Abstract
This project investigated the non-invasive iontophoretic delivery of novel triamcinolone acetonide amino acid prodrugs (TA-AA) synthesized in-house, to the eye and skin. Chapter 1 contains an exhaustive literature research on ocular iontophoresis. Chapter 2 describes the intracorneal iontophoresis of two TA-AA prodrugs, focusing on the quantitative (UHPLC-MS/MS) and qualitative (visualization by full field optical coherence tomography) evaluation of TA biodistribution. Chapter 3 presents the intracorneal distribution of 10 TA-AA prodrugs with analysis of the delivery of each individual prodrug in terms of its physicochemical properties. Chapter 4 describes the transscleral iontophoresis, using the entire eye globe, of selected TA-AA prodrugs and the quantification of TA in the different ocular compartments as a non-invasive alternative to ocular corticosteroid injections. Chapter 5 contains a detailed analysis of TA-AA prodrugs iontophoretic delivery into the skin as well as the co-iontophoretic delivery of TA-Arg (arginine) with the calcium channel blocker verapamil.
Development of new chemical and physical strategies to enhance topical drug delivery

THÈSE

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par

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Le Doyen

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To Paula
Keep Ithaka always in your mind.
Arriving there is what you are destined for.

not expecting Ithaka to make you rich.

Ithaka gave you the marvellous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won’t have fooled you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.

Constantine Petrou Cavafy
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Résumé

Le traitement local de maladies cutanées ou ophtalmiques offre l'avantage d'administrer le principe actif directement au site cible et de réduire les taux de médicament circulants qui peuvent conduire à des effets secondaires indésirables dans le cas d'une administration systémique. Aussi des procédures plus invasives telles que l'injection intravitréenne, qui diminuent notablement la compliance du patient, peuvent être évitées. Cependant, pour qu'un traitement local, oculaire ou cutané, soit efficace, les quantités de médicament thérapeutiques doivent être administrées dans un délai approprié. La question clé est donc l'optimisation de la cinétique d'administration des médicaments dans les organes cibles.

La peau est le bouclier protecteur du corps contre l'environnement externe: il empêche efficacement la pénétration de toute molécule étrangère. De même, les yeux sont efficacement protégés par un rinçage lacrymal constant et le réflexe de clignement, compliquant ainsi l'administration du médicament.

L'iontophorèse, une technique basée sur l'application d'un petit potentiel électrique, constitue une approche permettant d'améliorer considérablement la cinétique d’administration du médicament dans et au travers d'une membrane. Cette technique a été appliquée sur des barrières biologiques distinctes, telles que la peau, les ongles, les muqueuses et les yeux, qui, malgré leurs structures différentes, ont été surmontées de manière efficace par le transport électrique. Le chapitre I présente une recherche bibliographique exhaustive sur l'iontophorèse oculaire. Le principe de base de l'iontophorèse, ainsi que son applicabilité sur différents tissus oculaires (par exemple la cornée ou la sclère) ont été décrits au moyen de récentes études précliniques in vitro et in vivo. Un accent particulier a été mis sur les applications cliniques telles que l'iontophorèse cornéenne de la riboflavine pour la procédure de réticulation cornéenne et le traitement corticostéroïde pour le segment postérieur par iontophorèse transsclérale.

Les candidats idéaux pour l'iontophorèse sont des médicaments hydrophiles et chargés de faible poids moléculaire. Ces médicaments sont cependant une minorité puisque les molécules médicamenteuses...
classiques sont généralement plutôt lipophiles. Une approche pour adapter les propriétés physicochimiques de ces molécules pour le transport iontophorétique est leur modulation chimique transitoire, telle que la synthèse de prodrogues. Des prodrogues optimisées, ayant des propriétés physicochimiques appropriées, sont ensuite administrées dans les tissus, retransformées chimiquement ou enzymatiquement, générant ainsi le principe actif, capables d'exercer l'effet pharmacologique dans le tissu cible. Dans les chapitres suivants, l’administration ciblée de prodrogues d'acides aminés de l'acétonide de triamcinolone (TA-AA) par iontophorèse dans l'œil et la peau a été étudiée.

