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UNIVERSITÉ DE GENÈVE
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Modulating physicochemical properties of drugs to optimise topical delivery: Novel prodrugs of aciclovir to improve its cutaneous and ocular bioavailability using iontophoresis or supersaturated formulations

THÈSE

Présentée à la Faculté des Sciences de l'Université de Genève pour obtenir le grade de Docteur ès sciences, mention sciences pharmaceutiques

Par

Yong CHEN

De

Shijiazhuang (Chine)

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Thèse de ***Monsieur Yong CHEN***

intitulée :

"Modulating Physiochemical Properties of Drugs to Optimise Topical Delivery: Novel Prodrugs of Aciclovir to Improve its Cutaneous and Ocular Bioavailability Using Iontophoresis or Supersaturated Formulations"

La Faculté des sciences, sur le préavis de Monsieur Y. N. KALIA, professeur associé et directeur de thèse (Section des sciences pharmaceutiques), Monsieur L. SCAPOZZA, professeur ordinaire et co-directeur de thèse (Section des sciences pharmaceutiques), Monsieur N. LANGE, professeur associé (Section des sciences pharmaceutiques), Madame A. LÓPEZ CASTELLANO, professeure (CEU Universidad Cardinal Herrera, Departamento Farmacia - Seminario Salud, Moncada, Spain), Madame S. NICOLI, professeure (University of Parma, Department of Pharmacy, Parma, Italy), et Monsieur G. SCHWACH, docteur (F. Hoffmann-La Roche Ltd, Pharmaceuticals division, Basel, Switzerland), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 19 mars 2015

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N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

博學之，審問之，慎思之，明辨之，篤行之。

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Learn extensively, inquire thoroughly,

ponder carefully, distinguish clearly and practice devotedly.

«Book of Rites • Doctrine of the Mean»

You never get a second chance to make a good first impression.

Will Rogers (1879-1935)

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RÉSUMÉ

Une pharmacothérapie efficace repose sur la capacité à atteindre la concentration thérapeutique en principe actif dans le compartiment cible au moment approprié. Cela dépend de la vitesse d'administration, de la pharmacocinétique, des propriétés physicochimiques et de la puissance du principe actif. Un effort considérable est fourni dans la modulation des groupes substituants du principe actif ainsi, de sa structure moléculaire et ses propriétés chimiques dans le but d'améliorer ses propriétés pharmacologiques. Une modification chimique du principe actif peut également être utile en vue d'une administration particulière. C'est le cas des pro-drogues, où la molécule "optimisée pour son transport" va subir une biotransformation dans le corps pour libérer la substance pharmacologiquement active. Dans le cadre de ce projet, il a été décidé d'investiguer comment différentes pro-drogues peuvent améliorer l'administration topique de l'aciclovir (ACV) à travers une administration passive et active, électriquement-assistée au sein et à travers différentes barrières biologiques.

Le traitement topique des infections cutanées à herpès, (en particulier, l'herpès labial (*herpes labialis*)) constituerait une approche ciblée pour la thérapie, en diminuant les doses circulant dans le corps et en réduisant les risques d'insuffisance rénale. Cependant, dans ce cas l'efficacité clinique est faible et seulement peu de bénéfices ont été observés à la suite d'un traitement topique à l'ACV sous forme de crème ou d'onguent (5%) en comparaison au placebo du fait d'une pénétration réduite à travers la peau. La thérapie ophtalmique basée sur l'ACV est n'est guère meilleure et n'est efficace que sur des infections àvirus de l'herpès simplex (HSV) sur la partie superficielle de la cornée. Tandis que ce traitement est très peu utile lors d'infections du tissu profond de la cornée ou du segment postérieur.

L'administration cutanée de l'ACV, molécule polaire, faiblement soluble dans l'eau et dans l'huile, constitue un défi, du fait de la composition multilamellaire lipidique du stratum corneum qui impose une formidable barrière à la diffusion passive de l'ACV. De même, pour traiter des infections profondes de la cornée, l'ACV doit également franchir les jonctions épithéliales serrées riches en lipides. Bien que la sclére soit beaucoup plus perméable que la cornée, une clearance (élimination) rapide de la formulation rend quasi impossible

l'administration topique de l'ACV dans le segment postérieur de l'œil, vu que la vitesse de l'administration et les quantités administrée sont insuffisantes.

L'iontophorèse locale est un puissant outil pour l'administration de molécules hydrosolubles et chargées à travers des barrières biologiques telles que la peau. Cependant, étant donné que l'ACV est non chargé (pKa_1 2.27; pKa_2 9.25) à pH physiologiquement acceptable et avec sa mauvaise solubilité dans l'eau, ~ 0.2% à 25°C, il est loin d'être un candidat idéal pour iontophorèse. Toutefois, l'administration cutanée de quantités thérapeutiques d'ACV par iontophorèse a été rapportée dans la littérature mais pour des formulations à pH 3 ou 11. De manière similaire, le ganciclovir, un analogue de l'ACV, a été administré par iontophorèse à travers la membrane sclérale en utilisant son sel sodique dans une formulation à pH 11. Ces valeurs de pH sont au-delà du domaine physiologique acceptable et sont certainement irritants pour la peau ou la surface oculaire

Le but premier de cette thèse était d'étudier la possibilité de modifier les propriétés physicochimiques de l'ACV pour le rendre plus adapté à une application cutanée par iontophorèse. Les Chapitres 1 et 2 montrent que l'iontophorèse de courte durée de pro-drogues de l'ACV, étant des dérivés de type ester d'acide aminés soluble dans l'eau et ionisable, a permis d'atteindre rapidement des quantités d'ACV supra-thérapeutiques dans l'épiderme basal et pourrait permettre une approche efficace pour le traitement des infections récurrentes à HSV. Le deuxième but de cette thèse consistait à transférer le savoir-faire acquis lors de l'iontophorèse cutanée de l'ACV à une iontophorèse oculaire, où une courte durée de l'iontophorèse n'était pas uniquement capable d'administrer des quantités plus importantes d'ACV lors de perméation transcornéene et de déposition cornéenne, mais a aussi permis d'augmenter la vitesse et l'étendue de l'administration transsclérale de l'ACV en comparaison à une diffusion passive, comme le décrit le Chapitre 3. Bien que l'iontophorèse fût particulièrement efficace pour l'administration des pro-drogues à travers la peau et l'œil, une approche complémentaire est abordée au Chapitre 4. L'utilisation d'une pro-drogue lipophile de l'ACV a été investiguée dans le but de l'inclure dans des formulations supersaturées afin d'augmenter l'administration d'ACV. Cette étude s'est conclue par des résultats prometteurs.

Le Chapitre 1 décrit l'étude de l'administration iontophorétique topique d'une série de pro-drogues de type ester d'acide aminé de l'ACV comme moyen d'augmenter sa déposition dans la peau. L'objectif était d'investiguer l'administration iontophorétique topique d'une série de pro-drogues de type ester d'acide aminé de l'ACV (ACV-X, où ACV = aciclovir et X = Arg, Gly, Ile, Phe, Trp et Val) comme moyen d'augmenter l'administration cutanée, et plus particulièrement l'administration intraépidermique de l'ACV. Les pro-drogues nouvellement synthétisées ont été caractérisé par ^{1}H NMR et spectrométrie de masse à haute résolution. Des méthodes d'HPLC-UV ont été développées et validées pour la quantification de ces dernières. L'investigation de la stabilité en solution en fonction du pH ont montré que toutes les pro-drogues étaient relativement stables à pH 2.0 et pH 5.5 sur une durée de 8h mais étaient susceptibles à une hydrolyse importante à pH 7.4 et sous conditions alcalines (pH=10). Le transport passif de l'ACV et de ses pro-drogues (ACV-X) de solutions aqueuses (pH 5.5) durant 2h se situait en dessous de la limite de détection. L'iontophorèse de l'ACV (pH 5.5) à 0.5 mA/cm^2 pendant 2 h s'est conclue par une modeste déposition d'ACV dans la peau ($Q_{DEP,ACV}$, 4.6 ± 0.3 nmol/cm^2) tandis que l'iontophorèse des pro-drogues ACV-X sous les mêmes conditions a produit une augmentation significative dans la déposition cutanée des espèces d'ACV c'est-à dire $Q_{DEP, TOTAL} = Q_{DEP, ACV} + Q_{DEP, ACV-X}$. $Q_{DEP, TOTAL}$ pour ACV-Gly, ACV-Val, ACV-Ile, ACV-Phe, ACV-Trp et ACV-Arg était 412.8 ± 44.0, 358.8 ± 66.8, 434.1 ± 68.2, 249.8 ± 81.4, 156.1 ± 76.3 et 785.9 ± 78.1 nmol/cm^2, respectivement. L'étendue de la bioconversion de l'ACV-X en ACV dans la peau était élevée, atteignant des proportions d'ACV se situant entre 81 et 100%. Le ratio de rétention dans la peau qui mesure la sélectivité des espèces d'ACV pour la déposition par rapport à la perméation après administration iontophorétique des pro-drogues d'ACV-X dépendait de la vitesse de transport et de la susceptibilité des pro-drogues à l'hydrolyse. La déposition dans la peau d'ACV, ACV-Arg et ACV-Ile a été investiguée plus en détail en fonction de la densité du courant (0.125, 0.25 et 0.5 mA/cm^2) et de la durée du courant appliqué (5, 10, 30, 60 et 120 min). L'iontophorèse d'ACV-Arg et ACV-Ile à 0.25 mA/cm^2 pendant seulement 5 min a entraîné le dépôt de quantités appréciables d'ACV (36.4 ± 5.7 nmol/cm^2 et 40.3 ± 6.1 nmol/cm^2, respectivement), correspondant à des concentrations supra-thérapeutique contre HSV-1 ou -2 dans la peau. Les résultats démontrent que la biodisponibilité cutanée de l'ACV pourrait être significativement améliorée après l'iontophorèse de courte durée de pro-drogues ionisables et biolabiles ACV-X.

Le chapitre 2 compare la biodisponibilité cutanée après application topique de formulations commerciales d'aciclovir ou de penciclovir (PCV) à celle obtenue après iontophorèse de l'ACV ou de ses pro-drogues de type ester d'acide aminé. L'objectif était de déterminer la biodistribution cutanée de l'aciclovir, c'est à dire, la biodisponibilité en fonction de la profondeur dans la peau suite à l'administration iontophorétique de pro-drogues de l'ACV (ACV-X, où X = Arg, Ile ou Val). Les résultats ont été comparés à ceux obtenus avec des formulations commerciales d'aciclovir et de penciclovir, et à la suite d'iontophorèse l'ACV non modifié. La quantification des espèces moléculaires en fonction de la position dans la peau a été réalisée par congélation et réalisation de coupes fines au cryotome des échantillons de peau de façon à obtenir de fines lamelles de 100 um ou 20 um d'une épaisseur. Les molécules - ACV, ACV-X ou PCV - ont été extraites et quantifiées par UHPLC-MS/MS. L'administration passive de l'ACV ou du PCV après une application de 60 minutes à partir de crème et de pommade commerciales a donné lieu à une déposition cutanée modeste (le $Q_{DEP, ACV}$ et le $Q_{DEP, PCV} < 2$ nmol/cm^2). En outre, l'ACV et le PCV ont été trouvés principalement dans la couche cornée ou l'épiderme viable superficiel. Les niveaux de ces molécules dans les couches profondes de la peau (100 à 200 um), qui correspondent à l'épiderme basal où le virus se trouve, étaient négligeables. En revanche, l'iontophorèse de l'ACV-Ile ou ACV-Arg pendant seulement 10 min à 0.25 mA/cm^2 a occasionné une déposition d'espèces ACV à ces mêmes profondeurs de la peau (100-200 um): pour l'ACV-Ile – le $Q_{DEP, ACV}$ et le $Q_{DEP, ACV-Ile}$ étaient de 17.2 ± 6,9 nmol/cm^2 et de 8.2 ± 1.3 nmol/cm^2, respectivement; l'ACV-Arg était bio-converti complètement et seulement l'ACV à été récupéré (le $Q_{DEP, ACV}$ était de 41.2 ± 9.2 nmol/cm^2). Dans les études de biodistribution à haute résolution (lamelles de 20 um) une iontophorèse de courte durée (seulement 5 min) à 0.25 mA/cm^2 de l'ACV-Ile ou ACV-Arg a permis une déposition de quantités considérables d'espèces ACV dans l'épiderme basal et les couches voisines (100 à 160 um) (le $Q_{DEP, ACV}$ a été de 6.1 ± 1.7 nmol/cm^2 et de 4.0 ± 1.6 nmol/cm^2, respectivement). L'iontophorèse de l'ACV dans les mêmes conditions a donné lieu à une déposition négligeable dans ces mêmes couches. Les études ultérieures sur l'iontophorèse de l'ACV-Ile en utilisant un hydrogel ont révélé aucune différence statistiquement significative entre $Q_{DEP, TOTAL}$ sur peu porcine et humaine (13.4 ± 1.5 nmol/cm^2 et 12.4 ± 1.4 nmol/cm^2, respectivement). Afin d'améliorer la stabilité de l'ACV-Ile, de fins films secs à dissolution rapide ont été préparés. Ceux-ci ont été placés en contact avec des hydrogels conducteurs in situ juste avant l'iontophorèse. En plus de la stabilité améliorée, l'administration iontophorétique de l'ACV a été également supérieure à celle de l'hydrogel seul. Après iontophorèse d'ACV-Ile en utilisant des films à dissolution rapide, le $Q_{DEP, ACV}$

était de 29.1 ± 1.0 nmol/cm^2, et la déposition dans la zone de l'épiderme basale était de 2.7 ± 0.1 nmol/cm^2. Une iontophorèse courte de 5 min d'ACV-Ile à partir de solution aqueuse, d'un hydrogel ou d'un film à dissolution rapide conduit à des concentrations supra-thérapeutique de l'ACV (supérieure à l'IC_{50} de l'ACV contre le virus de l'herpès simplex 1 ou 2) dans l'épiderme basal et les couches adjacentes.

Le chapitre 3 décrit l'étude de l'administration transcornéenne par iontophorèse transsclérale de trois pro-drogues esters d'acide aminé de l'aciclovir - ACV-X, où X = Arg, Gly et Trp afin d'augmenter la biodisponibilité oculaire de l'ACV. La quantification de l'ACV et de chaque pro-drogue ACV-X a été réalisée par des méthodes UHPLC-MS/MS validées. Les pro-drogues ACV-X étaient biolabiles en présence d'estérases dans les yeux porcins frais ; cela a été démontré en comparant les demi-vies (h) des pro-drogues de l'ACV-X dans les extraits de tissus oculaires avec du PBS. Lors de l'utilisation soit de Zovirax® pommade (3%) soit d'une solution saturée, l'administration passive de l'ACV dans la cornée porcine après 1 h d'application était inférieure à la LOQ. La déposition de la cornée et la perméation après l'iontophorèse de l'ACV pendant 5 min suivie par la diffusion passive pendant 55 min ont été de 3.4 ± 1.0 nmol/cm^2 et 1.9 ± 0.3 nmol/cm^2, respectivement. L'iontophorèse d'ACV-Arg, d'ACV-Gly ou de ACV-Trp à partir de solutions de 5 mM a entrainé une déposition dans la cornée beaucoup plus élevée (21.5 ± 5.1, 14.1 ± 2.0 ou 5.3 ± 0.6 nmol/cm^2, respectivement) et une perméation transcornéene des espèces d'ACV également plus élevée (13.9 ± 1.6, 10.9 ± 1.8 ou 5.7 ± 0,5 nmol/cm^2, respectivement). La perméabilité accrue de la sclére a été mise en évidence par le fait que l'administration transsclérale passive de l'ACV en utilisant Zovirax® pommade (3%) ou la solution saturée après 30 min (4.9 ± 0.9 et 40.7 ± 3,7 nmol/ cm^2, respectivement) était supérieure à la perméation transcornéene après 60 min (< LOQ). Ces différences ont été accentuées par l'iontophorèse. La perméation transsclérale de l'ACV-Arg, l'ACV-Gly et l'ACV-Trp après une application de seulement 5 min était de 20.4 ± 3.8, 12.3 ± 0.3 ou 8.4 ± 0.4 nmol/cm^2, respectivement. Après 30 min, cette dernière à été augmentée à 345.3 ± 36.7, 179.9 ± 9.7 or 144.1 ± 6.8 nmol/cm^2, respectivement. L' utilisation de yeux porcins intacts avec une iontophorèse transsclérale d'ACV-Gly à 3.75 mA/cm^2 de 5 min a non seulement entraîné une déposition considérable d'espèces ACV dans la choroïde / rétine et le vitré (5.7 ± 2.3 et 11.7 ± 3.7 nmol/ cm^2, respectivement) mais aussi une concentration des espèces d'ACV moyenne significativement plus élevé que l' IC_{50} de l'ACV contre HSV-1 (< 0.22 nmol / ml), dans l'ensemble du globe oculaire (4.5 ± 1.6 nmol/cm^3) ce qui est très

prometteur pour le traitement des infections à HSV dans le segment antérieur ou postérieur de l'œil.

Le chapitre 4 décrit une méthode pour améliorer l'administration topique locale passive de l'aciclovir. L'objectif était d'utiliser une série de pro-drogues lipophiles d'aciclovir en combinaison avec la supersaturation pour augmenter la biodisponibilité cutanée de l'aciclovir. L'acétate d'aciclovir, le butyrate et l'hexanoate (ACV-Y, où Y = Ace, But et Hex) ont été synthétisés et caractérisés. Des méthodes UHPLC-MS / MS ont été développées et validées pour leur quantification. La solubilité de l'ACV et de chaque pro-drogue (ACV-Y) a été déterminée dans des mélanges de propylène glycol (PG) d'eau de différentes proportions. L'ACV et l'ACV-Ace n'étaient pas de bons candidats pour la préparation de formulations sursaturées. L'ACV-But n'était en mesure de créer qu'un degré modéré de sursaturation (DS) jusqu'à ~ 3. En revanche l'ACV-Hex, avec sa plus grande solubilité dans le PG et sa plus faible solubilité dans l'eau, a été en mesure de créer des DS très élevés, ~ 12. L'ACV-Hex en solution de PG à 10% a été choisi comme la meilleure combinaison pour créer des formulations supersaturées. En l'absence d' HPMC, la déposition cutanée d'espèces ACV était semblable à partir de toutes les formulations supersaturées d'ACV-Hex et n'était pas statistiquement différente de la valeur à partir d'une solution simplement saturée (0.27 ± 0.06 nmol/cm^2). Lorsque l' HPMC a été ajouté aux formulations supersaturées de DS 3 et 4, une augmentation de la déposition cutanée d'ACV espèce a été observée (2.55 ± 0.64 nmol/cm^2 et 4.90 ± 1.58 nmol/cm^2, respectivement). L'administration passive de l'ACV après l'application de la crème Zovirax® pendant 1 h a entraîné une déposition cutanée limitée (1.29 ±0.79 nmol/cm^2), et l'ACV à été trouvé principalement dans la partie superficielle de la peau. En outre, les quantités trouvées dans les tissus plus profonds de la peau (100-160 um), correspondants à l'épiderme basal où le virus est détecté, étaient négligeables. La formulation supersaturée d'ACV-Hex (DS = 4) a non seulement permis d'administrer des quantités plus élevées d'espèces ACV dans les couches profondes de la peau, mais aussi à mené à des niveaux supra-thérapeutiques des espèces ACV dans la zone ciblée, l'épiderme basale (espèces ACV 0.40 ± 0.12 nmol/cm^2, incl. 0.08 ± 0.03 nmol/cm^2 de l'ACV). L'exposition à l'ACV (l'ACV-Hex non converti n'a pas été considéré) de la zone cible était de 13.33 ± 5.01 nmol/cm^3, ce qui était beaucoup plus élevé que la IC_{50} de l'ACV contre le HSV-1. Cette formulation pourrait donc être utile dans les traitements topiques des infections à HSV.

SUMMARY

Effective pharmacotherapy relies on being able to attain therapeutic drug concentrations in the target compartment within the appropriate time-frame. This depends on the delivery kinetics, pharmacokinetics, drug physicochemical properties and potency. The substituent groups, chemical structure and molecular properties of a drug candidate undergo considerable fine-tuning in an effort to optimise its pharmacological properties. However, chemical modification can also be used to make a drug a better candidate for use with a particular delivery technique. This can be done through the use of prodrugs where the "transport-optimised" molecule will undergo biotransformation in the body to release the pharmacologically active substance. In this project it was decided to investigate how different prodrugs could be used to improve the topical delivery of aciclovir (ACV) through passive and active, electrically-assisted delivery into and across different biological barriers.

Topical ACV treatment of cutaneous herpes infections (in particular, *herpes labialis*) would constitute a targeted approach to therapy, reduce circulating drug levels and attenuate the risk of renal insufficiency. However, clinical efficacy is poor and only little or benefits have been observed following topical treatment with ACV cream or ointment (5%) vs. placebo due to poor skin penetration. Ophthalmic ACV therapy fares little better and is only effective against superficial corneal herpes simplex virus (HSV) infections. In contrast it is of little use against infections in deep corneal tissue or in the posterior segment.

Cutaneous delivery of polar, poorly water/oil soluble ACV is challenging, as the multilamellar lipidic composition of the skin stratum corneum imposes a formidable barrier for passive delivery of ACV. Similarly, to treat deep corneal infections, ACV is also required to pass the lipidic, tight epithelial junctions. Although sclera is much more permeable than cornea, rapid ocular clearance of formulations makes topical delivery into the posterior segment almost impossible, as the extent and rate of transscleral ACV input is insufficient.

Local iontophoresis provides a powerful tool to deliver hydrosoluble, charged molecules across biological barriers, e.g., skin. However, given that ACV is uncharged (pK_{a1} 2.27; pK_{a2}

9.25) at physiologically acceptable pH and with its poor water solubility, ~0.2% at 25 °C, it is far from being an ideal candidate for iontophoresis. Nevertheless, cutaneous delivery of therapeutic amounts of ACV by iontophoresis has been reported, but with formulations at pH 3 or 11. Similarly, ganciclovir, an analogue of ACV, has been iontophoretically delivered through scleral membrane using its sodium salt form with a formulation at pH 11. These pH values are outside the physiologically acceptable range and almost certainly strongly irritating to the skin or ocular surface.

The first aim of this thesis was to study the possibility of modifying the physicochemical properties of ACV to make it more suitable for cutaneous iontophoresis of ACV. Chapter 1 and 2 gave a clear answer that short-duration iontophoresis of water-soluble, ionisable amino acid ester prodrugs of ACV enabled supra-therapeutic levels of ACV to be achieved rapidly in the basal epidermis and may provide an effective approach to treat recurrent HSV infections. The second aim of the thesis was to transfer the experience from cutaneous iontophoresis of ACV to ocular iontophoresis, where short-duration iontophoresis was not only able to create much greater levels of transcorneal permeation and corneal deposition of ACV species but also significantly increased the rate and extent of transscleral ACV delivery as compared to passive diffusion, as shown in Chapter 3. Although iontophoresis was very successful in delivering the amino acid ester prodrugs into the skin and eye, a complementary approach using lipophilic ACV prodrugs for inclusion in supersaturated formulations for enhanced cutaneous ACV delivery was also investigated and gave encouraging results, as described in Chapter 4.

Chapter 1 describeds an investigation of the topical iontophoretic delivery of a series of amino acid ester prodrugs of aciclovir (ACV-X, where ACV=aciclovir and X=Arg, Gly, Ile, Phe, Trp and Val) (ACV) as a means to enhance cutaneous, and more specifically, intraepidermal delivery of ACV. The newly synthesised amino acid ester prodrugs were characterised by ^{1}H NMR and high resolution mass spectrometry. HPLC-UV methods were developed for their quantification and validated. Investigation of solution stability as a function of pH showed that all ACV-X prodrugs were relatively stable at pH 2.0 and pH 5.5 for up to 8 h but susceptible to extensive hydrolysis at pH 7.4 and under alkaline conditions (pH 10). No ACV-X hydrolysis was observed after contact for 2 h with the external surface of porcine stratum

corneum; however, there was significant hydrolysis following contact with the dermal surface of dermatomed porcine skin, in particular, for ACV-Arg. Passive transport of ACV and ACV-X prodrugs from aqueous solution (pH 5.5) during 2 h was below the limit of detection. Iontophoresis of ACV (pH 5.5) at 0.5 mA/cm^2 for 2 h led to modest ACV skin deposition ($Q_{DEP,ACV}$), 4.6 ± 0.3 nmol/cm^2. In contrast, iontophoresis of ACV-X prodrugs under the same conditions produced order of magnitude increases in cutaneous deposition of ACV species, that is, $Q_{DEP,TOTAL} = Q_{DEP,ACV} + Q_{DEP,ACV-X}$. $Q_{DEP,TOTAL}$ for ACV-Gly, ACV-Val, ACV-Ile, ACV-Phe, ACV-Trp and ACV-Arg was 412.8 ± 44.0, 358.8 ± 66.8, 434.1 ± 68.2, 249.8 ± 81.4, 156.1 ± 76.3 and 785.9 ± 78.1 nmol/cm^2, respectively. The extent of bioconversion of ACV-X to ACV in the skin was high and the proportion of ACV ranged from 81% to 100%. The skin retention ratio, a measure of the selectivity of ACV species for deposition over permeation after iontophoretic delivery of ACV-X prodrugs, was dependent on both the rate of transport and the susceptibility to hydrolysis of the prodrugs. Skin deposition of ACV, ACV-Arg and ACV-Ile was investigated further as a function of current density (0.125, 0.25 and 0.5 mA/cm^2) and the duration of current application (5, 10, 30, 60 and 120 min). Iontophoresis of ACV-Arg and ACV-Ile at 0.25 mA/cm^2 for only 5 min resulted in the deposition of appreciable amounts of ACV (36.4 ± 5.7 nmol/cm^2 and 40.3 ± 6.1 nmol/cm^2, respectively), corresponding to supra-therapeutic average concentrations in skin against HSV-1 or -2. The results demonstrated that cutaneous bioavailability of ACV could be significantly improved after the short-duration iontophoresis of ionisable, biolabile ACV-X prodrugs.

Chapter 2 compares cutaneous bioavailability following topical application of marketed aciclovir or penciclovir (PCV) formulations to that achieved after iontophoresis of ACV or its amino acid ester prodrugs. The objective was to determine the cutaneous biodistribution of aciclovir, that is, the bioavailability as a function of depth within the skin following topical iontophoretic administration of amino acid ester prodrugs of ACV (ACV-X, where X=Arg, Ile or Val). The results were compared to those obtained with marketed formulations of aciclovir and penciclovir, and following topical iontophoresis of ACV. Quantification of molecular species as a function of position in the skin was achieved by snap-freezing and cryotoming skin samples to obtain coarse or fine lamellae with a thickness of either 100 µm or 20 µm. The molecules – ACV, ACV-X or PCV – were extracted and quantified by validated UHPLC-MS/MS methods. Passive delivery of ACV or PCV from marketed cream and ointment formulations after application for 60 min resulted in modest cutaneous

deposition ($Q_{DEP,ACV}$ and $Q_{DEP,PCV}$ <2 nmol/cm^2). Moreover, ACV and PCV were found mainly in the stratum corneum or superficial viable epidermis. The levels in the deeper skin layers (100-200 μm), corresponding to the basal epidermis and adjacent area, where the virus would be found, were negligible. In contrast, iontophoresis of ACV-Ile or ACV-Arg for only 10 min at 0.25 mA/cm^2 resulted in much greater deposition of ACV species at the same skin depths (100-200 μm): for ACV-Ile – $Q_{DEP,ACV}$ and $Q_{DEP,ACV\text{-}Ile}$ were 17.2 ± 6.9 nmol/cm^2 and 8.2 ± 1.3 nmol/cm^2, respectively; in the case of ACV-Arg there was complete bioconversion and only ACV was recovered ($Q_{DEP,ACV}$ was 41.2 ± 9.2 nmol/cm^2). In the higher resolution biodistribution studies with 20 μm lamellae, short duration iontophoresis at 0.25 mA/cm^2 for only 5 min of ACV-Ile or ACV-Arg still enabled considerable amounts of ACV species to be delivered to the basal epidermis and neighbouring layers (100-160 μm) ($Q_{DEP,ACV}$ was 6.1 ± 1.7 nmol/cm^2 and 4.0 ± 1.6 nmol/cm^2, respectively). Iontophoresis of ACV under the same conditions resulted in negligible ACV deposition in these layers. Subsequent studies into the iontophoresis of ACV-Ile using a hydrogel revealed no statistically significant differences between $Q_{DEP,\,TOTAL}$ in full-thickness porcine and human skins (13.4 ± 1.5 nmol/cm^2 and 12.4 ± 1.4 nmol/cm^2, respectively). In order to improve ACV-Ile stability, dry fast dissolving thin film formulations were prepared. These were placed in contact with conducting hydrogels in situ immediately prior to iontophoresis. Furthermore, in addition to the improved stability, iontophoretic delivery of ACV was also superior to that from hydrogel alone. Following iontophoresis of ACV-Ile using fast dissolving films, $Q_{DEP,ACV}$ was 29.1 ± 1.0 nmol/cm^2, and the amount in the basal epidermis area was 2.7 ± 0.1 nmol/cm^2. Short duration iontophoresis for 5 min of ACV-Ile from aqueous solution, hydrogel or fast dissolving film resulted in supra-therapeutic concentrations of ACV in the basal epidermis and its adjacent area, superior to the IC_{50} of ACV against herpes simplex virus 1 or 2.

Chapter 3 describes a successful investigation into the transcorneal and transscleral iontophoretic delivery of three amino acid ester prodrugs of aciclovir – ACV-X, where X = Arg, Gly and Trp and as a means to increase ocular bioavailability of ACV. Quantification of ACV and each ACV-X prodrug was by ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS); the methods were validated. ACV-X prodrugs were biolabile in the presence of esterases in fresh porcine eyes which was demonstrated by comparing the half-lives (h) of ACV-X prodrugs in the extract solution of ocular tissues with that in simple PBS solution. Passive delivery of ACV across porcine cornea after 1 h was

<LOQ when using either Zovirax® ointment (3%) or saturated solution. Corneal deposition and permeation after ACV iontophoresis for 5 min followed by passive delivery for 55 min were 3.4 ± 1.0 nmol/cm^2 and 1.9 ± 0.3 nmol/cm^2, respectively. Iontophoresis of 5 mM solutions of ACV-Arg, ACV-Gly or ACV-Trp resulted in much higher corneal deposition (21.5 ± 5.1, 14.1 ± 2.0 or 5.3 ± 0.6 nmol/cm^2, respectively) and transcorneal permeation (13.9 ± 1.6, 10.9 ± 1.8 or 5.7 ± 0.5 nmol/cm^2, respectively) of ACV species. The increased permeability of the sclera was evidenced by the observation that passive transscleral delivery of ACV using Zovirax® ointment (3%) or saturated solution after 30 min (4.9 ± 0.9 and 40.7 ± 3.7 nmol/cm^2, respectively) was superior to transcorneal permeation after 60 min (<LOQ). The differences were accentuated by iontophoresis. Transscleral permeation of ACV-Arg, ACV-Gly and ACV-Trp after current application for only 5 min was 20.4 ± 3.8, 12.3 ± 0.3 or 8.4 ± 0.4 nmol/cm^2, respectively. After 30 min, it had increased to 345.3 ± 36.7, 179.9 ± 9.7 or 144.1 ± 6.8 nmol/cm^2, respectively. Using intact porcine eyes, five-minute transscleral iontophoresis of ACV-Gly at 3.75 mA/cm^2 resulted in not only considerable amounts of ACV species in the choroid/retina and vitreous humour (5.7 ± 2.3 and 11.7 ± 3.7 nmol/cm^2, respectively) but also significantly higher average ACV species concentration in the whole eye globe (4.5 ± 1.6 nmol/cm^3) than the IC_{50} of ACV against HSV-1 (< 0.22 nmol/ml), which was very promising for the treatment of HSV infections in either anterior or posterior segments of the eye.

Chapter 4 describes a method to enhance topical passive topical delivery of aciclovir.The objective was to use a series of lipophilic aciclovir prodrugs in combination with supersaturation to increase cutaneous bioavailability of aciclovir. Aciclovir acetate, butyrate and hexanoate (ACV-Y, where Y=Ace, But and Hex) were synthesised, characterised, and UHPLC-MS/MS methods for their quantification developed and validated. Solubility of ACV and each ACV-Y prodrug as a function of the proportion of propylene glycol (PG) in cosolvent mixtures of PG and water was determined. ACV and ACV-Ace were not good candidates to prepare supersaturated formulations. ACV-But was only able to create a moderate degree of supersaturation (DS) up to ~ 3. ACV-Hex, with the highest solubility in PG and lowest solubility in water, was able to create very high DS, ~ 12. ACV-Hex in 10% PG solution was selected as the best combination for creating supersaturated formulations. In the absence of HPMC, cutaneous deposition of ACV species was similar at all supersaturated ACV-Hex formulations and was not statistically different from the value using saturated

ACV-Hex solution (0.27 ± 0.06 nmol/cm^2). When HPMC was added, supersaturation at DS of 3 and 4 led to increased cutaneous ACV species deposition (2.55 ± 0.64 nmol/cm^2 and 4.90 ± 1.58 nmol/cm^2, respectively). Passive delivery of ACV after application of Zovirax® cream for 1 h resulted in limited cutaneous deposition (1.29 ±0.79 nmol/cm^2), and ACV was principally found in the superficial part of skin. Moreover, levels in the deeper skin tissue (100-160 μm), corresponding to the basal epidermis and adjacent area where the virus is found, were negligible. Supersaturated ACV-Hex formulation (DS=4) not only enabled a higher percentage of ACV species to be delivered into the deeper skin layers, but also resulted in supra-therapeutic levels of ACV species to be achieved in the targeted area, the basal epidermis (ACV species 0.40 ± 0.12 nmol/cm^2, incl. 0.08 ± 0.03 nmol/cm^2 of ACV). ACV exposure (the unconverted ACV-Hex was not included) in the target area was 13.33 ± 5.01 nmol/cm^3, which was much higher than the IC_{50} of ACV against HSV-1, 0.04-3.1 nmol/cm^3. This formulation might be of value in the treatment of topical HSV infections.

INTRODUCTION

Herpes virus infections

Herpes simplex virus (HSV) is a member of the herpesviridae family, in which HSV-1 and HSV-2 are the most serious human pathogens [1]. HSV establishes latency in sensory ganglia following primary infection, causing herpes recurrences that may persist for life [2]. HSV-1 infection is usually acquired during childhood and adolescence, and is commonly characterized by oral or facial lesions, keratitis and encephalitis, whereas HSV-2 infection is one of the most prevalent sexually transmitted infections worldwide, causing genital mucosa damage [1,3]. Although topical HSV infection is self-limited in most cases, with the lesions usually healing within 7-10 days [4], many patients are suffering from very frequent HSV recurrences. In immunocompromised individuals, HSV infection may be fatal if the disease develops to severe brain or liver infection [5]. **Table 1** summarized the agents used for the treatment of topical or systemic HSV infections.

