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Research paper

Permeation enhancer dodecyl 6-(dimethylamino)hexanoate increases transdermal and topical delivery of adefovir: Influence of pH, ion-pairing and skin species

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ABSTRACT

Adefovir (9-(2-phosphonomethoxyethyl)adenine) is an acyclic nucleoside phosphonate currently used for the treatment of hepatitis B. The aim of this study was to evaluate the effect of permeation enhancer DDAK (6-dimethylaminohexanoic acid dodecyl ester) on the transdermal and topical delivery of adefovir. In porcine skin, DDAK enhanced adefovir flux 42 times with maximum at pH 5.8 suggesting ion pair formation. DDAK increased thermodynamic activity and stratum corneum/vehicle distribution coefficient of adefovir, as well as it directly decreased the skin barrier resistance. Maximal flux was observed already at 2% adefovir + 1% DDAK. The results were confirmed in freshly excised human skin where DDAK enhanced adefovir flux 179 times to 8.9 μg/cm^2/h. This rate of percutaneous absorption would allow for reaching effective plasma concentrations. After the topical application, adefovir concentrated in the stratum corneum with low penetration into the deeper skin layers from either aqueous or isopropyl myristate vehicle without the enhancer. With 1% DDAK, adefovir concentrations in the viable epidermis and dermis were 33–61 times higher. These results offer an attractive alternative to established routes of administration of adefovir and other acyclic nucleoside phosphonates.

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1. Introduction

Adefovir (9-(2-phosphonomethoxyethyl)adenine, Fig. 1) is an acyclic nucleoside phosphonate with a broad-spectrum antiviral activity. In 2002, its bis(pivaloyloxymethyl) ester prodrug adefovir dipivoxil has been approved for treatment of hepatitis B in adult HBeAg-positive and negative patients as well as in adult patients with lamivudin-resistant HBV mutants, thereby providing a valuable alternative to current treatments. The major drawbacks of adefovir are its low bioavailability, accumulation in the kidney with dose-dependent nephrotoxicity and the requirement for a long-term therapy. Currently, the search for new prodrug types or delivery options to enhance adefovir bioavailability and improve its pharmacokinetic profile is one of the perspectives in this field. For recent reviews on adefovir, see Refs. [1,2].

Transdermal drug delivery offers an attractive alternative to established routes of administration [3,4]. In adefovir as well as in other chronic therapies, percutaneous application may be beneficial due to less frequent application and achievement of sustained plasma levels. Particularly in adefovir therapy of hepatitis B, substantial commitment from the patient is required as the discontinuation of the therapy may result in severe acute hepatitis exacerbation. Apart from its activity against hepatitis B virus, adefovir is active against various herpes viruses, and possesses a cytostatic, antiparasitic and immunomodulatory properties [1]. Thus, it is a candidate drug for local treatment of various skin diseases as well.

The major obstacle of the drug administration through the skin is the presence of the stratum corneum, the outermost layer of the skin, which provides an efficient barrier to drug penetration. However, the absorption of therapeutics either into the systemic circulation or to the deeper, viable skin layers may be increased by chemical substances known as transdermal permeation enhancers [5,6]. In our previous study [7], the effects of solvents of different polarity, donor pH and permeation enhancers including dodecanol, 1-dodecylazepan-2-one (Azone) [8], dodecyl 2-(dimethylamino)propionate (DDAIP) [9] and Transkarbam 12 (T12, 5-(dodecyloxycarbonyl)pentylammonium 5-(dodecyloxycarbonyl)pentylcarbamate) [10,11] on the transport of adefovir through and into the porcine skin were investigated. The highest adefovir flux (11.3 ± 3.6 μg/cm^2/h) and skin concentration (1549 ± 416 μg/g) was achieved with 1% T12 at pH 4. The activity of T12 at such acidic pH suggested that the presence of the unusual

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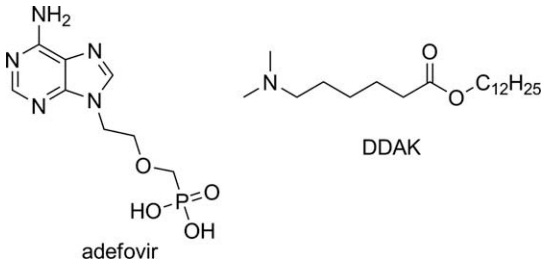

Fig. 1. The chemical structure of adefovir and the permeation enhancer DDAK.

carbamate polar head was unimportant in the enhancement of adefovir permeation, and T12 acted simply as a protonated 6-aminohexanoic acid ester. This brought us to a hypothesis that a structurally similar enhancer, 6-(dimethylamino)hexanoic acid dodecyl ester (DDAK, Fig. 1) [12], may possess the same or even better activity toward adefovir permeation. DDAK was designed by combining the important structural features of two well-known nontoxic enhancers – an ionizable dimethylamino polar head from DDAIP and 5-carbon linking group between the nitrogen and the ester group from T12. This enhancer showed an exceptional permeation-enhancing activity for a model drug theophylline [12].