Dans le chapitre II, l'iontophorèse intracornéenne de deux nouvelles prodrogues de TA-AA, TA-Arg et TA-Lys (arginine et lysine) a été étudiée, dans le but de développer une alternative efficace et rapide à la corticothérapie cornéenne. La synthèse des prodrogues a été réalisée en interne par des réactions de couplage en deux étapes à haut rendement (> 80%). Des méthodes analytiques pour la quantification des prodrogues de TA-AA et du médicament parent ont été développées par HPLC-UV et UHPLC-MS/MS. La solubilité et la stabilité aqueuse de TA-AA ont été mesurées: les prodrogues ont montré une solubilité aqueuse de 900 à 1000 fois supérieure à celle de l'acétonide de triamcinolone (TA). La liaison ester avec la chaîne latérale d'acides aminés a montré une stabilité pendant plusieurs heures à pH 5,5. Une iontophorèse anodique pendant 10 min à 3 mA/cm² a été réalisée in vitro en utilisant la cornée provenant d'yeux de porc fraîchement excisés. Aucune différence statistiquement significative dans la délivrance totale de TA n'a été observée à la suite de l'iontophorèse intracornéenne des différentes prodrogues de TA-AA (468,25 ± 59,70 et 540,85 ± 79,16 nmolTA/cm², de TA-Arg et TA-Lys, respectivement). Cependant, lorsque les deux prodrogues ont été comparées en termes de profils de biodistribution (distribution de TA dans la cornée en profondeur, obtenue par "tranchage couche par couche"), le dépôt de TA à la profondeur de 600 µm obtenu à partir de l'iontophorèse TA-Lys était 6 fois supérieur à TA-Arg. Cela a pu être expliqué par la différente susceptibilité à l'hydrolyse de deux prodrogues. TA-Arg a été hydrolysée beaucoup plus rapidement au contact des tissus cornéens, perdant ainsi le fragment chargé et donc la force motrice électrique. Au contraire, TA-Lys, avec sa stabilité
hydrolytique supérieure, pénétrerait profondément dans la cornée grâce à une électromigration maintenue. Des images par tomographie optique cohérente à plein champ (FFOCT) de la cornée ont été utilisées pour fournir des preuves visuelles indéniables de l’administration améliorée de TA après l’iontrophorèse de TA-Arg par rapport aux témoins passifs. Les profils de luminosité obtenus ont été comparés aux quantités de TA mesurées dans les lamelles corneennes (par UHPLC-MS/MS) et un excellent chevauchement entre l'évaluation (visuelle) qualitative et quantitative a été trouvé. Avec ces investigations, les avantages de l'iontrophorèse corneéenne des prodrogues de TA-AA par rapport à l'administration passive de TA ont été prouvés, représentant une alternative non invasive prometteuse pour la corticothérapie topique classique.