Therapy of topical HSV infections using aciclovir

Aciclovir (ACV, 9-[(2-hydroxyethoxy)methyl])-9H-guanine, *Zovirax*®), a guanosine analogue, is commonly used to treat topical HSV infections, e.g. *herpes labialis*, *herpes genitalis* and *herpes keratitis*. It is a very potent inhibitor of HSV-1, HSV-2 and varicella zoster virus. Its selectivity is also very high, which is due to the initial activation of ACV by phosphorylation by a herpes virus-specified thymidine kinase. Normal cells do not phosphorylate aciclovir to any significant degree. ACV monophosphate is subsequently converted to ACV triphosphate by host cell kinases, which is a more potent inhibitor of HSV DNA polymerases than cellular DNA polymerases [6]. Therefore, it is able to competitively prevent further viral DNA synthesis on one hand, it does not affect the normal cellular processes on the other hand.

In spite of the high potency and selectivity of ACV against HSV, the timing of ACV therapy is essential. Early initiation of ACV treatment can maximize its efficacy. Initiation of systemic ACV therapy within 24 h was found to prevent the establishment of viral latency and reduce the number of reactivable latent HSV [7]. Delay of ACV initiation by 1 day may result

in spread of herpetic lesions and prolong the course of disease [8]. Early ACV intervention and fast delivery is required.

Table 1 FDA approved therapeutic agents used for the treatment of HSV infections

Agents	**Route of administration**	**Main indications**
Aciclovir	Oral; Injection; Topical	Mucocutaneous HSV infection; Genital HSV infection
Penciclovir	Topical	Mucocutaneous HSV infection
Ganciclovir	Oral; Injection; Ophthalmic	HIV-infected host; CMV retinitis
Famciclovir	Oral	Herpes zoster in immunocompetent hosts; Recurrent genital HSV
Valaciclovir	Oral	Recurrent genital HSV; Herpes zoster in immunocompetent hosts
Valganciclovir	Oral	HIV-infected host; CMV retinitis
Trifluridine	Ophthalmic	Herpes simplex keratitis
Idoxuridine	Ophthalmic	Herpes simplex keratitis
Foscarnet	Injection	HIV-infected host; CMV retinitis

US FDA approved ACV dosage includes capsule/tablet/suspension for oral route, injectable for intravenous injection and cream/ointment for skin and lips. ACV ophthalmic cream is also commonly prescribed in Europe for the treatment of ocular HSV infections. Due to low oral

bioavailability (10-20 %), oral administration of ACV requires high daily strength, 800 mg 5 times a day at 4 hourly intervals for 7 days. This led to the development of valaciclovir (VCV, *Valtrex*®), its L-valyl ester prodrug, a substrate for the mammalian intestinal peptide transporters PEPT1 and PEPT2 [9]. Although this displays higher oral bioavailability (30-50%) [10], gram amounts of VCV have to be administered orally (1,000 mg three times a day). The risk of inducing inflammation or phlebitis at the site of injection [11] restricts injectable administration.

Topical ACV delivery to the basal epidermis where the virus is found would enable targeted therapy, reduce circulating drug levels and attenuate the risk of renal insufficiency [12]. Despite the therapeutic rationale and its highly specific activity against HSV-1 and HSV-2 in virus-infected cells, clinical studies of several topical ACV formulations gave mixed results. Therapeutic efficacy as measured by reducing healing time was reported with 5% ACV cream [13]; however, clinical failures were also seen [14]. ACV ointment (5%) seemed to have less or even no clinical benefit to skin infections [15]. Topical delivery of ACV into ocular anterior segment for the treatment of *herpes simplex keratitis* seems pretty straightforward, but it is only effective against superficial HSV infections, i.e. corneal *epithelial keratitis* [16], but not for deep infections, e.g., *herpes stromal keratitis* [17]. Poor penetration of ACV across lipidic stratum corneum or corneal epithelium led to the clinical inefficacy.

Iontophoresis

Iontophoresis technology has been successfully used in clinical medicine to achieve topical delivery of drugs in the past years. It is the application of a mild electric current on skin to enhance the delivery of water-soluble, ionized molecules [18]. By using an electrode of the same polarity as the charge on the molecule, the molecule is driven into the skin by the applied electric field. Although the use of iontophoresis has a long history, where the first proposal for the use of electric current in drug delivery date from the mid 18th century [19], the modern iontophoretic transdermal drug delivery started to achieve big success with the progress in the last decades in microelectronics and engineering processes from mid-1980s. More recently, the potential is being rediscovered for non-invasive transdermal systemic delivery of macromolecules, e.g., peptide, protein and nucleotides, which are normally

difficult to administer except by injections. Apart from its non-invasive property, it may provide increased delivery efficiency, faster onset of action, controllable manner of drug input and expanded range of actives that can be delivered via skin [18]. This technique has also been extensively investigated to deliver therapeutics into eye [20].

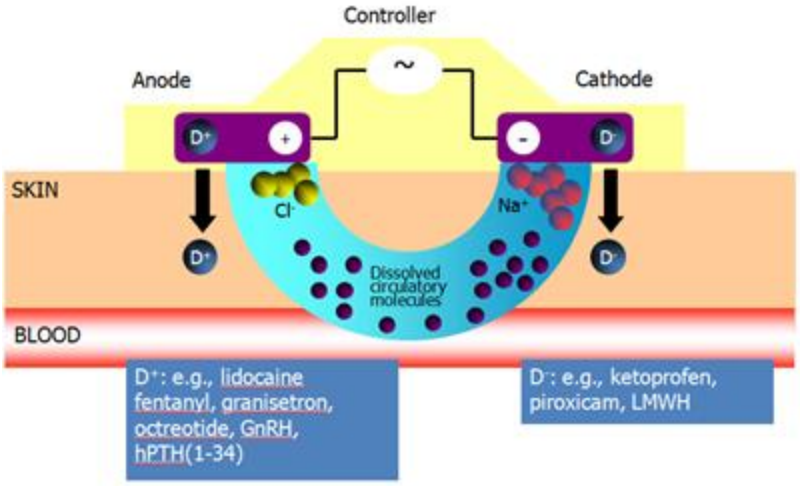

Figure 1. Schematic representation of iontophoresis

So far three iontophoretic systems are available on market-LidoS.ite™ (Vyteris Inc., Fairlawn, NJ, USA) (topical lidocaine delivery for local anesthesia), Ionsys™ (Alza Corporation, Mountain View, CA, USA) (systemic fentanyl for acute postoperative pain) and Zecuity™ (NuPathe Inc., Conshohocken, PA, USA) (systemic sumatriptan for migraine headaches). An US based company EyeGate Pharma is also developing a transscleral iontophoresis delivery system to deliver dexamethasone phosphate into the eye for the treatment of anterior uveitis.

The molecular transport during iontohoresis can be attributed to three component mechanisms: passive diffusion, electromigration (EM) and electroosmosis (EO). EM is the movement of the charged molecules in the presence of the applied electric field, whereas EO is the volume flow induced by the current flow [18]. EM is the principal transport mechanism. However, ACV (pK_{a1} 2.27; pK_{a2} 9.25) is uncharged under physiological conditions and with its poor water solubility, ~ 0.2 % at 25 °C, is far from being an ideal candidate for iontophoresis.

The prodrug strategy has been widely used to develop topical and transdermal formulations – most routinely in the case of corticosteroid esters. However, in the case of passive

administration, the objective is to increase molecular lipophilicity. Conversely, in the case of these “iontophoretic” prodrugs, the aim is to introduce polar ionisable moieties that increase hydrophilicity, aqueous solubility and most importantly, impart a charge to the molecule enabling it to benefit from iontophoresis. Once the charged prodrug is delivered into skin, the parent drug is released at or near the site of action relying on cutaneous esterase activity.

Objectives of the thesis

This thesis focuses on cutaneous and ocular delivery of ACV. In the first chapter, six water-soluble, charged amino acid ester prodrugs of ACV (ACV-X, where X=Gly, Val, Ile, Phe, Trp and Arg) were synthesized, characterized and their behaviour during cutaneous iontophoresis investigated. In the second chapter, two candidates (ACV-Arg and ACV-Ile) with the most appreciable skin delivery were subjected to the investigation of cutaneous biodistribution. ACV-Ile also formulated to hydrogel and fast dissolving film as ‘drug reservoir’ that could be integrated into an iontophoretic system. In the third chapter, the experience on cutanous ACV-X delivery using iontophoresis was transferred to ocular ACV delivery. In the fourth chapter, as a complementary strategy, lipophilic aliphatic acid ester prodrugs of ACV were synthesized and used in passive delivery into skin from supersaturated formulations.

The aims of this study were (i) to synthesise, purify and characterise prodrugs of ACV (ii) to develop and validate robust HPLC-UV or UPLC-MS/MS methods to quantify ACV and each prodrug simultaneously, (iii) to determine solution and enzymatic stability of the prodrugs, (iv) to compare passive and iontophoretic transport and optimise iontophoretic conditions and (v) to determine cutaneous or ocular bioavailability and biodistribution.

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Chapter 1

Synthesis, characterisation and topical iontophoretic delivery of ionisable, biolabile aciclovir prodrugs: a rational approach to improve cutaneous bioavailability

Synthesis, characterisation and topical iontophoretic delivery of ionisable, biolabile aciclovir prodrugs: a rational approach to improve cutaneous bioavailability

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ABSTRACT

The objective was to investigate the topical iontophoretic delivery of a series of amino acid ester prodrugs of aciclovir (ACV-X, where ACV=aciclovir and X=Arg, Gly, Ile, Phe, Trp and Val) (ACV) as a means to enhance cutaneous, and more specifically, intraepidermal delivery of ACV. The newly synthesised prodrugs were characterised by ^{1}H NMR and high resolution mass spectrometry. Analytical methods using HPLC-UV were developed for their quantification and validated. Investigation of solution stability as a function of pH showed that all ACV-X prodrugs were relatively stable at pH 2.0 and pH 5.5 for up to 8 h but susceptible to extensive hydrolysis at pH 7.4 and under alkaline conditions (pH 10). No ACV-X hydrolysis was observed after contact for 2 h with the external surface of porcine stratum corneum. However, there was significant hydrolysis following contact with the dermal surface of dermatomed porcine skin, in particular, for ACV-Arg. Passive transport of ACV and ACV-X prodrugs from aqueous solution after 2 h was below the limit of detection. Iontophoresis of ACV at 0.5 mA/cm^2 for 2 h led to modest ACV skin deposition ($Q_{DEP,ACV}$), 4.6 ± 0.3 nmol/cm^2. In contrast, iontophoresis of ACV-X prodrugs under the same conditions produced order of magnitude increases in cutaneous deposition of ACV species, that is, $Q_{DEP,TOTAL} = Q_{DEP,ACV} + Q_{DEP,ACV-X}$. $Q_{DEP,TOTAL}$ for ACV-Gly, ACV-Val, ACV-Ile, ACV-Phe, ACV-Trp and ACV-Arg was 412.8 ± 44.0, 358.8 ± 66.8, 434.1 ± 68.2, 249.8 ± 81.4, 156.1 ± 76.3 and 785.9 ± 78.1 nmol/cm^2, respectively. The extent of bioconversion of ACV-X to ACV in the skin was high and the proportion of ACV present ranged from 81% to 100%. The skin retention ratio, a measure of the selectivity of ACV species for deposition over permeation after iontophoretic delivery of ACV-X prodrugs, was dependent on both the rate of transport and the susceptibility to hydrolysis of the prodrugs. Skin deposition of ACV and ACV-Ile and ACV-Arg was investigated further as a function of current density (0.125, 0.25 and 0.5 mA/cm^2) and the duration of current application (5, 10, 30, 60 and 120 min). Iontophoresis of ACV-Arg and ACV-Ile at 0.25 mA/cm^2 for only 5 min resulted in the deposition of appreciable amounts of ACV (36.4± 5.7 nmol/cm^2 and 40.3 ± 6.1 nmol/cm^2, respectively), corresponding to supra-therapeutic average concentrations in skin against HSV-1 or -2. The results demonstrated that cutaneous bioavailability of ACV could be significantly improved after short-duration iontophoresis of ionisable, biolabile ACV-X prodrugs.

Keywords: aciclovir, prodrug, stability, iontophoresis, skin, selectivity, bioconversion

1. INTRODUCTION

Aciclovir (ACV, 9-[(2-hydroxyethoxy)methyl])-9H-guanine, Zovirax®) is commonly used to treat *herpes labialis*, but its poor and variable oral bioavailability (10-20%) decreases efficacy. This led to the development of valaciclovir (VCV, Valtrex®), the L-valyl ester of aciclovir, which is a substrate for the mammalian intestinal peptide transporters PEPT1 and PEPT2 [1]. Although this displays higher oral bioavailability (30-50%) [2], gram amounts of VCV have to be administered orally (1,000 mg three times a day) to treat an essentially local disorder. Topical ACV delivery to the basal epidermis where the virus is found would enable targeted therapy, reduce circulating drug levels and attenuate the risk of renal insufficiency [3].

Despite the therapeutic rationale and high specific activity against HSV-1 and HSV-2 in virus-infected cells, clinical studies of several topical ACV formulations against *herpes labialis* gave mixed results. Although a reduction in healing time was reported with 5% ACV cream [4], clinical failures were also seen [5]. Indeed, ACV ointment (5%) seemed to have less or even no clinical benefit [6,7]. Successful topical treatment relies on effective ACV delivery through intact skin since the epidermal pathology in the early stages of viral infection does not alter stratum corneum permeability [6]. Moreover, drug penetration must be sufficient to achieve therapeutic concentrations in the basal epidermis. Furthermore, drug therapy should be initiated in the prodrome or erythematous lesion stages [7,8,9]. Initiation of therapy more than 12 h after the appearance of symptoms, when the classical lesions such as papule, vesicle, ulcer or crust have been established, was observed to be ineffective and did not provide any benefit over the natural resolution of the infection [7,9,10]. Ultimately, effective treatment of *herpes labialis* needs early intervention and rapid delivery of sufficient drug to not only shorten the duration of the episode [9] but also to decrease virus entry into sensory neurons and the copy number of latent genome cloned. A smaller reservoir of latent virus would decrease its ability to reactivate periodically from latency [11]. However, ACV is not a good candidate for topical delivery. Its polarity (log D_{pH7} = -1.76) and sparing solubility in both aqueous and lipid media render its formulation difficult and limit its skin penetration. Poor bioavailability results in sub-inhibitory concentrations and delayed antiviral intervention in the basal epidermis [12].

Iontophoresis involves the application of a mild electric current and can be used to substantially increase the rate of transport of water soluble, charged molecules into and across

the skin [13,14]. However, ACV (pK_{a1} 2.27; pK_{a2} 9.25) is uncharged under physiologically acceptable conditions and has poor water solubility, ~0.2% at 25°C [15] – it is far from being an ideal candidate for iontophoresis. Although cutaneous delivery of therapeutic amounts of ACV by iontophoresis has been reported, it was achieved with formulations at pH 3 or 11 [16,17]. In contrast, we have previously shown that anodal iontophoresis of VCV, with a formulation pH of 5.24 and 5.65, was able to produce order of magnitude increases in ACV delivery [18]. Iontophoretic transport was facilitated by the positively charged valyl group, which was then cleaved by esterases to release ACV and valine during cutaneous transport.

The objective of the project was to investigate the electrotransport of a series of amino acid ester prodrugs of ACV (ACV-X, where X = Arg, Gly, Ile, Phe, Trp and Val) and to determine the influence of the different amino acid components on delivery. The specific aims of this study were (i) to synthesise, purify and characterise the amino acid ester prodrugs, (ii) to develop and validate robust HPLC-UV methods to quantify ACV and each prodrug simultaneously, (iii) to determine solution stability of the prodrugs as a function of pH and in the presence of skin, (iv) to compare passive and iontophoretic transport by measuring skin deposition and cumulative permeation of ACV species (that is, ACV and each ACV-X prodrug) and evaluate skin retention and total bioconversion ratios, and (v) to optimise iontophoretic conditions for milder and shorter current application.

2. MATERIALS AND METHODS

2.1. Materials

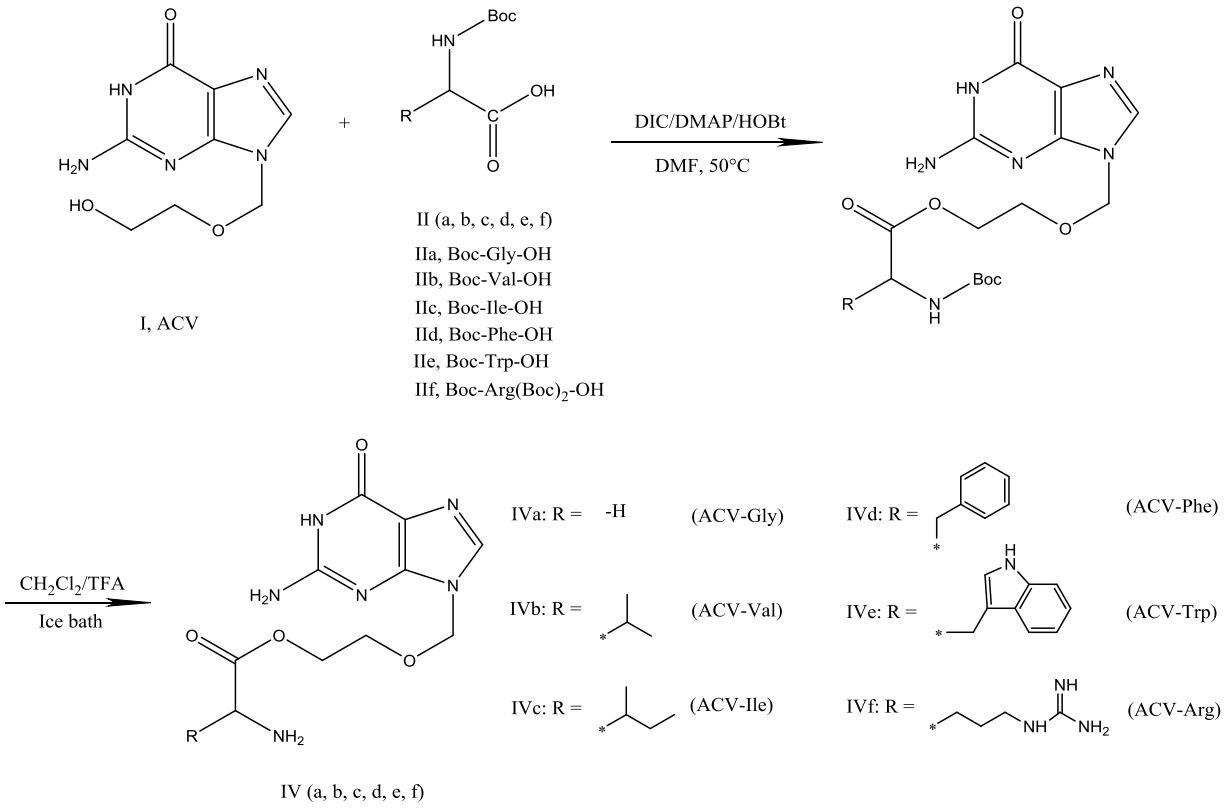
ACV was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). *N,N'*-Diisopropylcarbodiimide (DIC) was purchased from TCI (Tokyo, Japan). Boc-Gly-OH, , Boc-Ile-OH, Boc-Trp-OH, Boc-Val-OH and 1-hydroxybenzotriazole hydrate (HOBt) were supplied by Sigma-Aldrich (Buchs, Switzerland). Boc-Phe-OH was obtained from Acros (Hungary). Boc-Arg(Boc)$_2$-OH was purchased from Bachem AG (Bubendorf, Switzerland). *N, N'*- dimethylformamide (DMF, 99.8%, extra dry over molecular sieve, AcroSealÂ®) was supplied by Acros Organics (Geel, Belgium). 4-(dimethylamino) pyridine (DMAP) and 2-morpholino-ethanesulfonic acid monohydrate (MES) were obtained from Fluka (Buchs, Switzerland). ULC/MS grade acetonitrile (ACN) and PVC tubing (ID 3.17 mm; OD 4.97 mm) for preparing salt bridges were bought from VWR International AG (Dietikon, Switzerland). ULC/MS grade formic acid (FA) was purchased from Biosolve (Valkenswaard, Netherlands). Silver wire and silver chloride used for making electrodes were bought from

Sigma-Aldrich (St. Louis, US). All aqueous solutions were prepared using Milli-Q water (resistivity $>$ 18 MΩ.cm). All other chemicals were at least of analytical grade.

2.2. Prodrug synthesis

Amino acid ester prodrugs of ACV – ACV-Arg, ACV-Gly, ACV-Ile, ACV-Phe, ACV-Trp and ACV-Val, were prepared by a two-step synthesis as outlined in **Scheme I** starting from ACV using a method adapted from the literature [15,19,20].

Scheme I Synthesis of amino acid ester prodrugs of ACV



Briefly, *N*-Boc protected L-glycine, L-valine, L-isoleucine, L-phenylalanine, L- tryptophan or L-arginine (3.55×10^{-3} mol), DIC (0.48 g, 3.80×10^{-3} mol) and HOBt (0.48 g, 3.55×10^{-3} mol) were dissolved in DMF (10 ml) under N_2. The mixture was stirred for 2 h at 0°C, and was then added dropwise to a solution of ACV (0.8 g, 3.55×10^{-3} mol) and DMAP (0.005 g, 0.4×10^{-3} mol) in DMF (110 ml) at room temperature. The solution was stirred continuously and warmed up to 50°C. The reaction ran overnight. DMF was removed by rotary evaporation using a Rotavapor® (Büchi Labortechnik AG; Flawil, Switzerland) and the residue was placed on a silica gel column and eluted with 8:1:0.1~15:1:0.1 mixture of dichloromethane, methanol

and acetic acid to give the desired intermediate as a white solid. Deprotection of the intermediates was carried out using a 1:1 mixture of dichloromethane and trifluoroacetic acid (30 ml) in an ice bath for 2 h. After removal of the solvent by rotary evaporation, the resulting oil was then added dropwise to vigorously stirred cold diethyl ether (20 ml) and ACV-X prodrug was immediately precipitated. The precipitate was filtered with a Büchner funnel and then washed with cold diethyl ether (20 ml). The filter cake was dissolved in 10 ml of H_2O and lyophilised for 30 h using an Alpha® 2-4 LD Plus freeze dryer (Martin Christ GmbH; Osterode am Harz, Germany). A hygroscopic, fluffy white solid with a purity of >95 % (HPLC-UV, wavelength 254 nm) was obtained. The ^{1}H NMR spectrum was recorded on a Varian Gemini NMR spectrometer (300 MHz) at ambient temperature. The high resolution mass spectrum was acquired using an API QSTAR® Pulsar, ESI-Qtof mass spectrometer (Applied Biosystems; Rotkreuz, Switzerland).

I. (ACV): ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 3.46(m, 4 H, 12-H, 13-H), 4.67(s, 1 H, 13-OH), 5.34 (s, 2 H, 10-H), 6.46(s, 2 H, 2-NH_2), 7.81(s, 1 H, 8-H), 10.72(s, 1 H, 1-NH). HRESI-MS ($C_8H_{11}N_5O_3$+H): Calc. 226.0935; Exp. 226.0931.

IVa. (ACV-Gly): Yield: 54%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 3.71 (m, 2 H, 12-H), 3.80 (s, 2 H, 2′-H), 4.25 (m, 2 H, 13-H), 5.37 (s, 2 H, 10-H), 6.61 (s, 2 H, 2-NH_2), 7.91 (s, 1 H, 8-H), 8.29 (br s, 3 H, 2′-NH_3^+), 10.92 (s, 1 H, 1-NH). HRESI-MS ($C_{10}H_{15}N_6O_4$+H): Calc. 283.1149; Exp. 283.1141.

IVb. (ACV-Val): Yield: 61%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 0.88(m, 6 H, 4′-H, 5′-H), 2.05(m, 1 H, 3′-H), 3.71(m, 2 H, 12-H), 3.88(m, 1 H, 2′-H), 4.31(m, 2 H, 13-H), 5.36(s, 2 H, 10-H), 6.52(s, 2 H, 2-NH_2), 7.82(s, 1 H, 8-H), 8.18(br s, 3 H, 2′-NH_3^+), 10.72(s, 1 H, 1-NH). HRESI-MS ($C_{13}H_{21}N_6O_4$+H): Calc. 325.1618; Exp. 325.1628.

IVc. (ACV-Ile): Yield: 46%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 0.80(m, 6 H, 5′-H, 6′-H), 1.24(m, 2 H, 4′-H), 1.69(m, 1 H, 3′-H), 3.72(m, 2 H, 12-H), 3.87(m, 1 H, 2′-H), 4.32(m, 2 H, 13-H), 5.36(s, 2 H, 10-H), 6.64(s, 2 H, 2-NH_2), 7.87(s, 1 H, 8-H), 8.39(br s, 3 H, 2′-NH_3^+), 10.83(s, 1 H, 1-NH). HRESI-MS ($C_{14}H_{23}N_6O_4$+H): Calc. 339.1775; Exp. 339.1773.

IVd. (ACV-Phe): Yield: 31%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 3.04(d, 2 H, 3′-H), 3.61(m, 2 H, 12-H), 4.18(m, 2 H, 13-H), 4.30(m, 1 H, 2′-H), 5.33(s, 2 H, 10-H), 6.55(s, 2 H, 2-NH_2),

7.18-7.31(m, 5 H, 5′-H, 6′-H, 7′-H, 8′-H, 9′-H), 7.83(s, 1 H, 8-H), 8.44(br s, 3 H, 2′-NH_3^+), 10.72(s, 1 H, 1-NH). HRESI-MS ($C_{17}H_{21}N_6O_4$+H): Calc. 373.1618; Exp. 373.1621.

IVe. (ACV-Trp): Yield: 35%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 3.23(d, 2 H, 3′-H), 3.65(m, 2 H, 12-H), 4.18(m, 2 H, 13-H), 4.24(m, 1 H, 2′-H), 5.32(s, 2 H, 10-H), 6.54(s, 2 H, 2-NH_2), 7.00-7.46(m, 4 H, 9′-H, 10′-H, 11′-H, 12′-H), 7.22(m, 1 H, 5′-H), 7.83(s, 1 H, 8-H), 8.35(br s, 3 H, 2′-NH_3^+), 10.71(s, 1 H, 1-NH), 11.06(s, 1 H, 6′-NH). HRESI-MS ($C_{19}H_{22}N_7O_4$+H): Calc. 412.1727; Exp. 412.1729.

IVf. (ACV-Arg): Yield: 52%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 1.53(m, 2 H, 4′-H), 1.68(m, 2 H, 3′-H), 3.13(m, 5′-H), 3.71(m, 2 H, 12-H), 4.01(m, 1 H, 2′-H), 4.26(m, 2 H, 13-H), 5.37(s, 2 H, 10-H), 6.64(s, 2 H, 2-NH_2), 7.32(br s, 4 H, 7′-(NH_3^+)NH), 7.86(s, 1 H, 8-H), 7.93(m, 1 H, 6′-H), 8.34(br s, 3 H, 2′-NH_3^+), 10.86(s, 1 H, 1-NH). HRESI-MS ($C_{10}H_{15}N_6O_4$+H): Calc. 382.1945; Exp. 382.1947.

2.3. Analytical procedure

An ASI-100 auto-sampler equipped with a P680A LPG-4 pump and UVD 170U detector (formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach, Switzerland) was used to simultaneously quantify the amounts of ACV and each ACV-X prodrug permeated across and retained within the skin. The system was controlled by Chromeleon® Chromatography Management Software. The isocratic or gradient separations were achieved on a LiChrospher® (BGB Analytik AG; Boeckten, Switzerland) C18 reverse-phase analytical column (250×4.0 mm, 5 µm). A LiChrospher® guard column (10×4.0 mm, 5 µm) with the same packing material was mounted upstream from the analytical column. The column temperature was kept at 30°C, the flow rate was maintained at 1.0 ml/min, and the UV absorbance wavelength was set at 254 nm. The injection volume was 20 µl. The mobile phase comprised phase A, Na_2HPO_4 buffer (20 mM, pH 3.0), and phase B, acetonitrile. The composition of the mobile phase, the retention time for each compound, and the limit of quantification (LOQ) are shown in **Table 1**. The methods were validated with 3 replicates at 10, 50 and 100 µM and showed good intra-day precision and accuracy (**Table 2**). The specificity of the analytical methods was confirmed with respect to the peak resolution between ACV and each ACV-X prodrug, also between ACV species and endogenous substances extracted from porcine skin.

Table 1 HPLC conditions for quantification of ACV and ACV-X prodrugs.

Compound	Mobile Phase	Retention time (min)		LOQ (μM)
		ACV	Prodrug	
ACV	0~15.0 min 99 % A+1 % B	11.2	-	0.2
ACV-Gly		11.2	8.4	0.5
ACV-Val	0~15.5 min 99 % A, 1 % B 15.6~16.5 min 99 % A+1 % B to 92 % A+8 % B	11.5	22.0	0.5
ACV-Ile	16.6~27.6 min 92 % A+8 % B 27.7~28 min 92 % A+8 % B to 99 % A+1 % B 28~40 min 99 % A+1 % B	11.5	25.0	0.5
ACV-Phe	0~15.5 min 99 % A, 1 % B 15.6~16.5 min 99 % A+1 % B to 90 % A+10 % B 16.6~27.6 min 90 % A+10 % B	12.0	25.3	1.0
ACV-Trp	27.7~28 min 90 % A+10 % B to 99 % A+1 % B 28~40 min 99 % A+1 % B	12.0	26.6	1.0
ACV-Arg	0~14.0 min 98.7 % A+1.3% B	11.0	9.6	2.0

Table 2 Precision and accuracy of the analytical methods used to quantify ACV and ACV-X prodrugs

	Theoretical concentration (μM)	Experimental concentration (μM)	Precision (%)[a]	Accuracy (%)[b]
ACV	10	9.83 ± 0.04	0.41	98.30
	50	51.34 ± 0.29	0.56	102.68
	100	102.49 ± 0.68	0.66	102.49
ACV-Gly	10	9.61 ± 0.06	0.62	96.10
	50	48.23 ± 0.51	1.05	96.46
	100	96.39 ± 0.79	0.82	96.39
ACV-Val	10	9.81 ± 0.05	0.51	98.10
	50	50.74 ± 0.43	0.85	101.48
	100	103.43 ± 0.80	0.77	103.43
ACV-Ile	10	10.77 ± 0.07	0.65	107.70
	50	49.02 ± 0.39	0.79	98.04
	100	102.56 ± 1.01	0.98	102.56
ACV-Phe	10	9.59 ± 0.10	1.04	95.90
	50	47.49 ± 0.33	0.69	94.98
	100	94.23 ± 0.69	0.73	94.23
ACV-Trp	10	10.65 ± 0.10	0.94	106.50
	50	52.83 ± 0.51	0.96	105.66
	100	99.17 ± 0.97	0.98	99.17
ACV-Arg	10	11.51 ± 0.10	0.87	115.10
	50	53.77 ± 0.47	0.87	107.54
	100	108.15 ± 1.03	0.95	108.15

[a]Precision = (SD/mean) x 100.

[b]Accuracy = (obtained concentration/theoretical concentration) x 100

2.4 Physicochemical properties

2.4.1 Log D, p*K*a and aqueous solubility

The log D values at pH 5.5 (log $D_{pH\ 5.5}$) and the pKa values of ACV and ACV-X prodrugs were predicted using ACD/ChemSketch. The aqueous solubility of ACV and ACV-X prodrugs was measured in MES buffer (10 mM, pH 5.5). Excess ACV or prodrug was added to 200 μl MES buffer and vortexed at ambient temperature for 2 h. Then, the samples were

centrifuged at 12,000 rpm for 15 min. An aliquot (10 µl) was taken from the supernatant and diluted 1:1,000,000 by volume with fresh MES buffer, which was then subjected to HPLC analysis. The extremely high solubility of ACV-Arg meant that it was not possible to obtain a saturated solution.

2.4.2 Stability

2.4.2.1 Effect of pH

The stability of ACV-X prodrugs as a function of pH was investigated by dissolving each prodrug (100 µM) into 4 buffers at different pH: i) Glycine buffer at pH 2.0 (10 mM glycine); ii) MES buffer at pH 5.5 (10 mM MES monohydrate); iii) Phosphate buffered saline at pH 7.4 (136.9 mM NaCl, 2.7 mM KCl, 1.7 mM KH_2PO_4 and 8.1 mM Na_2HPO_4) and iv) Na_2CO_3 buffer at pH 10.0 (10 mM Na_2CO_3). Aliquots were withdrawn at regular time intervals over 2 h. The natural logarithm of the remaining fraction of each ACV-X prodrug (ln ACV-X) was plotted as a function of time (t). The first order rate constant (k_{obs}) of hydrolysis was estimated from the slope of the regression curve and the corresponding half-life ($t_{1/2}$) calculated ($t_{1/2} = ln2/k_{obs}$). All experiments were performed in triplicate at 8°C.

2.4.2.2 Effect of skin tissue

The effect of skin metabolism on the stability of ACV-X prodrugs was examined by (i) placing 1 ml of a 5 mM solution of ACV-X prodrug in MES buffer (pH 5.5) in contact with the external surface of porcine stratum corneum and (ii) exposing 1 ml of a 100 µM solution of each ACV-X prodrug in PBS buffer (pH 7.4) to the dermal surface of dermatomed porcine skin for 2 h. Aliquots were withdrawn at regular intervals. Experiments were performed in triplicate at 32°C. The remaining fraction of ACV-X was plotted as a function of time (t). All experiments were performed in triplicate.

2.5 Transport studies

2.5.1 Skin source

Fresh porcine ears were supplied from a local abattoir. The skin was processed (~750 µm) with an air dermatome (Zimmer Holdings Inc; Münsingen, Switzerland). Skin disks (diameter 26 mm) were punched out from skin membranes and stored at −20°C. Porcine ear skin has been extensively investigated and is considered to be a good research model for human skin [21,22,23].

2.5.2 Iontophoretic set-up

Iontophoretic transport studies were conducted to quantify skin deposition and cumulative permeation of ACV ($Q_{DEP,ACV}$ and $Q_{PERM,ACV}$) and each ACV-X prodrug ($Q_{DEP,ACV\text{-}X}$ and $Q_{PERM,ACV\text{-}X}$). Passive transport experiments using the same set-up but in the absence of current served as the control. The dermatomed skin was clamped in vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland) and allowed to equilibrate for 30 min before transport experiments. The area of skin exposed to the donor formulation was ~0.8 cm^2. The anodal and cathodal (receiver) compartments were filled with 20 and 10 ml of PBS solution (pH 7.4), respectively. The receiver solution was continuously stirred, and the temperature was maintained at 32°C using a dynamic water bath system. Ag and AgCl electrodes were placed in the anodal and receiver compartments, respectively. Salt bridge assemblies (3 % agarose in 0.1 M NaCl) were employed to electrically connect the physically isolated anodal and donor compartments. ACV and ACV-X prodrugs were prepared at a concentration of 5.0 mM in MES buffer (10 mM, pH 5.5). 1 ml of solution was pipetted into each donor compartment and current application initiated. Two series of iontophoretic transport studies were carried out: (i) to investigate the effect of current on molecular transport using three constant currents, 0.125, 0.25 and 0.5 mA/cm^2, applied for 120 min and (ii) to investigate the effect of duration of iontophoresis on molecular transport, the current density was fixed at 0.25 mA/cm^2 and the application time decreased to 60, 30, 10 and 5 min. Current was applied using a APH 1000 M power generator (Kepco Inc; Flushing, US). All transport studies were carried out in quintuplicate. In all experiments the donor was not occluded.