The aim of this work was to evaluate the effect of the permeation enhancer DDAK on the flux of adefovir and its concentration in the porcine skin *in vitro*. The optimal pH and the concentration of both the drug and the enhancer applied on the skin were determined. Furthermore, the potential mechanisms of DDAK action including its influence on the adefovir solubility and stratum corneum/vehicle distribution coefficient and its direct effect on the skin barrier were studied. The best results obtained in the porcine skin were subsequently evaluated in freshly excised human skin.

2. Materials and methods

2.1. Chemicals

Adefovir [13] and permeation enhancer dodecyl 6-dimethylaminohexanoate (DDAK) [12] were synthesized as described previously. Their structure and purity was confirmed by IR and NMR spectra. KH_2PO_4, NaH_2PO_4, Na_2HPO_4 and NaCl were purchased from LachNer (Neratovice, Czech Republic). Isopropyl myristate (IPM) was purchased from Kulich (Hradec Králové, Czech Republic). Ultrapure water was obtained using Milli-Q Water Filtration System (Millipore, Bedford, MA). All other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.2. Skin

Porcine ears were purchased 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 was then immersed in 0.05% sodium azide solution in saline for 5 minutes for preservation. The skin fragments were stored at –20 °C up to 2 months.

Human skin from Caucasian female patients, who had undergone abdominal plastic surgery ($n = 4$), was used. The procedure has been approved by the Ethics Committee of the University Hospital Hradec Králové, Czech Republic (No. 200609 S09P), and conducted according to the Declaration of Helsinki Principles. Before use, the subcutaneous fatty tissue was removed by a scalpel.

2.3. Donor samples and adefovir solubility

Donor samples were prepared by stirring an appropriate amount of adefovir (0.5%, 1%, 2%, 3%, and 5%, respectively) in 1 ml of phosphate buffer (PB) either with or without DDAK (0.5%, 1%, 2%, and 3%, respectively). 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 the application on the skin.

For the determination of adefovir solubility in the donor solvent, an excess of adefovir was added to the pertinent solvent either with or without DDAK, pH was adjusted and the suspension was allowed to equilibrate. After 48 h, the suspensions were centrifuged at 10,000*g* for 5 min; the supernatant was withdrawn, diluted with phosphate-buffered saline (PBS) at pH 7.4 if needed, and analyzed by HPLC (see below). Three replicates were performed in each solvent.

2.4. Permeation experiments

2.4.1. Porcine skin

The skin permeability of adefovir was evaluated *in vitro* using the Franz diffusion cells [14]. 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 diffusion area was 1 cm^2. The acceptor compartment of the cell was filled with PBS at pH 7.4 with 0.03% sodium azide as a preservative, and was allowed to equilibrate in a 32 °C water bath for 30 min. The precise volume of the acceptor compartment (ca 18 ml) was measured for each cell and included into the calculations. Before application of the donor samples, the skin integrity was checked by measuring electrical resistance by an LCR meter 4080 (Conrad Electronic, Hirschau, Germany). The resistance was 5–30 and 15–45 kΩ/cm^2 in porcine and human skin, respectively. The donor sample (150 μl) was applied on the skin surface, and the donor compartment was occluded with a cover glass. The acceptor phase was stirred at 600 rpm at 32 °C throughout the experiment. Samples of the acceptor phase (600 μl) were withdrawn at predetermined intervals over 48 h, each time being replaced with fresh acceptor phase, and analyzed by HPLC, see below. The cumulative amount of adefovir 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 *x*-axis.

2.4.2. Porcine skin, pretreatment with DDAK

In the pretreatment experimental setup, 150 μl of 1% DDAK in PB at pH 5.8 was applied on the porcine skin in the Franz diffusion cell. The control skin fragments received 150 μl of PB at pH 5.8 without the enhancer to eliminate the effect of hydration and pH. After 2 h, the donor sample was removed. This application time has been widely used for enhancer pretreatment, e.g. [15]. The skin fragments that were exposed to DDAK were washed three times with 0.5 ml PBS to remove the residual enhancer, and were gently blotted dry. Then, 150 μl of 2% adefovir in PB at pH 5.8 was applied onto the skin and the permeation conducted as described above.

2.4.3. Human skin

The experiment with human skin was performed like in Section 2.4.1, except for the acceptor phase, which was 10 mM HEPES-buffered Hanks balanced salt solution at pH 7.4 without phenol red containing 50 μg/ml gentamicin. This acceptor phase was previously shown to maintain skin viability and metabolic activity during storage and permeation experiment [16]. Samples of the acceptor phase were collected over 72 h to achieve steady state flux.