Suite à ces résultats très encourageants avec TA-Arg et TA-Lys pour l'iontrophorèse intracorneéenne, au **chapitre III** le nombre de nouvelles prodrogues a été augmenté. Le but était d'étudier l'influence de dix chaînes latérales d'acides aminés différentes (alanine (Ala), arginine (Arg), asparagine (Asn), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), méthionine (Met) et valine (Val)) sur les propriétés physicochimiques des prodrogues de TA-AA et leur transport iontrophorétique dans la cornée. Les expériences d'iontrophorèse ont été conduites *in vitro* sur des globes oculaires porcins entiers en utilisant le dispositif iontrophorétique commercialisé (IONTOFOR-CXL, SOOFT Italia S.p.A, 5 min 1,8 mA/cm²) conçu pour l'utilisation humaine. Les deux prodrogues de TA-AA (TA-Arg et TA-Lys) avec double charge positive étudiés dans le chapitre précédent ont été parmi les meilleurs prodrogues administrées (649.71 ± 52.19 et 729.53 ± 49.28 nmolTA/g respectivement). Cependant, malgré leur mobilité électrique (la plus élevée; établie par électrophorèse capillaire (déterminée expérimentalement pour tous les prodrogues)), TA-Arg et TA-Lys n'ont pas surpassé d'autres prodrogues tels que TA-Ala et TA-Gly qui portaient seulement une charge positive. Ces résultats indiquent que d'autres propriétés physicochimiques, en plus de la charge, jouent un rôle clé dans l'efficacité du transport iontrophorétique. Des caractéristiques telles qu’un poids moléculaire élevé et un haut potentiel de lipophilie apparaissent comme des facteurs défavorables pour l'iontrophorèse des
prodrogues de TA-AA. Les candidats de charge optimale se sont avérés être les plus petites prodrogues de TA-AA (TA-Ala et TA-Gly) avec une distribution de la charge superficielle relativement grande et une faible tendance à interagir avec le tissu. La biodistribution intracornéenne de TA après 5 min d'iontophorese de TA-Gly a montré un dépôt TA supra-thérapeutique dans toutes les couches cornéennes, reconfirmant une fois de plus que cette approche est une option de traitement valable pour le traitement des tissus cornéens.

Au chapitre IV, l’utilité de quatre de prodrogues de TA-AA (TA-Ala, TA-Arg, TA-Ile et TA-Lys) dans l'iontophorese transsclérale a été étudiée. Le traitement non invasif avec iontophorese à court terme a été testé en tant qu’alternative potentielle aux injections intravitréennes ou sous la gaine de Tenon de corticostéroïdes utilisées dans la pratique clinique pour les états inflammatoires du segment postérieur. Une intensité de courant de 3 mA/cm² pendant 10 minutes a été appliquée à la solution de prodrogues de TA-AA sur la sclère de globes oculaires porcins fraîchement énucléés. Successivement les différents tissus oculaires (cornée, humeur aqueuse, sclère, corps ciliaire, épithélium pigmentaire rétinien (RPE), rétine neurale et humeur vitrée) ont été séparés et la quantité de médicament déposée a été quantifiée par UHPLC-MS/MS. En comparaison à l'administration passive de TA ou des solutions TA-AA pendant 10 min, l'iontophorese transsclérale a permis une augmentation de 14 à 30 fois dans le dépôt total de TA, mettant en évidence la supériorité de la technique active d'administration de médicament. Cependant, l’étude de la distribution après application a mis en évidence des différences nettes entre les prodrogues. Ces comportements spécifiques aux prodrogues étaient liés à leurs propriétés physicochimiques. Une fois de plus, la nature lipophile de TA-Ile était contre-productive pour un transport iontophorétique efficace, la distribution en profondeur de TA-Arg était de nouveau suspectée d'être freinée par le taux d'hydrolyse élevé, alors que la tendance prédominante de TA-Lys à se lier à la mélanine a retardé sa distribution passive dans les membranes. Néanmoins, en mettant les résultats dans un contexte cliniquement pertinent, la concentration de TA dans l’humeur vitrée suivant l’iontophorese de TA-Lys a été doublée par rapport à la concentration maximale rapportée après
l'injection sous la gaine de Tenon in vivo. Afin de mieux adapter l'iontophorèse transsclérale, pour cibler les tissus profonds du segment postérieur, l'effet du temps d'application (5 ou 20 min) a été étudié par l'administration d'une charge totale égale (1,44 C, TA-Ala). La quantité totale de TA a été trouvée statistiquement similaire pour les deux temps d'application (211,38 ± 37,32 et 202,51 ± 35,97 nmol/g après 5 min et 20 min). Cependant, le temps d'application de 20 minutes à une densité de courant de 1,5 mA/cm² a permis d'obtenir un dépôt de médicament environ 6 fois supérieur dans la rétine et l'humeur vitrée. Ces résultats peuvent être considérés comme très avantageux pour le traitement par exemple des troubles rétiniens. Grâce à ces découvertes, l'administration par ionophorèse de TA-AA s'est révélée être une alternative non invasive intéressante aux injections de corticostéroïdes oculaires.

