPMPDAP by the above method.

Solubility in Donor and Acceptor Phases and Skin Extraction Recovery

The solubility values of the studied antivirals in the donor and acceptor phases are given in Table I. The addition of DDAK increased the solubilities of the HDP prodrugs by an order of magnitude and decreased the solubilities of (*S*)-cHPMPDAP and (*S*)-8-azaHPMPDAP by 9 and 17%, respectively. However, all of the donor samples, with or without 1% DDAK, were applied at their maximum thermodynamic activity. Sink conditions were maintained throughout the permeation studies, even for the most lipophilic drug, HDP-(*R*)-PMPDAP. This is likely because these lysolipid HDP prodrugs still contain one negative charge at physiological pH. In fact, the distribution coefficient (logD) values at pH 7 for these HDP prodrugs are 2.86 and 2.51 for HDP-(*R*)-PMPDAP and HDP-(*S*)-HPMPDAP (calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02).

The recovery of the drug extraction from the skin was tested at three levels (1 nmol, 10 nmol, and 50 nmol). At the medium and high concentrations, the recoveries were greater than 85% for all drugs and greater than 80% at the low concentration.

Transdermal Delivery of 2,6-Diaminopurine ANP Antivirals

The permeation profiles of the studied drugs with and without the penetration enhancer DDAK are shown in Fig. 2. In general, the flux of the studied ANPs through intact human skin was low, reaching values of 0.50–1.40 nmol/cm^2/h, and the lysolipid HDP prodrugs were not detected in the acceptor phase at all (Table II). No significant difference was found between PMPDAP enantiomers. The introduction of a hydroxyl group to the linker between the 2,6-diaminopurine base and oxygen in (*S*)-HPMPDAP decreased its permeation through the skin by more than twofold compared to PMPDAP isomers, while its skin concentration remained approximately the same. Given the presence of a free phosphonate in both compounds, this effect was surprisingly strong.

Consistent with this effect, masking the hydroxyl group in (*S*)-cHPMPDAP by cyclization diminished its negative influence and yielded a flux value similar to PMPDAP. This cyclic compound (which is actually a phosphonate monoester) showed approximately 5-fold lower relative skin retention than the free phosphonates. The values of relative skin retention, *i.e.*, the transdermal/dermal ratios, were calculated as the ratio of the amount of drug that permeated the skin and the amount that was retained in the skin at 72 h (Table II). Substituting the methine group in position 8 of the purine ring of (*S*)-HPMPDAP for nitrogen to generate (*S*)-8-azaHPMPDAP did not have any significant influence on its skin flux compared to the parent (*S*)-HPMPDAP, but the skin retention of (*S*)-8-azaHPMPDAP was similar to the cyclic compound (Table II). The lysolipid HDP prodrugs were found at 12–15 nmol per cm^2 of the skin.

Next, we tried to increase the drug flux using the penetration enhancer DDAK. Indeed, DDAK increased the flux of the studied 2,6-diaminopurine-based antivirals by 16–31 times, to 14.1–36.3 nmol/cm^2/h (Fig. 2 and Table II). No differences were found between the flux and skin concentration of (*R*)- and (*S*)-PMPDAP. In the presence of the additional hydroxyl group in (*S*)-HPMPDAP and (*S*)-8-azaHPMPDAP, the enhancer acted similarly well (ER values of 27 and 31, respectively), but DDAK was not as effective for the cyclic (*S*)-HPMPDAP (ER = 16). Interestingly, the transdermal/dermal ratios in the presence of DDAK were ~10 for (*S*)-HPMPDAP and its derivatives; these values are approximately 2-fold lower than those of PMPDAP.

While DDAK failed to increase the permeation of the lysolipid HDP prodrugs into the acceptor phase in measurable quantities, it increased their skin concentration by 2-3.7-fold.

Cutaneous Absorption of 2,6-Diaminopurine ANP Antivirals

The above results suggested that the lysolipid prodrugs may be good candidates for targeted cutaneous delivery. To investigate the dermal delivery of 2,6-diaminopurine antivirals in greater detail, we applied the donor samples at 25 μl/cm^2 for 8 h, and analyzed the drug content in individual skin layers.