2.5. Concentration of adefovir in the skin

2.5.1. Porcine skin

At the end of the permeation experiment (48 h), the diffusion cells were dismounted, and the skin surface was washed three times with 0.5 ml PB to remove the residual donor samples. 1 cm^2 of the skin exposed to the donor compartment was punched out, blotted dry, and precisely weighed. Then, it was placed into a vial with a stirring bar and extracted with 5.0 ml PBS at 32 °C for 48 h.

2.5.2. 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 homogeneous pressure (1.5 kg per 1 cm^2) for 5 s, and then was 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" from a plastic foil with a 1 cm^2 opening was attached to the skin fragment. Due to 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 adefovir amount in the stratum corneum. Then, 1 cm^2 of the remaining tissue was punched out, wrapped into an aluminum foil and exposed 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. Each sample was extracted as described above for the porcine skin, the tape strips and epidermis with 1 ml PBS each and the dermis with 5 ml PBS. The extract was filtered and analyzed by HPLC. Skin concentration, expressed as μg of adefovir per gram of the tissue, was calculated by dividing adefovir amount by the respective skin weight. The efficiency of adefovir extraction from the skin was previously validated, and was over 96% [17].

2.6. Stratum corneum/donor solvent distribution coefficient

The relative stratum corneum/donor solvent distribution coefficient was determined by a modified method of Kiptoo [18]. Stratum corneum sheets were prepared by trypsin treatment as described elsewhere [19], and were dried in vacuo. Before the experiment, SC sheets of ca 10 mg were precisely weighed and hydrated in 1 ml of saline with 0.03% sodium azide at 32 °C. After 48 h, SC was withdrawn and blotted dry on a filter paper. 10 μg/ml adefovir solution either with or without DDAK (5 μg/ml) in the pertinent solvent was added to each SC sheet (1 ml of the solution per 10 mg of the stratum corneum), and was allowed to equilibrate for 24 h at 32 °C. The sample was centrifuged at 10,000*g* for 5 min, and the concentration of adefovir in the supernatant was determined by HPLC (c_{24}). The same solution without SC was treated likewise (c_0). The distribution coefficient D was determined as follows: $D = (c_0 - c_{24})/c_{24}$. The concentration used in this experiment was different from those in the diffusion 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 [18].

2.7. HPLC conditions

The samples were analyzed with a system consisting of a Shimadzu LC-20AD high-pressure pump, Shimadzu SIL-20AC autosampler (Kyoto, Japan), LCD 2083 UV detector (Ecom, Prague, Czech Republic), and CSW v. 1.7 for Windows integrating software (Data Apex, Prague, Czech Republic). A LiChroCART 250-4 column with Purospher STAR, RP 18e, 5 μm (Merck, Darmstadt, Germany) with a LiChroCART 4–4 guard column with the same sorbent at 40 °C was used for separation of adefovir. The mobile phase consisted of 10 mM KH_2PO_4 and 2 mM Bu_4NHSO_4 at pH 6.0 with 7% acetonitrile at a flow rate of 1.5 ml/min. The detector wavelength was set at 260 nm, and the volume of injection was 20 μl. The method was previously validated [17].

2.8. Data treatment

Enhancement ratio (ER) was calculated as a ratio of the permeation characteristics, either flux or skin concentration, with or without the enhancer. The data are presented as means ± SD, n is given in the pertinent figures. Statistical significance was determined using t-test or Rank Sum Test, where appropriate, for evaluation of the effect of the enhancers and ANOVA or ANOVA on Ranks with Dunn's post-test for multiple comparisons.

3. Results

3.1. DDAK increases both adefovir flux and skin concentration with an optimum at pH 5.8

The flux of 2% adefovir through porcine skin plotted against pH of the donor sample is shown in the upper panel of Fig. 2. Without an enhancer, the adefovir permeation through the skin was low