Dans le dernier chapitre V, les prodrogues de TA-AA en combinaison avec l'iontophorèse ont été testés pour surmonter la barrière de la couche cornée, pour le traitement local efficace des affections cutanées telles que les cicatrices chéloïdes ou hypertrophiques. Une iontophorèse à court terme de 20 min à 0,5 mA/cm² sur TA-Ala, TA-Arg et TA-Lys a pu administrer une quantité supra-thérapeutique de TA jusqu'à 1 mm de profondeur dans la peau. Une distribution aussi profonde des médicaments est très souhaitable étant donné l'épaississement du tissu cicatriciel. De plus, l'administration de TA par ionophorèse de TA-Ala s'est révélée linéairement dépendante de la densité de courant et du temps d'application. Par la suite, la posologie du traitement pourrait être adaptées et optimisées en fonction des besoins des patients et de la progression de la maladie. Une fois de plus, comme on l’a vu dans les chapitres précédents, le transport iontophorétique des prodrogues de TA-AA s'est révélé influencé par leurs propriétés physicochimiques. Etonnamment, dans le cas de l'iontophorèse dermique, les prodrogues de TA-AA à double charge positive (TA-Arg et TA-Lys) étaient nettement surpassées par les prodrogues de TA-AA avec une seule charge positive. Ce phénomène a été attribué à la tendance accrue de TA-Arg et TA-Lys à interagir avec le tissu cutané, influençant ainsi non seulement le transport électroosmotique (testé par co-ionophorèse du paracétamol) mais aussi le transport électromigratoire (testé par iontophorèse de la lidocaïne). Il est supposé que la double charge positive
entraînait le transport iontophorétique, ainsi que celui des molécules co-administrées. Ceci a été clairement observé dans les expériences co-iontophorétiques de TA-Arg avec le vérapamil (VER) (un autre principe actif utilisé pour le traitement des cicatrices hypertrophiques et chéloïdes), initialement conçues pour fournir un traitement en profondeur du tissu cicatriciel limitant l'exposition systémique à TA. Cependant, il a été observé qu'une concentration élevée de TA-Arg dans la formulation (au-dessus de 25%) empêchait le bon transport de VER. Ce n'est que lorsque la concentration de TA-Arg a été réduite à 5% que le dépôt de TA principalement superficiel a été atteint en combinaison avec des concentrations de VER thérapeutiquement pertinentes sur l'échantillon de peau (1 mm). En résumé, l'iontophorèse des prodrogues de TA-AA ainsi que la co-iontophorèse avec VER se sont révélés appropriés dans certaines conditions pour le traitement profond et non invasif des maladies dermatiques.
Summary

The local treatment of skin or ophthalmic conditions offers the advantage over systemic drug delivery to administer the active principle directly on the target site and to reduce the circulating drug levels which can lead to undesired secondary effects. Also, more invasive procedures such as intravitreal injection, which notably decrease the patient compliance, can be avoided. However, for a local treatment, eye or skin, to be effective, therapeutic drug amounts have to be delivered in an appropriate timeframe. Key question therefore is the optimization of the drug delivery kinetics into the target organs. Skin is the body’s protective shield from the external environment; it prevents effectively the delivery of any foreign molecules; similarly, also the eyes are effectively protected by the constant lacrimal outwash and the blinking reflex, complicating therefore successful drug delivery.

One approach to dramatically enhance the drug penetration kinetics into a membrane is iontophoresis, a technique based on the application of a small electric potential. This technique was found suitable to distinct biological barriers, such as skin, nail, mucosa and eye, which despite their different structures, were found effectively overcome by the electrical driven transport. Chapter I contains an exhaustive literature research on ocular iontophoresis. The basic principle of iontophoresis, as well as its applicability on different ocular tissues (e.g. cornea or sclera) was described by means of recent preclinical in vitro and in vivo studies. Special focus was set on the clinical applications such as the corneal iontophoresis of riboflavin for the corneal cross-linking procedure and corticosteroid treatment for the posterior segment via transscleral iontophoresis.