The free and cyclic phosphonates were detected at ~5 nmol per cm^2 of the full-thickness skin, while the lysolipid prodrugs reached 2-4-fold higher values (Fig. 3a). For all ANPs, at least half of this amount was found in the SC, *i.e.*, the skin barrier. To measure the approximate concentrations of ANPs in the skin, the drug amounts were divided by the respective skin layer weight (the tissue density was considered to be ~1 g/ml). This simple calculation revealed that all compounds reached a concentration of 0.9–13.6 mM in the SC within 8 h (Fig. 3b). The concentrations of the lysolipid prodrugs in the SC were up to one order of magnitude higher than those of the free

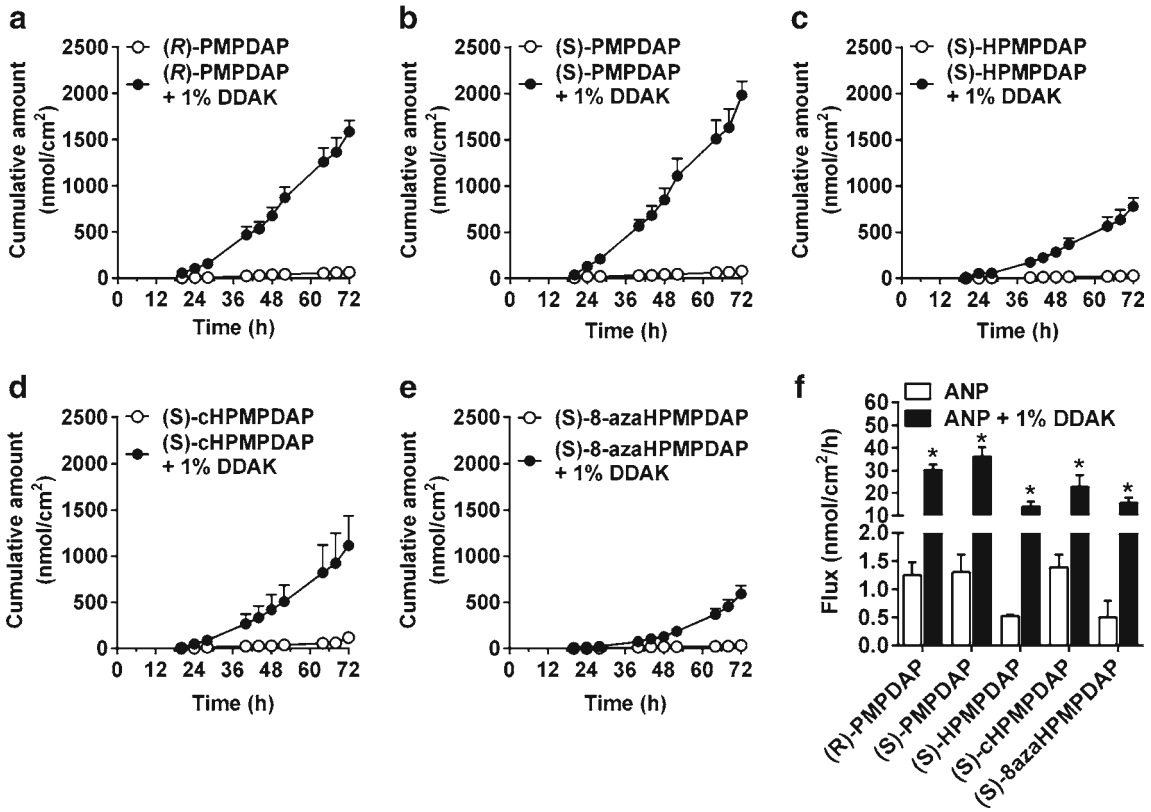

Fig. 2 Transdermal Delivery of 2,6-Diaminopurine Antivirals. The permeation profiles of ANPs, including (*R*)-PMPDAP (**a**), (*S*)-PMPDAP (**b**), (*S*)-HPMPDAP (**c**), (*S*)-cHPMPDAP (**d**), and (*S*)-8-azaHPMPDAP (**e**) through human skin *in vitro*. The drugs were applied at 60 mM in a 6:4 (v/v) mix of PG/PBS at pH 6, with and without 1% of the penetration enhancer DDAK at 100 μl/cm^2. The flux values are given in (**f**) * indicates a statistically significant difference between the samples with and without DDAK at $p < 0.05$ ($n \geq 3$). The lysolipid prodrugs HDP-(R)-PMPDAP and HDP-(S)-HPMPDAP were not detected in the acceptor phase.