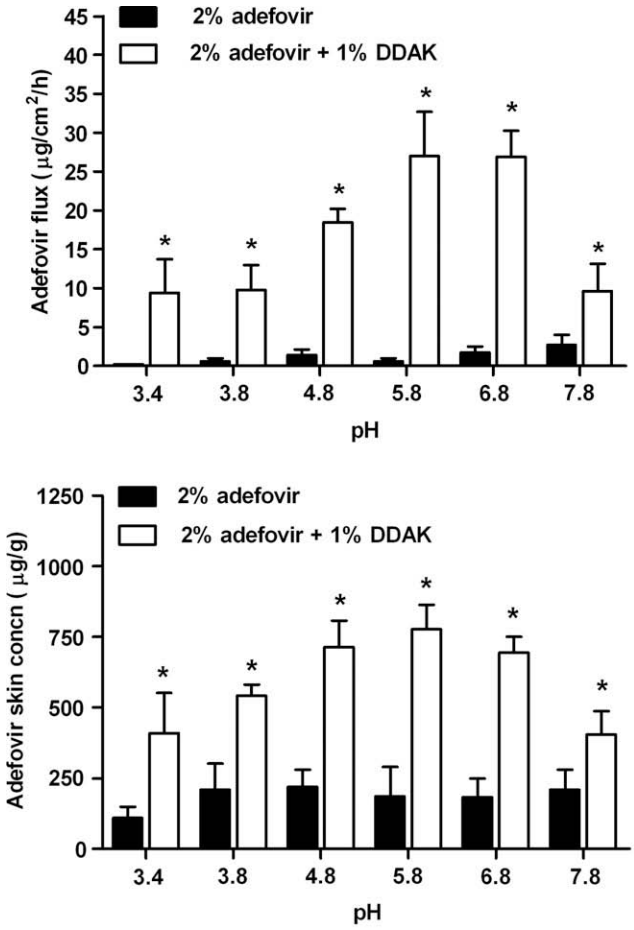

Fig. 2. Adefovir flux through (upper panel) and its concentration in the porcine skin (lower panel) either with or without the permeation enhancer DDAK plotted against the donor pH. Means ± SD, $n = 4$ (2 donors), except for pH 5.8, where $n = 12$ (6 donors). * Indicates significant difference between the value with or without DDAK.

and relatively insensitive to the donor pH as already shown in our previous study [7]. Addition of 1% permeation enhancer DDAK into the donor sample increased adefovir flux through the skin significantly at all pH values (Fig. 2). The maximum activity was observed at pH 5.8, where DDAK increased adefovir flux 42 times to 27 μg/cm^2/h.

The skin concentration of adefovir, expressed as μg adefovir per g of the tissue, is shown in the lower panel of Fig. 2. In the first part of this study using porcine skin, only the concentration in the whole skin was determined. The skin concentration of adefovir significantly increased in the presence of the permeation enhancer DDAK up to 778 μg/g. As in the transdermal transport, the pH dependence was parabolic with an optimum at pH 5.8.

3.2. At pH 5.8, DDAK increases both adefovir thermodynamic activity and its stratum corneum/vehicle distribution

The solubility of adefovir in the donor samples either with or without the permeation enhancer DDAK plotted against donor pH is shown in the upper panel of Fig. 3. Below pH 4, most of adefovir was present in the form of a zwitterion associated with low aqueous solubility. Thus, the donor samples at pH 3.4 and 3.8 were applied onto the skin as saturated adefovir suspensions at its maximal thermodynamic activity. Addition of 1% DDAK into the donor sample had no effect on adefovir solubility/thermodynamic activity at these pH values.

The solubility of adefovir markedly increased with the formation of a monoanion (pK_a = 4.2) up to 120 mg/ml. Because such concentration may be associated with a risk of local toxicity, a compromise between the maximal thermodynamic activity and therapeutic safety had to be accepted. All donor samples were prepared by dispersing 20 mg adefovir in 1 ml of the solvent; this concentration is indicated as a dashed line in the upper panel of Fig. 3. Thus, the donor samples at pH 4.8 and above were used as non-saturated solutions. Addition of 1% DDAK significantly decreased adefovir solubility at pH 5.8 and above. This means that the thermodynamic activity of 20 mg/ml non-saturated adefovir was higher in the presence of DDAK, which explains a part of its permeation-enhancing activity. At pH 5.8, i.e. the optimum activity of DDAK, most of adefovir was in the form of a hydrogenphosphonate monoanion, and the tertiary amino group of DDAK was protonated. Thus, the formation of an ion pair between the enhancer and the drug may account for both the decreased solubility and the higher transport.

Another possible mechanism of action of a permeation enhancer is an influence on the partitioning (distribution) of the compound between the stratum corneum, and the vehicle. The relative distribution coefficients were determined using an isolated porcine stratum corneum and the values are shown in the lower panel of Fig. 3. Although this method is very simple, it can be used for a rapid assessment whether there is any effect of an enhancer or not. According to these results, DDAK was able to increase adefovir distribution between the stratum corneum and the vehicle at pH from 4.8 to 6.8, which is consistent with the observed maximum of adefovir flux and the suggested formation of an ion pair.

3.3. Pretreatment with DDAK decreases the skin barrier resistance

In a subsequent experiment, porcine skin was pretreated with DDAK to investigate whether DDAK enhancing activity was associated only with the indirect effects described above, i.e. the increase of adefovir thermodynamic activity and its stratum corneum/vehicle distribution. First, DDAK was applied onto the skin for 2 h. Then, it was removed, and adefovir without the enhancer was applied. This experiment yielded adefovir flux of 16 μg/cm^2/h and skin concentration of 436 μg/g (Fig. 4). These values were approximately half of those obtained after co-administration with DDAK

