Supporting Information

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SI Mechanism of Penetration of Avicins

The permeation of avicins (*Av*s; *Av*G and *Av*D) cannot be explained based on classic mechanisms and models. The predicted permeability of *Av*s based on the equation of Potts and Guy (1) or related models (2) is negligible. Furthermore, the predicted value based on the hydrophilic pore pathways is between 10^{-4} cm/h and 10^{-5} cm/h (3), a number smaller than the experimentally observed value. In theory, it is possible that the measured permeabilities are influenced by free or labile iodide. However, this scenario is unlikely because the unreacted iodine, the most likely source of free iodine, is removed during the purification process. In addition, trace amounts of sodium iodide, if present in the donor, should have a negligible contribution to the measured permeability. Because 81% of the iodination on the *Av* molecule occurs on the hydrocarbon residues (outer monoterpene and triterpene core), the formation of labile iodine can be ruled out because the stratum corneum (SC) pH is acidic and the carbon–iodine bond is stable in acidic and neutral pH because it is not susceptible to nucleophilic attack. Because the proteases in skin are specific to esters of fatty acids, ceramides, and cholesterol, the formation of free or labile iodine due to enzymatic hydrolysis of *Av*s in the SC can be ruled out.

The precise origin of the high permeability of *Av*s requires further investigation. The triterpene core is small and highly hydrophobic. Thus, its permeation in the absence of any saccharides would be very high (estimated permeability on the order of 10^{-3} cm/h). At the same time, the saccharide residues, especially the glucosamines, have been shown to possess peculiar skin permeation properties on their own. Specifically, glucosamine sulfate has been shown to permeate the skin at a rate higher than that expected based on its hydrophilic nature (4). Conjugates of ibuprofen and ketoprofen with *N*-acetyl glucosamine (NAG) have been proposed for transdermal delivery of these drugs (5). Hyaluronic acid, a polymer of glucuronic acid and NAG , has been demonstrated to penetrate the skin and enhance the penetration of various molecules, including growth hormones into skin (6). Hyaluronan-based formulations have been used for topical delivery of drugs (7) and have been shown to enhance partitioning of drugs, such as diclofenac, into skin. It is possible that a combination of a highly hydrophobic and permeable core, such as the triterpene, connected with saccharide units capable of permeating the skin on their own could be responsible for the unusual permeation properties of *Av*s. Studies performed with *Av* fragments are consistent with this hypothesis (Table 3).

SI Materials and Methods

Octanol-Water Partitioning Coefficient Studies. To determine the octanol-water partition coefficient ($K_{o/w}$), *AvD* was used as a model system. Because *Av*D and *Av*G only differ by a single oxygen atom at the monoterpene residue, their $K_{o/w}$ values are expected to be comparable. One milliliter of ^{125}I-*Av*D in PBS (1 mg/mL) was transferred to a glass vial to which 1 mL of 1-octanol was added. The system was then vortexed vigorously for 5 min to ensure thorough mixing of the two phases. The ensuing milky white emulsion was allowed to separate into the two constituent phases over a 24-h period following which 100 μL of each phase was carefully removed using a micropipette and transferred to a scintillation vial filled with 5 mL of scintillation mixture and then counted in a gamma counter. The $K_{o/w}$ was then determined as the ratio of [*Av*D in octanol]/[*Av*D in water].

SC-Water Partitioning Coefficient Studies. The SC skin from the pig ear was separated from the epidermis by the heat-stripping method. Skin from the pig ear was used for this study because the SC is much more easily removed in comparison to skin from the abdominal region. Briefly, the full-thickness skin from the pig ear was isolated as described earlier and then immersed in a water batch set at 70 °C for 2 min. Following this, the SC was separated from the epidermis by gently nudging it using a spatula. The isolated SC was then carefully lifted onto a blotting paper for drying. The dried SC was then carefully weighed using a microbalance (SC mass ranged from 44–52 mg) until a steady weight was attained and was then transferred into a vial containing 1.5 mL of ^{125}I-labeled *Av*D in PBS (1 mg/mL) and incubated for 24 h. As in the measurement of $K_{o/w}$, these studies were carried out using only *Av*D, because *Av*D and *Av*G differ by only a single oxygen atom at the monoterpene residue; therefore, their lipophilicity should be comparable. Following this period, the SC was carefully removed and washed two times in 1 mL of PBS by gentle immersion and swirling to remove excess liquid and was then placed in a 10-mL scintillation vial filled with 5 mL of scintillation mixture and counted in a Perkin–Elmer gamma counter. The washes were then pooled with the original solution in contact with the SC and counted as described above. The SC/water partition coefficient ($K_{SC/w}$) was then calculated assuming a density of 1 g/cm^3 for the SC and the weight of the SC before the start of the experiment. The $K_{SC/w}$ was then determined as follows:

$$K_{SC/w} = \frac{[Concentration\ \ AvD\ \ in\ \ Skin]}{[Concentration\ \ AvD\ \ in\ \ Solution]}. \qquad \textbf{[S1]}$$

Determination of the Amount of AvD in the Skin Under Conditions in Which the Donor Concentration Is Held Constant. Because *Av*s exhibit high affinity to skin lipids, the concentration of *Av*s in full-thickness skin at steady state was determined using skin from pig ear. A 1-inch × 1-inch piece of skin was mounted in a side-side diffusion cell; the donor compartment was filled with 1.5 mL of ^{125}I-labeled *Av*D in PBS (1 mg/mL), and the receiver compartment was filled with 1.5 mL of PBS. Throughout the 24-h period, the donor concentration was held constant at 1 mg/mL by periodically replacing the entire donor volume at all time points [t =8 h, which is around lag phase and after the lag phase (t = 9, 12, 15, 18, 21)]. The establishment of stable donor concentrations over t9–t24 was confirmed by measuring the radioactivity of the donor compartment before and after sampling at a given time point. At 24 h, the donor and receiver compartment were removed and fresh PBS was added to rinse off any loosely associated residual radioactivity. The skin was then allowed to dry overnight under ambient conditions, following which it was removed and counted in a gamma counter. The experiment was carried out using biological triplicates (n = 3). The fraction of *Av*s from the donor compartment sequestered in skin at steady state was then calculated as follows:

$$\chi_{Avs,\ Skin} = \left(\frac{Mass\ \ of\ \ AvD\ \ in\ \ Skin\ \ at\ \ t24}{Mass\ \ in\ \ Donor\ \ at\ \ t0\ \ - Mass\ \ of\ \ AvD\ \ in\ \ Skin\ \ at\ \ t24}\right). \qquad \textbf{[S2]}$$

The concentration of *Av*s in the donor compartment during steady state (C_{ss}) was then calculated as follows:

$$C_{ss} = C \times \left(1 - \chi_{Avs,\,Skin}\right). \qquad \textbf{[S3]}$$

Partitioning of Estradiol in Presence of Avs. Partitioning of ^{3}H-estradiol from water into isopropylmyristate (IPM) was determined in the presence of increasing amounts of *Av*-rich extract F094 (1 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL). One milliliter of IPM and 1 mL of ^{3}H-estradiol (10 mg/mL) solution in water or *Av*s enriched with ddH_2O were added to a microcentrifuge tube, and the phases were well mixed together by vigorous vortexing for 10 min and then allowed to equilibrate for 12 h. The samples were then centrifuged for 6 min at $125 \times g$ to thoroughly separate the phases (top phase, IPM; bottom phase, water). The concentration of ^{3}H-estradiol in the respective phases was determined by scintillation counting of the water phase and the IPM phase separately, and the IPM/water partition coefficient was calculated as the ratio of the radioactivity in the IPM phase over the water phase.

1. Potts RO, Guy RH (1992) Predicting skin permeability. *Pharm Res* 9(5):663–669.
2. Mitragotri S (2002) A theoretical analysis of permeation of small hydrophobic solutes across the stratum corneum based on Scaled Particle Theory. *J Pharm Sci* 91(3):744–752.
3. Mitragotri S (2003) Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. *J Control Release* 86(1):69–92.
4. Kanwischer M, et al. (2005) Evaluation of the physicochemical stability and skin permeation of glucosamine sulfate. *Drug Dev Ind Pharm* 31(1):91–97.
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6. Yang JA, et al. (2012) Transdermal delivery of hyaluronic acid–human growth hormone conjugate. *Biomaterials* 33(25):5947–5954.
7. Brown MB, Jones SA (2005) Hyaluronic acid: A unique topical vehicle for the localized delivery of drugs to the skin. *J Eur Acad Dermatol Venereol* 19(3):308–318.