phosphonates. This is in line with their much higher lipophilicity due to the presence of the HDP chain.

In the viable epidermis (the epidermal layers without SC), the concentrations of the free and cyclic ANPs reached 24.5–

Table II Parameters of the Transdermal Delivery of 2,6-diaminopurine Antivirals

Drug	DDAK (1%)	Cumulative amount permeated at 72 h (nmol/cm^2)	Flux (nmol/cm^2/h)	ER	Amount retained in the skin at 72 h (nmol/cm^2)	Transdermal/dermal ratio
(*R*)-PMPDAP	–	65 ± 13	1.25 ± 0.23		74 ± 35	0.9
	+	1586 ± 120*	30.2 ± 2.3*	24	92 ± 15	17.2
(*S*)-PMPDAP	–	78 ± 10	1.31 ± 0.31		98 ± 42	0.8
	+	1982 ± 149*	36.3 ± 4.1*	28	100 ± 15	19.8
(*S*)-HPMPDAP	–	28 ± 1	0.53 ± 0.02		77 ± 12	0.4
	+	777 ± 95*	14.1 ± 2.1*	27	85 ± 9	9.1
(*S*)-cHPMPDAP	–	118 ± 34	1.40 ± 0.22		24 ± 3	4.9
	+	1115 ± 324*	22.7 ± 5.3*	16	108 ± 18*	10.3
(*S*)-8-azaHPMPDAP	–	32 ± 14	0.50 ± 0.29		25 ± 2	1.3
	+	592 ± 88*	15.7 ± 2.5*	31	64 ± 4*	9.3
(*R*)-HDP-PMPDAP	–	n.d.	–		15 ± 8	< 1
	+	n.d.	–	–	55 ± 43	< 1
(*S*)-HDP-HPMPDAP	–	n.d.	–		12 ± 3	< 1
	+	n.d.	–	–	24 ± 24	< 1

* indicates a statistically significant difference between the sample with and without DDAK at $p < 0.05$ ($n \geq 3$)