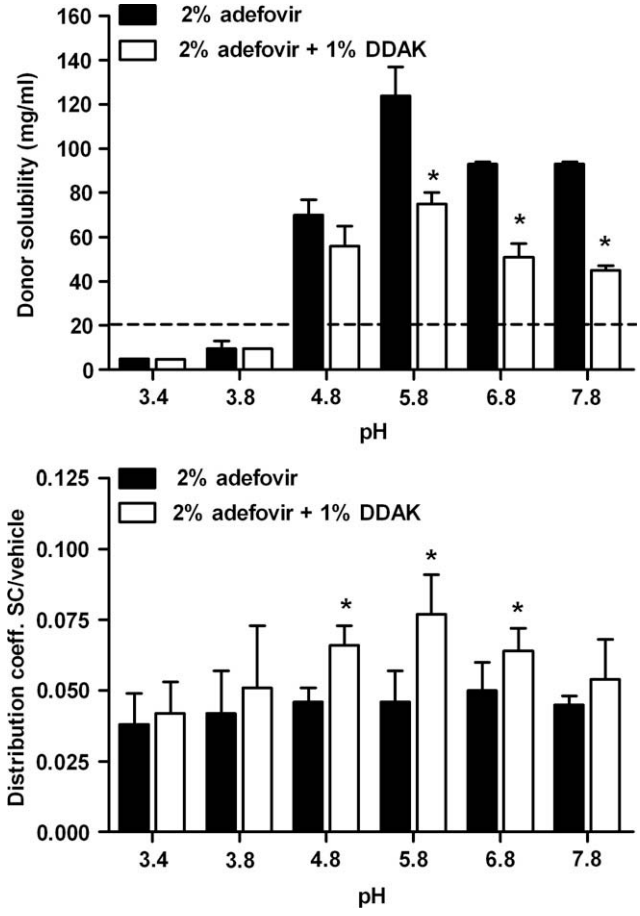

Fig. 3. Adefovir solubility in the donor sample (upper panel) and its relative distribution coefficient between the porcine stratum corneum and the donor solvent (lower panel) either with or without the permeation enhancer DDAK plotted against the donor pH. Means ± SD, n = 4. * Indicates significant difference between the value with or without DDAK. Dashed line at 20 mg/ml represents adefovir amount in the donor sample; i.e. the samples at pH 3.4 and 3.8 were suspensions and those at pH 4.8 and above were non-saturated solutions.

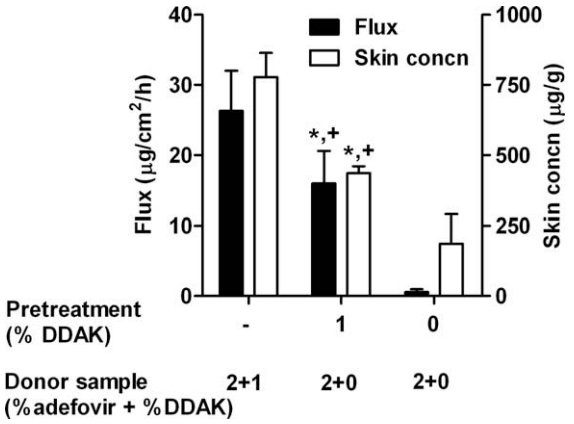

Fig. 4. The potency of DDAK to increase adefovir flux through and its concentration in the porcine skin as a pretreatment (middle columns) compared with its co-application with adefovir (left columns) and a pretreatment with pure solvent (right columns). In both pretreatment and co-application, PB at pH 5.8 was used as the solvent. Means ± SD, n = 4 (2 donors), except for the co-application sample, where n = 12 (6 donors). * Indicates significant difference between the pretreatment with or without DDAK, and + indicates significant difference between the pretreatment and co-application protocol.

but still significantly higher than those without the enhancer. Because this experimental setup excluded any interaction of DDAK and adefovir in the vehicle, we may assume that direct interaction with the skin barrier structures also plays a role in the permeation-enhancing activity of DDAK.

3.4. Effect of adefovir and DDAK concentration on adefovir flux and skin absorption

To find the optimal composition of the donor sample for the human skin experiment, the concentration of both the drug and the enhancer in PB at pH 5.8 was varied. Fig. 5A shows adefovir flux plotted against the concentration of adefovir applied onto the skin either with or without DDAK. Without the enhancer, adefovir flux increased with increasing donor concentration of the drug, in accordance with its increasing thermodynamic activity. However, when 1% DDAK was present in the vehicle, adefovir flux reached a plateau at 2% drug applied on the skin. When the concentration of adefovir in the vehicle was kept at 2% and the amount of DDAK was varied, the maximal flux was observed already at 1% DDAK present in the donor sample (Fig. 5B).

Unlike the transdermal flux of adefovir, its skin absorption steadily increased with increasing adefovir concentration in the vehicle either with or without the enhancer (Fig. 5C). At 2% adefovir in the donor sample, the maximal skin absorption occurred already at 0.5% DDAK (Fig. 5D).