2.5.3 Sample preparation

A sample (1 ml) was withdrawn from each receiver compartment upon termination of current application. The diffusion cells were dismantled and the skin was washed with running water to remove any residual formulation on the surface. The formulation-exposed area was cut into small pieces and extracted for 3 h with 0.1% formic acid (this was used to deactivate skin esterases). Given that ACV-X prodrugs are too polar to penetrate the skin passively and are relatively unstable in contact with dermis, it was not possible to validate their extraction efficiency. However, it was assumed that the extraction method was efficient since ACV and ACV-X prodrugs are highly soluble in acidic solution, and the previously reported incubation time to extract ACV from human, porcine and rabbit skin was less than 1 h [12,24,25]. The skin extraction medium and the samples from the receiver compartment were centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through an Exapure® nylon filter

membrane with the pore size of 0.22 µm (Alys Technologies; Bussigny-près-Lausanne, Switzerland). All the samples were subjected to HPLC-UV analysis.

2.6 Data analysis

Data were expressed as the mean ± SD. Outliers determined using the Dixon test were discarded. Results were evaluated statistically using either analysis of variance (ANOVA) or Student's t-test. Student Newman Keuls test was used when necessary as a post-hoc procedure. The level of significance was fixed at $p = 0.05$.

3. RESULT AND DISCUSSIOIN

3.1 Synthesis of ACV-X prodrugs

A series of amino acid ester prodrugs of ACV was successfully synthesised via Steglich esterification. DIC was preferred to *N*,*N*′-dicyclohexylcarbodiimide (DCC) as the coupling reagent since the reaction by-product, *N*,*N*′-diisopropylurea, was soluble in most organic solvents and thus easier to remove than the DCC by-product, *N*,*N*′-dicyclohexylurea [26,27]. Moreover, being a liquid rather than a waxy solid, DIC was also easier to handle. HOBt was used as a racemisation suppressant since DMAP was suspected to lead to slight racemisation of the L-amino acid during esterification [19,28]. All products gave unambiguous ^{1}H NMR assignments, accurate m/z $[M+H]^+$ and showed good purity. ^{1}H NMR spectra, showed that the peak due to the proton of the -OH group in ACV (δ 4.67 ppm) was absent in the spectra of each prodrug. Proton deshielding of the 13-H (immediately adjacent to the -OH group) was also observed: ACV δ 3.44, ACV-Gly δ 4.25, ACV-Val δ 4.31, ACV-Ile δ 4.32, ACV-Phe δ 4.18, ACV-Trp δ 4.18 and ACV-Arg δ 4.26, which was due to the electron-withdrawing (-I) inductive effect of the newly formed ester group. The protonated primary amine from the amino acid moiety was also seen at low field (δ > 8) because all prodrugs were obtained as the TFA salt. The m/z $[M+H]^+$ values of the prodrugs showed that the absolute value of the difference between observed and calculated value was less than 6×10^{-4} units. The purity of all prodrugs was determined by HPLC-UV and was > 95 %.

3.2 Investigation of ACV-X prodrug physicochemical properties

3.2.1 Log *D*, p*K*a and aqueous solubility

The log D, pKa and aqueous solubility of ACV and ACV-X prodrugs are shown in **Table 3**. The predicted log $D_{pH\ 5.5}$ of ACV is -1.48, and the log $D_{pH\ 5.5}$ of most of the ACV-X prodrugs are lower than that of ACV; for example, ACV-Arg has a very low log $D_{pH\ 5.5}$ value -6.08,

which is attributed to the introduction of two polar and charged groups – the primary amine group and the guanidyl group. ACV-Phe has a comparable log $D_{pH\ 5.5}$ to ACV because the conjugation with a phenylalanine introduced both a hydrophilic primary amine group and a lipophilic phenyl group. According to the Henderson-Hasselbalch equation, all prodrugs are > 90% ionised and positively charged at pH 5.5 (approximate skin surface pH) – this enables them to benefit from electromigration during iontophoresis which is a far more efficient electrotransport mechanism than electroosmosis [13,18]. Furthermore, although the aqueous solubility of ACV at pH 5.5 is limited, ~ 9.2 mM, all of the ACV-X prodrugs were very soluble at this pH. ACV-Gly, ACV-Val, ACV-Ile, ACV-Phe, ACV-Trp and ACV-Arg had solubility enhancements of ~56, 48, 38, 33, 6 and >90-fold (by molar concentration), respectively, as compared to ACV. Indeed, ACV-Arg was so soluble that it was not possible to determine its solubility limit, which was >820 mM. The solubility enhancement can be attributed to the effect of hydrogen-bonded solvation of the ionisable group(s) [29].

Table 3 Physicochemical properties of ACV and ACV-X prodrugs at pH 5.5

Compound	M.W. [a]	Charge:Mass ratio ($\times 10^3$)	log D	pKa	Solubility (mM)
ACV	225.2	0	-1.48	2.55, 9.35	9.2
ACV-Gly	282.3	3.54	-3.30	2.53, 7.20, 9.34	512.2
ACV-Val	324.3	3.08	-2.64	2.54, 7.75, 9.34	441.3
ACV-Ile	338.4	2.96	-2.16	2.54, 7.78, 9.34	347.8
ACV-Phe	372.4	2.69	-1.22	2.54, 6.93, 9.34	304.4
ACV-Trp	411.4	2.43	-0.73	2.54, 6.70, 9.34	51.3
ACV-Arg	381.4	5.24	-6.08	2.54, 7.28, 9.34, 13.36	> 820

[a] free type

3.2.2 Stability

The investigation into the effect of pH and porcine skin on the stability of ACV-X prodrugs showed that the ACV-X prodrugs were converted to ACV by both chemical and enzymatic hydrolysis.

The remaining unhydrolysed fraction of each prodrug (ACV-X) after 8 h, k_{obs} (h^{-1}) and $t_{1/2}$ (h) for the pH stability experiments are shown in **Table 4**. The rate of chemical hydrolysis appeared to be pH-dependent. All ACV-X prodrugs were more stable in acidic solution. At

Table 4 pH stability of ACV-X prodrugs: remaining fraction of each prodrug (%ACV-X) after 8 h, k_{obs} (h^{-1}) and $t_{1/2}$ (h)

Compound	pH	Remaining fraction of prodrug (%ACV-X)	k_{obs} (h^{-1})	$t_{1/2}$ (h)
ACV-Gly	2.0	100.8 ± 0.5	—	—
	5.5	99.4 ± 3.4	—	—
	7.4	76.5 ± 0.7	0.0368 ± 0.0010	18.8 ± 0.5
	10.0	20.6 ± 1.1	0.2106 ± 0.0070	3.3 ± 0.1
ACV-Val	2.0	100.4 ± 0.5	—	—
	5.5	98.9 ± 1.5	—	—
	7.4	92.5 ± 0.6	0.0093 ± 0.0005	74.5 ± 4.0
	10.0	79.2 ± 0.1	0.0288 ± 0.0003	24.1 ± 0.2
ACV-Ile	2.0	99.6 ± 0.1	—	—
	5.5	99.3 ± 0.2	—	—
	7.4	94.9 ± 0.2	0.0069 ± 0.0003	101.1 ± 4.4
	10.0	86.0 ± 0.3	0.0191 ± 0.0003	36.4 ± 0.6
ACV-Phe	2.0	99.8 ± 0.6	—	—
	5.5	98.3 ± 0.1	—	—
	7.4	76.9 ± 0.4	0.0325 ± 0.0006	21.4 ± 0.4
	10.0	20.6 ± 0.6	0.1960 ± 0.0048	3.5 ± 0.1
ACV-Trp	2.0	100.1 ± 0.9	—	—
	5.5	99.5 ± 0.5	—	—
	7.4	78.8 ± 0.9	0.0287 ± 0.0005	24.2 ± 0.4
	10.0	36.9 ± 0.9	0.1322 ± 0.0160	5.3 ± 0.6
ACV-Arg	2.0	100.0 ± 0.5	—	—
	5.5	100.8 ± 2.1	—	—
	7.4	77.5 ± 1.8	0.0318 ± 0.0029	22.0 ± 2.2
	10.0	24.1 ± 0.1	0.1770 ± 0.0002	3.9 ± 0.0

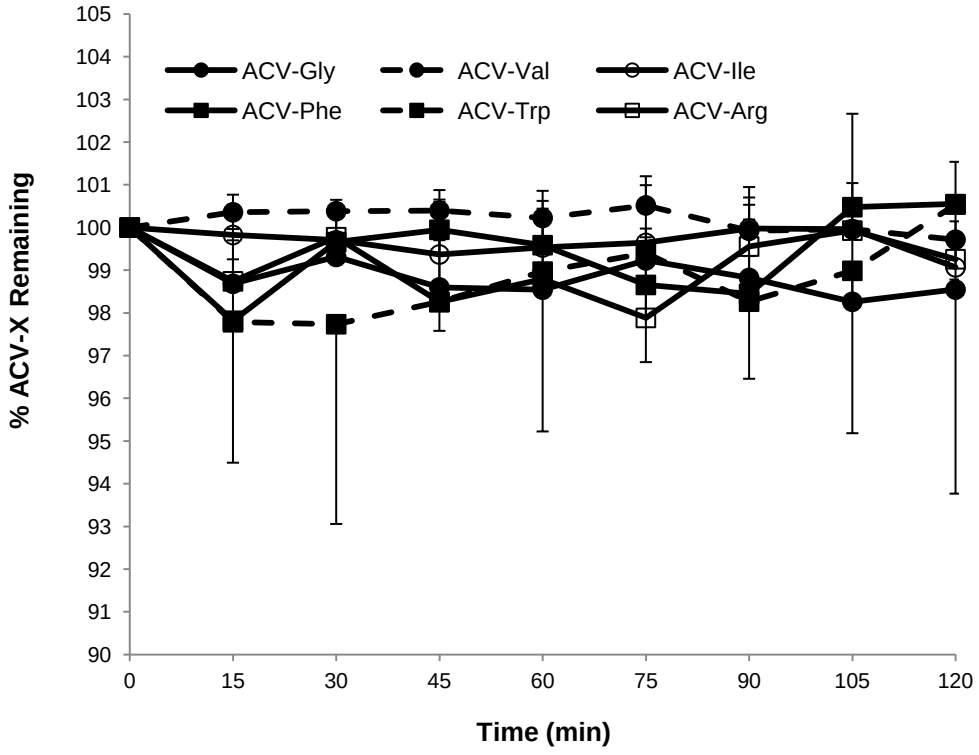
pH 2.0 and pH 5.5 more than 98.5% of the prodrugs was unchanged after 8 h at 8°C, and the $t_{1/2}$ was not measurable. However, incubation at pH 7.4 and pH 10.0 for 8 h at 8°C resulted in appreciable hydrolysis of certain prodrugs – the unchanged fraction was 75-95% and 20-85%,

respectively. The prodrugs were clearly more susceptible to base-catalysed chemical hydrolysis; increased concentrations of nucleophilic hydroxyl ions at higher pH increased the extent of hydrolysis. Among the six ACV-X prodrugs, ACV-Arg was quite labile at pH 7.4 and pH 10.0 ($t_{1/2}$ 21.9 h and 3.9 h, respectively), and ACV-Ile showed the highest stability at either pH ($t_{1/2}$ 100.4 h and 36.3 h, respectively). Therefore, the pH of all the formulations selected for use in the transport studies was 5.5, which ensured relative stability of the ACV prodrugs and protected the skin from irritation.

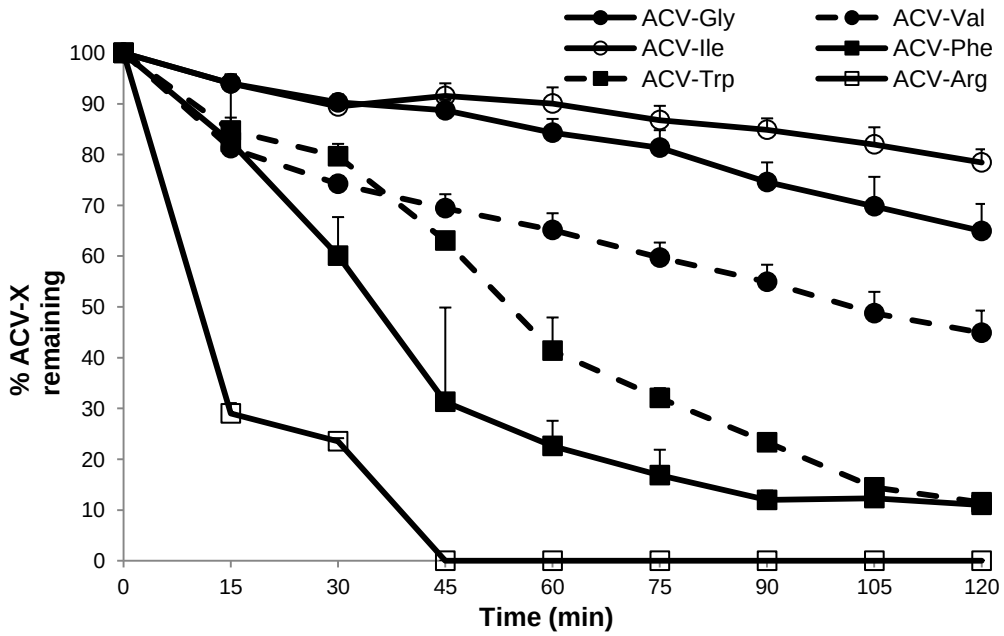
All ACV-X prodrugs were very stable after incubation with the external surface of porcine stratum corneum for 2 h (**Figure 1a**), but they were extensively hydrolysed to ACV and the respective amino acid when in contact with the dermal surface of dermatomed porcine skin for 2 h (**Figure 1b**).

For example, ACV-Arg underwent complete bioconversion after exposure to the dermis for 45 min, whereas negligible reactivity was found following contact with the stratum corneum for 2 h. It has been reported that very limited enzymatic activity was observed in the keratinized stratum corneum [30], in contrast, considerable amounts of non-specific esterase and other phase I metabolic enzymes were found in dermis [31]. The enzymatic hydrolysis of ACV-X prodrugs by dermis was also prodrug-dependent. In contrast to ACV-Arg, which was extremely susceptible to biotransformation in contact with the dermis, only 21.5 % of ACV-Ile was hydrolysed after exposure to dermis for 2 h. The different hydrolytic rates must be attributed to the different structures of the substrates; for example, steric hindrance encountered by the esterase must have been influenced by the amino acid moiety [32].

The prodrug strategy has been widely used to develop topical and transdermal formulations – most routinely in the case of corticosteroid esters. However, in the case of passive administration, the objective is to increase molecular lipophilicity. Conversely, in the case of these “iontophoretic” prodrugs, the aim is to introduce polar ionisable moieties that increase hydrophilicity, aqueous solubility and most importantly, impart a charge to the molecule enabling it to benefit from iontophoresis. Once the charged prodrug is delivered into skin, the parent drug is released at or near the site of action relying on cutaneous esterase activity.



(a)



(b)

Figure 1. Solution stability of ACV-X prodrugs in the presence of **(a)** external surface of porcine epidermis (32°C, 2 h) and **(b)** dermis (32°C, 2 h). (Mean ± SD; n=3)

3.3 Transport studies

3.3.1 Passive delivery

The passive diffusion of ACV and ACV-X prodrugs into and across porcine skin from 5 mM of aqueous solution for 2 h was negligible, neither skin retention nor cumulative permeation was quantifiable. The amount of ACV present in skin was inferior to 0.25 nmol/cm^2, since the LOQ of ACV was 0.2 μM and the volume of skin extraction medium was 1 ml. As indicated in Section 3.2.1, ACV has poor oil/water solubility, which limits its partitioning into the highly lipidic intercellular space in the keratinized stratum corneum. Formulation strategies have been extensively described to improve the cutaneous ACV bioavailability either by incorporation of chemical enhancers such as DMSO [33,34] and high quantities of propylene glycol [35] or by fabrication of lipid- or polymer-based formulations such as microemulsions [36], liposomes [37], microparticles [24] and nanoparticles [38]; however, the risk of skin irritation or complicated preparative procedures may hinder their clinical development.

3.3.2 Iontophoretic delivery of ACV

Deposition of ACV ($Q_{DEP,ACV}$) after iontophoresis of a 5 mM solution at pH 5.5 for 2 h at 0.125, 0.25 and 0.5 mA/cm^2 was 0.9 ± 0.3, 2.1 ± 0.3 and 4.6 ± 0.3 nmol/cm^2, respectively. Cumulative permeation of ACV ($Q_{PERM,ACV}$) was below the LOQ. Given that ACV is essentially neutral at or close to physiologically acceptable pH conditions (**Table 3**), it is transported by electroosmosis, a solvent volume flow induced by the movement of ions across a charged membrane [13]. Electroosmosis is a less effective transport mechanism than electromigration, thus there is only a modest increase in ACV delivery [25]. Given the pKa of its ionisable groups, iontophoretic enhancement of ACV delivery by electromigration can only be observed with formulations at a pH outside the physiological range. For example, at pH 3 where ACV was present at ~20 % as a cation, anodal iontophoresis for 7 h at 0.5 mA/cm^2 using human skin *in vitro* resulted in epidermal and dermal ACV concentrations in the range of 80-150 μg/cm^3 (corresponding to 8-15 μg of ACV per cm^2, as the thickness of the skin was ~1.0 mm) [16]. Similarly, at pH 11, where ACV was ~ 99 % deprotonated and present as an anion, cathodal iontophoresis for 10 min at 0.2 mA/cm^2 in rats *in vivo* resulted in skin levels of 27.27 ± 3.53 μg/cm^2 [17].

3.3.3 Iontophoretic delivery of ACV-X prodrugs

Iontophoresis of ACV-X prodrugs at 0.5 mA/cm^2 for 2 h produced order of magnitude increases in cutaneous deposition of ACV species as compared to either passive diffusion or

iontophoresis of ACV (Q_{DEP} = $Q_{DEP,ACV}$ + $Q_{DEP, ACV-X}$) (ACV-Gly: 412.8 ± 44.0, ACV-Val: 358.8 ± 66.8, ACV-Ile: 434.1 ± 68.2, ACV-Phe: 249.8 ± 81.4, ACV-Trp: 156.1 ± 76.3, and ACV-Arg: 785.9 ± 78.1 nmol/cm^2, respectively) (**Figure 2**). It should also be emphasised that all tested prodrug formulations were easily prepared in aqueous solution at a patient-friendly pH 5.5. The total delivery of ACV species ($Q_{DEP , ACV}$ + $Q_{PERM, ACV}$ + $Q_{DEP, ACV-X}$ + $Q_{PERM, ACV-X}$), transport efficiency (transport number x 100%) and delivery efficiency (fraction of the applied dose delivered x 100%) after iontophoresis of ACV-X prodrugs for 2 h was shown in **Table 5**.

Iontophoresis of di-protonated ACV-Arg resulted in the greatest amount of skin deposition of ACV at the three current densities (**Figure 2**). Iontophoresis of the two aromatic amino acid ester prodrugs, ACV-Trp and ACV-Phe, led to the lowest skin deposition of ACV species. Iontophoresis of ACV-Gly, ACV-Val and ACV-Ile delivered intermediate, comparable levels of ACV species into skin. Di-protonated ACV-Arg had the highest charge-mass ratio among the six prodrugs, therefore, its electromigration was presumably the highest under a certain electric potential gradient. Mono-protonated, ACV-Trp and ACV-Phe had lower charge-mass ratio and thus presumably had lower electric mobility. Moreover, the aromatic rings may also have facilitated hydrophobic interactions with the skin microenvironment. ACV-Val and ACV-Ile had slightly higher charge-mass ratios than ACV-Trp and ACV-Phe but displayed much greater deposition in skin. They enabled the highest level of cumulative permeation of ACV species (**Figure 2**).

The mechanisms used to explain iontophoretic transport across the skin consider the ions to be stable and to remain unchanged during transit. Obviously, this was not the case here – ACV-X prodrugs underwent extensive hydrolysis during cutaneous transport. More esterase-sensitive prodrugs, such as ACV-Arg, were readily hydrolysed after entering the viable epidermis, lost the amino acid promoiety and remained in the skin. Prodrugs, such as ACV-Val and ACV-Ile, which were less susceptible to enzymatic hydrolysis, retained their charged moiety and were able to electromigrate further into the skin before cleavage of the ester linkage. ACV-Trp and ACV-Phe were also resistant to esterase activity but the aromatic amino acid side-chains facilitated interactions with the skin and hindered transport. These observations provide an insight into the rational design of prodrugs for iontophoresis and their optimization for either topical or transdermal delivery based on the stability of linkage to the charged moiety. Comparatively stable prodrugs might have a greater probability of reaching

Figure 2a

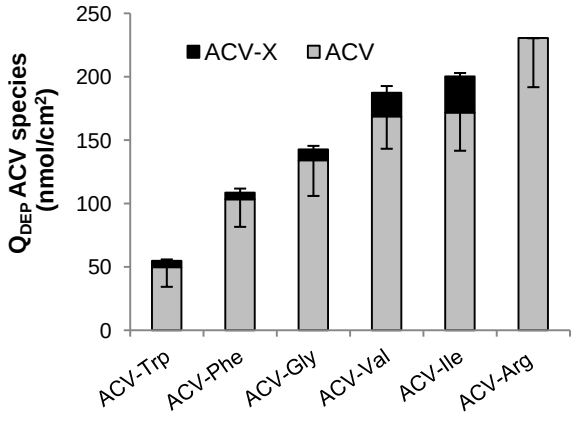


Figure 2b

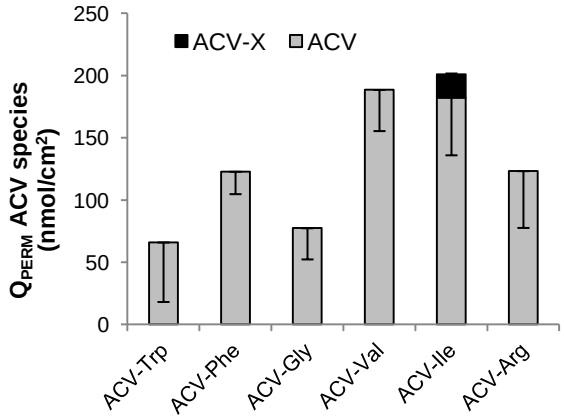


Figure 2c

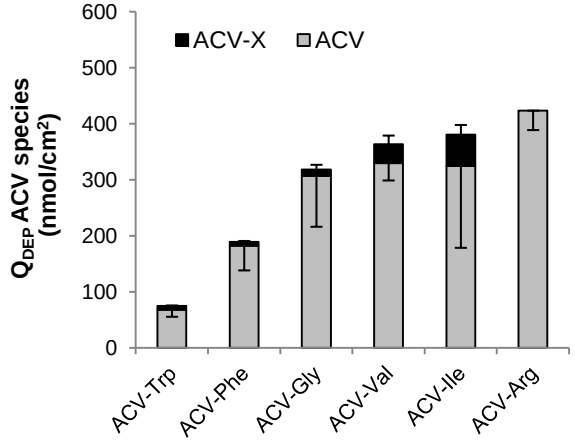


Figure 2d

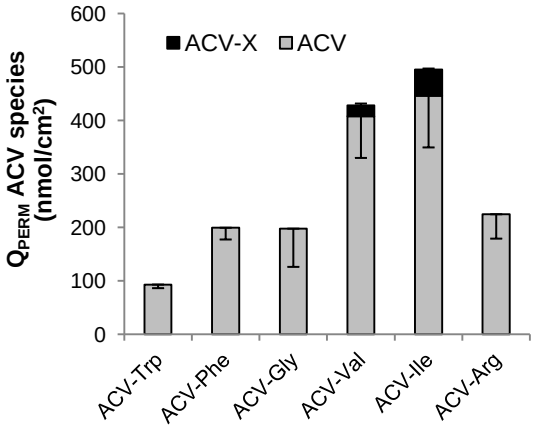


Figure 2e

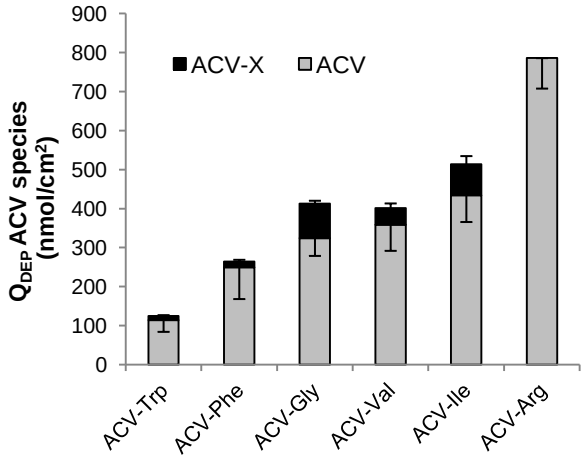


Figure 2f

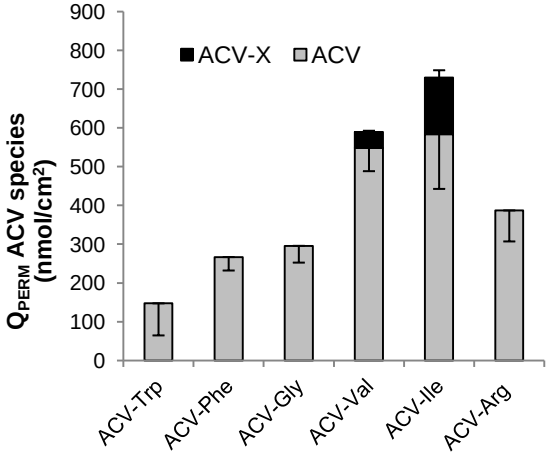


Figure 2. Delivery of ACV and ACV-X following iontophoresis of ACV-X prodrugs (5 mM, pH 5.5) at **(a, b)** 0.125, **(c, d)** 0.25 and **(e, f)** 0.5 mA/cm^2 for 2 h. Data are given in terms of amounts deposited in the skin ($Q_{DEP} = Q_{DEP,ACV} + Q_{DEP,\ ACV\text{-}X}$) **(a, c, d)** and the cumulative permeation ($Q_{PERM} = Q_{PERM,\ ACV} + Q_{PERM,\ ACV\text{-}X}$) **(b, d, f)** of ACV species. (Mean ± SD; n=5)

the systemic circulation during iontophoresis, i.e., better suited to transdermal delivery and systemic action. In contrast, enzymatically labile prodrugs would be more likely to undergo hydrolysis earlier during cutaneous transit, release uncharged parent drug that would be retained within the membrane. They may be considered as better suited for the treatment of dermatological diseases. However, the intrinsic stability (i.e., ease of formulation) and molecular properties that influence potential interactions along the transport pathways must also be considered in conjunction with these "guidelines" based on the stability of the linkage between the parent molecule and prodrug moiety.

Table 5 Total delivery of ACV species ($Q_{DEP,\ ACV} + Q_{PERM,\ ACV} + Q_{DEP,\ ACV\text{-}X} + Q_{PERM,\ ACV\text{-}X}$) (nmol/cm^2), transport efficiency (%) and delivery efficiency (%) after iontophoresis of ACV-X prodrugs for 2 h.

	Current (mA/cm^2)	Total delivery (nmol/cm^2)	Transport efficiency (%)	Delivery efficiency (%)
ACV-Gly	0.125	220.1 ± 29.3	2.36	3.52
	0.25	515.5 ± 123.6	2.76	8.25
	0.5	708.4 ± 71.2	1.80	11.33
ACV-Val	0.125	375.9 ± 22.7	4.02	6.01
	0.25	791.3 ± 103.9	4.23	12.66
	0.5	1034.6 ± 110.8	2.62	16.55
ACV-Ile	0.125	401.3 ± 62.0	4.30	6.42
	0.25	875.9 ± 341.1	4.69	14.01
	0.5	1243.4 ± 150.6	3.15	19.89
ACV-Phe	0.125	231.5 ± 28.1	2.48	3.70
	0.25	388.3 ± 51.5	2.08	6.21
	0.5	530.5 ± 86.4	1.34	8.49
ACV-Trp	0.125	120.8 ± 53.0	1.29	1.93
	0.25	167.4 ± 16.4	0.90	2.68
	0.5	303.5 ± 102.4	0.77	4.86
ACV-Arg	0.125	353.8 ± 40.2	7.57	5.66
	0.25	647.6 ± 33.3	6.93	10.36
	0.5	1172.9 ± 104.2	5.95	18.77

The skin retention ratio ($Q_{DEP,\ ACV} + Q_{DEP,\ ACV\text{-}X}$ /($Q_{DEP,\ ACV} + Q_{DEP,\ ACV\text{-}X} + Q_{PERM,\ ACV} + Q_{PERM,\ ACV\text{-}X}$) x 100%), was used to represent the selectivity for deposition over permeation

(**Figure 3**). The skin retention ratio of ACV-Arg after iontophoresis for 2 h at any given current was statistically significantly higher than those of the other prodrugs. For example, at 0.5 mA/cm^2 iontophoresis of ACV-Arg, ACV-Gly, ACV-Trp, ACV-Phe, ACV-Val ACV-Ile resulted in skin retention ratios of 67.1 ± 5.0%, 58.3 ± 3.8%, 51.9 ± 4.6%, 48.3 ± 9.4%, 42.9 ± 3.3% and 41.6 ± 5.8%, respectively. The skin retention ratio of ACV species did not seem to be affected by the applied current density (**Figure 3**).

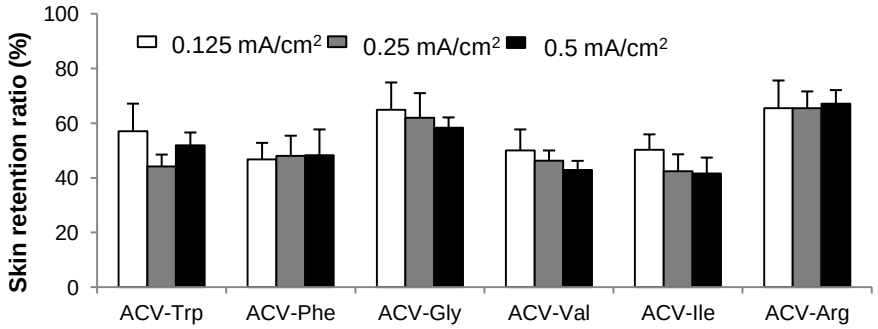


Figure 3. Skin retention of total ACV species (sum of ACV and ACV-X) is given by the ratio of the amounts deposited to the total delivery ($Q_{DEP,\ ACV} + Q_{DEP,\ ACV\text{-}X}$ /($Q_{DEP,\ ACV} + Q_{DEP,\ ACV\text{-}X} + Q_{PERM,\ ACV} + Q_{PERM,\ ACV\text{-}X}$) x 100%) after iontophoresis of ACV-X prodrugs (5 mM, pH 5.5) at 0.125, 0.25 and 0.5 mA/cm^2 for 2 h. (Mean ± SD; n=5)

The bioconversion ratio ($Q_{DEP,\ ACV} + Q_{PERM,\ ACV}$ /($Q_{DEP\ ,\ ACV} + Q_{PERM,\ ACV\ +}\ Q_{DEP,\ ACV\text{-}X} + Q_{PERM,\ ACV\text{-}X}$) x 100%) after iontophoresis delivery of the ACV-X prodrugs is shown in **Figure 4**. ACV-Arg was the most biolabile prodrug, complete bioconversion (100%) was achieved during ACV-Arg iontophoresis, which was statistically significantly higher than that of any of the other ACV-X prodrugs. In contrast, ACV-Ile, the most enzymatically stable prodrug, had the lowest total bioconversion ratio.

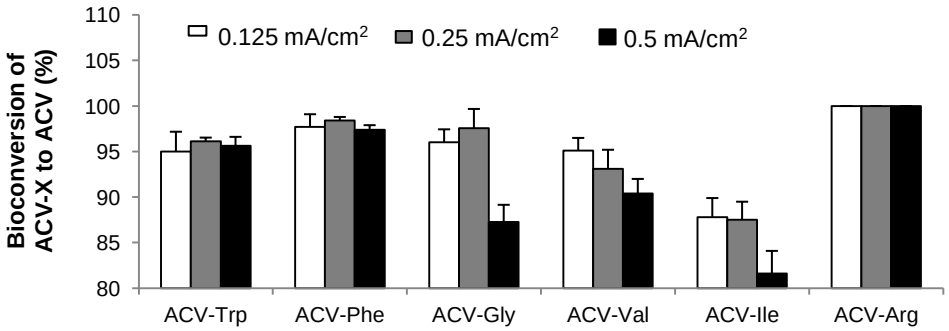
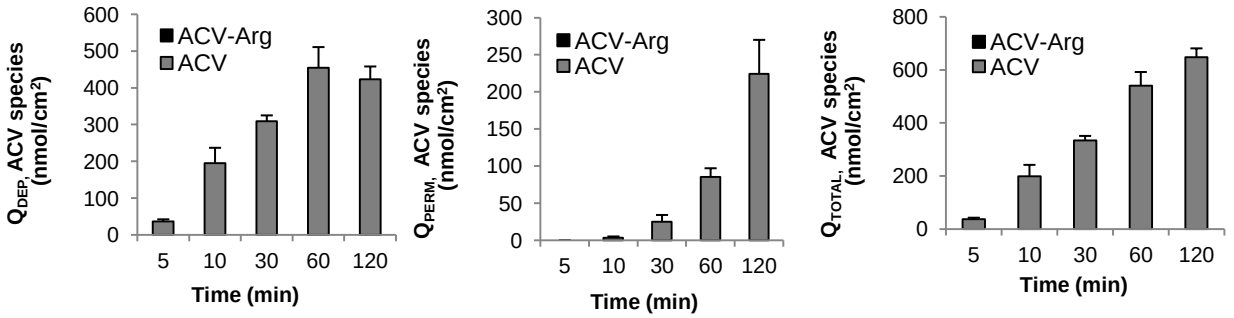


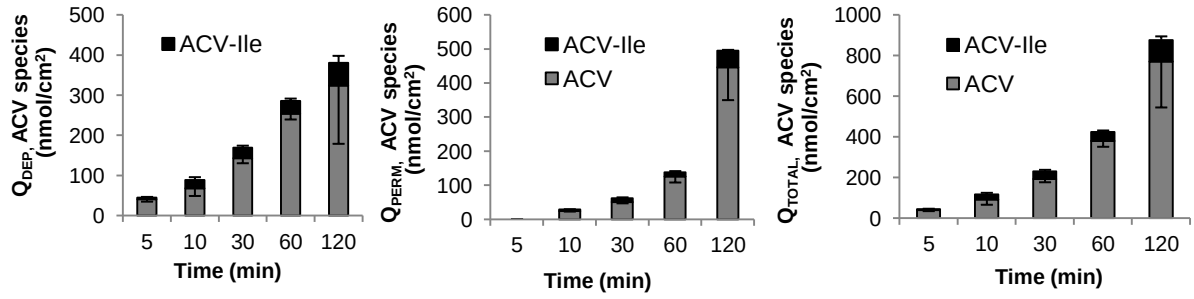
Figure 4. Bioconversion of ACV-X to ACV after iontophoresis of ACV-X prodrugs (5 mM, pH 5.5) at 0.125, 0.25 and 0.5 mA/cm^2 for 2 h. This is given by ($Q_{DEP,\ ACV} + Q_{PERM,\ ACV}$ /($Q_{DEP\ ,\ ACV} + Q_{PERM,\ ACV\ +}\ Q_{DEP,\ ACV\text{-}X} + Q_{PERM,\ ACV\text{-}X}$) x 100%). (Mean ± SD; n=5)

It was decided to investigate the iontophoretic transport of ACV-Arg and ACV-Ile more closely. The variation of the relative contributions of deposition (Q_{DEP}) and permeation (Q_{PERM}) to the total iontophoretic delivery of ACV-Arg and ACV-Ile as a function of the duration of current application are shown in **Figure 5** and **Figure 6**, respectively. Given the rapid bioconversion of ACV-Arg to ACV, it is not surprising to observe that total delivery was dominated by deposition of ACV ($Q_{DEP, ACV}$). A greater absolute amount and percentage of the applied ACV-Ile permeated across the skin as a function of time; this was consistentwith the greater resistance to hydrolysis of the ester linkage in ACV-Ile. After iontophoresis of ACV-Arg for 60 min, $Q_{DEP, ACV}$ was 454.9 ± 55.9 nmol/cm^2, and no ACV-Arg was observed. In the case of ACV-Ile, $Q_{DEP, ACV}$ + $Q_{DEP, ACV-ILE}$ was 285.5 ± 22.1 nmol/cm^2. The profiles started to plateau which may have indicated that deposition was a saturable process, perhaps due to saturation of cutaneous esterases resulting in intact ionised prodrug reaching the receiver compartment [39].