n.d. not detected, *ER* enhancement ratio

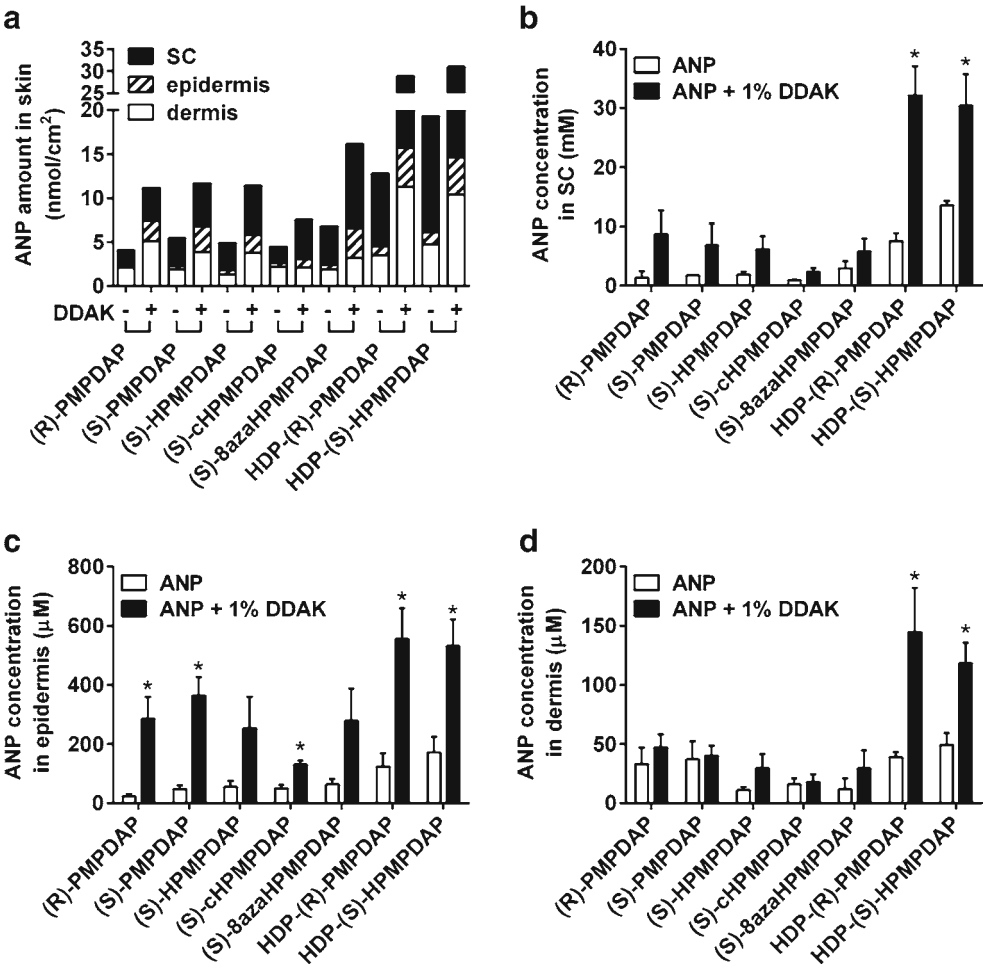

Fig. 3 Topical Delivery of 2,6-Diaminopurine Antivirals. The amounts of the studied antivirals per cm^2 of human skin (**a**) and their respective concentrations in SC (**b**), viable epidermis (**c**), and dermis (**d**) after an 8-h *in vitro* penetration. The drugs were applied at 60 mM in a 6:4 (v/v) mix of PG/PBS at pH 6, with and without 1% of the penetration enhancer DDAK, at 25 $\mu l/cm^2$. * indicates a statistically significant difference between the samples with and without DDAK at $p < 0.05$ ($n \geq 3$).

64.6 μM, and the HPD prodrugs were detected at 124–172 μM (Fig. 3c). In the dermis, all ANPs were detected at tens of μM (Fig. 3d).

When the penetration enhancer DDAK was co-applied with the ANPs (at 1% w/v, which corresponds to 250 $\mu g/cm^2$), the concentrations of the two lysolipid prodrugs increased in all skin layers, reaching ~30 mM in the SC, ~ 500 μM in the epidermis and over 100 μM in the dermis. The free and cyclic phosphonates also reached higher concentrations in the presence of DDAK, but this elevation was only statistically significant in viable epidermis (2.6 – 11.6 times). Using this setup, none of the drugs was detected in the acceptor compartment.

During the *in vitro* permeation experiments, we did not detect any hydrolysis of these HDP esters into their parent phosphonates. This is consistent with the fact that these prodrugs can only be cleaved by phospholipase C (12).

DISCUSSION

ANPs have become a key class of clinically efficacious antiviral nucleoside derivatives. They display a broad antiviral activity, a unique mode of action, a low resistance profile, and controllable toxicity (mainly nephrotoxicity) (7). The phosphonate group in ANPs allows for the bypass of the first phosphorylation step required for activation of nucleosides to their triphosphates, but limits the permeability of these drugs through biological membranes. Thus, improving their delivery into the human body and to target organs is still an active task.