3.5. Adefovir flux across human skin is 8.9 ± 2.4 μg/cm²/h

To verify the results obtained using the frozen-and-thawed porcine skin, adefovir transport through the freshly excised human skin from patients who had undergone plastic surgery was investigated. Adefovir in PB at pH 5.8 either with or without DDAK and in isopropyl myristate (IPM), respectively, was selected. 2% adefovir flux through the human skin without an enhancer was an order of magnitude lower than that through the porcine skin. However, the addition of 1% DDAK increased adefovir flux 179 times up to 8.9 ± 2.4 μg/cm²/h.

Similar to the porcine skin, there was no significant difference in adefovir flux through human skin from a sample containing 2%, 3%, and 5% adefovir, respectively, when applied together with 1% DDAK (Fig. 6).

3.6. DDAK increases adefovir concentration in viable epidermis and dermis

The human skin from the permeation experiment was separated into the stratum corneum, viable epidermis and dermis, and adefovir amount in each skin layer was determined. Fig. 7 presents the amount of adefovir absorbed in 1 cm² of the individual skin layers after the application of adefovir in PB at pH 5.8 and in IPM, respectively. IPM was selected according to our previous results where high accumulation of adefovir in the skin with low systemic absorption was observed [7,17]. However, this study showed that most of adefovir applied in this lipophilic vehicle was concentrated in the stratum corneum with very low penetration into the viable skin layers (Fig. 7).

Similar distribution pattern of adefovir in the skin layers was produced by the aqueous adefovir solutions without the enhancer. By taking the weight of the skin fragments into consideration, the concentrations of adefovir in the epidermis reached 25–64 μg/g from the donor samples containing from 2% to 5% adefovir. The respective concentrations in the dermis were 8–19 μg/g. It should be noted that all these values were achieved at steady state and represent the saturation concentrations. Thus, the in-use skin con-

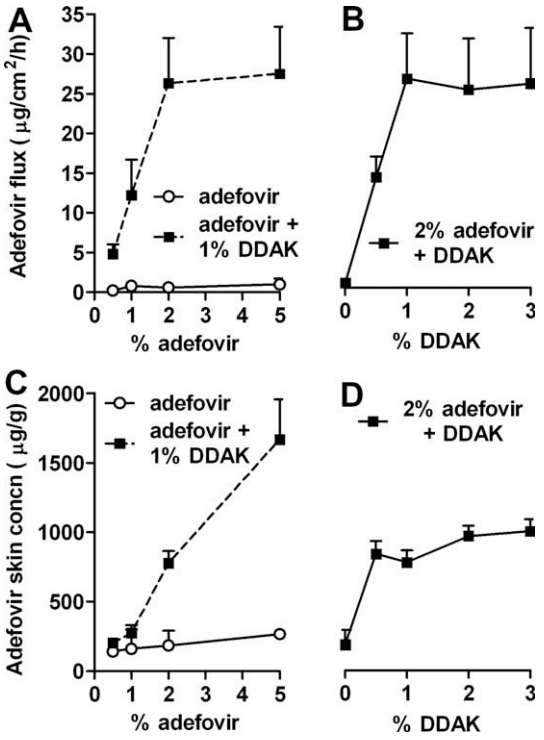

Fig. 5. The effect of concentration of adefovir and the permeation enhancer DDAK in the donor sample at pH 5.8 on either adefovir flux (A and B) or its concentration in the porcine skin (C and D). In A and C, the transport characteristics of adefovir are plotted against the concentration of adefovir either with or without 1% DDAK. In B and D, the transport characteristics of adefovir are plotted against the concentration of DDAK in the presence of 2% adefovir. Means ± SD, $n = 4$ (2 donors), except for 2% adefovir and 1% DDAK samples, where $n = 12$ (6 donors).

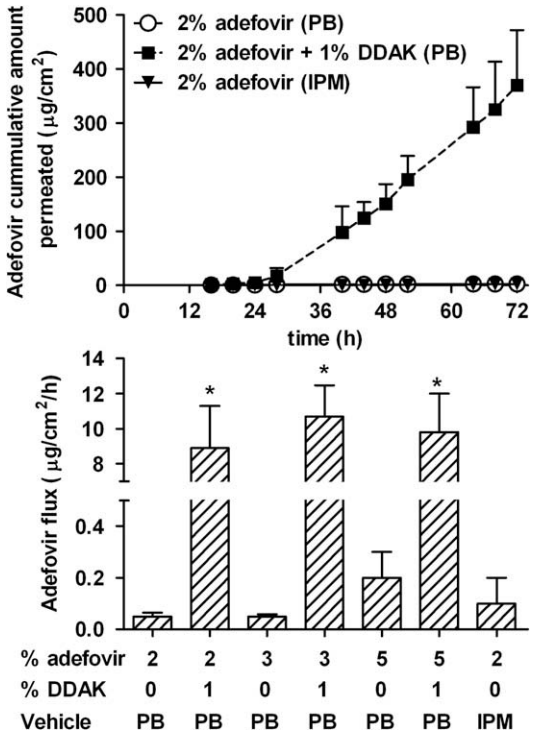

Fig. 6. The permeation profile (upper panel) and the flux of adefovir (lower panel) through the human skin; effects of adefovir concentration, DDAK and the vehicle. Means ± SD, $n = 3$–4 (2 donors), except for 2% adefovir samples, where $n = 12$ (6 donors). * Indicates significant difference between the value with or without DDAK.