(a) **(b)** **(c)**

Figure 5. Skin deposition (Q_{DEP}) **(a)**, cumulative permeation (Q_{PERM}) **(b)** and total delivery (Q_{PERM}) **(c)** as a function of the duration of iontophoresis at 0.25 mA/cm^2 for ACV-Arg (Mean ± SD; n=5)



(a) **(b)** **(c)**

Figure 6. Skin deposition (Q_{DEP}) **(a)**, cumulative permeation (Q_{PERM}) **(b)** and total delivery (Q_{PERM}) **(c)** as a function of the duration of iontophoresis at 0.25 mA/cm^2 for ACV-Ile (Mean ± SD; n=5)

The cutaneous deposition of ACV ($Q_{DEP, ACV}$) and corresponding concentration of ACV in the skin (C_{ACV}) following iontophoresis of ACV-Arg and ACV-Ile as function of time are shown in **Figure 7**. There is considerable variability in the reported IC_{50} of ACV against HSV-1 and HSV-2, 0.01-0.7 μg/ml and 0.01-3.2 μg/ml, respectively [19], which is equivalent to 0.04-3.1 nmol/ml and 0.04-14.2 nmol/ml, respectively. The average cutaneous concentrations of ACV after iontophoresis of ACV-Ile and ACV-Arg at each time were orders of magnitude greater than IC_{50} of ACV against HSV-1 and HSV-2. For example, iontophoresis of ACV-Arg and ACV-Ile at 0.25 mA/cm^2 for only 5 min resulted in the deposition of appreciable amounts ACV (36.4± 5.7 nmol/cm^2 and 40.3 ± 6.1 nmol/cm^2, respectively), corresponding to supra-therapeutic levels of ACV to be achieved in the skin (485.2.3 ± 75.9 nmol/ml and 537.4 ± 80.7 nmol/ml, respectively).

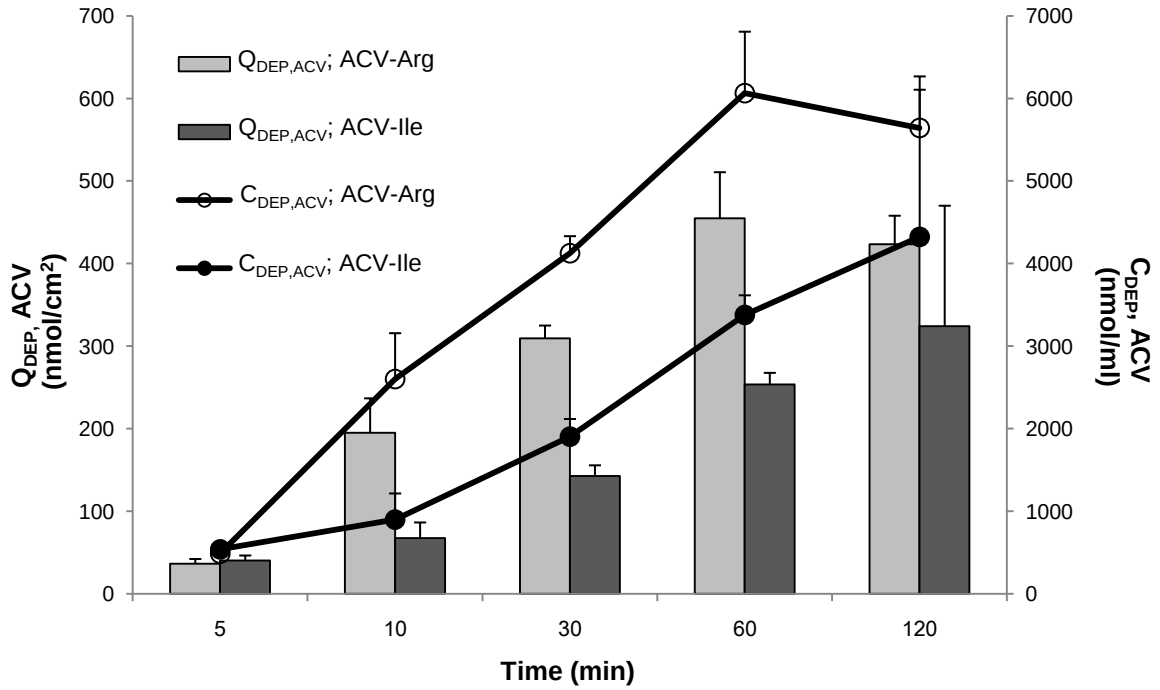


Figure 7. Skin deposition of ACV ($Q_{DEP, ACV}$) (columns) and corresponding concentration $C_{DEP, ACV}$ (circles) observed as a function of the duration of iontophoresis at 0.25 mA/cm^2 for ACV-Arg and ACV-Ile (nmol/cm^2). (Mean ± SD; n=5)

4. CONCLUSION

Prodrugs have been widely used to increase the topical bioavailability of molecules [40] in the skin. For example, mono- and di-ester derivatives of corticosteroid esters are routinely used to increase lipophilicity by masking C17 and C21 hydroxyl groups and so facilitate partition into the intercellular lipid matrix in the stratum corneum. In contrast, prodrugs for topical

iontophoresis were designed (i) to increase hydrosolubility and (ii) to introduce ionisable group(s). Improved percutaneous transport of dehydroepiandrosterone [41] and dexamethasone [42,43] was observed when the drugs were conjugated to ionisable, biolabile promoieties, to form charged, water-soluble prodrugs, and delivered across skin by iontophoresis. However, this is not always the case [44]. The results described in this first part of the study confirmed that iontophoresis of ionised amino acid ester prodrugs of ACV was an extremely effective means to increase cutaneous delivery of ACV. The next step was to optimise the iontophoretic conditions and formulation and to demonstrate that topical iontophoresis not only increased absolute amounts in the skin but enabled targeted intraepidermal delivery to the deep epidermis where the virus is found. This is described in the next paper.

5. ACKNOWLEDGEMENTS

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Chapter 2

Cutaneous biodistribution of ionisable, biolabile aciclovir prodrugs after short duration topical iontophoresis: demonstrating targeted intra-epidermal delivery

Cutaneous biodistribution of ionisable, biolabile aciclovir prodrugs after short duration topical iontophoresis: demonstrating targeted intra-epidermal delivery

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ABSTRACT

The objective was to determine the cutaneous biodistribution of aciclovir (ACV), that is, the bioavailability as a function of depth within the skin following topical iontophoretic administration of amino acid ester prodrugs of ACV (ACV-X, where X=Arg, Ile or Val). The results were compared to those obtained with marketed formulations of aciclovir and penciclovir (PCV), and following topical iontophoresis of ACV. Quantification of molecular species as a function of position in the skin was achieved by snap-freezing and cryotoming skin samples to obtain coarse or fine lamellae with a thickness of either 100 μm or 20 μm. The molecules – ACV, ACV-X or PCV – were extracted and quantified by validated UHPLC-MS/MS methods. Passive delivery of ACV or PCV from marketed cream and ointment formulations after application for 60 min resulted in modest cutaneous deposition ($Q_{DEP,ACV}$ and $Q_{DEP,PCV}$ $<$2 nmol/cm^2). Moreover, ACV and PCV were found mainly in the stratum corneum or superficial viable epidermis. The levels in the deeper skin layers (100-200 μm), corresponding to the basal epidermis and adjacent area, where the virus would be found, were negligible. In contrast, iontophoresis of ACV-Ile or ACV-Arg for only 10 min at 0.25 mA/cm^2 resulted in much greater deposition of ACV species at the same skin depths (100-200 μm): for ACV-Ile – $Q_{DEP,ACV}$ and $Q_{DEP,ACV\text{-}Ile}$ were 17.2 ± 6.9 nmol/cm^2 and 8.2 ± 1.3 nmol/cm^2, respectively; in the case of ACV-Arg there was complete bioconversion and only ACV was recovered ($Q_{DEP,ACV}$ was 41.2 ± 9.2 nmol/cm^2). In the higher resolution biodistribution studies, with 20 μm lamellae, short duration iontophoresis at 0.25 mA/cm^2 for only 5 min of ACV-Arg or ACV-Ile still enabled considerable amounts of ACV species to be delivered to the basal epidermis and neighbouring layers ($Q_{DEP,ACV}$ was 4.0 ± 1.6 nmol/cm^2 and 6.1 ± 1.7 nmol/cm^2, respectively). Iontophoresis of ACV under the same conditions resulted in negligible ACV deposition in these layers. Subsequent studies into the iontophoresis of ACV-Ile using a hydrogel revealed no statistically significant differences between $Q_{DEP,ACV}$ in full-thickness porcine and human skins (13.4 ± 1.5 nmol/cm^2 and 12.4 ± 1.4 nmol/cm^2, respectively). In order to improve ACV-Ile stability, dry fast dissolving thin film formulations were prepared. These were placed in contact with conducting hydrogels in situ immediately prior to iontophoresis. In addition to the improved stability, iontophoretic delivery of ACV-Ile from the thin film was also superior to that from hydrogel alone. Following iontophoresis of ACV-Ile using fast dissolving films, $Q_{DEP,ACV}$ was 29.1 ± 1.0 nmol/cm^2, and the amount in the basal epidermis area was 2.7 ± 0.1 nmol/cm^2. Short duration iontophoresis for 5 min of ACV-Ile from aqueous solution, hydrogel or fast dissolving film

resulted in supra-therapeutic concentrations of ACV in the basal epidermis and its adjacent area.

Keywords: aciclovir, prodrug, distribution, iontophoresis, hydrogel, fast dissolving film

1. INTRODUCTION

Aciclovir (ACV) was developed for the treatment of herpes simplex virus (HSV) infections; however, poor cutaneous bioavailability of topical ACV formulations resulted in limited efficacy [1,2]. We have shown that topical iontophoresis of amino acid ester prodrugs of ACV (ACV-X, where X = Arg Gly, Ile, Phe, Trp and Val) was extremely effective in improving cutaneous ACV delivery as the positively charged ACV-X prodrugs were able to electromigrate into skin under the applied electric field and that ACV was released from the corresponding ACV-X prodrug following cleavage of the ester linkage by cutaneous esterases (Chapter 1 and 2). Moreover, the patient-friendly compositions (pH 5.5; no chemical enhancers), ease of preparation (simple buffered solution of ACV-X prodrug), mild current densities (0.125, 0.25 and 0.5 mA/cm^2) and short-duration current application (as short as 5 min) provided an appreciable added value to the strategy.

These preliminary studies demonstrated short-duration iontophoresis of ACV-X prodrugs enabled higher average concentrations of ACV in skin than its IC_{50} against HSV-1 and 2. However, it is essential to determine where the ACV species were deposited and how much was distributed in the different skin layers. It is known that although ACV can be released from topical formulations, it accumulates in the stratum corneum (SC), failing to penetrate down to the basal epidermis, the target compartment. The cutaneous biodistribution of ACV needs to be known to a high resolution in order to determine whether the amounts of ACV delivered into the basal epidermis and adjacent area, where the virus is found, are sufficient to improve its therapeutic effect.

Conventional approaches to investigate cutaneous bioavailability involve the use of tape-stripping to isolate layers of the SC, trypsin digestion to separate the SC from the viable epidermis and the use of heat separation to tease apart the latter from the dermis. However, the procedures involved may affect drug distribution and/or prodrug integrity – moreover, once beyond the SC, the resolution is limited [7]. Microdialysis can also be used for in site measurements of drug levels but reproducible manual insertion of the microdialysis probe to a consistent depth which is just around the basal epidermis is a challenge [8]. To overcome the technical limitations mentioned above and to improve the vertical resolution, a more sophisticated approach was used in this study to determine the biodistribution of ACV species in skin [9,10]. After iontophoresis, the skins were frozen and cryotomed to produce a series of lamellae (each with a nominal thickness of 20 μm), followed by extraction of deposited ACV

and the corresponding ACV-X prodrug from each lamella and simultaneous quantification of relevant compounds by UHPLC-MS/MS. This enabled a vertical quantification of ACV and ACV-X as a function of position within the skin to a nominal resolution of 20 µm.

The objectives of this study were (i) to develop and validate robust UHPLC-MS/MS methods for the quantification of ACV, PCV and ACV-X prodrugs (X=Ile, Val and Arg), (ii) to determine cutaneous bioavailability and biodistribution after topical application of marketed ACV-containing products (Zovirax® cream and ointment) and a penciclovir (PCV) cream (Penvir®), (iii) to compare these to the levels of ACV observed after iontophoresis of ACV, ACV-Arg or ACV-Ile (iv) to develop hydrogel and fast dissolving films as more realistic formulations that might serve as drug reservoirs for iontophoretic systems, and (v) to evaluate the potential clinical relevance of the results.

2. MATERIAL AND METHODS

2.1 Material

Aciclovir (ACV) was purchased from Sequoia Research Product Ltd. (Pangbourne, UK). Penciclovir (PCV) was obtained as a kind gift from ANAWA Trading SA (Wangen, Switzerland). Zovirax® cream (ACV 5% w/w), Zovirax® ointment (ACV 3% w/w) and Fenivir® cream (PCV 1% w/w) were purchased from a local pharmacy store. L-argininyl, L-isoleucyl and L-valyl esters of ACV, i.e. ACV-Arg, ACV-Ile and ACV-Val, were synthesised in-house (Chapter 1). The chemical structures of ACV, PCV, ACV-Arg, ACV-Ile and ACV-Val are shown in **Figure 1**. 2-Morpholino-ethanesulfonic acid monohydrate (MES) was purchased from Fluka BioChemika (Hungary). Carbopol® 980 was purchased from Noveon Inc. (Ohio, USA). Triethanolamine was obtained from Sigma-Aldrich (Buchs, Switzerland). Hydroxypropyl methylcellulose (HPMC, E3) was obtained from Dow Chemical Company (Michigan, US). Glycerin (85%) was purchased from Hanseler AG (Herisau, Switzerland). ULC/MS grade acetonitrile (ACN) and PVC tubing (ID 3.17 mm; OD 4.97 mm) were bought from VWR International AG (Dietikon, Switzerland). ULC/MS grade formic acid (FA) was purchased from Biosolve (Valkenswaard, Netherlands). Silver wire and silver chloride used for making electrodes were supplied by Sigma (St. Louis, US). All aqueous solutions were prepared using Milli-Q water (resistivity $>$ 18 MΩ.cm). All other chemicals were at least of analytical grade.

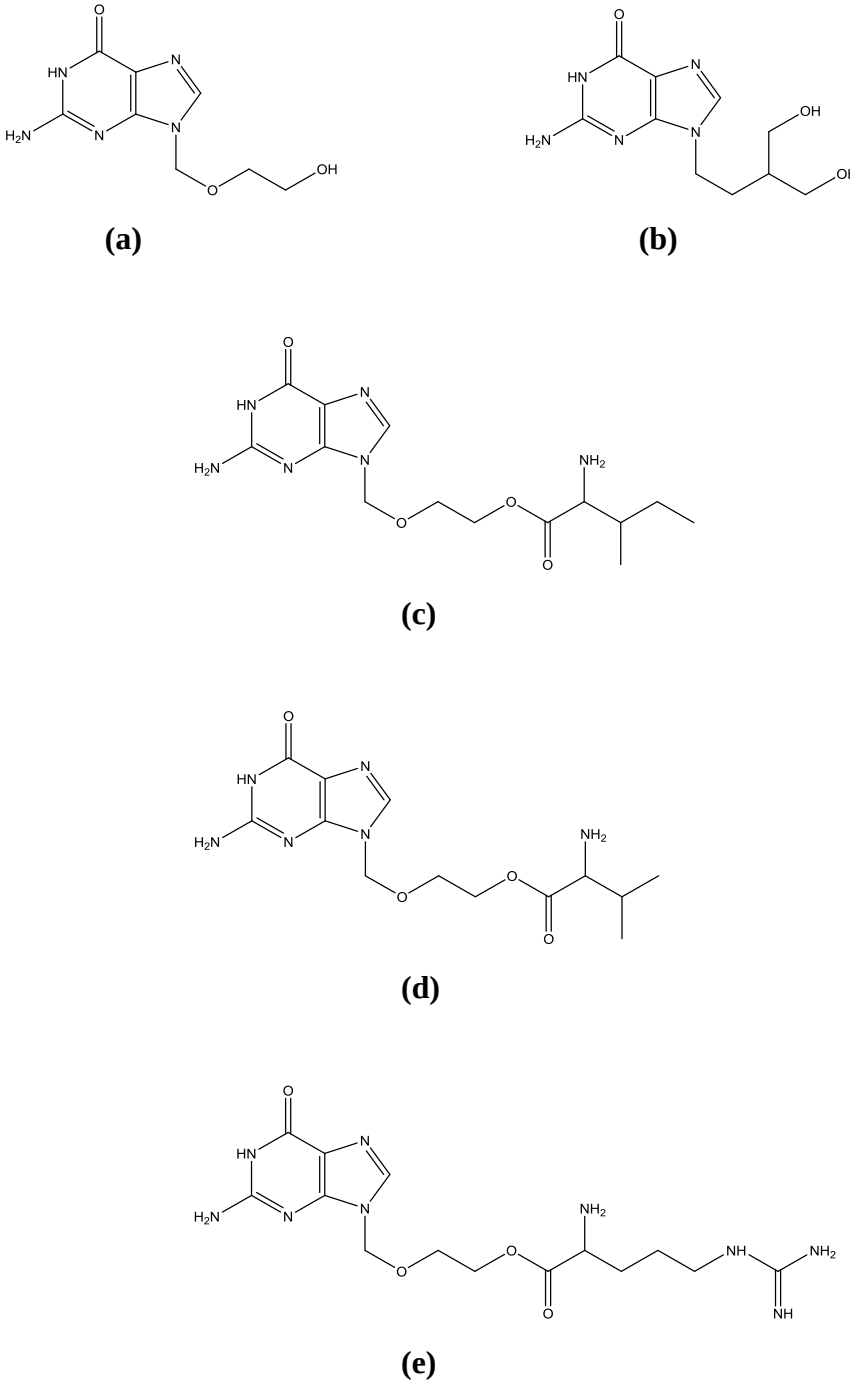


Figure 1. Chemical structure of (a) ACV, (b) PCV, (c) ACV-Ile, (d) ACV-Val and (e) ACV-Arg.

2.2 Skin source

Fresh porcine ears were obtained from a local slaughterhouse (CARRE; Rolle, Switzerland). Full-thickness porcine skin (1.2-1.4 mm) was excised from the outer region of the ear and separated from the underlying cartilage with a scalpel. Human skin samples were collected immediately after surgery from (i) the Department of Plastic, Aesthetic and Reconstructive

Surgery, Geneva University Hospital (Geneva, Switzerland), (ii) Clinique Vert-Pré (Geneva, Switzerland) and (iii) Hôpital de la Tour (Geneva, Switzerland). Full-thickness human skin (1.3-1.5 mm) was obtained after removing subcutaneous fat. Skin membranes were cleaned with running water, punched out into round disks (diameter 26-32 mm), wrapped in Parafilm® and stored at −20°C. Prior to use, skin samples were thawed in saline for 10 min. The studies utilising human skin samples were approved by (i) the Central Committee for Ethics in Research (CER: 08-150 (NAC 08-051); Geneva University Hospital) and (ii) the Commission d'Ethique pour la Recherche Clinique en Ambulatoire (Protocol 10-25; Association des Médecins du Canton de Genève et Société Médicale).

2.3 Analytical methods

Quantification of ACV, ACV-Arg and ACV-Ile was done by UHPLC-MS/MS using a Waters Acquity® UPLC® system coupled to a Waters Xevo® TQ triple quadrupole mass spectrometer. The chromatographic system comprised a binary solvent manager, a sample manager and a column oven. The isocratic separation of ACV and each ACV-X prodrug was achieved with a Waters XBridge® BEH C18 (50 × 2.1 mm, 2.5 μm) reverse phase column maintained at 30°C, with a Waters XBridge® BEH C18 (5 × 2.1 mm, 2.5 μm) guard column placed upstream. The mobile phase consisted of different proportions of 0.1 % formic acid and acetonitrile and the flow rate was 0.2 ml/min (**Table 1**). The temperature of the sample manager was maintained at 8°C. The injection volume was 5 μl. Nitrogen and argon were used as the drying gas and collision gas, respectively. ESI positive mode (ES^+) and multiple reaction monitoring mode (MRM) were employed on the triple quadrupole mass spectrometer to ionise, select and analyse the ions of interest (**Table 1**). The data processing was performed using MassLynx® software. The methods were validated with 3 replicates at 100, 500 and 1,000 nM and showed good intra-day precision (RSD < 1 %) and accuracy (> 92 %), as shown in **Table 2**.

2.4 Cutaneous biodistribution following passive or iontophoretic delivery

2.4.1 The set-up

Iontophoretic and passive transport studies were conducted using full-thickness porcine or human skin. Passive diffusion experiments using the same set-up but in the absence of current served as controls. Full-thickness skin was clamped in vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland) and allowed to equilibrate for 30 min before starting the transport experiments. The permeation area was 0.8 cm^2. The anodal and cathodal (receiver) compartments contained 20 and 10 ml of PBS solution (pH 7.4), respectively. The receiver

Table 1 UPLC-MS/MS methods for ACV, PCV and ACV-X prodrugs quantification.

Analytical Task	Compounds	Chromatography		Mass spectrum					
		Mobile phase	Elution time (min)	Capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)	Parent ion	Daughter ion	LOQ (nM)
ACV-Val transport study	ACV-Val	95% A/ 5% B	2.23	2.5	17	18	325.2	152.0	20
	ACV		1.10				226.2		10
ACV-Ile transport study	ACV-Ile	95% A/ 5% B	2.41	2.5	31	19	339.3		20
	ACV		1.10				226.2		10
ACV-Arg transport study	ACV-Arg	99% A/ 1% B	1.31	2.5	27	20	382.2		50
	ACV		2.46				226.2		10
ACV transport study	ACV	99% A/ 1% B	2.46	2.5	27	20	226.2		10
PCV transport study	PCV	98% A/ 2% B	1.04	2.5	31	19	254.2		20

* Mobile phase A: 0.1 % formic acid. Mobile phase B: acetonitrile

Table 2 Precision and accuracy of the analytical methods used to quantify ACV, PCV and ACV-X prodrugs

	Theoretical concentration (nM)	Experimental concentration (nM)	Precision (%)[a]	Accuracy (%)[b]
ACV	100	104.66 ± 0.71	0.68	104.66
	500	508.23 ± 3.89	0.77	101.65
	1000	1018.87 ± 6.33	0.62	101.89
PCV	100	107.29 ± 0.65	0.61	107.29
	500	512.21 ± 2.79	0.54	102.44
	1000	1023.89 ± 5.29	0.52	102.39
ACV-Val	100	98.21 ± 0.78	0.79	98.21
	500	489.94 ± 3.01	0.61	97.99
	1000	978.78 ± 5.89	0.60	97.88
ACV-Ile	100	97.44 ± 0.61	0.63	97.44
	500	493.25 ± 3.59	0.73	98.65
	1000	988.30 ± 3.91	0.40	98.83
ACV-Arg	100	95.24 ± 0.78	0.82	95.24
	500	471.94 ± 4.01	0.85	94.39
	1000	921.01 ± 8.81	0.96	92.10

[a]Precision = (SD/mean) x 100.

[b]Accuracy = (obtained concentration/theoretical concentration) x 100

solution was stirred continuously, and the temperature maintained at 32°C using a water bath. Ag and AgCl electrodes were inserted into the anodal and cathodal compartments, respectively. Salt bridge assemblies (3 % agarose in 0.1 M NaCl) were prepared using PVC tubing and employed to electrically connect the physically isolated anodal and donor compartments [11]. The donors were not occluded. A constant current, 0.25 mA/cm^2, was supplied using an APH 1000 M power generator (Kepco Inc; Flushing, US).

Upon completion of the transport experiments, the diffusion cells were dismantled and the surface of skin samples cleaned with running water and wet cotton to remove any residual formulation. The skin surface in contact with the formulations was punched out, fixed in an embedding medium, and flattened by application of slight pressure using a glass slide. It was

then snap-frozen in isopentane cooled by liquid N_2 and cryotomed using a Microm HM 560 Cryostat (Thermo Scientific; Walldorf, Germany) to obtain a series of lamellae (see below for thickness – either 20 µm or 100 µm). The lamellae were individually extracted with 0.1 % formic acid for 3 h (it also deactivated the cutaneous esterases). The mixture was centrifuged at 12,000 rpm for 15 min and the supernatant filtered through an Exapure® nylon filter membrane with a pore size of 0.22 µm (Alys Technologies; Bussigny-près-Lausanne, Switzerland). All samples were analysed by UHPLC-MS/MS.

2.4.2 Passive delivery of ACV and PCV from marketed semi-solid formulations

Zovirax® cream (40 mg, 11.10 µmol/cm^2), Zovirax® ointment (40 mg, 6.66 µmol/cm^2) or Fenivir® cream (40 mg, 1.97 µmol/cm^2) were placed in the donor compartment. All formulations were spread evenly across porcine skin surface and left in place for 60 min. Upon completion of the experiments, 10 lamellae each with a thickness of 20 µm were obtained from each skin sample using the method described above, followed by extraction and quantification. Experiments were performed in triplicate.

2.4.3 Iontophoretic delivery of ACV-X prodrugs from aqueous solution

Aqueous solutions of ACV-Arg and ACV-Ile were prepared at a concentration of 5 mM in MES buffer (10 mM, pH 5.5). 1 ml of ACV-X prodrug solution (6.25 µmol/cm^2) was pipetted into each donor compartment and current was applied for 10, 30 or 60 min. Upon completion of the experiments, 4 lamellae each with a thickness of 100 µm were obtained from each skin sample as described above. ACV, ACV-Arg or ACV-Ile were quantified in each lamella to determine the "coarse" biodistribution as a function of position and the duration of iontophoretic current application. More precise cutaneous biodistribution profiles were determined following iontophoresis of ACV or each ACV-X prodrug for 5 min. Each skin sample was cryotomed to obtain a total of 10 lamellae each with a thickness of 20 µm, followed by extraction and quantification. Experiments were performed in quintuplicate.

2.4.4 Short duration iontophoretic delivery of ACV-X from hydrogel and thin film formulations

2.4.4.1 Preparation of ACV-Ile containing hydrogel

Hydrogels have been extensively used as drug reservoirs in iontophoretic patches [12]. Here, a Carbopol® hydrogel was prepared to incorporate ACV-Ile. Briefly, Carbopol® 980 powder was slowly dispersed in water at 40°C. After swelling for 30 min, mild agitation was initiated

until the polymeric solution became transparent. The solution was then cooled to room temperature, followed by addition of ACV-Ile and two preservatives, methylparaben and propylparaben. The pH of the mixture was adjusted with 10% triethanolamine solution to 5.5. A transparent, viscous hydrogel was formed. The composition (w/w) of the hydrogel was 0.22% ACV-Ile, 1.07% Carbopol® 980, 0.26% methyparaben and 0.03% propylparaben.

2.4.4.2 Preparation of ACV-Ile / ACV-Val containing fast dissolving film

A novel drug reservoir for iontophoresis, comprising a fast dissolving film, which can dissolve or disintegrate rapidly when hydrated [13], was prepared by the solvent-casting method. It was envisaged that an ACV-X containing fast dissolving film could be hydrated either by water or a water-rich material, e.g. a blank hydrogel. Before use, the ACV-X prodrug film was combined with the hydrogel (with the film in contact with the skin) and was rapidly hydrated and current application initiated. The films were prepared using HPMC as the film forming polymer; it was gradually poured and dissolved in water (50°C) with constant blending (600 rpm). After sufficient swelling, the polymeric solution was cooled down to room temperature. Glycerin, acting as the film plasticizer, and ACV-Ile were subsequently added to the solution with mild stirring. Given its similar physical chemical properties to ACV-Ile, it was also decided to test ACV-Val. The mixture was then left to stand for 2 h to remove any bubbles. The optimised compositions (w/v) in film casting solution were 1.57% ACV-X, 8% HPMC and 3% glycerin. Five ml of the mixture was cast onto a plastic Falcon® Petri dish with a diameter of 88 mm and area of 60 cm^2 (BD Company; Franklin Lakes, US) and dried overnight in a ventilated fume hood at room temperature. The Petri dish was covered with a piece of filter paper to avoid contamination. The films were carefully peeled off from the bottom of the Petri dish, punched into disks with a diameter of 16 mm (area ~2 cm^2) as one dosage unit, sealed into sachets and stored in a desiccator. The ACV-Ile or ACV-Val content in the fast dissolving films was ~1.3 mg/cm^2. To determine the disintegration time of the fast dissolving film, a film disk was put onto a piece of light blue blotting paper (VWR; Radnor, US). One drop of distilled water was added onto the film disk. The water drop started to hydrate the film from the top to the bottom. When the hydration process reached the bottom, the light blue paper was fully moistened and changed the colour from light blue to dark blue. The disintegration time was recorded from the time when the water drop was added onto the film disk to the time when the colour of the blotting paper changed to dark blue.

2.4.4.3 Stability studies

ACV-Ile containing hydrogels and fast dissolving films underwent stability testing. The hydrogels were kept at 4°C and the fast dissolving films were stored in a desiccator at room temperature over 1 month. Samples were taken every 5 days to determine the extent of hydrolysis of ACV-Ile. The fraction of ACV-Ile remaining (%ACV-Ile) was plotted as a function of time (*t*). Experiments were performed in triplicate.

2.4.4.4 Iontophoretic protocol

Iontophoresis of ACV-Ile from the Carbopol® hydrogels at a current density of 0.25 mA/cm^2 for 5 min was carried out using full-thickness porcine or human skin. 1 g of hydrogel containing ACV-Ile was placed in each donor compartment (permeation area ~0.8 cm^2, ACV-Ile loading 6.1 μmol/cm^2). Salt bridges assemblies were used to connect the hydrogel and the anodal compartment. After completion of the experiments, each skin sample was cryotomed to obtain 10 lamellae each with a thickness of 20 μm, followed by extraction and quantification. Experiments were performed in quintuplicate.

Iontophoresis of ACV-Ile or ACV-Val from fast dissolving films at a current density of 0.25 mA/cm^2 for 5 min was conducted using full-thickness porcine skin. A disk of the film containing either ACV-Ile or ACV-Val was placed into each donor compartment (permeation area ~2 cm^2, ACV-Ile loading 2.9 μmol/cm^2, ACV-Val loading 3.0 μmol/cm^2). While the thin film was in contact with the skin surface, 1 g of blank Carbopol® 980 hydrogel, serving as the electrically conductive medium and the hydrating material, was placed on top of the film. The blank Carbopol® 980 hydrogel was prepared as described in Section 2.4.4.1, except no active compound was added. Salt bridge assemblies were used to connect the electrically conductive hydrogel and the anodal compartments. Upon completion of the experiments, each skin sample was cryotomed to yield 10 lamellae each with a thickness of 20 μm, followed by extraction and quantification. Experiments were performed in triplicate.

2.5 Statistics

All the data were expressed as the mean ± SD. Dixon test was used to determine outliers. Results were evaluated statistically using either analysis of variance (ANOVA) or Student's t-test. Student Newman Keuls test was also performed when necessary as a post-hoc procedure. The level of significance was fixed at $p = 0.05$.

3. RESULTS AND DISCUSSION

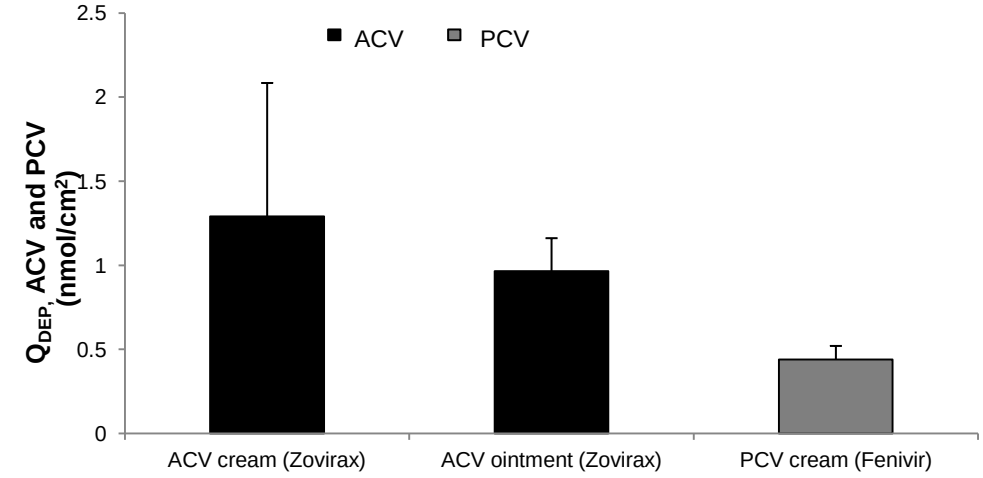
3.1 Passive delivery from commercial formulations

Unsatisfactory clinical outcomes seen with ACV cream or ointment have been attributed to insufficient delivery of ACV to the basal epidermis, the target site of HSV infections [2,14,15]. In the present study, cryosectioning combined with UHPLC-MS/MS was used in order to obtain a cutaneous biodistribution profile of ACV or PCV following topical application of marketed formulations. This gave a more precise assessment of cutaneous bioavailability as a function of position within the epidermis and upper dermis after topical drug application, as compared to other methods, e.g., tape-stripping or heat-separation.