Ideal candidates for iontophoresis are low molecular weight, charged hydrophilic drug molecules. These drugs however are in the minority since classical drug molecules are usually rather lipophilic. One approach to adapt the molecules physicochemical properties for the iontophoretic transport is their temporary chemical modulation, such as synthesis of prodrugs. Optimised prodrugs, with suitable
physicochemical properties, will then be delivered into the tissues, chemically or enzymatically retransformed to the active principle and able to exercise the effect in the target tissue. In the following chapters the targeted delivery of amino acid prodrugs of triamcinolone acetonide (TA-AA) via iontophoresis into the eye and skin were investigated.

In Chapter II the intracorneal iontophoresis of two novel TA-AA prodrugs, TA-Arg and TA-Lys (arginine and lysine) was investigated, with the aim to develop an efficient short duration and deep corneal corticosteroid treatment alternative. Prodrug synthesis was performed in house by high yielding (>80%) two-step coupling reactions. Analytical methods for the quantification of the TA-AA prodrugs and the parent drug were developed by HPLC-UV and UHPLC-MS/MS. TA-AA aqueous solubility and stability were characterised: prodrug showed a 900 to 1000-fold higher aqueous solubility compared to the parent drug triamcinolone acetonide (TA) and presented a stability of the ester bond with the amino acid sidechain over several hours at pH 5.5. Anodal iontophoresis for 10 min at 3 mA/cm² was performed in vitro using the cornea from freshly excised porcine eyes. No statistically significant difference in total TA delivery was observed following TA-AA prodrugs intracorneal iontophoresis (468.25 ± 59.70 and 540.85 ± 79.16 nmol TA/cm², from TA-Arg and TA-Lys, respectively). However, when depth corneal biodistribution profiles, obtained by “layer by layer” slicing procedure, of the two TA-AA prodrugs were compared TA deposition in depth of 600 µm obtained from TA-Lys iontophoresis was 6-fold higher compared to TA-Arg. This was suggested to be determined by the TA-AA prodrugs distinct susceptibility to hydrolysis- TA-Arg was found much quicker hydrolysed in contact with the corneal tissues, losing the charged moiety and thus the electrical driving force. TA-Lys on the contrary, with its superior hydrolytic stability was thought to penetrate down to deep cornea thanks to sustained electromigration. Corneal full field optical coherence tomography (FFOCT) images were used to provide striking visual evidence of the enhanced delivery of TA after TA-Arg iontophoresis as compared to passive controls. The obtained luminosity profiles were compared to the amounts of TA measured in the corneal lamellae (by UHPLC-MS/MS) and found
an excellent overlap in between the (visual) qualitative and quantitative evaluation. With these investigations the advantages of corneal iontophoresis of TA-AA prodrugs over passive TA delivery were proven, representing a non-invasive and promising alternative for conventional topical corticosteroid therapy.