Given the potential of transdermal delivery of the studied 2,6-diaminopurine-derived ANPs, the first results seemed to be rather discouraging; their flux values through intact human skin were in the nanomolar or subnanomolar range. In an effort to increase these unsatisfactory permeabilities, we combined these antivirals with the penetration enhancer DDAK (16). We selected this amino acid-based penetration enhancer because of its high activity (13–15) and favorable properties including low toxicity (17), biodegradability by esterases, and reversible action (16). The enhancer concentration was based on a previous work showing that 1% DDAK is sufficient to exert its full potential. The donor samples were maintained at pH 6 because this was the optimum pH for the enhancing action of DDAK for a related ANP adefovir (14). We also included PG as a co-enhancer because this solvent acted synergistically with amphiphilic enhancers (17,29–31)

including DDAK (16). This is likely because PG and amphiphilic enhancers act on different targets in the skin barrier (17).

Indeed, DDAK increased the flux of these drugs through the skin by up to 31-fold, resulting in values that may be attractive for potential transdermal delivery. The effect of DDAK on the donor solubility of the studied drugs was mixed: it slightly decreased in solubility of the highly hydrophilic phosphonates, which may be caused by a partial ion pairing with the basic tertiary nitrogen of DDAK. On the other hand, DDAK increased the solubility of the HDP esters probably due to its amphiphilic nature that may change the solvent properties of the donor sample by acting as a surfactant. Probably the most interesting combination of antiviral efficacy and relatively high skin flux was observed for (*R*)-PMPDAP, which is a 2-amino congener of tenofovir. Tenofovir is currently the most successful drug for the treatment of HIV infection; nevertheless, there is still a necessity for new drugs to overcome the development of HIV resistance (22). (*R*)-PMPDAP is a promising anti-HIV candidate because it is even more potent against retroviruses than tenofovir (18,19). We found that the flux of (*R*)-PMPDAP through human skin in the presence of 1% DDAK reached 30.2± 2.3 nmol/cm^2/h. Thus, the amount delivered from a 30 cm^2 patch over 24 h would be approximately 22 μmol. For a comparison, a daily dose of a structurally related ANP, tenofovir disoproxil fumarate, is 300 mg (= 472 μmol). Its bioavailability is 25%, so 118 μmol of tenofovir daily is enough for an effective treatment of HIV infection. Because (*R*)-PMPDAP is approximately 30 times more effective than tenofovir (the concentrations required to inhibit HIV-induced cytopathicity in MT-4 cells by 50% were 0.17 μM and 5.9 μM, respectively (19)), as little as 4 μmol of (*R*)-PMPDAP daily could be enough for the treatment of AIDS. Thus, the possibility to deliver over 20 μmol/day of (*R*)-PMPDAP *via* a medium-size transdermal patch should provide enough room for appropriate adjustments to the patch, *e.g.*, a controlled release to overcome variability of the skin barrier; (*R*)-PMPDAP can benefit from a sustained transdermal delivery.

The skin absorption of (*S*)-PMPDAP was very similar to its (*R*)- enantiomer; its flux in the presence of DDAK reached 36.3 ±4.1 μg/cm^2/h. However, the therapeutic potential of (*S*)-PMPDAP is limited because this compound is generally 15- to 40-fold less effective against HIV than its (*R*)-isomer (19).

HDP-(*R*)-PMPDAP is a lysolipid prodrug of (*R*)-PMPDAP with 400 times higher anti-HIV-1 activity *in vitro* (EC_{50}= 2.24 nM). Unfortunately, we did not detect this drug under the skin. Although the distribution coefficient of this prodrug at physiological pH is lower than 3, it is probably too large (MW over 500 g/mol) for effective transport and its lysolipid character makes its affinity for cell membranes very high. We cannot exclude the possibility that a certain amount of this drug below the detection limit permeated through the skin. However, such an amount would be much more than 400 times less than that of its parent drug (*R*)-PMPDAP (1586± 120 nmol at 72 h), making the higher activity of this prodrug useless. On the other hand, the skin concentration of HDP-(*R*)-PMPDAP was high, suggesting that this prodrug has a potential as a topical therapy, for example, as an intravaginal drug for the prevention of HIV infection as described for tenofovir (32), assuming that the vaginal tissue concentrations of HDP-(*R*)-PMPDAP would be similar to those observed in the skin.