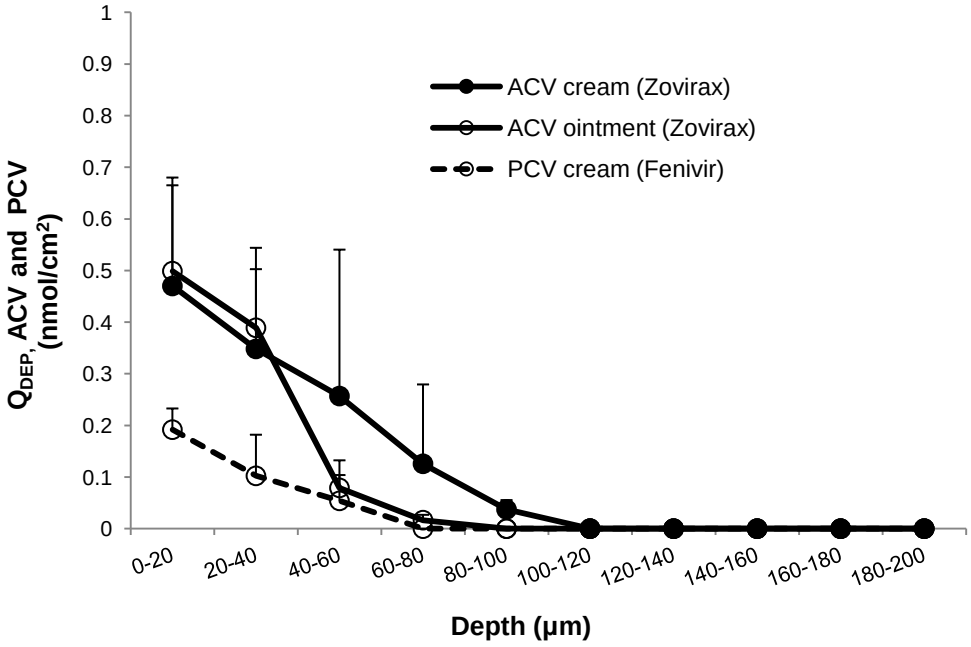
Zovirax® cream, Zovirax® ointment or Fenivir® cream only resulted in very limited drug deposition in full-thickness porcine skin over 60 min, 1.3 ± 0.8, 1.0± 0.2 and 0.4 ± 0.1 nmol/cm^2, respectively (**Figure 2a**). The biodistribution profiles following application of Zovirax® cream and ointment showed that ACV was deposited principally in the stratum corneum and upper epidermis (**Figure 2b**). In the case of Zovirax® ointment, ACV levels decreased rapidly after this point; Zovirax® cream enabled slightly deeper ACV penetration but amounts were <0.1 nmol/cm^2 at a depth of 80-100 μm. The biodistribution profile of PCV following application of Fenivir® showed that PCV levels dropped to <0.1 nmol/cm^2 at a depth of 40-60 μm. The amount of ACV or PCV adjacent to the basal epidermis (100-160 μm) was below the LOQ. These results demonstrated that poor intraepidermal bioavailability was the cause for the lack of clinical efficacy observed with these formulations.

Prolonged topical application of ACV or PCV cream can increase cutaneous bioavailability; for example, application of ACV and PCV cream on human skin *in vitro* for 24 h led to modest drug levels up to a depth of 100 μm [18]. However, successful treatment of topical HSV infections depends on both the amount of drug present in the target compartment and the rate of delivery, as ACV needs to be present at the target site in a narrow therapeutic time window, i.e. within 8-12 h after the onset of symptoms [19,20]. Given the poor penetration of ACV or PCV, it is unlikely that either drug could be delivered at a sufficient rate during the therapeutic time window and so alter the course of the disease. Unsurprisingly, the pharmacokinetic profiles of ACV in the basal epidermis following topical administration have shown that it was poorer than that following daily oral administration (at albeit high doses of 200-800 mg five times a day) [17]. This explains the better pharmacological efficacy observed following oral administration, although high systemic exposure of ACV also

increases the risk of potential kidney damage [21]. Efficient topical ACV formulations with better delivery kinetics would address this clinical unmet need.



(a)



(b)

Figure 2. Delivery of ACV or PCV in full-thickness porcine skin after application of ACV cream (Zovirax®), ACV ointment (Zovirax®) or PCV cream (Fenivir®) for 60 min **(a)** Total deposition of ACV ($Q_{DEP,ACV}$) and PCV ($Q_{DEP,PCV}$); **(b)** Cutaneous biodistribution of ACV and PCV as a function of position to a depth of 200 µm. (Mean ± SD; n=3).

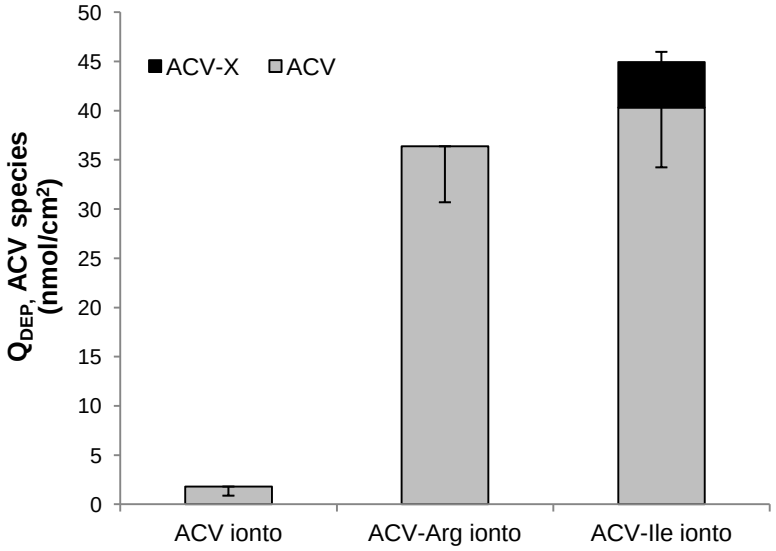
3.2 Iontophoretic delivery of ACV-X prodrugs from aqueous solution

In contrast to the poor passive ACV skin delivery from the marketed formulations, iontophoresis of ACV-Arg or ACV-Ile for 60 min from aqueous solution resulted in much greater skin deposition of ACV species (the sum of the amount of ACV ($Q_{DEP,ACV}$) and each prodrug ($Q_{DEP,ACV\text{-}X}$)): 375.3 ± 66.0 and 302.4 ± 45.3 nmol/cm^2 were observed up to 400 μm, respectively (**Table 3**). This very high deposition provided ample room to reduce the duration of current application: iontophoresis for 10 and 30 min also resulted in appreciable skin deposition of ACV species. Iontophoresis of ACV-Arg and ACV-Ile for 10 min resulted in ACV skin deposition 174.7 ± 44.7 nmol/cm^2 and 97.9 ± 25.3 nmol/cm^2 in the first 400 μm of the skin, which was ~130- and ~70-fold higher than ACV deposition after application of ACV 5 % cream for 60 min. More importantly, it enabled much deeper ACV skin penetration. In contrast to passive delivery of ACV, where drug was retained in the stratum corneum and upper epidermis, appreciable amounts of ACV species were observed in the second lamella (100-200 μm) which may be considered to correspond approximately to the basal epidermis and its adjacent area – the target compartment for ACV. For example, iontophoretic delivery of ACV-Arg and ACV-Ile for 10 min resulted in ACV species deposition in this area, of 41.2 ± 9.2 and 25.4 ± 8.1 nmol/cm^2, whereas a negligible amount of ACV was found after application of ACV cream or ointment for 60 min.

The duration of iontophoresis was further reduced to 5 min and the resolution of the ACV/ACV-X prodrug cutaneous biodistribution profile was improved by taking finer 20 μm lamellae. Following iontophoresis of ACV-Ile or ACV-Arg from aqueous solution for 5 min, the amounts of ACV species deposited in full-thickness porcine skin were 44.9 ± 7.0 and 36.4 ± 5.7 nmol/cm^2, respectively, which were ~25 and ~20 times higher than that after iontophoresis of ACV (1.8 ± 0.9 nmol/cm^2) (**Figure 3a**). The ACV species distribution profile in the first 200 μm of the skin is shown in **Figure 3b**. Considerable levels of ACV species were present at the target area (100-160 μm) after iontophoresis of ACV-Arg and ACV-Ile and, 4.0 ± 1.6 nmol/cm^2 and 6.5 ± 1.7 nmol/cm^2, respectively.

Table 3 Iontophoretic delivery of ACV-X (X=Ile and Arg) prodrugs for 10, 30 and 60 min from aqueous solution

Duration of Iontophoresis (min)		**ACV species deposition in each lamella** (nmol/cm^2)								**Sum of the amount up to 400 µm**
		0~ 100 µm		**100~200 µm**		**200~300 µm**		**300~400 µm**		
		ACV	ACV-X	ACV	ACV-X	ACV	ACV-X	ACV	ACV-X	ACV+ACV-X
ACV-Ile	10	25.5 ± 10.5	13.6 ± 5.3	17.2 ± 6.9	8.2 ± 1.3	12.8 ± 4.0	6.4 ± 1.3	9.8 ± 2.7	4.3 ± 1.6	97.9 ± 25.3
	30	62.1 ± 12.9	40.0 ± 12.1	27.2 ± 11.4	12.0 ± 5.8	21.3 ± 4.8	8.6 ± 5.0	20.2 ± 4.6	6.4 ± 2.5	197.6 ± 21.3
	60	77.4 ± 8.5	40.8 ± 20.1	49.9 ± 12.9	19.5 ± 7.9	41.8 ± 4.7	16.3 ± 3.3	41.4 ± 6.0	15.3 ± 3.8	302.4 ± 45.3
ACV-Arg	10	73.9 ± 39.2	0	41.2 ± 9.2	0	35.0 ± 7.2	0	24.6 ±15.0	0	174.7 ± 44.7
	30	78.8 ± 19.6	0	56.6 ± 6.8	0	43.2 ± 4.4	0	41.7 ± 7.4	0	220.3 ± 16.0
	60	155.5 ± 43.0	0	94.5 ± 32.6	0	68.0 ± 15.6	0	57.4 ± 6.3	0	375.3 ± 66.0



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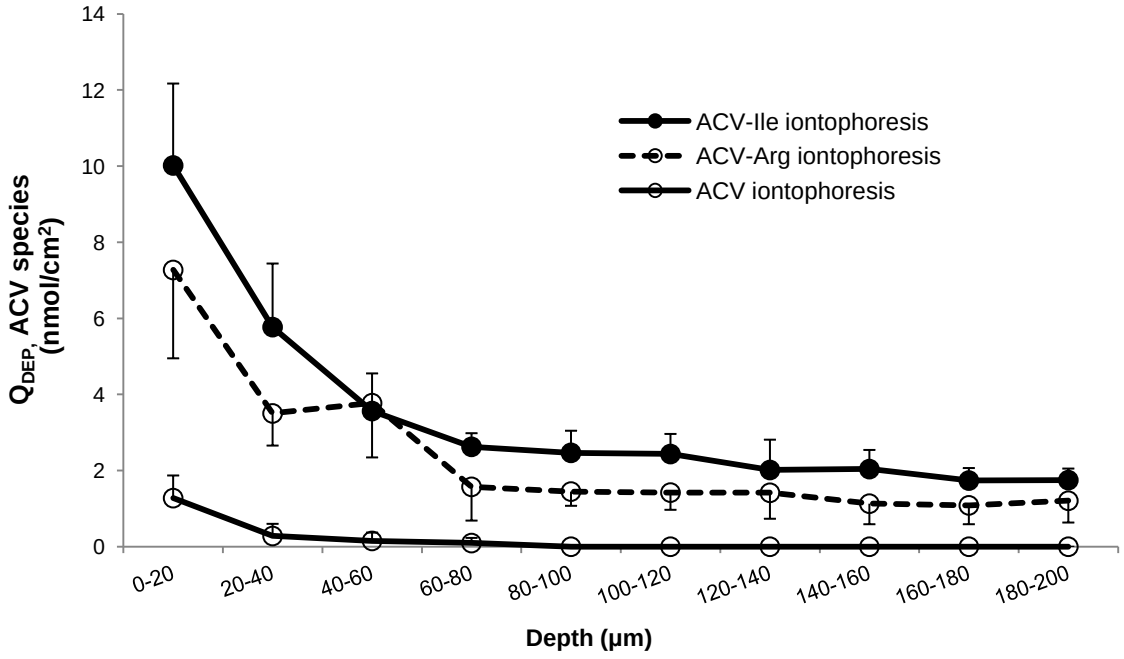


(b)

Figure 3. Delivery of ACV and ACV-X following iontophoresis of ACV, ACV-Arg or ACV-Ile (5 mM) in 10 mM MES buffer (pH 5.5) at 0.25 mA/cm^2 for 5 min **(a)** Total deposition of ACV, ACV-Arg (complete bioconversion of ACV-Arg to ACV) and ACV-Ile; **(b)** of ACV species. (Mean ± SD; n=5)

3.4 Iontophoresis using hydrogel

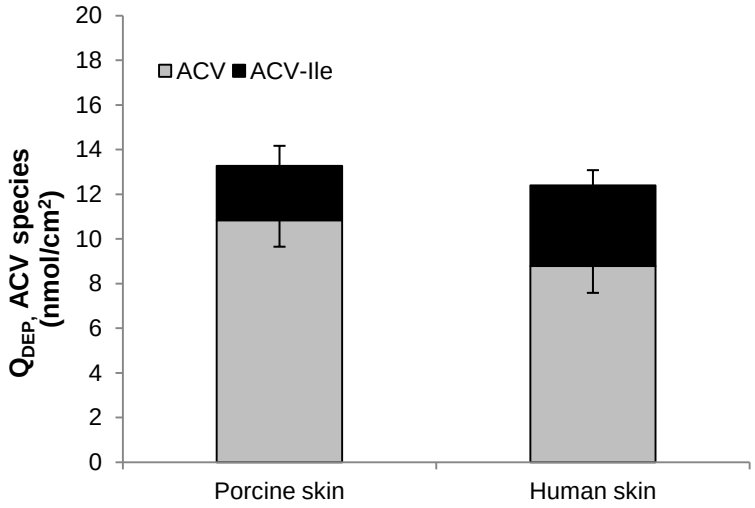
Short-duration iontophoresis of ACV-X prodrugs from aqueous solution certainly showed promising results; however, a solution formulation is not convenient for practical use.

Hydrogels are easily-prepared, semi-solid formulations, which can be conveniently incorporated into an iontophoretic patch, serving as a base/reservoir of active compounds in dermal [12], ocular [22] and buccal [23] drug delivery. They have been employed in US FDA approved pre-filled iontophoretic systems, e.g., Lidosite® (Vyteris Inc.; Fair Lawn, United States), where Ag/AgCl electrodes and a lidocaine hydrochloride/epinephrine dispersion were incorporated into the hydrogel matrix patch [24]. It was decided to develop the hydrogel system for ACV-Ile rather than ACV-Arg since ACV-Ile was i) more chemically stable than ACV-Arg, which was advantageous for storage, and ii) much less hygroscopic, which made its handling and storage much easier. A viscous, transparent Carbopol® hydrogel was used to formulate ACV-Ile (0.22 % w/w) for use in subsequent experiments.

Short-duration iontophoresis for 5 min at 0.25 mA/cm^2 using the ACV-Ile hydrogel resulted in statistically equivalent deposition of ACV species in full thickness porcine and human skin (13.3 ± 1.5 and 12.4 ± 1.4 nmol/cm^2, respectively) (**Figure 4a**). Although less ACV species deposition was observed than after iontophoresis of an aqueous solution of ACV-Ile under almost equivalent conditions (ACV-Ile loading dose: aqueous solution 6.25 μmol/cm^2; hydrogel 6.1 μmol/cm^2) , the cutaneous distribution profile demonstrated appreciable delivery up to 200 μm, including the basal epidermis (100-160 μm) (**Figure 4b**), where 2.1 ± 0.7 and 1.3 ± 0.3 nmol/cm^2 of ACV species were found in porcine and human skin, respectively

3.5 Iontophoresis using fast dissolving film

Although iontophoresis using the hydrogel systems enabled appreciable delivery of ACV-X prodrugs and was a step towards a more "real-world" formulation, the high water content might decrease the stability of the ester prodrugs and render their long-term storage difficult. To overcome this problem, either stabilising agents can be added to form complexes with the esters [25] or the water content must be reduced. Fast dissolving films are novel dosage forms that have been investigated in recent years for paediatric and geriatric oral administration [26]. This solid dosage form can disintegrate or dissolve rapidly in saliva after application [27]. Advantages including fast disintegration, limited water content, ease of handling and convenient dosing, make them attractive carriers for the ACV-X prodrugs. ACV-Ile and ACV-Val, another prodrug with a moderately hydrophobic side chain, were formulated into this dosage form, and the content of ACV-Ile or ACV-Val was ~1.3 mg/cm^2 of the film; the disintegration time of the films was 38.0 ± 20.2 s.



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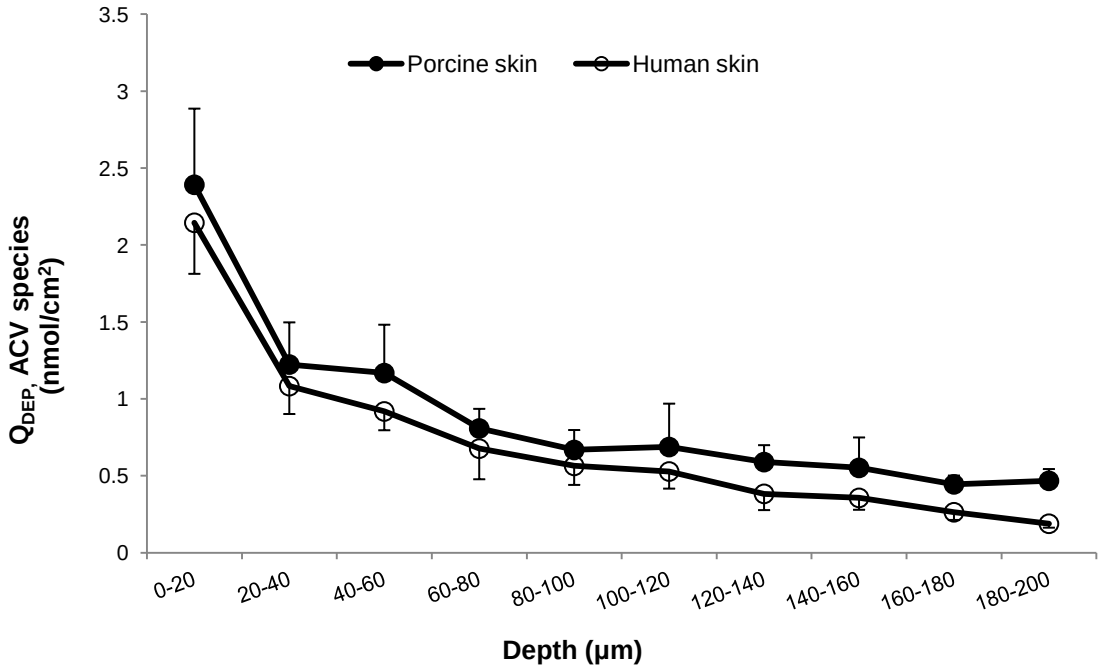


(b)

Figure 4. Comparison of **(a)** total deposition (Q_{DEP}) and **(b)** cutaneous biodistribution of ACV species in full-thickness porcine and human skin after iontophoresis at 0.25 mA/cm^2 of ACV-Ile from a hydrogel for 5 min. (Mean ± SD; n=5)

ACV-Ile showed considerable stability within the fast dissolving film after storage for 1 month at room temperature (~99% remained intact). Slightly greater ACV-Ile hydrolysis was observed with the hydrogel with ~ 93% of the ester remaining intact after storage for 1 month at 4°C but preservatives were also added to prevent bacterial contamination (**Figure 5**).

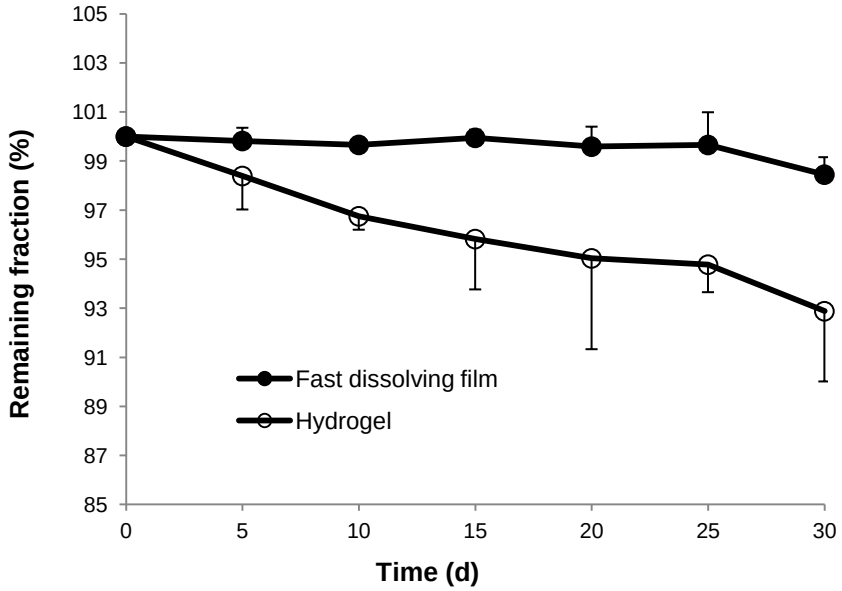
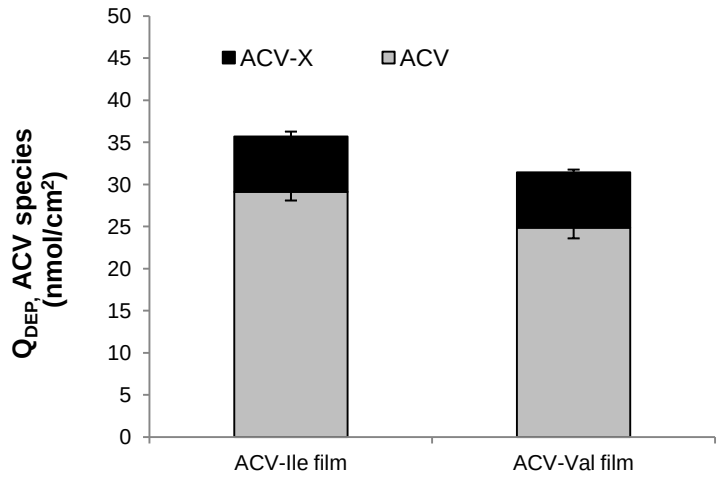


Figure 5. Stability profile of ACV-Ile in hydrogel or fast dissolving film over 1 month at room temperature (Mean ± SD; n=3)

Blank Carbopol® hydrogel (1 g) was used as the hydrating material as well as a conductor, which was connected to the anodal compartment via a salt bridge. Following iontophoresis of ACV-Ile or ACV-Val for 5 min using the fast dissolving film, 35.7 ± 2.0 or 31.4 ± 2.0 nmol/cm^2 of total ACV species was observed in full-thickness porcine skin (**Figure 6a**). Despite a 2-fold lower loading of ACV-Ile than the hydrogel (2.9 μmol/cm^2 and 6.1 μmol/cm^2, respectively), the fast dissolving film enabled a ~2.7 fold increase in cutaneous deposition of ACV species under the same iontophoretic conditions. The amount of ACV species deposited in the basal epidermis and its adjacent area (100-160 μm) after iontophoresis of ACV-Ile or ACV-Val was 3.2 ± 0.1 and 3.1 ± 0.1 nmol/cm^2, respectively (**Figure 6b**). The excellent iontophoretic delivery of ACV-X prodrugs might be attributed to the intimate contact with skin tissue.



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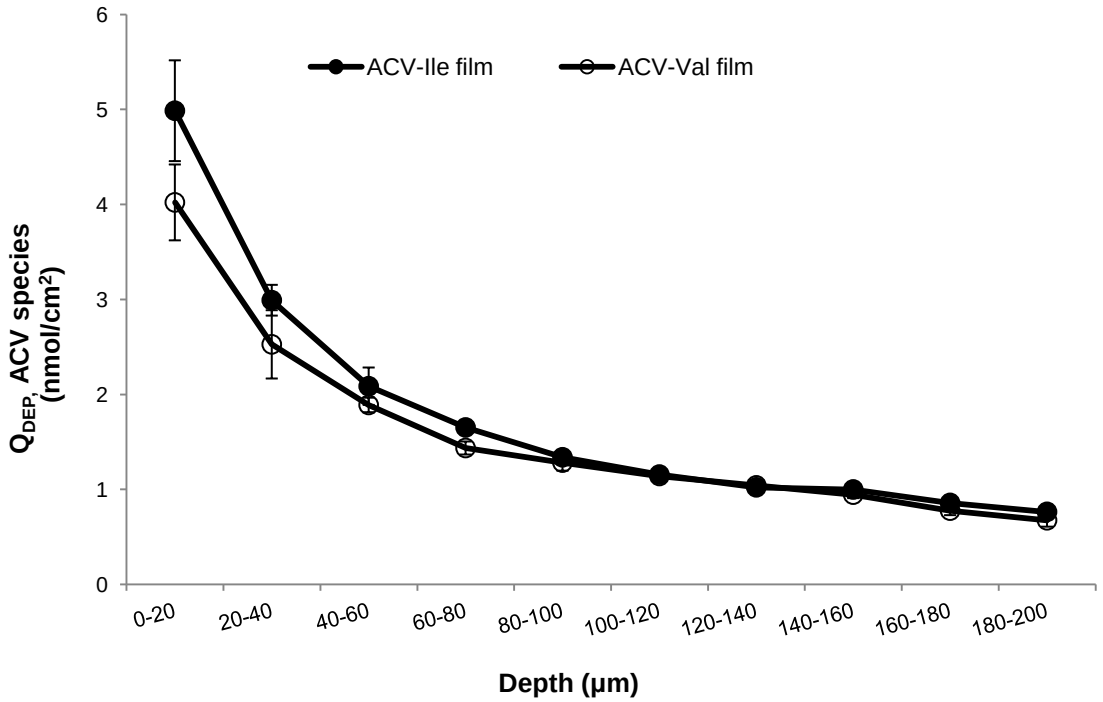

(b)

Figure 6. Comparison of **(a)** total deposition (Q_{DEP}) and **(b)** cutaneous biodistribution of ACV species after iontophoresis at 0.25 mA/cm^2 of ACV-Ile or ACV-Val from fast dissolving films for 5 min. (Mean ± SD; n=3)

3.6 Clinical relevance

The poor clinical efficacy of ACV cream or ointment for the treatment of herpes infections has resulted in much work being done to address the unmet need [28], including the

development of various novel topical ACV delivery systems [29]. However, only a few of them have discussed ACV levels in the basal epidermis [6,10,15,30,31].

Iontophoresis of ACV-Ile from aqueous solution, hydrogel or fast dissolving film for 5 min resulted in high levels of ACV species deposited in the target area, 6.5 ± 1.7, 2.1 ± 0.7 and 3.2 ± 0.1 nmol/cm^2, respectively, which was principally present as ACV, 6.1 ± 1.5, 1.8 ± 0.5 and 2.7 ± 0.1 nmol/cm^2, respectively (**Figure 7**). Converted to concentration units, ACV exposure in the target area was 1,018 ± 247, 303 ± 91 and 455 ± 7 nmol/cm^3, respectively. Given that the IC_{50} of ACV against HSV-1 and HSV-2, 0.04-3.1 and 0.04-14.2 nmol/cm^3, respectively [32] (Beauchamp *et al*., 1992), is orders of magnitude lower than the ACV concentrations achieved in the target area following iontophoresis of ACV-Ile for only 5 min, it is hypothesised that the fast, efficient topical ACV delivery described here would be a promising approach for the successful treatment of topical HSV infections.

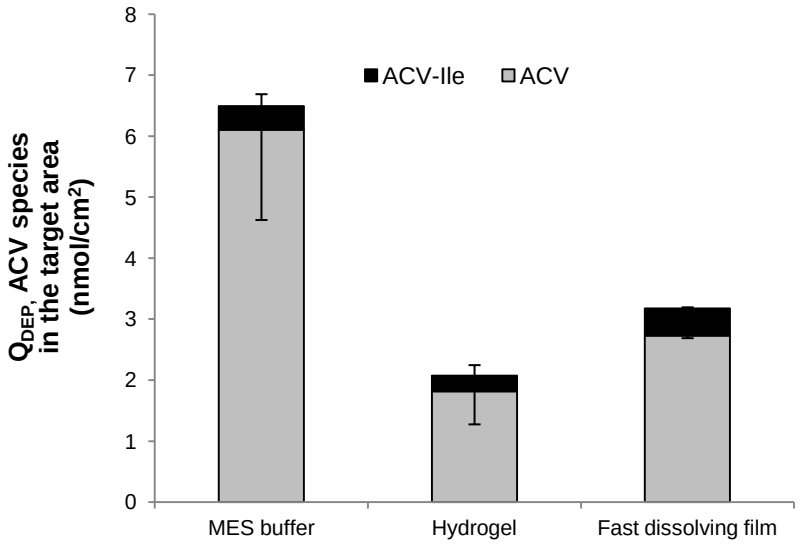

Figure 7. Deposition (Q_{DEP}) of ACV species in the basal epidermis and its adjacent area (100-160 μm) after iontophoresis at 0.25 mA/cm^2 of ACV-Ile from MES buffer, hydrogel or a fast dissolving film for 5 min. (Mean ± SD; n=3 or 5)

4. CONCLUSION

The cutaneous biodistribution of ACV species following iontophoresis of amino acid ester prodrugs (ACV-Ile, ACV-Val or ACV-Arg) was investigated using cryosectioning in conjunction with UHPLC-MS/MS. The amount of ACV in the basal epidermis and its adjacent area (100-160μm) following iontophoresis of ACV-X prodrugs for only 5 min was

orders of magnitude greater than that observed after topical application of Zovirax® or Penvir®. Iontophoretic delivery of ACV-Ile using two clinically acceptable formulations, a hydrogel and a fast dissolving film, enabled appreciable levels of ACV species in the basal epidermis. In summary, short-duration iontophoresis of water-soluble, ionisable amino acid ester prodrugs of ACV enabled supra-therapeutic levels of ACV to be achieved rapidly in the basal epidermis and may provide an effective approach to treat recurrent HSV infections. Moreover, hydrolysis of the ACV-X prodrug only releases ACV and a natural amino acid.

Clinical trials have been conducted using a "pen-like" handheld iontophoretic device with a central drug reservoir in the headpiece to hold 250 mg dose of aciclovir 5% cream (Uquifa®) which was applied through a bandage-like applicator for the treatment of *herpes labialis* [33]. Clinical observations suggested superiority over placebo cream (same composition except aciclovir was replaced with titanium dioxide). Since there was no comparison with passive aciclovir cream, it is not possible to determine whether superiority was due to the presence of the drug or the combination of iontophoresis and the aciclovir containing formulation. However, it is clear that given its physicochemical properties – in particular lack of charge at physiologically acceptable conditions – ACV is a poor iontophoretic candidate.

It should be possible to develop a much more efficient iontophoretic device incorporating "wet" or "dry" formulations of an amino acid ester prodrug of ACV. Future studies will focus on the optimisation of the formulation and the design and development of an applicator.

5. ACKNOWLEDGEMENTS

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Chapter 3

Short duration ocular iontophoresis of ionisable aciclovir prodrugs: a new approach to treat herpes simplex eye infections

Short duration ocular iontophoresis of ionisable aciclovir prodrugs: a new approach to treat herpes simplex eye infections

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ABSTRACT

The objective was to investigate transcorneal and transscleral iontophoretic delivery of three amino acid ester prodrugs of aciclovir (ACV) – ACV-X, where X = Arg, Gly and Trp – as a means to increase ocular bioavailability of ACV. Quantification of ACV and each ACV-X prodrug was by ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS); the methods were validated. ACV-X prodrugs were biolabile in the presence of esterases in fresh porcine eyes. Passive delivery of ACV across porcine cornea after 1 h was below LOQ when using either Zovirax® ointment (3%) or saturated ACV solution. Corneal deposition and permeation after ACV iontophoresis for 5 min followed by passive delivery for 55 min were 3.4 ± 1.0 nmol/cm^2 and 1.9 ± 0.3 nmol/cm^2, respectively. Iontophoresis of 5 mM solutions of ACV-Arg, ACV-Gly and ACV-Trp resulted in much higher corneal deposition (21.5 ± 5.1, 14.1 ± 2.0 and 5.3 ± 0.6 nmol/cm^2, respectively) and transcorneal permeation (13.9 ± 1.6, 10.9 ± 1.8 and 5.7 ± 0.5 nmol/cm^2, respectively) of ACV species. The higher permeability of the sclera as compared to the cornea was evidenced by the observation that passive transscleral delivery of ACV using Zovirax® ointment (3%) or saturated solution after 30 min (4.9 ± 0.9 nmol/cm^2 and 40.7 ± 3.7 nmol/cm^2, respectively) was superior to transcorneal permeation after 60 min, which was below the LOQ. The differences were accentuated by iontophoresis. Transscleral permeation of ACV-Arg, ACV-Gly and ACV-Trp and after current application for only 5 min was 20.4 ± 3.8, 12.3 ± 0.3 and 8.4 ± 0.4 nmol/cm^2, respectively. After 30 min, it had increased to 345.3 ± 36.7, 179.9 ± 9.7 and 144.1 ± 6.8 nmol/cm^2, respectively. Using intact porcine eyes, five-minute transscleral iontophoresis of ACV-Gly at 3.75 mA/cm^2 resulted in not only considerable amounts of ACV species in the choroid/retina and vitreous humour (5.7 ± 2.3 and 11.7 ± 3.7 nmol/cm^2, respectively) but also significantly higher average concentration of ACV species in the whole eyeball (4.5 ± 1.6 nmol/cm^3) than the IC_{50} of ACV against ocular HSV-1 (< 0.22 nmol/ml), which was a very promising result for the treatment of HSV infections in either anterior or posterior segments of the eye.