Following these very encouraging results with TA-Arg and TA-Lys for intracorneal iontophoresis, in Chapter III the number of novel prodrugs was increased. The aim was to investigate the influence of ten different amino acid sidechains (alanine (Ala), arginine (Arg), asparagine (Asn), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), methionine (Met) and valine (Val)) on the physiochemical properties of the TA-AA prodrugs and their correspondent iontophoretic transport into the cornea. The iontophoretic experiments were conducted in vitro on entire porcine eye globes using the marketed iontophoretic device (IONTOFOR-CXL; SOOFT Italia S.p.A; 5 min 1.8 mA/cm²) designed for the use on humans. The two double positively charged TA-AA prodrugs (TA-Arg and TA-Lys) studied in the previous chapter were among the best delivered prodrugs (649.71 ± 52.19 and 729.53 ± 49.28 nmol_TA/g respectively). However, despite their highest electrical mobility established by capillary zone electrophoresis (determined experimentally for all prodrugs), TA-Arg and TA-Lys did not outperform other single positively charged prodrugs like TA-Ala and TA-Gly. These findings implied that other physicochemical properties, in addition to charge, play key importance in the iontophoretic transport efficiency. Features like high molecular weight and lipophilicity potential were found disfavouring factors for the TA-AA prodrugs iontophoresis. Optimal charge transporters were found to be the smallest TA-AA prodrugs (TA-Ala and TA-Gly) with relative large surface distribution of the charge and low tendency to interact with the tissue. The intracorneal biodistribution of TA following 5 min short term iontophoresis of TA-Gly revealed supra-therapeutic TA deposition in all corneal layers, reconfirming once more the approach as valuable treatment option for the effective corticosteroid treatment of the corneal deep tissue.
In Chapter IV, four TA-AA prodrugs (TA-Ala, TA-Arg, TA-Ile and TA-Lys) were further investigated for their relevance in transscleral iontophoresis. The non-invasive short term iontophoretic treatment was tested as potential alternative to the intravitreal or periocular corticosteroid injections used in clinics for posterior segment inflammatory conditions. A current intensity of 3 mA/cm$^2$ for 10 min was applied to TA-AA prodrug solution on the sclera of freshly enucleated porcine eye globes. Successively the different ocular tissues (cornea, aqueous humor, sclera, ciliary body, choroid and retinal pigmented epithelium (RPE), neural retina and vitreous humor) were separated and deposited drug amount quantified by UHPLC-MS/MS. Compared to passive drug administration of the TA-AA solutions for 10 min, the transscleral iontophoresis achieved a 14 to 30-fold increment in the total drug deposition, evidencing the superiority of the active drug delivery technique. However, TA-AA iontophoretic delivery into ocular tissues and the further distribution following application evidenced net differences in between the prodrugs. These prodrug specific behaviours were related to their physicochemical properties. Once more the lipophilic nature of TA-Ile was shown contra-productive for efficient iontophoretic transport, TA-Arg iontophoretic depth distribution was again suspected to be hampered by the high hydrolysis rate, whereas TA-Lys predominant tendency to bind to melanin was found to delay its passive distribution into the membranes. Nevertheless, putting the results in a clinically relevant picture, the concentration of TA in the vitreous humor following TA-Lys iontophoresis was found double compared to maximal concentration reported following sub Tenon injection in vivo. In order to further tailor the transscleral iontophoresis, for targeting deep posterior segment tissues, the effect of application time (5 or 20 min) was investigated by the delivery of equal total charge (1.44 C, TA-Ala). Total TA delivery was found statistically similar for both application times (211.38 ± 37.32 and 202.51 ±35.97 nmol$_{TA}$/g following 5 min and 20 min). However, the increased application time of 20 min at a current density of 1.5 mA/cm$^2$ achieved a ~6-fold increased drug deposition into the retina and vitreous humor; results which were thought of great advantage for the treatment of e.g. retinal disorders. By means of these findings, TA-AA iontophoretic delivery was shown as valuable non-invasive alternative to ocular corticosteroid injections.
In the last Chapter V TA-AA prodrugs efficacious delivery in combination with iontophoresis was tested to overcome the stratum corneum barrier, for the effective local treatment of skin conditions such as keloid or hypertrophic scars. Short term iontophoresis of 20 min at 0.5 mA/cm² on TA-Ala, TA-Arg, and TA-Lys was shown to deliver supra-therapeutic amount of TA down to 1mm of depth into the skin. Such profound drug distribution is most desirable given the increase specimen of the scar tissue. Moreover, TA delivery by TA-Ala iontophoresis was found to be linearly depending on current density and application time. Herewith the treatment schedule and intensity could be adapted and optimized according to the patients’ needs and progression of the disease. Once more as seen in the previous chapters, the iontophoretic delivery of TA-AA prodrugs was found distinct according to their physicochemical properties. It was most astonishingly that in the case of dermal iontophoresis double positively charged TA-AA prodrugs (TA-Arg and TA-Lys) were clearly outperformed by the single positively charged TA-AA prodrugs. This phenomenon was attributed to the increased tendency of TA-Arg and TA-Lys to interact with the skin tissue, thereby not only influencing the electroosmotic transport (tested by acetaminophen co-iontophoresis) but also the electromigratory transport (tested by lidocaine iontophoresis). The double positively charged prodrugs herewith were thought to hinder their proper iontophoretic delivery, as well as the delivery of co-administered molecules. This was seen clearly in the co-iontophoretic experiments performed of TA-Arg with verapamil (VER, another active principle used for the treatment of hypertrophic and keloid scars), which were initially designed to provide a deep treatment of the scar tissue limiting TA systemic exposure. However, it was seen that high TA-Arg concentration in the formulation (above 25%) avoided effective VER delivery. Only when the concentration of TA-Arg was reduced to 5% mostly superficial TA deposition was achieved in combination with therapeutically relevant concentrations of VER all over the skin specimen (1mm). In summary, TA-AA iontophoresis as well as co-iontophoresis with VER were found suitable under certain conditions for the profound and non-invasive treatment of the dermal layer.
FOREWORD