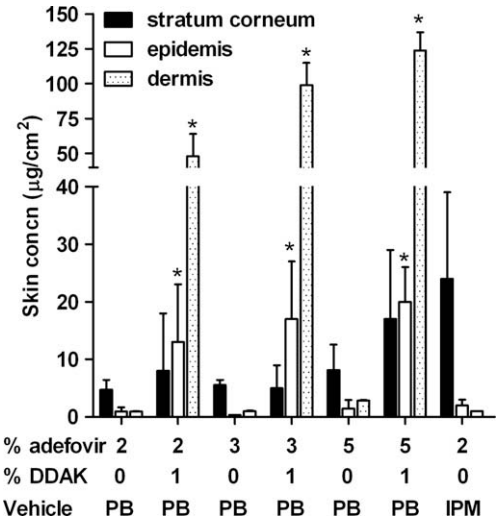

Fig. 7. The amount of adefovir in the human skin layers – the stratum corneum, viable epidermis and dermis. Mean ± SD, n = 3–4 (2 donors), except for 2% adefovir samples, where n = 12 (6 donors). * Indicates significant difference between the value with or without DDAK.

centrations would be lower. Nevertheless, the addition of 1% DDAK into the donor sample resulted in a significantly higher adefovir penetration into the viable skin layers (Fig. 7). The adefovir concentration in the epidermis expressed in μg per gram of the tissue was 822–3060 μg/g and 488–1466 μg/g in the epidermis and dermis, respectively, from 2% to 5% adefovir donor samples with 1% DDAK. The adefovir amount absorbed in the skin increased with increasing concentration of the drug applied on the skin, as in the porcine skin.

4. Discussion

The aim of this work was to study the effect of a transdermal permeation enhancer DDAK on adefovir delivery through and into the skin. For the initial in vitro experiments, porcine skin was selected because it is easy to obtain and its permeability is close to the human skin [20,21].

Our hypothesis that DDAK will enhance both the flux of adefovir and its absorption into deeper skin layers was fully confirmed. Importantly, the highest adefovir flux values were more than twice higher than those reached with the permeation enhancer T12 in our previous study [7]. The action of these structurally related enhancers differed also in their pH dependence. While T12 was unable to enhance adefovir flux at neutral and basic pH [7], DDAK increased adefovir flux at all the evaluated pH values thereby allowing for an appropriate formulation adjustment. The maximal delivery of adefovir both through and into the skin was achieved at pH around 6, which also contrasts with T12, where the highest activity was observed at pH 4. Although the reasons for these differences are unknown (they may be connected with the difference in basicity and steric hindrance of the nitrogen), DDAK was clearly superior in enhancing adefovir flux through the porcine skin.

The reason for the exceptional permeation-enhancing activity of DDAK was investigated further using the porcine skin. Generally, an enhancer may act by a direct influence of the skin barrier structures, preferably a reversible one, or by an indirect influence of the drug behavior in the vehicle [5,22]. According to these results, the indirect effects, both on the adefovir solubility and on its stratum corneum/vehicle distribution coefficient, contribute to the permeation-enhancing action of DDAK, at least at around pH 6, i.e. its maximal activity. When considering the chemical structure of adefovir and DDAK and their ionization constants, the formation of an ion pair may be a reasonable explanation of the observed behavior, including the decreased aqueous solubility, increased distribution coefficient and greater flux through the lipophilic skin barrier. These species, where ions are in close proximity and their charges are masked or shielded by the low dielectric constant of hydrocarbon moieties of the functional groups, display a lower hydrophilicity than the two ions considered separately and offer unusual behavior for an ionic species, such as high solubility in apolar solvents or increased partition toward a lipid phase [23]. The formation of ion pairs is also known to increase skin penetration; see Sarveiya [24] and references therein. On the other hand, this could hardly explain the DDAK activity at lower pH where adefovir is in the form of a zwitterion.

In order to find whether the interactions between DDAK and adefovir in the vehicle are the only determinants of the DDAK enhancing activity at pH 5.8, the skin was pretreated with the enhancer. Because the enhancer retained a part of its activity under this application protocol, we may conclude that DDAK acts by the direct influence of the skin barrier as well. This assumption is supported by the DDAK ability to enhance the skin permeation of other drugs, which are not capable of ion-pairing with DDAK (unpublished results). Moreover, the donor sample containing 2% adefovir (73 mmol) and 1% DDAK (30 mmol), i.e. theoretically 30 mmol of the potential ion pair, produced almost the same flux as the sample containing 2% adefovir and 2% DDAK, i.e. with double (60 mmol) concentration of the potential ion pair. The formation of an ion pair may be inhibited due to high dielectric constant of the donor solution. Other studies on the behavior of this permeation enhancer are in progress.