Keywords: Aciclovir, prodrug, stability, ocular, iontophoresis, biodistribution

1. INTRODUCTION

Herpes simplex virus, particularly HSV-1, can affect various components of the human eye, causing *herpes simplex keratitis* [1], *uveitis* [2], *retinitis* [3] or *endophthalmitis* [4]. Furthermore, after primary infection, HSV-1 can remain latent in the trigeminal ganglion and reactivate periodically, causing cumulative damage to ocular tissues or even leading to blindness [5].

Aciclovir (ACV) is used for the treatment of *herpes simplex* eye infections [6] but it displays only modest clinical efficacy [7]. ACV has poor and variable oral bioavailability (10-20%), and gram amounts must be taken daily, which can affect renal function [8]. Topical administration of ACV would enable targeted therapy and reduce circulating drug levels, but it is also not without problems.

Topical delivery of ACV into the anterior segment for the treatment of *herpes simplex keratitis* seems pretty straightforward, but it is only effective against superficial HSV infections, i.e. corneal *epithelial keratitis* [9], but not for deep infections, e.g., *herpes stromal keratitis* [10]. The cornea is comprised of an outer lipophilic epithelium, followed by a hydrophilic stroma and an endothelial layer [11]. The tight epithelial junctions and the water-rich matrix of the stroma (90% water content) present biological barriers to hydrophilic and lipophilic molecules, respectively [12]. ACV is a polar (log D_{pH7} -1.76), poorly water soluble molecule [13], whose transport is restricted efficiently by the corneal epithelium [14]. This translates to sub-therapeutic levels of ACV in the stroma, endothelium, aqueous humour and iris. Topical treatment of HSV infections in the posterior segment is challenging as intraocular bioavailability following topical administration is $\leq$ 10% [15]. Thus, achieving therapeutic drug levels in the posterior segment is extremely difficult [16].

Many topical formulations of ACV have been developed with the aim of increasing ocular ACV bioavailability [17,18,19]. However, either the risk of ocular irritation or complicated preparation techniques may limit their clinical development. Lipophilic prodrugs of ACV have been proven to increase corneal transport passively through improved partitioning [14]. More recent observations have identified amino acid or oligopeptide transporters present within the cornea and retina [20]. These were exploited to facilitate the delivery of amino acid ester prodrugs or oligopeptide prodrugs of ACV into the anterior segment [21] and ganciclovir (GCV), an ACV analogue, for the treatment of retina CMV infections, into the

posterior segment [22]. Although the function of these ATP-dependent transporters in pathological conditions needs to be investgated, this is an interesting approach for convenient and safe delivery of ACV or GCV into ocular tissue.

Iontophoresis involves the application of an electric field across a biological barrier in order to facilitate electromigration of ions; electrotransport of neutral molecules is also enhanced across permselective membranes by electroosmosis, a secondary mechanism which is much less effective than electromigration [23]. The presence of the potential gradient enables much greater transdermal drug delivery than simple passive diffusion [24]. It has been proven to be an effective, non-invasive method to enhance ocular bioavailability of many drugs [25]. Although iontophoresis has been investigated as a means to increase corneal delivery of ACV [26], given that it is uncharged (pK_{a1} 2.27; pK_{a2} 9.25) at physiologically acceptable pH and with its poor water solubility, ~0.2% at 25 °C [13], it is far from being an ideal candidate for iontophoresis. GCV has similar physicochemical properties to ACV but its sodium salt was delivered iontophoretically through scleral membrane [27]. Although GCV anions can benefit from electromigration, unlike the parent neutral molecule, the high pH value (pH 11 at 50 mg/ml) of GCV sodium solution (FDA website: http://www.accessdata.fda.gov/drugsatfda_ docs/ label/2006/ 019661s030lbl.pdf) is almost certainly an irritant to the ocular surface.

We have previously shown that anodal iontophoresis of valaciclovir, the L-valyl ester prodrug of ACV, from aqueous solutions with patient-friendly pH values (5.24 and 5.65), was able to produce order of magnitude increases in cutaneous ACV delivery – iontophoretic transport was facilitated by its positive charge and the prodrug was hydrolysed by cutaneous esterases [28]. We have recently reported the synthesis and cutaneous iontophoresis of a series of ACV-X prodrugs (Chapter 1 and 2) and in this project we wanted to use these ACV-X prodrugs to overcome the challenges posed by ocular ACV delivery. The aims were (i) to develop and validate robust UHPLC-MS/MS methods for the (bio)pharmaceutical analysis of three amino acid ester prodrugs of ACV (ACV-X, where X = Arg, Gly and Trp), (ii) to determine solution stability of the prodrugs in the presence of different ocular tissues, (iii) to compare passive and iontophoretic transport through excised porcine cornea and sclera, (iv) to study the effect of current density and drug concentration on transscleral iontophoresis of ACV-X prodrugs and (v) to investigate ocular biodistribution of ACV species after transscleral iontophoresis.

2. MATERIALS AND METHODS

2.1. Materials

ACV was bought from Sequoia Research Product Ltd. (Pangbourne, UK). ACV ophthalmic ointment (Zovirax®, 3%) was purchased from a local pharmacy. L-argininyl, L-glycyl and L-tryptophanyl ester of ACV, i.e. ACV-X (where X=Arg, Gly and Trp) prodrugs, were synthesised in-house. 2-Morpholino-ethanesulfonic acid monohydrate (MES) was purchased from Fluka. Silver wire and silver chloride were bought from Sigma (Buchs, Switzerland). PVC tubing (ID 3.17 mm; OD 4.97 mm) and ULC/MS grade of acetonitrile (ACN) were bought from VWR International (Dietikon, Switzerland). ULC/MS grade formic acid was purchased from Biosolve B.V. (Valkenswaard, Netherlands). All aqueous solutions were prepared using Milli-Q water (resistivity ＞ 18 MΩ.cm). All other chemicals were at least of analytical grade.

2.2. Preparation of aciclovir prodrugs

ACV-X prodrugs were synthesised via a Steglich esterification; structures (**Figure 1**) were ascertained by high resolution mass spectrometry and ^{1}H-NMR and the purity determined with HPLC-UV (＞95%). The physicochemical properties of ACV-X prodrugs, i.e. Log *D* and p*K*a, were predicted using software from ACD/Labs. Aqueous solubility was measured in MES buffer (10 mM, pH 5.5) (**Table 1)**. All prodrugs were shown to have much greater aqueous solubility than ACV. Moreover, all prodrugs are positively charged at physiologically acceptable pH; therefore, they are much better candidates for iontophoresis.

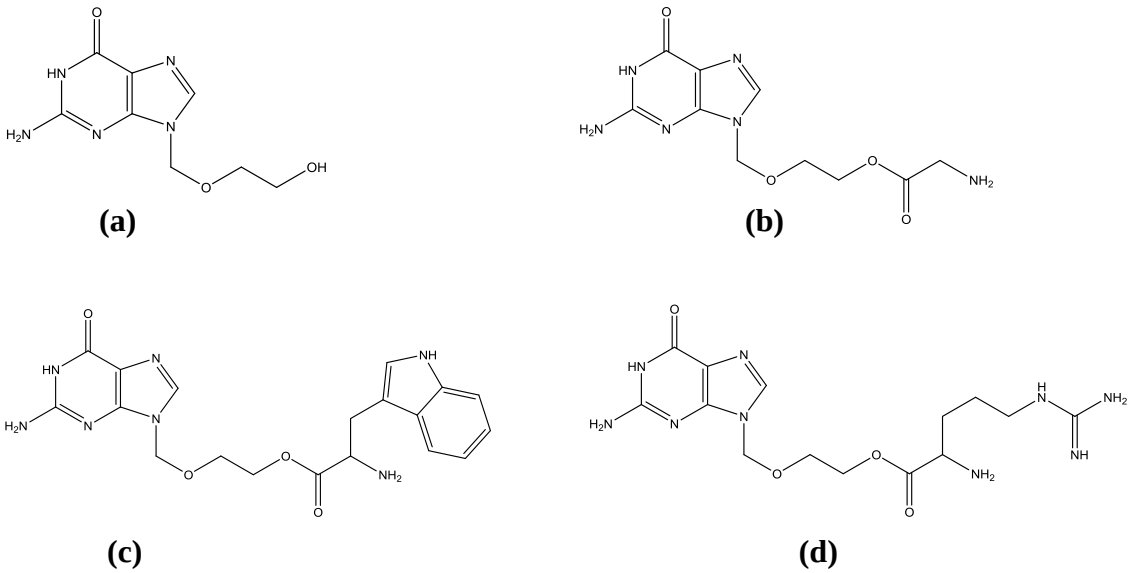


Figure 1. The chemical structures of **(a)** ACV, **(b)** ACV-Gly, **(c)** ACV-Trp and **(d)** ACV-Arg

2.3 Preparation of ocular tissues

Fresh porcine eyes were obtained from a local abattoir immediately after sacrifice of the animals (40-60 kg). Eye globes were rinsed with saline to remove any trace of blood, followed by removal of adherent muscle with a scalpel. The entire eye globes or individual excised tissues were used for transport studies. Fresh excised cornea was obtained by cutting along the sclera-limbus junction and used for transport studies within 3 h of harvesting. To obtain excised sclera, the iris-ciliary body and lens were removed, the vitreous humor was squeezed out and the choroid/retina tissue underlying the sclera was curetted. Each scleral cup was cut into two pieces. The sclera was stored in a freezer (– 20°C) and used in transport studies within 1 week. For the stability studies, in addition to the cornea and sclera, the choroid/retina tissue and vitreous humor were also harvested, and all ocular tissues were used within 3 h of harvesting.

2.4 Stability studies

The stability of ACV-X prodrugs was evaluated in the presence of freshly excised porcine eye tissues, a) sclera, b) choroid/retina, c) vitreous humor and d) cornea. The cornea and sclera were cut into small pieces. Each part of the ocular tissue was collected separately in a vial, to which 10 ml of PBS solution (pH 7.4) was added. The mixture was subjected to vigorous magnetic stirring for 1 h at room temperature, followed by centrifugation at 4,000 rpm for 5 min. The supernatant was collected and used for the stability studies to investigate ACV-X hydrolysis. Each ACV-X prodrug solution (1 mM, 1 ml) was added into the supernatant (9 ml). The theoretical concentration of each ACV-X prodrug was 100 μM. The solution was stirred and kept at 32°C. Aliquots were withdrawn at pre-determined time points during 1 h. The natural logarithm of fraction remaining of each ACV-X prodrug (*ln %RF*) was plotted as a function of time (*t*). The first order rate constant of hydrolysis (k_{obs}) was estimated from the slope of the regression curve, and the corresponding half-life ($t_{1/2}$) calculated ($t_{1/2} = ln2/k_{obs}$). All experiments were performed in triplicate.

2.5 Transport studies *in vitro*

2.5.1 The set-up

Two-compartment vertical diffusion cells with two sampling arms in the receiver compartment and a cross-sectional diffusional area of ~0.8 cm^2 (Glass Technology; Geneva, Switzerland) were used for the *in vitro* transport studies. The outer surface of the cornea or sclera faced the donor compartment. After clamping the fresh porcine cornea or sclera

between the two halves of the diffusion cells, the receiver compartment was filled with 10 ml of PBS solution at pH 7.4 and kept under magnetic stirring. The temperature was maintained at 32°C using a dynamic water bath system. For the iontophoretic delivery experiments, Ag electrodes were placed in the anodal compartments (filled with 20 ml of PBS solution at pH 7.4) which were connected to the donor compartments via salt bridge assemblies (3% agarose in 0.1 M NaCl). AgCl electrodes were placed in the cathodal receiver compartments. A constant current was applied from a power generator (APH 1000 M, Kepco Inc, Flushing, United States). The same set-up but in the absence of an applied electric current served as the control.

2.5.2 Transcorneal delivery of ACV or ACV-X prodrugs

MES buffer (10 mM, pH 5.5) was used to prepare all aqueous formulations. For passive delivery, the following formulations were applied for 1 h: ACV ophthalmic ointment (50 mg) and ACV, ACV-Gly or ACV-Trp saturated solution (0.4 ml). For short-duration iontophoretic delivery, ACV, ACV-Arg ACV-Gly or ACV-Trp solution (5 mM, 0.4 ml) were used and a constant current (0.5 mA/cm^2) was supplied for 5 min followed by passive delivery for another 55 min. An aliquot (1 ml) was withdrawn from the receiver and replaced with the same volume of fresh PBS solution after 5, 10, 15, 20, 30, 40, 50 and 60 min. Upon completion of the experiment, the diffusion cells were dismantled, and the residual formulation on the cornea was removed under running water or using a wet cotton bud. There is little information concerning methods to extract ACV from corneal tissue. Although incubation for 1 h in 1 N NaOH solution at 60°C was used as an extraction medium [18], this was not an option for our studies since ACV-X prodrugs underwent extensive base-catalyzed chemical hydrolysis. Given that ACV and ACV-X prodrugs are highly soluble in acidic solution and ACV-X prodrugs are relatively stable under these conditions (at least for several hours) (paper submitted), the corneas were cut into small pieces and extracted by incubation with 0.1 % formic acid (FA) solution for 3 h (FA was used to deactivate the ocular esterases). The extraction medium was centrifuged at 12,000 rpm for 15 min and the supernatant was withdrawn for analysis. All experiments were performed in quintuplicate.

2.5.3 Transscleral delivery of ACV or ACV-X prodrugs

As above, MES buffer (10 mM, pH 5.5) was used to prepare the aqueous formulations. For passive delivery, ACV ophthalmic ointment (50 mg), ACV, ACV-Arg, ACV-Gly or ACV-Trp solution (9 mM, 0.4 ml) were applied in each donor compartment for 30 min. For

iontophoretic delivery, only the aqueous solution of ACV or ACV-X prodrugs with the same volume and concentration was used, and a constant current (1.25 mA/cm^2) was applied for 30 min. The effect of concentration and the current density on transscleral delivery of ACV-Gly was also evaluated: i) ACV-Gly solutions with concentrations of 1, 5 and 9 mM at a constant current density of 1.25 mA/cm^2, ii) constant current densities of 0.63, 1.25 and 3.75 mA/cm^2 at a fixed ACV-Gly concentration of 5 mM. Samples (1 ml) were taken from the receiver compartment after 5, 10, 15, 20 and 30 min and subjected to centrifugation at 12,000 rpm for 15 min. The supernatant was taken for analysis. All experiments were performed in quintuplicate.

2.6 Ocular distribution after short-duration transscleral iontophoresis

2.6.1 Experimental set-up

A novel custom-made diffusion cell was developed to investigate ocular distribution after short-duration transscleral iontophoresis of ACV-X prodrugs *ex vivo*. In this assembly, a "conventional" donor compartment (permeation area ~0.8 cm^2) was combined with a 'base' (i.e. a receiver in a "conventional" system) that was adapted to the geometry of the eye globe and provided the necessary support. In these experiments, PBS solution (pH 7.4, 30 ml), served as an electrically conductive medium for the return electrode (AgCl), was added to the base, and an intact fresh porcine eye globe was placed on the contact surface. The donor was placed on the scleral tissue using a little slight pressure and the permeation zone was close to the sclera-limbus junction. The whole system was fixed with a customized clamp and bracket. The set-up for the control experiments was the same as that for iontophoresis but in the absence of current application. All experiments were performed in quadruplicate.

2.6.2 Transport studies *ex vivo*

Ocular delivery and biodistribution following short-duration transscleral iontophoresis were investigated using ACV-Gly. Briefly, ACV-Gly solution (9 mM, 0.4 ml) was put into each donor, and a constant current (1.25 or 3.75 mA/cm^2) was applied for 5 min. After switching off the power generator, the system was rapidly dismantled and the surface of the eye was washed with running water for 10 s. The eye was dissected to obtain the aqueous humor, cornea, sclera, choroid/retina and vitreous humor, which were then separately extracted with 0.1% FA solution for 3 h. The extraction medium was centrifuged at 12,000 rpm for 15 min, and the supernatant was withdrawn for analysis. All experiments were performed in quadruplicate.

2.7 Analytical procedure

ACV and ACV-X prodrugs were analysed using a Waters® Acquity® UPLC® system, coupled with a Waters® Xevo® TQ triple quadrupole mass spectrometer (UHPLC-MS/MS) system and a Waters® XBridge® BEH C18 (50 × 2.1 mm, 2.5 μm) reverse phase column, maintained at 30 °C. The injection volume was 5 μl. A Waters® XBridge® BEH C18 (5 × 2.1 mm, 2.5 μm) guard column was mounted upstream from the analytical column. The mobile phase was a mixture of 0.1% formic acid aqueous solution and ULC/MS grade ACN, and the flow rate was 0.2 ml/min (specific compositions given in **Table 2**). The Xevo® triple quadrupole mass spectrometer, with ESI positive mode (ES^+) and multiple reaction monitoring mode (MRM) was used to ionise, select and analyse the ions of interest. Nitrogen and argon were used as the drying gas and collision gas, respectively. The data processing was performed using MassLynx® software. The chromatography and mass spectrometry parameters are shown in **Table 2**. The analytical methods were validated with 3 replicates at 100, 500 and 1,000 nM and showed acceptable intra-day precision and accuracy, as shown in **Table 3**.

2.8 Data analysis

Data were expressed as the mean ± SD. Results were evaluated statistically using analysis of variance (ANOVA) or Student's t-test. Student-Newman-Keuls (SNK) test was used when necessary as a post-hoc procedure. The level of significance was fixed at $\alpha = 0.05$.

3. RESULT AND DISCUSSIOIN

3.1 Stability

The therapeutic effect of the ACV prodrugs depends on the effective cleavage of the amino acid moiety, as this is the prerequisite for phosphorylation of the ACV terminal hydroxyl group by viral thymidine kinase and human phosphorylase - ACV triphosphate is the active form that inhibits viral DNA replication [29]. Since the three amino acid ester prodrugs of ACV were relatively stable at physiological pH (Chapter 1 and 2), the cleavage of the amino acid moieties was envisaged to depend principally on the enzymatic activity of esterases present in different ocular tissues [30].

The first order rate constant (k) for hydrolysis of the ACV-X prodrugs and the corresponding half-life ($t_{1/2}$) in the extract of different ocular tissues are presented in **Table 4**. The enzymatic stability of ACV-X prodrugs in each extract of ocular tissue, as measured by $t_{1/2}$, decreased in

Table 2 UPLC-MS/MS methods for ACV and ACV-X prodrugs quantification.

Analytical Task	Compounds	Chromatography		Mass spectrum					
		Mobile phase	Elution time (min)	Capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)	Parent ion	Daughter ion	LOQ (nM)
ACV transport study	ACV	99% A 1% B	2.46	2.5	27	20	226.2	152.0	10
ACV-Gly transport study	ACV-Gly	99% A 1% B	1.44	2.5	25	17	283.2		20
	ACV		2.43				226.2		10
ACV-Trp transport study	ACV-Trp	90% A 10% B	2.77	2.5	28	20	412.2		50
	ACV		0.97				226.2		10
ACV-Arg transport study	ACV-Arg	99% A 1% B	1.31	2.5	27	20	382.2		50
	ACV		2.46				226.2		10

* Mobile phase A: 0.1 % formic acid. Mobile phase B: acetonitrile

Table 3 Precision and accuracy of the analytical methods used to quantify ACV and ACV-X prodrugs

	Theoretical concentration (nM)	Experimental concentration (nM)	Precision (%)[a]	Accuracy (%)[b]
ACV	100	104.66 ± 0.71	0.68	104.66
	500	508.23 ± 3.89	0.77	101.65
	1000	1018.87 ± 6.33	0.62	101.89
ACV-Gly	100	95.82 ± 0.43	0.45	95.82
	500	485.77 ± 4.14	0.85	97.15
	1000	973.01 ± 6.89	0.71	97.30
ACV-Trp	100	98.96 ± 0.22	0.22	98.96
	500	480.25 ± 3.10	0.65	96.05
	1000	945.91 ± 7.12	0.75	94.59
ACV-Arg	100	95.24 ± 0.78	0.82	95.24
	500	471.94 ± 4.01	0.85	94.39
	1000	921.01 ± 8.81	0.96	92.10

[a]Precision = (SD/mean) x 100.

[b]Accuracy = (obtained concentration/theoretical concentration) x 100

the order ACV-Gly＞ACV-Trp＞ACV-Arg. Obviously, the different hydrolytic rates were due to the different structures of the substrates.

Prodrug hydrolysis rates were found to vary between different ocular tissues suggesting that esterase activity was not uniform. The shortest $t_{1/2}$ of ACV-X prodrugs was observed in the presence of choroid/retina, and the longest $t_{1/2}$ was found with vitreous humour. The same observations were also reported with ganciclovir prodrugs in rabbit eyes [31].

Although ACV-X prodrugs are hydrolysed by the esterases present in ocular tissue, the rate and extent of bioconversion is region-dependent; therefore, in the following sections describing the results of studies on transcorneal and transscleral delivery, the amount of each ACV-X prodrug and the amount of ACV that was regenerated from the corresponding ACV-X prodrug were combined and defined as the 'ACV species' (ACV species = amount of ACV + amount of ACV-X prodrug). The incomplete bioconversion of amino acid ester prodrugs [32], aliphatic ester prodrugs [33] and phosphate ester prodrugs [34] following ocular delivery

Table 4 First-order hydrolysis rate constants ($k \times 10^3$ min^{-1}) and half lives (h) of ACV-X prodrugs in the extracts of ocular tissues

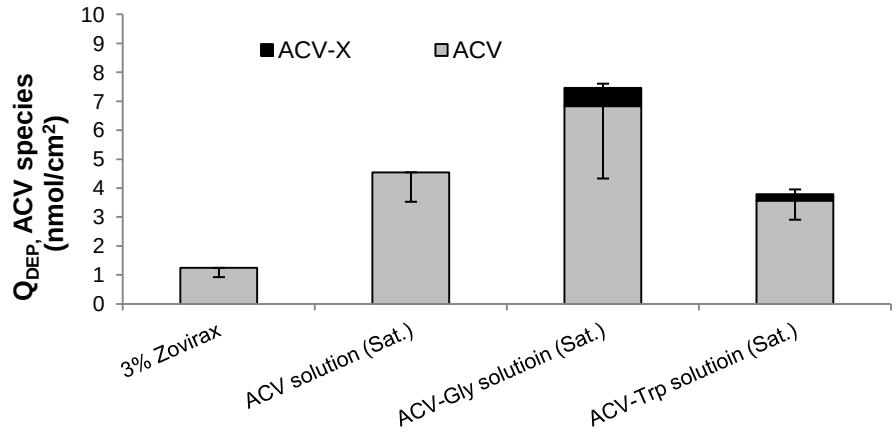
	ACV-Gly		ACV-Trp		ACV-Arg	
	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)
Sclera	6.87 ± 0.50	1.69 ± 0.13	9.80 ± 1.07	1.19 ± 0.12	11.43 ± 0.66	1.01 ± 0.06
Choroid/Retina	7.37 ± 0.61	1.58 ± 0.13	11.13 ± 0.98	1.05 ± 0.09	18.83 ± 0.17	0.61 ± 0.01
Vitreous humour	2.13 ± 0.34	5.54 ± 0.82	3.43 ± 0.42	3.41 ± 0.40	5.37 ± 0.74	2.19 ± 0.30
Cornea	5.97 ± 1.08	1.99 ± 0.32	11.90 ± 1.60	0.99 ± 0.13	13.47 ± 0.17	0.86 ± 0.01

has been previously reported, and the amounts of prodrug and newly regenerated parent drug in ocular tissue were also summed to represent the total drug delivery.

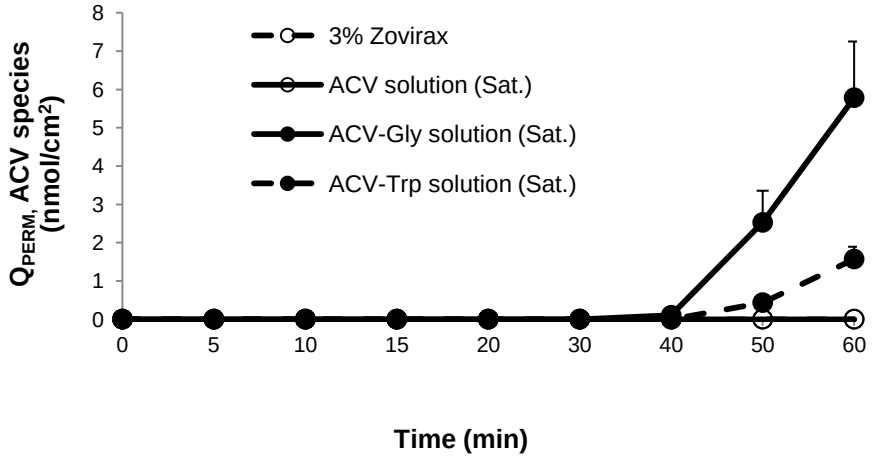
3.2 Transcorneal delivery

In principle, conventional ophthalmic formulations, e.g. eye drops and ointment, should enable targeted therapy of corneal diseases. However, Zovirax® ophthalmic ointment (3%) shows limited efficacy in the treatment of corneal HSV infections. In order to quantify corneal delivery of ACV, its deposition and permeation within excised cornea were determined after application of either 50 mg of Zovirax® ointment (3%) or 0.4 ml of ACV saturated solution (ACV dose per unit area of 8.3 or 4.5 μmol/cm^2, respectively) for 1 h. Compared with passive delivery of ACV from the ointment, greater corneal deposition of ACV species was achieved using saturated solutions of ACV, ACV-Gly and ACV-Trp (4.5 ± 1.0, 7.5 ± 2.6 and 3.8 ± 0.8 nmol/cm^2, respectively cf. 1.2 ± 0.3 nmol/cm^2 of ACV after application of Zovirax® ointment) (**Figure 2a**). Moreover, ACV levels in the receiver were below the LOQ (10 nM) after application of Zovirax® ointment or saturated ACV solution, meaning that permeation was <0.125 nmol/cm^2. In contrast, passive diffusion of ACV-Gly or ACV-Trp from saturated aqueous solution, with much higher loading doses (256.0 and 25.6 μmol/cm^2, respectively), resulted in more than 46- and 12-fold increases in ACV permeation (5.8 ± 1.5 and 1.6 ± 0.3 nmol/cm^2, respectively) (**Figure 2b**). The total permeated amounts of ACV species upon completion of the experiments were shown in **Figure 2c**.

The enhanced corneal delivery of ACV species from saturated solutions of ACV-X prodrugs can be attributed to the increased concentration gradient, as the aqueous solubility of ACV-Gly and ACV-Trp was 55.7- and 5.6- greater than that of ACV (**Table 1**). The transport might also be facilitated by an oligopeptide transporter in the corneal epithelium; it has been reported that these might still be active in excised cornea [35].



(a)



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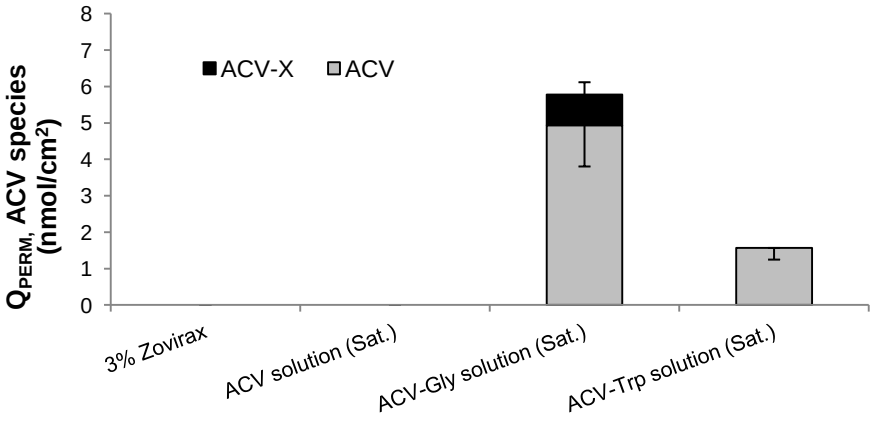
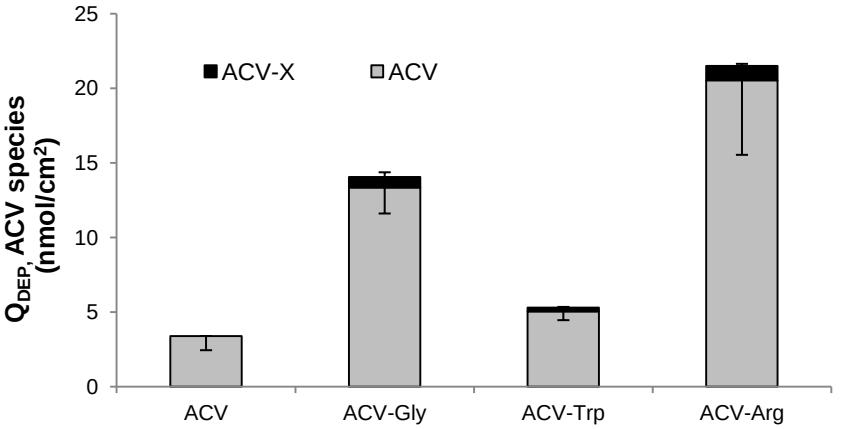


(c)

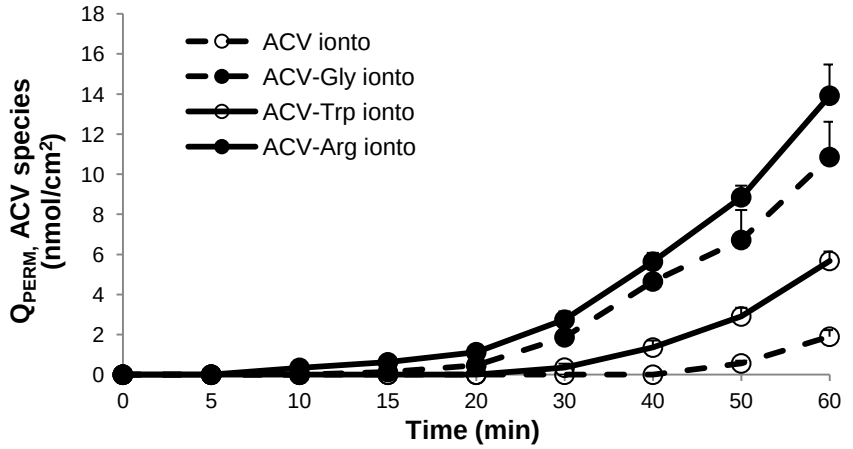
Figure 2. Corneal deposition **(a)**, transcorneal permeation profiles **(b)** and total permeated amounts **(c)** of ACV species after application of 3% Zovirax® or saturated solutions of ACV, ACV-Gly or ACV-Trp for 1 h. (Mean ± SD; n=3)

Although transcorneal passive delivery of ACV-X prodrugs from their saturated solutions was much better than delivery of ACV from Zovirax® ointment, the concentrations in the formulations were not suited to practical use. Transcorneal iontophoresis is able to deliver appreciable drug amounts to the anterior segment and thus has potential for the treatment of anterior segment diseases [25]. Nevertheless, the current density and the duration of application have to be carefully controlled, as the use of extreme iontophoretic conditions, e.g. 21 mA/cm^2 for 15 min, can result in corneal toxicity [36]. Therefore, in the transport experiments described below, a dosage regimen combining an initial very short-duration, mild iontophoresis (5 min, 0.5 mA/cm^2) with a 55 min post-iontophoretic passive delivery, was used to deliver ACV-X prodrugs from 5 mM solutions. This was designed to mimic a possible clinical scenario where administration of an initial iontophoretic bolus loading dose, by medical personnel, was followed by patient self-medication, e.g. using eye drops for continuous instillation.

Electrically-assisted delivery of ACV-Arg and ACV-Gly enabled considerable amounts of corneal deposition of ACV species (21.5 ± 5.1 nmol/cm^2 and 14.1 ± 2.0 nmol/cm^2, respectively), which was much higher than that after passive or electrically-assisted delivery of ACV itself (3.4 ± 1.0 nmol/cm^2) (**Figure 3a**). Iontophoresis of ACV-Gly or ACV-Trp (using 5 mM solutions) followed by the 55 min passive diffusion resulted in cumulative permeation of ACV species of 10.9 ± 1.8 or 5.7 ± 0.5 nmol/cm^2, corresponding to ~1.9- and ~3.6-fold increases over passive delivery using saturated solutions which were 102.4 or 13.3 times more concentrated than those used in iontophoresis (**Figure 3b**). Moreover, higher permeation ACV species (13.9 ± 1.6 nmol/cm^2) was observed following iontophoresis of ACV-Arg, which should be attributed to the stronger electromigratioin of the di-protonated of ACV-Arg than that of the mono-protonated ACV-Gly and ACV-Trp with the same electric potential gradient. For comparison, electrically-assisted delivery of ACV resulted in permeation of 1.9 ± 0.3 nmol/cm^2. The total permeated amounts of ACV species upon completion of the experments were shown in **Figure 3c**. Superior delivery of ACV species into and across the cornea after short-duration iontophoresis of ACV-X prodrugs should result in appreciable ACV levels in the aqueous humour *in vivo* which should be an advantage in treating HSV-1 infections in the anterior segment.