The second series of antivirals, bearing hydroxyl in the linking chain between the phosphonate and the 2,6-diaminopurine base, has a different spectrum of antiviral activity. (*S*)-HPMPDAP is one of the most potent ANP-based antipoxvirus agents. It was highly effective against vaccinia virus (VV) infections, and in inhibition of camelpox virus and Orf virus replication in organotypic epithelial raft cultures (33–35). Its effect was comparable to or higher than that of cidofovir, the only licensed drug with antipoxvirus activity, currently marketed for the treatment of CMV retinitis in AIDS patients and recommended as a second-line treatment of adverse reactions of smallpox vaccination (36). However, when comparing our *in vitro* permeability of (*S*)-HPMPDAP (14.1 nmol/cm^2/h) and cidofovir dosing (5 mg/kg infusion once a week), the potential of reaching such doses by transdermal delivery is rather low. Considering the antiviral activities of (*S*)-HPMPDAP against herpesviruses and its concentrations found in epidermis, the treatment of skin infections would be possible. However, for the targeted cutaneous treatment of such viral infections, its prodrug HDP-(*S*)-HPMPDAP seems to be more advantageous, given its low systemic absorption. The substitution of methine in position 8 in the purine ring for nitrogen in (*S*)-8-azaHPMPDAP has no effect on the permeability of such drug through and into the skin.

(*S*)-cHPMPDAP is a cyclic prodrug of (*S*)-HPMPDAP with similar antiviral activities. Consistent with masking the polar groups, the skin flux of (*S*)-cHPMPDAP was 2.6-fold higher than that of its parent drug. However, DDAK was almost twofold less potent as in the parent (*S*)-HPMPDAP, leading to moderate flux of 22 nmol/cm^2/h. This is consistent with our previous study suggesting that a certain portion of the enhancing activity of DDAK is connected with ion-pair formation of ANPs (14).

HDP-(*S*)-HPMPDAP is another member from the studied 2,6-diaminopurine class of ANPs whose application on the skin has a high clinical potential. HDP-(S)-HPMPDAP has a spectrum of activity that is similar to its parent drug (*S*)-HPMPDAP but is effective in the nanomolar or subnanomolar range (23). The potency of the alkoxyalkyl (*e.g.,* HDP) prodrugs of ANPs is improved because they disguise as lysophospholipids and have high affinity for cellular membranes (12). The retention of this prodrug in the viable epidermis is high, even without the penetration enhancer DDAK, and its systemic absorption seems to be negligible. In fact, the concentrations of this

drug in viable epidermis were so high that much lower dosing would actually be necessary. A possible explanation why DDAK failed to increase the permeation of the HDP esters through the skin may be the affinity of the HDP chain for cellular membranes or that the concentration of the HDP esters that crossed the skin was still below the detection limit. Although this favorable absorption profile with a high cutaneous/systemic ratio certainly needs to be confirmed *in vivo* using more sensitive analytical techniques, we assume that this drug is an ideal candidate for the topical treatment of sensitive viral skin infections.

CONCLUSION

In this work, we investigated both prodrug and enhancer approaches for transdermal and topical delivery of 2,6-diaminopurine ANP antivirals. The combination of antivirals with the penetration enhancer DDAK proved to be a viable approach for transdermal delivery, especially in the case of the anti-HIV effective drug (*R*)-PMPDAP. On the other hand, lysolipid-like HDP prodrugs of these antivirals, especially HDP-(*S*)-HPMPDAP, reached high concentrations in viable epidermis without significant systemic absorption. Therefore, by using penetration enhancers or lysolipid prodrugs it is possible to effectively target systemic diseases by the transdermal route or cutaneous pathologies by topical delivery.

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