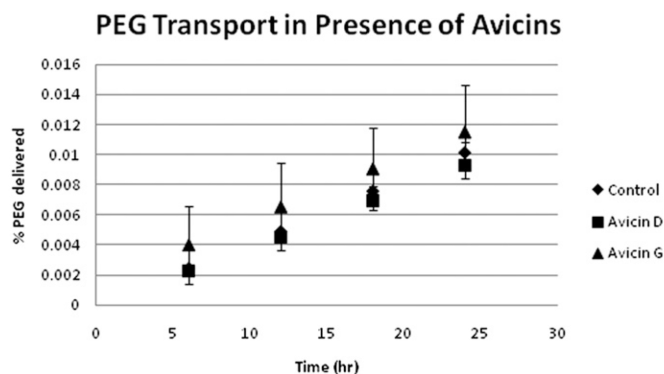

Fig. S1. FITC-polyethylene glycol (PEG) transdermal transport from *Av* formulations. The average ± SD is reported. Comparisons between control PEG transport and PEG + *Av*D or PEG + *Av*G are not statistically different ($P > 0.1$).

Table S1. Thermal transitions of stripped human SC before and after exposure to *Av*s were measured

Human skin transition*	Transition temperatures of untreated skin, °C	Transition temperatures of *Av*-treated skin, °C
I	44–48	44–48
II	52–57	Not observed
III	65–68	65–68
IV	72–78	72–78
—	—	90–95

Note that after interaction with *Av*s, the SC lipid transitions between 52 °C and 57 °C are no longer observed; instead, a new transition in the range of 90–95 °C emerges, which may be attributed to regions of SC lipids where *Av*s could have intercalated and accumulated. The human skin transition was classified according to the methods of Al-Saidan et al. (1) and Gay et al. (2).

1. Al-Saidan SM, Barry BW, Williams AC (1998) Differential scanning calorimetry of human and animal stratum corneum membranes. *Int J Pharm* 168(1):17–22.
2. Gay CL, Guy RH, Golden GM, Mak VH, Francoeur ML (1994) Characterization of low-temperature (i.e., < 65 degrees C) lipid transitions in human stratum corneum. *J Invest Dermatol* 103 (2):233–239.

Table S2. ***Av*** **transport across full-thickness pig skin in the presence of** ***Av*****-rich extract F094 (*n* = 9)**

Donor compartment	Permeability $\times 10^{-3}$, cm/h	Lag time, h	Enhancement factor
Av D	1.67 ± 0.48	8.63 ± 1.16	NA
*Av*D + 4 mg/mL *Av*-rich extract	1.99 ± 0.24	8.41 ± 0.66	1.19 ± 0.09
*Av*D + 8 mg/mL *Av*-rich extract	2.57 ± 0.94	7.74 ± 0.59	1.55 ± 0.13
*Av*D + 16 mg/mL *Av*-rich extract	2.98 ± 0.42*	7.31 ± 1.41	1.79 ± 0.20
Av G	1.66 ± 0.73	10.31 ± 0.61	NA
*Av*G + 4 mg/mL *Av*-rich extract	2.21 ± 0.63	10.88 ± 0.55	1.34 ± 0.10
*Av*G + 8 mg/mL *Av*-rich extract	3.07 ± 0.86*	10.45 ± 0.42	1.86 ± 0.31
*Av*G + 16 mg/mL *Av*-rich extract	2.69 ± 0.44*	10.22 ± 1.01	1.62 ± 0.23

^{125}I-labeled *Av*s were used for the transport studies. The enhancement factor is a comparative measure within each experiment and is the ratio of the permeability of the *Av*s at higher concentrations to the permeability of *Av*s at 1 mg/mL. The average ± SD is reported, with statistically significant values denoted. NA, not applicable. **P* < 0.1.

Table S3. Partitioning of estradiol from water into IPM in presence of ***Av*****-rich extract F094**

Concentration of *Av*-rich extract F094, mg/mL	Estradiol $K_{IPM/w}$ (± SD)	Enhancement in $K_{IPM/w}$ in the presence of F094, %
0	1.91 ± 0.42	NA
1	1.99 ± 0.14	5
5	3.31 ± 0.22	74
10	1.82 ± 0.92	−5
20	1.68 ± 0.30	−12

$K_{IPM/w}$, partitioning of estradiol from water into IPM; NA, not applicable.