(a)



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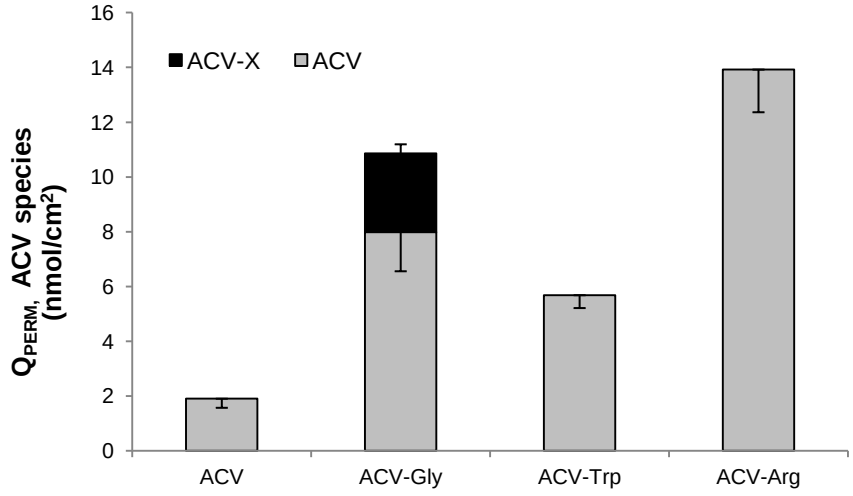


(c)

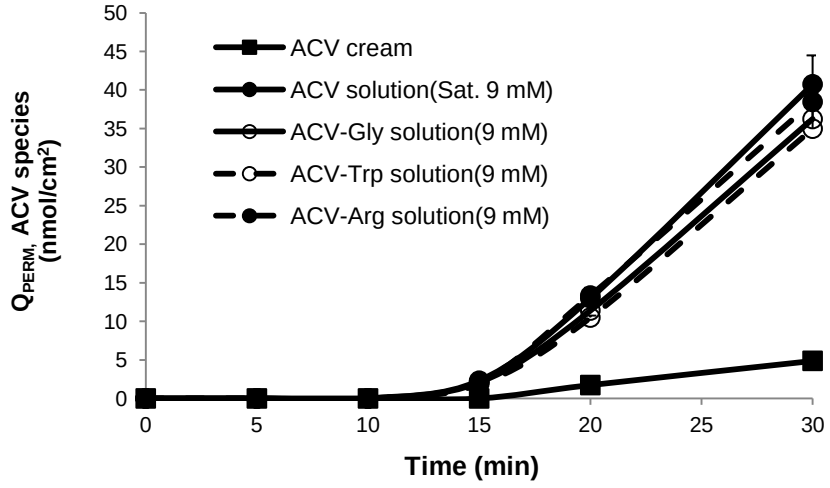
Figure 3. Corneal deposition **(a)**, transcorneal permeation profiles **(b)** and total cumulative amounts **(c)** of ACV species after iontophoresis of ACV, ACV-Gly or ACV-Trp for 5 min, followed by passive delivery for 55 min. (Mean ± SD; n=5)

3.3 Transscleral delivery

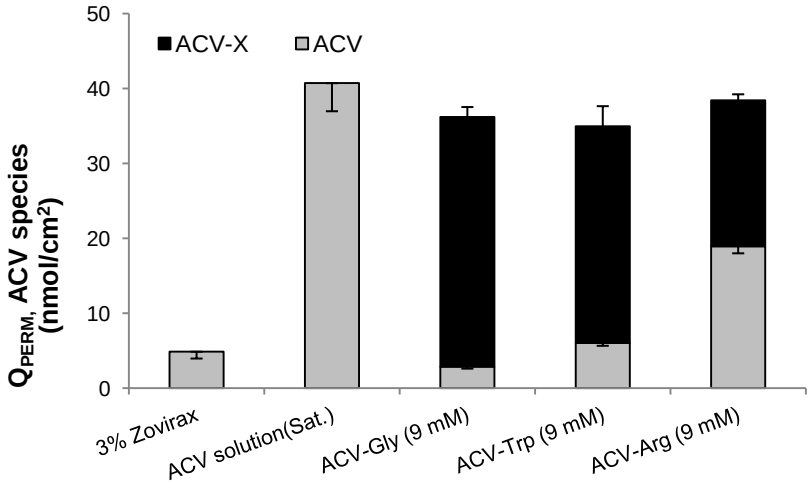
Despite the absence of the retinal pigmented epithelium barrier and the lack of choroidal blood flow [37], the excised sclera is still widely used to develop topical ophthalmic formulations [25,38,39], as the amount of drug permeated across the sclera can be regarded as the drug input into a 'central compartment' before further distribution into the choroid, retina and vitreous humour. Given the difficulty in procuring human sclera, excised porcine sclera is considered to be an excellent surrogate [40].

Transscleral cumulative permeation of ACV after application of Zovirax® ophthalmic ointment (3%) for 30 min (8.3 μmol/cm^2) was 4.9 ± 0.9 nmol/cm^2 (**Figure 4a**), which was much more efficient than corneal permeation; however, ACV was not detected until the 20 min time-point. Passive application of ACV or ACV-X prodrug solutions (9 mM) for 30 min (dose 4.5 μmol/cm^2) enabled much higher permeation (30-40 nmol/cm^2 of ACV species was found in the receiver compartment), but they were still unable to deliver detectable amounts of ACV species in the receiver compartment within 15 min. Given that the residence time of topically applied formulations is limited to a few minutes or even a few seconds due to solution drainage, blinking, tear film, tear turnover, induced lacrimation, etc. [41], passive delivery of ACV or ACV-X prodrugs from either ointment or solutions was deemed insufficient to enable therapeutic levels of ACV species to be achieved beneath the sclera before the formulations were washed away physiologically. The total permeated amounts of ACV species upon completion of the experments were shown in **Figure 4b**.

Transscleral iontophoresis has been considered as a good potential alternative to intravitreal injections or systemic administration for the treatment of posterior ocular disorders [25]. An anti-CMV/HSV agent, foscarnet, with two negative charges, was delivered intraocularly through sclera using this technique [42], which might be very useful for aciclovir-resistant ocular HSV infections [43]. However, retinal toxicity was observed [44], which was most likely due to the very high current density employed (1 mA for 10 minutes with a 0.19 mm^2 probe).



(a)

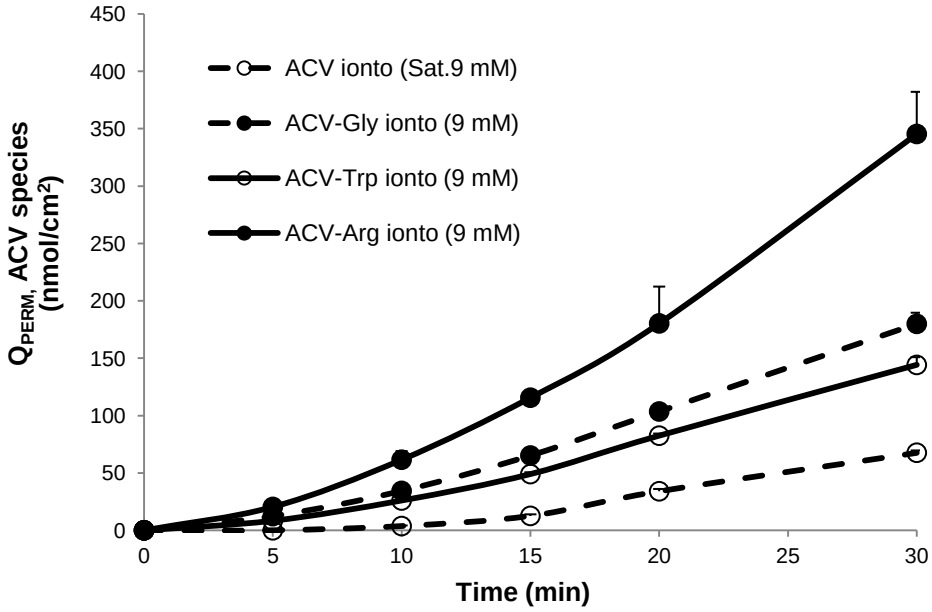


(b)

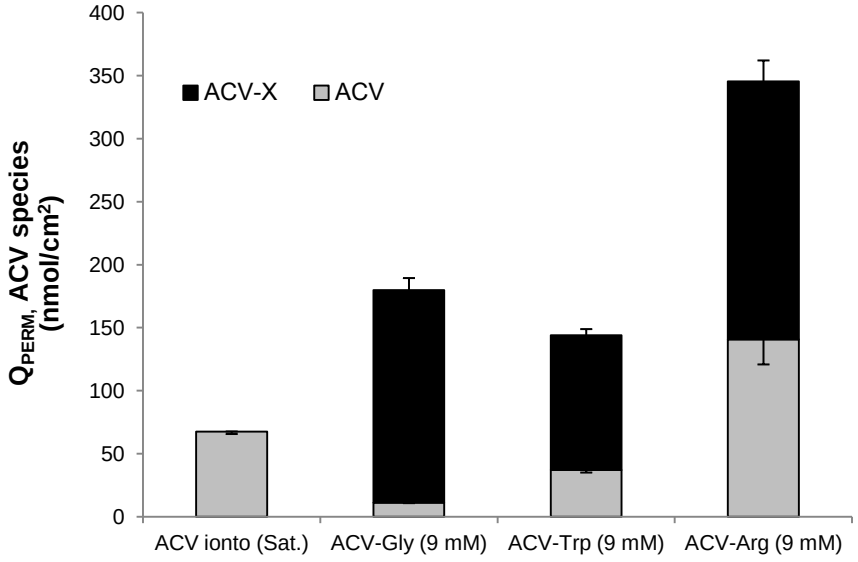
Figure 4. Transscleral permeation profiles **(a)** and total cumulative amounts **(b)** of ACV species after passive delivery of ACV or ACV-X prodrugs during 30 min. (Mean ± SD; n=5)

In the present study, a mild iontophoretic current of 1.25 mA/cm^2, was used to investigate transscleral delivery of ACV-X prodrugs (dose 4.5 μmol/cm^2). Iontophoresis for 30 min resulted in cumulative permeation of ACV species as compared to passive administration (**Figure 5a**) – 345.3 ± 36.7, 179.9 ± 9.7 or 144.1 ± 6.8 nmol/cm^2 for ACV-Arg, ACV-Gly and ACV-Trp, respectively. More importantly, after iontophoresis of ACV-Arg, ACV-Gly and ACV-Trp only 5 min, 20.4 ± 3.7, 12.3 ± 0.3 or 8.4 ± 0.4 nmol/cm^2 of ACV species were found in the receiver compartment demonstrating the superior rate of delivery. Compared with passive delivery of ACV/ACV-X prodrugs or iontophoresis of ACV itself where negligible amounts of ACV was delivered across the sclera before 5 min, short duration transscleral iontophoresis of ACV-X prodrugs might open the way to deliver ACV species

non-invasively across sclera, and thereby target intraocular HSV infections, particularly those in the choroid [45]. The total permeated amounts of ACV species upon compeletion of the experiments were shown in **Figure 5b**. The bioconversion was low, which should be attributed to the fact that sclera presented small amounts of esterases (**Table 4**) and the *in vitro* conditions could decrease the original enzymatic activity.



(a)



(b)

Figure 5. Transscleral permeation profiles **(a)** and total cumulative amounts **(b)** of ACV species after iontophoretic delivery (1.25 mA/cm^2) of ACV or ACV-X prodrugs during 30 min. (Mean ± SD; n=5)

3.4 The effect of concentration and current density on transscleral iontophoresis

Modulation of the current density and the drug concentration can be used to individualise and to adapt treatment to the disease state [46]. In addition, it enables side-effects of transscleral iontophoresis, e.g. the irritation to retina, to be attenuated. Constant current transscleral iontophoresis of ACV-Gly at a current density of 1.25 mA/cm^2 for 30 min and using concentrations of 1, 5 or 9 mM, resulted in cumulative ACV species ($Q_{PERM,\ ACV\ species}$ = $Q_{PERM,\ ACV\text{-}Gly}$ + $Q_{PERM,\ ACV}$) permeation of 21.9 ± 1.9, 95.7 ± 8.1 and 179.9 ± 9.7 nmol/cm^2, respectively, and a transscleral flux of 0.8 ± 0.1, 3.5 ± 0.3 or 6.7 ± 0.4 nmol cm^{-2} min^{-1}, respectively (**Figure 6**).

Figure 6a

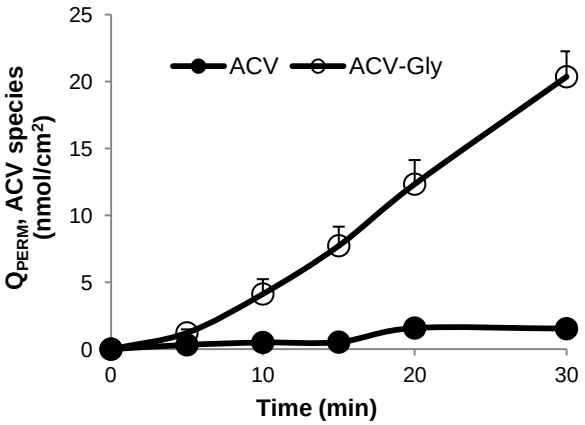


Figure 6b

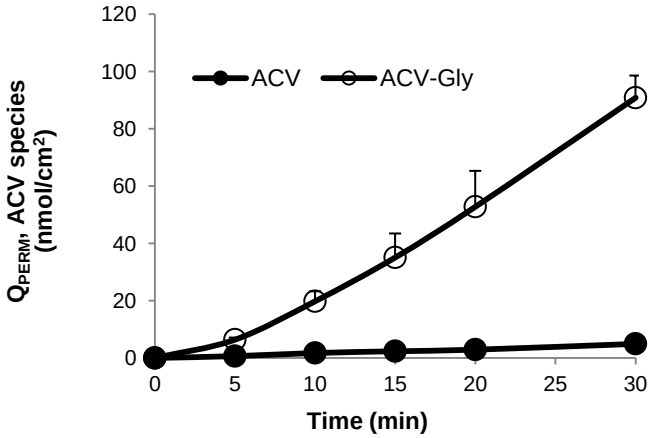


Figure 6c

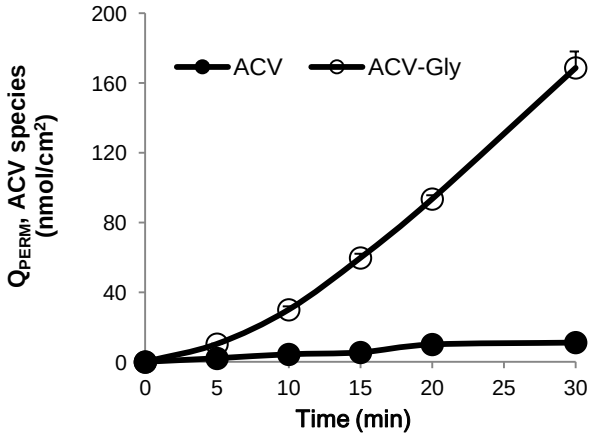


Figure 6d

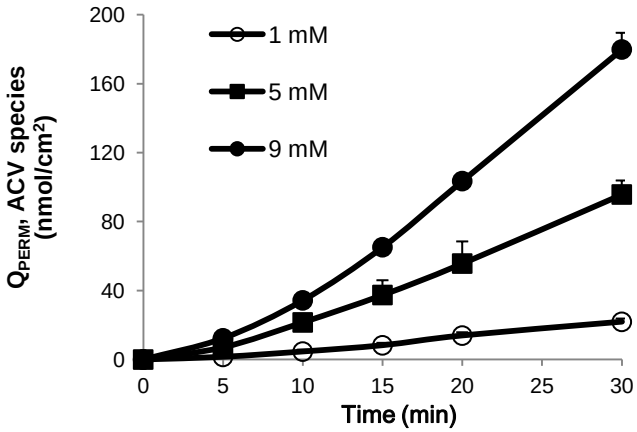


Figure 6. Effect of concentration on transscleral permeation of ACV and ACV-Gly following iontophoresis of 1 mM **(a)**, 5 mM **(b)** or 9 mM **(c)** solution of ACV-Gly at 1.25 mA/cm^2; the total permeation of ACV species was calculated by the summed amounts of ACV-Gly and ACV **(d)**. (Mean ± SD; n=5)

At a fixed concentration of ACV-Gly of 5 mM, the current density was varied (0.63, 1.25 or 3.75 mA/cm^2); the cumulative permeation of ACV species ($Q_{PERM, ACV\ species} = Q_{PERM, ACV\text{-}Gly} + Q_{PERM, ACV}$) after 30 min was 46.0 ± 7.1, 95.7 ± 8.1 and 293.6 ± 24.1 nmol/cm^2, respectively, and the transscleral flux was 1.7 ± 0.3, 3.5 ± 0.3 and 11.0 ± 0.9 nmol cm^{-2} min^{-1}, respectively (**Figure 7**). The results showed that transscleral flux was linearly dependent upon the concentration (r^2=0.9983) and the current density (r^2=1.0000). Similar behaviour was reported following transscleral iontophoresis of other small molecules [47]. The ability of transscleral iontophoresis to precisely control intraocular drug input rates is an advantage over other delivery strategies.

Figure 7a

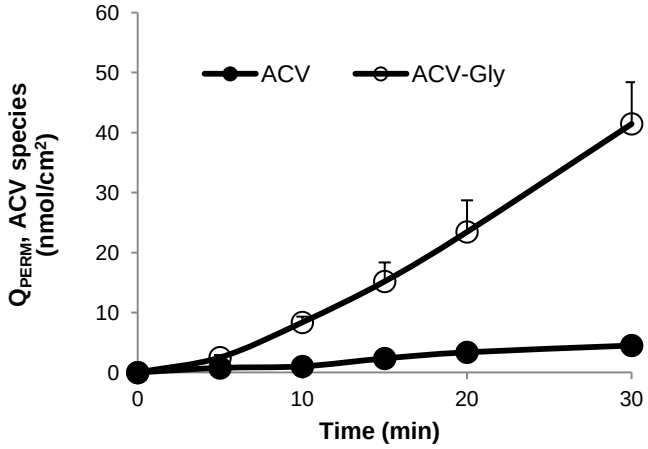


Figure 7b

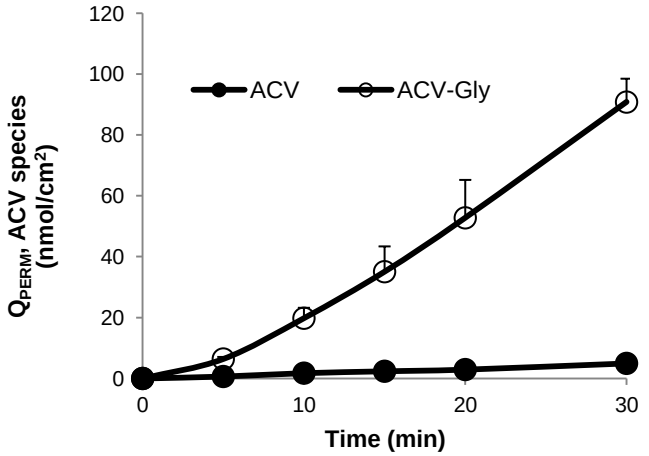


Figure 7c

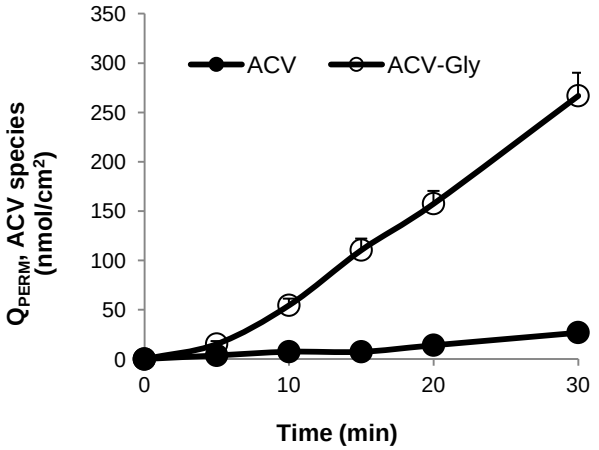


Figure 7d

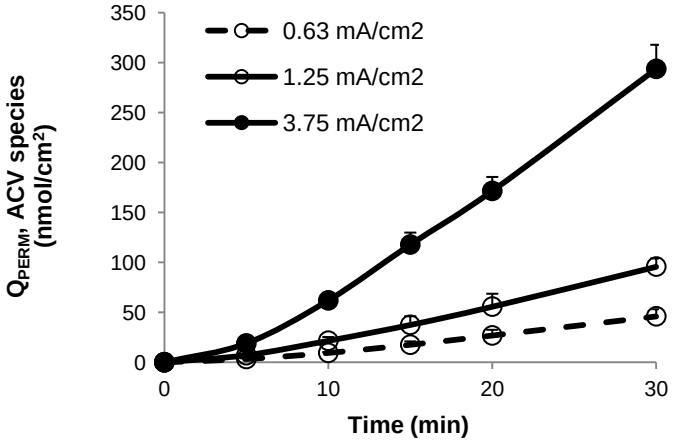


Figure 7. Effect of current density on transscleral permeation of ACV and ACV-Gly following iontophoresis of a 5 mM solution of ACV-Gly at 0.63 mA/cm^2 **(a)**, 1.25 mA/cm^2 **(b)** or 3.75 mA/cm^2 **(c)**; the total permeation of ACV species was calculated by the summed amounts of ACV-Gly and ACV **(d)**. (Mean ± SD; n=5)

3.5 Biodistribution studies *ex vivo*

The results above have demonstrated the superiority of transscleral iontophoresis of ACV-X prodrugs over passive delivery. A more realistic model would consider the fate of ACV species after short-duration iontophoresis across the sclera – in other words, the ocular biodistribution of ACV species. A useful *ex vivo* model for ocular iontophoresis using intact fresh porcine eyes, has been recently reported and used for biodistribution studies [48].

Using custom-made apparatus, passive delivery of ACV-Gly for 5 min enabled deposition of 5.6 ± 1.1 nmol/cm^2 of ACV species in the sclera but neither ACV-Gly nor ACV was observed in deeper ocular tissues (**Figure 8**). Iontophoresis of ACV-Gly (1.25 mA/cm^2) for 5 min

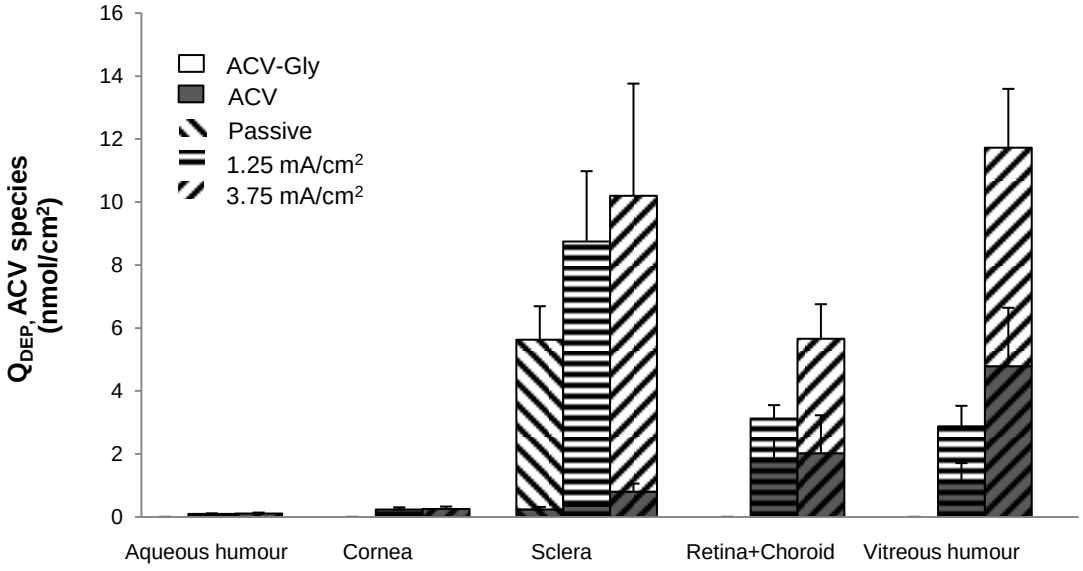


Figure 8. Comparison of ocular biodistribution of ACV species after passive and transscleral iontophoretic administration at of ACV-Gly (9 mM) for 5 min. Iontophoretic transport measured at 1.25 mA/cm^2 and 3.75 mA/cm^2. (Mean ± SD; n=4)

delivered more ACV species into sclera (8.7 ± 2.3 nmol/cm^2), which showed no statistically significant difference from scleral deposition after passive delivery. More significantly, considerable amounts of ACV species were found in choroid/retina and vitreous humour, 3.1 ± 1.1 and 2.9 ± 1.2 nmol/cm^2, respectively. Upon increasing the current density from 1.25 to 3.75 mA/cm^2, ACV species deposition in choroid/retina and vitreous humour were 5.7 ± 2.3 and 11.7 ± 3.7 nmol/cm^2, respectively. Transscleral iontophoresis of ACV-Gly only enabled small amounts of ACV species to be deposited in the cornea and aqueous humour. The sum of the amount of ACV species in choroid/retina, vitreous, cornea and aqueous humour, i.e. delivered across the sclera, after ACV-Gly iontophoresis at 1.25 and 3.75 mA/cm^2 for 5 min

was 6.3 ± 2.4 or 17.7 ± 6.1 nmol/cm^2 respectively, which was comparable to the amount of ACV species delivered after 5 min using excised tissues., 7.0 ± 1.0 or 18.9 ± 2.5 nmol/cm^2, respectively.

The average concentration of ACV species in the whole eye (incl. sclera) could be roughly estimated by dividing the amounts of ACV species in the whole eye by the volume of the porcine eyes, ~ 5 cm^3, therefore, the average concentration of ACV species were 2.4 ± 0.8 or 4.5 ± 1.6 nmol/cm^3, following five-minute transscleral iontophoresis of ACV-Gly at 1.25 or 3.75 mA/cm^2, respectively. The majority of isolates from ocular herpes simplex virus were very sensitive to ACV (IC_{50} < 0.5 μg/ml, corresponding to 0.22 nmol/ml) [49]. Given this value was much lower than the average concentration of ACV species after five-minute iontophoresis at 1.25 and 3.75 mA/cm^2, we believe that short-duration transscleral iontophoresis of ionisable ACV-Gly is a promising approach for the successful local treatment of HSV infections in posterior segments. Moreover, as the concentration used here was only 5 mM, a higher concentration would definitely enable greater amounts of ACV species to be delivered.

The half-life of ACV in the vitreous was determined to be very short after a single intravitreal dose (Albino rabbits ~ 3 h; Pigmented rabbits ~ 8 h), which was attributed to the retinal transport system [4]. It means frequent intravitreal injections are required to maintain therapeutic levels of ACV. Iontophoresis of ACV-X prodrugs, although seems not able to prolong the half-life of ocular ACV residence, provides an efficient, non-invasive method to deliver ACV into posterior segment, by which frequent administration is feasible. A better patient compliance and clinical efficacy might be achieved.

4. CONCLUSION

Poor ocular bioavailability of many molecules that were applied topically was partially attributed to their physicochemical properties, e.g. low water solubility and/or low corneal partitioning rate. To optimise intraocular drug input, prodrug strategies were extensively investigated [30]. Amino acid ester prodrugs of ACV (ACV-X, where X = Arg, Gly and Trp) have much higher aqueous solubility than ACV due to the ionisable amino acid moieties. All ACV-X prodrugs were biolabile and hydrolysed by esterases present in ocular tissue. The extent of hydrolysis was different in the different compartments. Among the separated tissues, the choroid/retina showed the highest level of esterase activity. Although the high aqueous

solubility of ACV-X prodrugs enhanced passive transcorneal delivery of ACV species, short-duration iontophoresis resulted in significantly greater transcorneal permeation and corneal deposition of ACV species using less concentrated prodrug solutions. Passive delivery of ACV through porcine sclera resulted in much greater permeation than that in corneal delivery due to the high intrinsic permeability of scleral tissue; however, from a practical point of view, the comparatively long diffusion time and slow transport would make successful intraocular delivery challenging due to fast ocular surface clearance *in vivo*. In contrast, transscleral iontophoresis of ACV-X prodrugs enabled both greater levels of ACV to be achieved and a much faster rate of delivery. More importantly, as evidenced by the results with ACV-Gly, transscleral iontophoresis enabled the ocular input to be controlled and modulated, which may faciliate individualised therapy. Biodistribution studies further confirmed the superiority of short-duration iontophoresis, as the average ACV species concentration in the eye globes following five-minute iontophoresis of ACV-Gly was higher than the IC_{50} of ACV against HSV-1. In summary, iontophoresis of these water-soluble ionisable ACV-X prodrugs may prove to be useful for the treatment of HSV infections in anterior and posterior segments.

5. ACKNOWLEDGEMENTS

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Chapter 4

Supersaturated formulations of lipophilic aciclovir prodrugs to improve targeted intra-epidermal delivery of aciclovir

Supersaturated formulations of lipophilic aciclovir prodrugs to improve targeted intra-epidermal delivery of aciclovir

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ABSTRACT

The objective was to use lipophilic aciclovir (ACV) prodrugs in combination with supersaturation to increase cutaneous bioavailability of aciclovir. Aciclovir acetate, butyrate and hexanoate (ACV-Y, where Y = Ace, But and Hex) were synthesised and characterised. Analytical methods using UHPLC-MS/MS were developed for their quantification and validated. Solubility of ACV and each ACV-Y prodrug as a function of the proportion of propylene glycol (PG) in cosolvent mixtures of PG and water was determined. ACV and ACV-Ace were found to be poor candidates for supersaturated formulations. ACV-But was only able to create a moderate degree of supersaturation (DS) up to ~3. ACV-Hex, with the highest solubility in PG and lowest solubility in water, was able to create very high DS, of upto ~12 and ACV-Hex in 10% PG solution was selected as the best system for creating stable supersaturated formulations. In the absence of HPMC, there was no statistically significant difference in cutaneous deposition of ACV species between saturated (0.27 ± 0.06 nmol/cm^2) and supersaturated ACV-Hex formulations. When HPMC was added, formulations with DS=3 or 4 led to increased cutaneous deposition of ACV species (the amount of ACV + the amount of ACV-Hex), 2.55 ± 0.64 and 4.90 ± 1.58 nmol/cm^2, respectively. Passive delivery of ACV after application of Zovirax® cream for 1 h resulted in limited cutaneous deposition (1.29 ± 0.79 nmol/cm^2), and ACV was principally found in the superficial part of the skin. Moreover, levels in the deeper skin (100-160 μm), corresponding to the basal epidermis and adjacent area where the virus is found, were negligible. The supersaturated ACV-Hex formulation (DS=4) enabled a higher percentage of ACV species to be delivered to deeper skin layers and also resulted in supra-therapeutic levels of ACV species the basal epidermis (ACV species 0.40 ± 0.12 nmol/cm^2, incl. 0.08 ± 0.03 nmol/cm^2 of ACV). ACV concentration (the unconverted ACV-Hex was not included) in the target area was 13.33 ± 5.01 nmol/cm^3, which was much higher than the IC_{50} of ACV against HSV-1, 0.04-3.1 nmol/cm^3. This formulation should be useful for the treatment of topical HSV-1 infections.

Keywords: aciclovir, prodrug, solubility, cosolvent, supersaturation, skin, anti-HSV

1. INTRODUCTION

Herpes simplex virus (HSV) is an enveloped, double-stranded DNA virus, which belongs to the *herpes viridae* family. It can be classified into two serotypes, HSV-1 and HSV-2 [1]. Orofacial infections and encephalitis, caused predominantly by HSV-1, and genital infections, caused predominantly by HSV-2, may result in significant morbidity and mortality [2]. Both HSV-1 and HSV-2 establish latent forms in sensory neurons and, upon reactivation by triggers such as emotional stress, high fever and ultraviolet light, the virus descends through the sensory nerve axons [3], causing lesions at or near the point of entry into the body. This latency increases the pathogenicity of HSV and renders successful treatment difficult.

Aciclovir (ACV), an acyclic purine nucleoside analogue, is a highly potent inhibitor of HSV and has very low toxicity for normal host cells [4]. The high selectivity has made ACV as one of the most widely used agents for the treatment of local or systemic HSV infections; however, its use is not without problems. Low and variable oral bioavailability (10-20%) [5] reduces clinical efficacy, and the risk of inducing inflammation or phlebitis at the site of injection [6] restricts parenteral administration. Topical ACV delivery to the basal epidermis where the reactivated virus is found would enable targeted therapy, reduce circulating drug levels and attenuate the risk of renal insufficiency [7]. However, only limited or modest benefits were observed following topical treatment with ACV cream or ointment (5%) vs. placebo [8,9] due to poor skin penetration [10]. Moreover, topical ACV therapy provided no clinical efficacy against recurring HSV infections [8]. ACV has high polarity (log D_{pH7} -1.76) and limited aqueous solubility, ~ 0.2% at 25°C [11], making it difficult to formulate. Moreover, it partitions poorly into the intercellular lipidic matrix of the stratum corneum. Poor topical bioavailability translates to sub-inhibitory concentrations and delayed antiviral intervention in the basal epidermis, the target compartment for chemotherapy [12].

Prodrug strategies have been extensively explored to increase transdermal drug delivery [13]. In contrast to the majority of cases, where prodrugs were synthesised to increase the lipophilicity and hence enhance the partition into stratum corneum and the diffusion through the “brick and mortar" structure [14], we have successfully delivered a series of water-soluble, biolabile amino acid ester prodrugs of aciclovir into deep skin tissue by application of an ‘active’ drug delivery technique, iontophoresis [15], the application of a mild electric current on biological tissue (Chapter 1 and 2). Although it enabled supra-therapeutic levels of ACV to be achieved in the basal epidermis within a very short duration, it is not possible to

customize the size and shape of an iontophoretic patch for all types of topical herpes infections (the area is unknown). Therefore, there is still a need to develop an efficient formulation for passive delivery.

Passive permeation of a molecule through the stratum corneum (SC) can be enhanced by increasing its thermodynamic activity in the formulation [16]. In a supersaturated system the molecule is at a concentration above its solubility limit and this increases molecular transport. The steady state flux of a molecule across the SC can be expressed using Fick's First Law as:

$$J_{SS} = \frac{D_{SC} K_{SC,formulation}}{L_{SC}} \cdot c_{formulation} = \frac{D_{SC} c_{sat,SC}}{L_{SC}} \cdot \frac{c_{formulation}}{c_{sat,formulation}}$$

where D_{SC} represents the molecule's diffusivity in the SC, $K_{SC,formulation}$ its partition coefficient, (given by the ratio of the concentration at saturation in the SC ($c_{sat,SC}$) to the corresponding value in the formulation $c_{sat,formulation}$), L_{SC} is the diffusional pathlength and $c_{formulation}$ is the concentration of the molecule in the formulation [17].

Supersaturated systems can be prepared using different techniques including the use of a cosolvent [18], evaporation of a volatile solvent [19] and rapid solubility changes with temperature or pH [20]. The cosolvent method involves mixing a solution of a molecule in one solvent in which the molecule is soluble with another solvent in which the molecule has much lower solubility, and is best for highly lipophilic molecules [21].

The aims of this study were (i) to synthesise, purify and characterise a series of lipophilic aliphatic acid ester prodrugs of ACV – aciclovir acetate, butyrate and hexanoate (ACV-Y, where Y=Ace, But and Hex), (ii) to develop and validate robust UHPLC-MS/MS methods to quantify ACV and the respective ACV-Y prodrugs, (iii) to determine solubility of the prodrugs in cosolvent systems, (iv) to prepare physically stable supersaturated formulations at different degrees of saturation (DS), (v) to measure skin deposition of ACV species (ACV and ACV-Y) after application of supersaturated formulations and (vi) to determine the cutaneous biodistribution of ACV species in the skin as a function of position by cryosectioning.