Regarding the enhancer concentration, 1% was sufficient to reach both the maximal adefovir flux and its accumulation in the skin, which is advantageous as the systemic exposure of DDAK would be low. Such low concentrations suggest a mechanism of action involving the perturbation of the stratum corneum intercellular lipids. The flux of adefovir without the enhancer increased with increasing adefovir concentration applied on the skin in accordance with the increasing thermodynamic activity of the drug in the aqueous solution. The same was true for the adefovir skin absorption with or without DDAK. On the other hand, the flux of adefovir with 1% DDAK reached its maximum at 2% adefovir in the donor vehicle. This finding was rather surprising as the sink conditions were maintained in all the experiments. Thus, the concentration dependence was evaluated again using the human skin with 2%, 3% and 5% adefovir but yielded essentially the same result.

Although there is a substantial similarity between the human and porcine skin, the effect of permeation enhancers may differ [11,20]. Thus, we evaluated the most promising donor samples using freshly excised human skin obtained from patients who had undergone plastic surgery. The receptor buffer was changed to maintain the skin viability as high as possible. The HEPES-buffered Hanks balanced salt solution with gentamicin was selected as its use has been well documented in prolonging the viability of skin post-excision [16].

The adefovir flux without the enhancer through the human skin was an order of magnitude lower than that through the frozen-and-thawed porcine skin. The lower flux was accompanied with higher lag times, due to which the experiment had to be prolonged to 72 h to reach the pseudo steady state. Interestingly, although the adefovir flux through the human skin was lower, the effect of the enhancer DDAK was more than four times higher (ER = 179) in the human skin than in the porcine one (ER = 42). This observation suggests that the skin barrier toward penetration of such a hydrophilic substance like adefovir is stronger but more susceptible to the action of DDAK in viable human skin. As DDAK is an amphiphilic enhancer possessing a hydrophilic polar head and a

lipophilic tail, it is likely to insert into the stratum corneum lipid layers and fluidize them. Hence, the more rigid and less permeable these lipid lamellae are, the higher relative enhancement may be caused by the insertion of an enhancer. However, the detailed mechanism of action of DDAK within the stratum corneum remains to be elucidated. Apart from the difference in the absolute enhancement values, the concentration–effect relationship seems to be the same, supporting the utility of the porcine skin in initial screening of the permeation enhancers.

Regarding the question whether the flux of 8.9 μg/cm^2/h may lead to clinically relevant plasma concentrations, we can estimate the following: After an oral administration of single doses of adefovir dipivoxil 10 mg to chronic hepatitis B patients or healthy subjects, the maximum observed adefovir concentrations in plasma occur at a median 0.76–1.75 h following dosing, with mean values ranging from 17.5 to 21.3 ng/ml. Over 90% of the dose is eliminated during 24 h with the mean area under the curve ($AUC_{0-\infty}$) ranging from 178 to 210 ng/h/ml. The oral bioavailability has been estimated to be approximately 60% [2]. The steady state plasma concentration, c_{SS}, achieved after transdermal administration can be calculated using the equation $J^* A = c_{SS}{}^* CL$, where the flux of the drug J through the patch area A is proportional to c_{SS} and clearance CL. By substitution of the values $J = 8.9$ μg/cm^2/h, $A = 30$ cm^2, CL ≈ 200 ml/h/kg [25] and 80 kg weight we can estimate the $c_{SS} \approx 17$ ng/ml, which is comparable even to the peak plasma concentration after oral administration. Thus, using the permeation enhancer DDAK, clinically relevant steady plasma levels following transdermal administration of adefovir could be achieved. Thus, the transdermal route of administration offers an interesting alternative to the oral adefovir prodrug and merits further evaluation using suitable in vivo pharmacokinetics model.

We have also evaluated the effect of DDAK on the retention of adefovir in the skin. In the initial experiments using the porcine skin, only the overall concentration was determined. Subsequently, selected donor samples were studied in more detail using the human skin. Without an enhancer, adefovir was concentrated mainly in the stratum corneum, and its penetration into the viable epidermis and dermis was low. Nevertheless, the addition of 1% DDAK to 2% adefovir solution at pH 5.8 resulted in a 33 and 61 times higher adefovir concentration in the viable epidermis and dermis, respectively. Although the assumption that adefovir can be used in local treatment of various skin diseases is rather theoretical, the strong penetration-enhancing activity of DDAK strongly encourages further studies on both adefovir and other acyclic nucleoside phosphonates.

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