2. MATERIALS AND METHODS

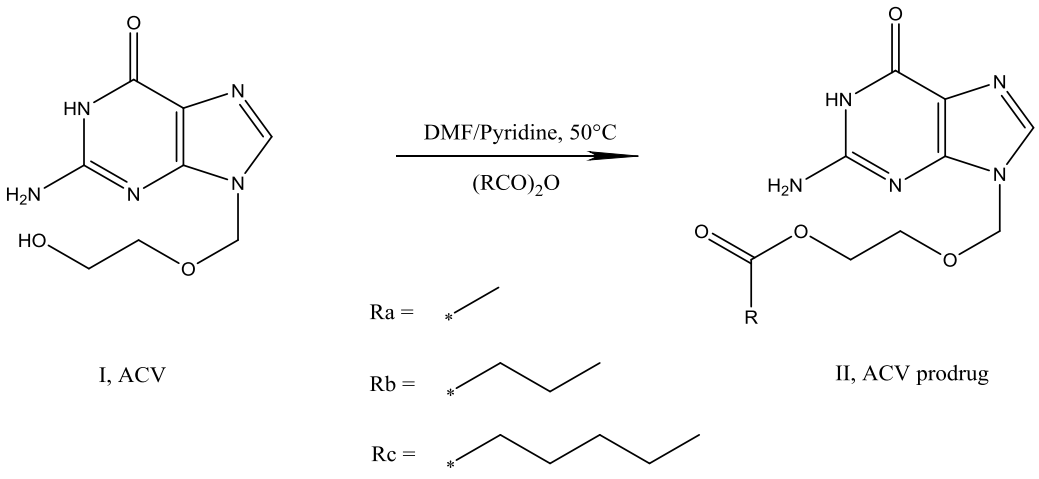
2.1. Materials

ACV was purchased from Sequoia Research Product Ltd. (Pangbourne, UK). Zovirax® cream (ACV 5% w/w) was purchased from a local pharmacy. Acetic, butyric and hexanoic anhydrides were bought from Sigma-Aldrich (Buchs, Switzerland). *N, N'*-dimethylformamide (DMF, 99.8%, extra dry over molecular sieve, AcroSealÂ®) was supplied by Acros Organics (Geel, Belgium). Hydroxypropyl methylcellulose (HPMC, E3) was obtained from Dow Chemical Company (Michigan, US). Propylene glycol (PG) was obtained from Fluka (Buchs, Switzerland). ULC/MS grade of acetonitrile (ACN) was obtained from VWR (Dietikon, Switzerland) and the same grade of formic acid (FA) was purchased from Biosolve B.V. (Valkenswaard, Netherlands). All aqueous solutions were prepared using Milli-Q water (resistivity ＞ 18 MΩ.cm). All other chemicals were at least of analytical grade.

2.2. Prodrug synthesis

Three aliphatic acid ester prodrugs of aciclovir – aciclovir acetate (ACV-Ace), butyrate (ACV-But) and hexanoate (ACV-Hex) were synthesised starting from ACV using the corresponding anhydrides of the carboxylic acids as acylating agents, as outlined in **Scheme I**.

Scheme I Synthesis of aliphatic acid ester prodrugs of ACV



Briefly, ACV (1.0 g, 4.4×10^{-3} mol) was mixed with DMF (105 ml), and the mixture was continuously stirred and warmed up to 75°C to completely dissolve ACV. The temperature was then decreased to 50°C, followed by dropwise addition of an anhydrous pyridine solution (30 ml) of either acetic (2.3 g, 22.2×10^{-3} mol) or butyric (3.5 g, 22.2×10^{-3} mol) or hexanoic anhydride (4.8 g, 22.2×10^{-3} mol). The reaction was run for 15-24 h, and the process was monitored by thin layer chromatography (TLC Silicagel 60 F 254, Merck; Darmstadt, Germany) and an API *150EX*® LC/MS System (Shimadzu Corporation; Kyoto, Japan). Upon

completion of the reaction, the solvent was removed by rotary evaporation using a Rotavapor® (Büchi Labortechnik AG; Flawil, Switzerland) and the residue was applied to a silica gel column and eluted with 10:1 to 20:1 mixture of dichloromethane and methanol to give the final product as a white solid. The purity (>98%) was determined by HPLC-UV (wavelength 254 nm), the structures were confirmed by ^{1}H-NMR spectroscopy (Varian® Gemini NMR 300 MHz spectrometer), and the molecular weight was determined by mass spectrometry (API QSTAR® Pulsar, ESI-Qtof mass spectrometer (Applied Biosystems; Rotkreuz, Switzerland). The log P of ACV and ACV-Y prodrugs were predicted using ChemBioDraw Ultra Software.

Three ACV-Y prodrugs were successfully synthesised with unambiguous ^{1}H NMR assignments and accurate m/z $[M+H]^{+}$. The spectrums showed that the peak of terminal hydroxyl group that appeared within ACV (δ 4.67 ppm) was absent in the spectra of each prodrug. Proton deshielding of the 13-H (immediately adjacent to the -OH group) was also observed: ACV δ 3.46, ACV-Ace δ 4.07, ACV-But δ 4.10, ACV-Hex δ 4.10, demonstrating successful acylation on the terminal hydroxyl group (electron-withdrawing (-I) inductive effect of the newly formed ester group). The m/z $[M+H]^{+}$ values of the prodrugs showed that the absolute value of the difference between measured and predicted value was less than 5×10^{-4} units.

I. (ACV): ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 3.46(m, 4 H, 12-H, 13-H), 4.67(s, 1 H, 13-OH), 5.34 (s, 2 H, 10-H), 6.50 (s, 2 H, 2-NH_2), 7.80(s, 1 H, 8-H), 10.63 (s, 1 H, 1-NH). HRESI-MS ($C_8H_{11}N_5O_3$+H): Calc. 226.0935; Exp. 226.0931.

IIa (ACV-Ace): Yield: 82%. ^{1}H NMR (DMSO-*d6*, 300 MHz): 1.94(s, 3 H, 2′-H), 3.65(m, 2 H, 12-H), 4.07(m, 2 H, 13-H), 5.33(s, 2 H, 10-H), 6.54(s, 2 H, 2-NH_2), 7.80(s, 1 H, 8-H), 10.67(s, 1 H, 1-NH). HRESI-MS ($C_{10}H_{13}N_5O_4$+H): Calc. 268.1040; Exp. 268.1041.

IIb (ACV-But): Yield: 51%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 0.86(m, 3 H, 4′-H), 1.49(m, 2 H, 3′-H), 2.21(m, 2 H, 2′-H), 3.67(m, 2 H, 12-H), 4.10(m, 2 H, 13-H), 5.34(s, 2 H, 10-H), 6.52(s, 2 H, 2-NH_2), 7.81(s, 1 H, 8-H), 10.63(s, 1 H, 1-NH). HRESI-MS ($C_{12}H_{17}N_5O_4$+H): Calc. 296.1353; Exp. 296.1353.

IIc (ACV-Hex): Yield: 77%. ^{1}H NMR (DMSO-*d6*, 300 MHz): δ 0.85(m, 3 H, 6′-H), 1.24(m, 4 H, 4′-H, 5′-H), 1.47(m, 2 H, 3′-H), 2.22(m, 2 H, 2′-H), 3.67(m, 2 H, 12-H), 4.10(m, 2 H, 13-H), 5.34(s, 2 H, 10-H), 6.51(s, 2 H, 2-NH_2), 7.84(s, 1 H, 8-H), 10.62(s, 1 H, 1-NH). HRESI-MS ($C_{14}H_{21}N_5O_4$+H): Calc. 324.1666; Exp. 324.1661.

2.3. Analytical procedure

ACV and ACV-Y prodrugs were quantified by UHPLC-MS/MS using a Waters Acquity® UPLC® system coupled to a Waters Xevo® triple quadrupole mass spectrometer. The UPLC® system comprised a binary solvent manager, a sample manager and a column oven. The separation of ACV and each ACV-Y prodrug was achieved with a Waters XBridge® BEH C18 (50 × 2.1 mm, 2.5 µm) reverse phase column maintained at 30°C, with a Waters XBridge® BEH C18 (5 × 2.1 mm, 2.5 µm) pre-column. The mobile phase consisted of a 7:3 mixture of 0.1 % formic acid and acetonitrile and the flow rate was 0.2 ml/min. The temperature of the sample manager was maintained at 8°C. The injection volume was 5 µl. Nitrogen and argon were used as the drying gas and collision gas, respectively. ESI positive mode (ES^+) and multiple reaction monitoring mode (MRM) were employed (**Table 1**). Data processing was performed using MassLynx® software. The methods were validated with 3 replicates at 100, 500 and 1,000 nM and showed good intra-day precision (RSD < 1 %) and accuracy (> 95 %), as shown in **Table 2**.

2.4 Supersaturated formulations

2.4.1 Solubility studies

The saturated solubility curves for ACV and the ACV-Y prodrugs in PG/H_2O cosolvent mixtures (varying from 100% water to 100% PG) were constructed. Briefly, excess ACV or each ACV-Y prodrug was added to the cosolvent mixtures and stirred for 3 h at 32°C. Then, the solutions were centrifuged at 10,000 rpm for 15 min. The supernatant was filtered through a 0.22 µm Exapure® nylon filter membrane (Alys Technologies; Bussigny-près-Lausanne, Switzerland), followed by appropriate dilutions with a 3:7 mixture of methanol and water. All samples were analysed by UHPLC-MS/MS.

2.4.2 Cosolvent method

Supersaturated systems were prepared by the cosolvent method using PG and water [22]. To choose the best candidate for preparing supersaturated formulations, the maximum degree of saturation (DS_{max}) of ACV or ACV-Y prodrugs was calculated by dividing the concentration

Table 1 UPLC-MS/MS methods for ACV and ACV-Y prodrugs quantification.

Compounds	Elution time (min)	Parameters					
		Capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)	Parent ion	Daughter ion	LOQ (nM)
ACV	1.01	2.5	31	19	226.2	152.0	10
ACV-Ace	1.67				268.1		10
ACV-But	2.02				296.1		10
ACV-Hex	2.83				324.2		15

Table 2 Precision and accuracy of the analytical methods used to quantify ACV and ACV-Y prodrugs

	Theoretical concentration (nM)	Experimental concentration (nM)	Precision (%)[a]	Accuracy (%)[b]
ACV	100	102.38 ± 0.84	0.82	102.38
	500	505.74 ± 2.31	0.46	101.15
	1000	1013.07 ± 5.90	0.58	101.31
ACV-Ace	100	100.93 ± 0.69	0.68	100.93
	500	494.68 ± 3.99	0.81	98.94
	1000	985.10 ± 6.37	0.65	98.51
ACV-But	100	98.37 ± 0.94	0.96	98.37
	500	490.75 ± 4.46	0.91	98.15
	1000	978.23 ± 7.39	0.76	97.82
ACV-Hex	100	95.70 ± 0.87	0.91	95.70
	500	481.85 ± 4.79	0.99	96.37
	1000	975.44 ± 6.38	0.65	97.54

[a]Precision = (SD/mean) x 100.
[b]Accuracy = (obtained concentration/theoretical concentration) x 100

of the drug in the solution by its solubility in the cosolvent mixture [23], where the condition offering the highest DS_{max}, i.e. the largest capacity to form supersaturated systems. In the next step, supersaturated formulations were prepared by mixing a solution of the candidate with either water or 1% HPMC aqueous solution. The physical stability of the supersaturated systems was evaluated at 32°C during 1 h after preparation. The presence of crystals in the solutions was detected by optical microscopy.

2.5 Transport studies

2.5.1 Skin source

Fresh porcine ears were supplied from a local slaughterhouse (CARRE; Rolle, Switzerland). Full-thickness porcine skin (1.2-1.4 mm) was excised from the outer region of the ear and separated from the underlying cartilage with a scalpel. Skin membranes were cleaned with running water, punched out into round disks (diameter 26-32 mm), wrapped in Parafilm® M laboratory wrapping film (Sigma-Aldrich; Buchs, Switzerland) and stored at −20°C. Prior to use, skin samples were thawed in saline for 10 min.

2.5.2 Skin penetration studies

Transport experiments were conducted using vertical Franz diffusion cells (Glass Technology, Geneva, Switzerland), with a cross-sectional diffusional area of ~ 2.0 cm^2. After clamping the full-thickness porcine skin between the two halves of the diffusion cells, the receiver compartment was filled with 10 ml of PBS solution at pH 7.4 and kept under constant agitation by magnetic stirring. The temperature was maintained at 32°C using a dynamic water bath system. 1 ml of 0.9% NaCl solution was pipetted into each donor compartment and the skin was equilibrated for 20 min. After the equilibration period, the saline solution was removed and 0.5 ml of ACV-Hex formulation with DS of 0.5, 1, 2, 3 or 4 was added into each donor. The donors were then occluded with Parafilm®. After 1 h, an aliquot (1 ml) was withdrawn from each receiver compartment and the diffusion cells were dismantled. Each skin sample was washed with running water to remove any residual formulation on the surface, it was then cut into small pieces which were placed in a mixture of methanol and water (5 ml, 6:4) for 3 h to extract deposited ACV or ACV-Y prodrug. The skin extraction medium and the samples from the receiver compartment were centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through an Exapure® nylon filter membrane with a pore size of 0.22 µm (Alys Technologies; Bussigny-près-Lausanne, Switzerland). All samples were analysed by UHPLC-MS/MS. All transport studies were carried out in sextuplicate.

2.6 Biodistribution studies

Upon completion of transport experiments using the formulations with DS of 1 and 4, the skin in contact with formulations was punched out (diameter), fixed in an embedding medium, and flattened by application of a light pressure using a glass slide. It was then snap-frozen in isopentane cooled by liquid N_2 and cryotomed using Microm HM 560 Cryostat (Thermo Scientific; Walldorf, Germany) to obtain 10 lamellae each with a thickness of 20 µm. Each lamella was extracted with a mixture of methanol and water (1 ml, 6:4) for 2 h, followed by centrifugation, filtration and UHPLC-MS/MS analysis as described above. Cutaneous biodistribution of aciclovir after application of Zovirax® cream (100 mg, 11.10 µmol/cm^2) for 1 h was also determined, using the same methods as for the supersaturated formulations.

2.6 Data analysis

Data were expressed as the mean ± SD. Outliers determined using the Dixon test were discarded. Results were evaluated statistically using either analysis of variance (ANOVA) or

Student's t-test. Student Newman Keuls test was used when necessary as a post-hoc procedure. The level of significance was fixed at $\alpha = 0.05$.

3. RESULT AND DISCUSSION

3.1 Solubility studies

The predicted log *P* values of the ACV-Y prodrugs (ACV-Ace (-1.43) ＜ACV-But (-0.36) ＜ ACV-Hex (0.48)) showed that increased aliphatic chain-length increased lipophilicity, which increased solubility in PG (6.8 ± 0.1, 51.4 ± 2.1 or 53.4 ± 14.2 mM, respectively) but decreased solubility in H_2O (5.0 ± 0.1, 2.4 ± 0.1 and 0.5 ± 0.0 mM, respectively) (**Figure 1**). All prodrugs had lower aqueous solubility than ACV (10.3 ± 0.1 mM); however, ACV (Log P value -1.66) had moderate solubility in PG (28.4 ± 0.1 mM).

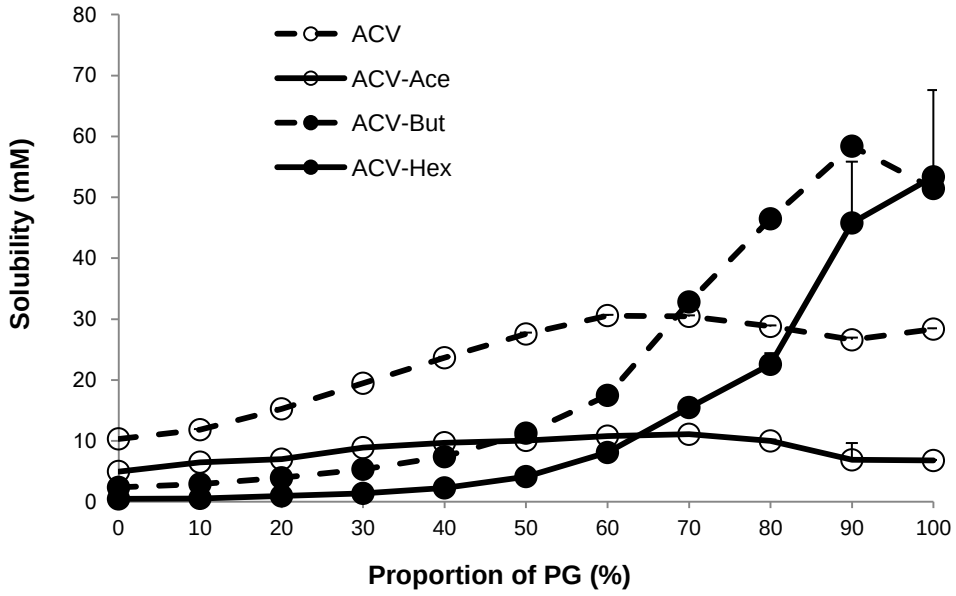


Figure 1 Solubility of ACV and ACV-Y prodrugs in PG / water mixtures presented as a function of the proportion of PG

The variation of DS as a function of the proportion of PG in the mixture is shown in **Figure 2**. It was not possible to create supersaturated mixtures of ACV and ACV-Ace. ACV-But gave better results and it was possible to create a supersaturated mixture with DS≈3, where the proportion of PG was 20-40%. ACV-Hex, with the highest solubility in PG and lowest solubility in water, was able to create supersaturated systems with the highest DS (DS_{max}). No statistically significant difference was observed in the DS_{max} achieved using 10%, 20% and 30%

PG content (10.5 ± 3.2, 12.3 ± 4.6 and 12.0 ± 3.4, respectively). Therefore, ACV-Hex in 10% PG solution was selected as the best candidate for creating supersaturated formulations since it enabled very high DS_{max} and the low PG content decreased the risk of skin irritation.

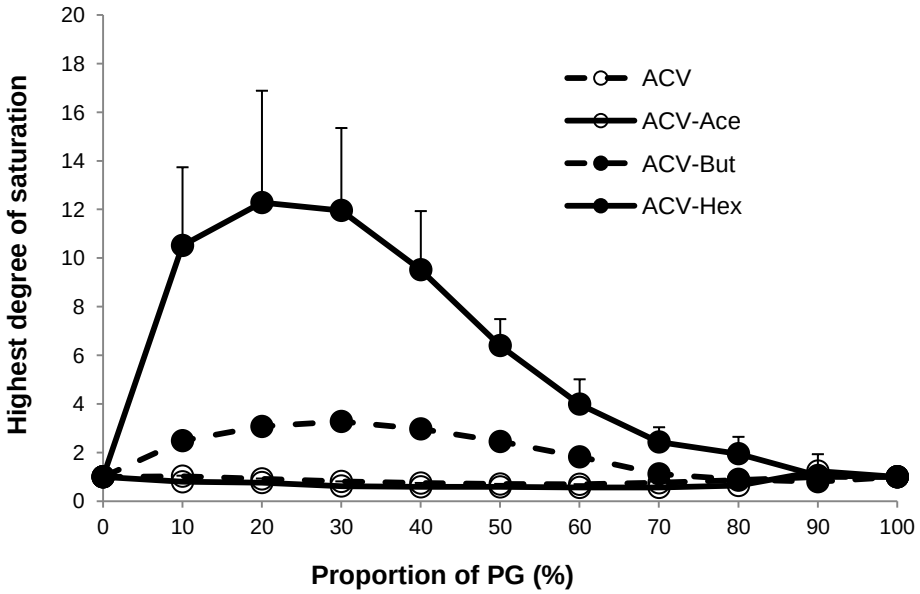


Figure 2 Degree of saturation of ACV and ACV-Y prodrugs in PG / water mixtures presented as a function of the proportion of PG

3.2 Creation of supersaturated formulations

Supersaturated formulations were prepared by mixing a saturated solution of ACV-Hex PG solution with either water or 1% HPMC aqueous solution. The cosolvent method, unlike the solvent evaporation or heating-cooling methods, is a controlled method for the creation of supersaturated systems [24]. Supersaturated systems are by definition thermodynamically unstable and tend to crystallize by spontaneous nucleation and return to the more thermodynamically stable state [25], therefore, antinucleants such as polymers were added to the supersaturated system to increase its physical stability. In this study, two series of ACV-Hex formulations (10% PG:90% H_2O) with DS from 0.5-4 were prepared – with or without HPMC. The stability of the formulations was checked by optical microscopy 1 h after preparation. All formulations were transparent when DS was set at 0.5 and 1; however, crystals were observed in the formulations with DS⩾2, where HPMC was not added (**Table 3**). In contrast, all formulations with HPMC remained clear after 1 h of preparation even with the highest DS of 4, suggesting that HPMC significantly increased the physical stability of the ACV-Hex supersaturated systems. HPMC-stabilised formulations with DS=5 were prepared;

however, precipitation was apparent immediately after mixing the solutions. Therefore, only the formulations with DS from 0.5-4 were used for the skin penetration studies.

Table 3 The conditions used for ACV-Hex skin transport studies

Degree of saturation	ACV-Hex conc. (mM)	The amount of application on skin (μmol/cm^2)	Antinucleant polymer	Physical stability after 1 h
0.5	0.28	0.07	–	Transparent
1	0.56	0.14	–	Transparent
2	1.12	0.28	–	Crystals
3	1.69	0.42	–	Crystals
4	2.25	0.56	–	Crystals
0.5	0.28	0.07	1% HPMC	Transparent
1	0.56	0.14	1% HPMC	Transparent
2	1.12	0.28	1% HPMC	Transparent
3	1.69	0.42	1% HPMC	Transparent
4	2.25	0.56	1% HPMC	Transparent

3.3 Skin penetration studies

Neither ACV nor ACV-Hex was found in the receiver compartment after application of any of the formulations for 1 h. In the absence of HPMC, skin deposition of ACV species at DS=0.5 was 0.12 ± 0.03 nmol/cm^2 and increased to 0.27 ± 0.06 nmol/cm^2 at DS=1. Although Eq 1 relates the flux to the DS, it seemed also to hold here as the 2-fold increase in DS produced a corresponding increase in deposition. However, there were no further statistically significant increases in skin deposition of ACV species at DS⩾2, (0.30 ± 0.03, 0.41 ± 0.17 or 0.54 ± 0.30 nmol/cm^2, at DS of 2, 3 and 4, respectively) (**Figure 3**). These results suggested that the formulation was no longer supersaturated, which was attributed to ACV-Hex crystallisation.

In the presence of HPMC, ACV-Hex formulations with DS of 0.5, 1 or 2 gave comparable deposition of ACV species delivered into skin (0.18 ± 0.04, 0.27 ± 0.06 and 0.30 ± 0.18 nmol/cm^2, respectively). However, the formulations at DS 3 and 4 produced significant increases in skin deposition of ACV species (2.55 ± 0.64 and 4.90 ± 1.58 nmol/cm^2, respectively) (**Figure 3**). It appeared that the 1% HPMC stabilised supersaturated

formulations were able to create and maintain a high degree of supersaturation and hence increase cutaneous delivery of lipophilic ACV-Hex.

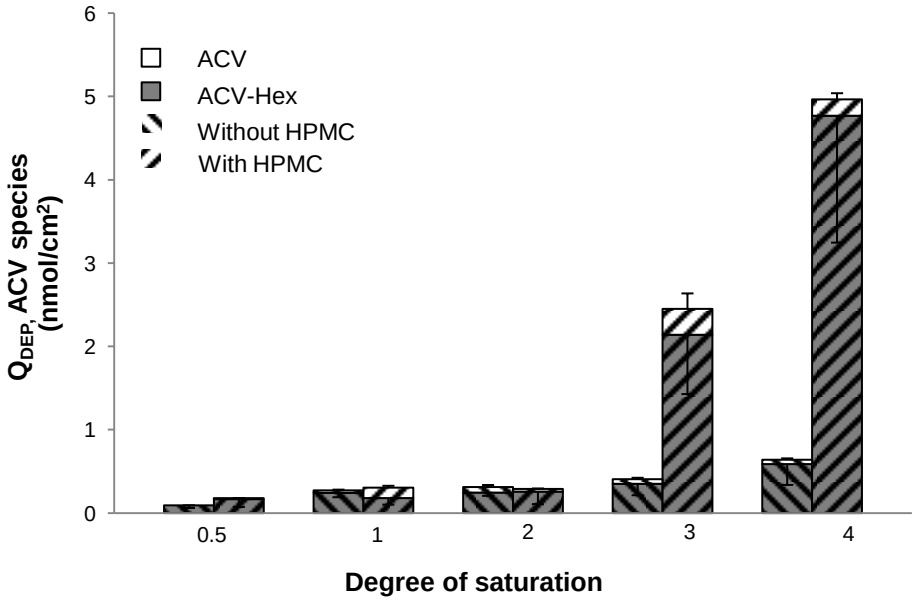


Figure 3 Cutaneous deposition of ACV and ACV-Hex after application of supersaturated formulations of ACV-Hex without or with addition of HPMC (Mean ± SD; n=6)

The therapeutic effect of ACV-Y prodrugs relies on the effective cleavage of the aliphatic acid moiety, as this is the prerequisite for phosphorylation of the ACV terminal hydroxyl group by viral thymidine kinase and human phosphorylase - ACV triphosphate is the active form that inhibits viral DNA replication [26]. The cleavage of aliphatic acid moiety is dependent principally on the enzymatic activity of the esterases present in the skin. In this study, the cutaneous bioconversion of ACV-Hex to ACV was incomplete at the 1 h time-point; only 12.2 ± 7.3 % and 4.1± 1.6 % of ACV-Hex was converted to ACV after application of HPMC stabilised supersaturated formulations with DS of 3 and 4, respectively. However, it has been reported that *in vivo* enzymatic hydrolysis rate constants of some aliphatic acid ester prodrugs of ACV were significantly greater than those observed *in vitro* [14]. Therefore, the *in vitro* permeation studies may underestimate the prodrug bioconversion rate *in vivo* most probably due to a decrease in enzymatic activity during skin processing and storage.

3.4 Biodistribution study

Saturated (DS=1) and supersaturated (DS=4) ACV-Hex formulations with HPMC and were prepared and used for the investigation of the cutaneous biodistribution of ACV species after

formulation application for 1 h. The results were compared with those of Zovirax® cream (**Figure 4**).

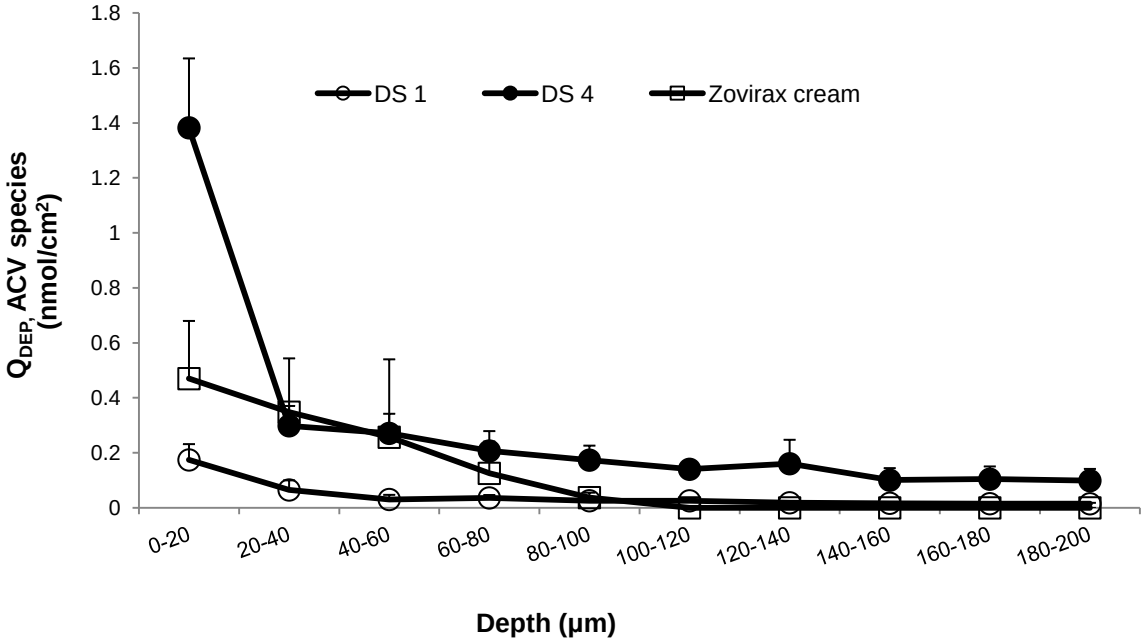


Figure 4 Cutaneous biodistribution of ACV species after application of saturated (DS=1), supersaturated (DS=4) formulations of ACV-Hex or Zovirax cream for 1 h (Mean ± SD; n=3)

It should be noted that the dose of ACV applied using Zovirax® cream (dose 11.10 µmol/cm^2) was significantly higher than with ACV-Hex formulations at DS=1 and DS=4 (0.14 and 0.56 µmol/cm^2, respectively), i.e. nearly ~80- and ~20-fold greater. Despite this, ACV deposition from Zovirax® cream (1.29 ±0.79 nmol/cm^2) was only 4-fold greater than that from the ACV-Hex formulation at DS=1 (0.27 $\pm$ 0.06 nmol/cm^2) and significantly less than that from the formulation at DS=4 (4.90 $\pm$ 1.58 nmol/cm^2).

More importantly, appreciable levels of ACV species were observed in the target area – the deep epidermis (100-160 µm). Almost no ACV was found in this area using Zovirax® cream. This was consistent with earlier findings, which showed that although ACV was able to partition into the SC from Zovirax® cream, negligible amounts reached the basal epidermis [10]. In contrast, the supersaturated ACV-Hex formulation resulted in appreciable levels of ACV species deposited in this area, 0.40 ±0.12 nmol/cm^2, where 0.08 ±0.03 nmol/cm^2 of ACV was present. The ACV concentration, (the unconverted ACV-Hex was not included) in the target area was 13.33 ± 5.01 nmol/cm^3. Given that the IC_{50} of ACV against HSV-1 0.04-3.1 nmol/cm^3[27], is much lower than the ACV concentration achieved in the target area

following application of the ACV-Hex supersaturated formulation (DS=4) for 1 h, this formulation should be valuable for further development to treat topical HSV infections, e.g., *herpes labialis*. As described above, prodrug bioconversion could be higher *in vivo*; therefore, ACV concentrations might be even greater making this approach potentially useful for the treatment of HSV-2 infections (IC_{50} 0.04-14.2 nmol/cm^3), e.g., *herpes gentitalis*.

4. CONCLUSION

Three aliphatic acid ester prodrugs of ACV were synthesised and characterised (ACV-Ace, ACV-But and ACV-Hex). ACV-Hex was the best candidate to create supersaturation formulations using 10%PG:90% H_2O mixtures and containing 1% HPMC. Best results were achieved using a formulation at DS=4. The skin transport enhancement was attributed to the higher thermodynamic activity of the molecule in the vehicle. Cutaneous biodistribution showed that supersaturated ACV-Hex formulation not only enabled more ACV to be delivered in the deeper skin layers, but also resulted in supra-therapeutic level of ACV to be achieved in the targeted area, the basal epidermis. The bioconversion from ACV-Hex to ACV was low in this *in vitro* study, but much higher cutaneous enzymatic activity was observed in *in vivo* condition [28]. The strategy of using prodrugs designed for use in the development of supersaturated systems might be of interest for the improving the efficacy of topical ACV therapy. Moreover, marketed topical ACV creams incorporate ~40% propylene glycol to enhance the penetration across stratum corneum [29]. Compared to this high quantity of PG, only 10% PG was used in our formulation, which should also reduce the risk of skin irritation.

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CONCLUSION

In this study, six water-soluble, ionised amino acid ester prodrugs of ACV were synthesised, characterised and their analytical methods developed and validated. Compared with passive cutaneous delivery of ACV and its prodrugs, iontophoresis significantly enhanced ACV delivery into porcine skin. Cutaneous transport of ACV-X prodrugs under iontophroesis was facilitated by electromigration, where the charged prodrugs were electrically driven into skin tissue and regenerate the parent drug, ACV, by hydrolysis with cutaneous esterases at or close to the site of herpes infections. The cutaneous biodistribution of ACV species showed that iontophoresis of ACV prodrugs for only 5 min was able to produce orders of magnitude ACV levels in basal epidermis superior to that observed after topical application of *Zovirax*® or *Penvir*®. Two clinically acceptable formulations, a hydrogel and a fast dissolving film were developed to incorporate ACV-X prodrugs. Iontophoretic delivery of ACV-Ile using either hydrogel or fast dissolving film enabled appreciable levels of ACV species in the whole skin as well as in the target area. Short-duration iontophoresis was also able to create great levels of transcorneal permeation and corneal deposition of ACV species. Similarly, transscleral iontophoresis of ACV prodrugs enabled great amount and fast speed of ACV species input. Biodistribution studies confirmed the superiority using short-duration iontophoresis (3.75 mA/cm^2), as the average ACV species concentration in intact eyes following 5 minute iontophoresis of ACV-Gly was higher than the IC_{50} of ACV against HSV-1. As a complementary strategy to iontophoresis of water-soluble, charged prodrugs of ACV, three lipophlic prodrugs of ACV were prepared and formulated into supersaturated formulations to enhance passive delivery of ACV into skin. Supersaturation led to increased cutaneous ACV hexonate deposition, which was attributed to the higher thermodynamic activity of the molecule in the vehicle. Cutaneous biodistribution showed that supersaturated ACV-Hex formulation not only enabled higher percentage of ACV species to be delivered in the deeper skin layers, but also resulted in supra-therapeutic level of ACV species to be achieved in the targeted area, the basal epidermis.

PRESENTATIONS

1. Poster presentation: Yong Chen, Dhaval Kalaria, Maria Lapeteva, Ingo Alberti and Yogeshvar N. Kalia. Cutaneous biodistribution of aciclovir following short-duration iontophoresis of water-soluble, ionizable prodrugs. *The 13th European Symposium on Controlled Drug Delivery, Egmond aan Zee, The Netherlands, April 16-18, 2014.*

2. Podium Presentation: Yong Chen, Yogeshvar N. Kalia. Electrically-assisted topical delivery of aciclovir and its biolabile prodrugs into cutaneous and ocular tissue. *The Ph.D Day 2014, Archamps, France, Jun 13th, 2014. (**First-Prize winner**)*

3. Podium Presentation: Yong Chen, Yogeshvar N. Kalia. New concept to treat herpes simplex keratitis and uveitis: iontophoretic delivery of water-soluble, biolabile aciclovir prodrugs into ocular tissue. *The 11th International Symposium on Ocular Pharmacology and Therapeutics, Reykjavik, Iceland, Jun19-22, 2014.*

4. Podium presentation: Yong Chen, Yogeshvar N. Kalia. Supersaturation and iontophoresis: Complementary strategies to increase cutaneous delivery of lipophilic and hydrophilic aciclovir prodrugs. *Skin Forum 14th Annual Meeting Percutaneous penetration - measurement, modulation and modeling, Prague, Czech, Sept 4-5, 2014.*

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The time is flying, but the emotion is lasting. While finishing the 50th month of my Ph.D study, I am still keeping a very clear image about the first few days when I just arrived at Geneva and registered as a Ph.D student at UNIGE. I also remembered the first smell of our lovely building Pavillon Ansermet – the delicate fragrance of coffee. That was really impressive for a person who came from a tea-drinking country. At that moment, I suddently realized that I was going to start my 'dreaming journey'.

Fortunately, I am keeping clear during this journey, because my Ph.D project is the best coffee. The first taste of my coffee was not pleasant, to be honest, but finally I got the real fragrance from it, which is because I have my thesis director Prof. Yogeshvar N. Kalia's excellent guidance. Prof. Kalia not only brought me to the field of topical drug delivery and led me to explore interesting knowledge in it, but also gave me enough freedom to think and try. I really appreciate his guidance, patience and great support. I also thank the co-director of my thesis, Prof. Leonardo Scapozza, for his great encouragement during my study.

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