- Challenges in ophthalmic and dermal drug delivery
This foreword sets the framework for the problematics of drug delivery to different organs (eye and skin), which were the targeted barriers in this research. The coverage is intentionally brief since it aims to provide a basic understanding of the topic and the difficulties which will be discussed more into detail in the further manuscript.

1. Anatomy of the eye and drug delivery barriers

![Human eye globe cross-section](image)

**Figure 1.** Human eye globe cross-section, evidencing the tissue components and physical barriers for drug delivery, reproduced with permission from [1].
Drug delivery to the eye is a peculiar challenge due to the efficient isolation of the organ, not only from the external environment but also from the body own blood circulation - as a defence mechanism to assure the visual function [2] [3]. From an anatomical point of view, the fragile structure defined as eye globe is included in the orbital cavity of the scalp. Posterior part of the eye globe is bolstered into fat and muscular tissue, whereas the external part of the eye is covered by the eyelids. The eye globe itself is commonly divided into two segments, namely anterior (cornea, aqueous humor, iris-ciliary body, and lens) and posterior (sclera, choroid, retina, vitreous humor, optic nerve) [4].

Figure 1 shows an ocular crosscut exposing the different tissues composing the two ocular compartments and summarizes the physical barriers which were found of major role in the ocular drug delivery [1]. With the evident complexity of the organ, drug delivery will be affected by the different static and dynamic barriers which hamper the administration of therapeutically relevant drug amounts.

1.1 Challenges for anterior segment drug delivery

Most common treatment option for anterior segment diseases is the topical administration, usually in the form of eye drops. Although it is widely practiced in clinics with large patient compliance, the success rate is limited given the very low bioavailability of the active principle - only 1-7% of the applied drug will reach the aqueous humor [5].

Eye drop instillation provokes the blinking and lacrimal reflex, which dilutes and eliminates the formulation applied within seconds. Most of the eye drop liquid (30-50 µl) will be directly spilled on cheeks or eyelashes, given that the capacity of fluid storage in the cul-de-sac is largely overwhelmed [6]. The remaining formulation will be rapidly evacuated through the lacrimal drainage system via the nasolacrimal duct or by the absorption of the conjunctiva (Figure 2).
**Figure 2** Schematic summary of the faith of topically instilled drugs, the thickness of the arrows represents the relative important of each phenomenon on the drug distribution form eye drops. Reproduced and adapted with permission from [7]

Only a small volume of the formulation will get in contact with the cornea which is the target membrane for the treatment of most anterior segment diseases. The cornea itself, however, represents a challenging membrane for drug penetration given its tri layer structure (epithelium, stroma, and endothelium; interfaced by Bowman’s and Descent’s membrane). The main rate limiting membrane was found to be the outermost epithelium with the intercellular tight junctions and its high lipophilicity [8]. The permeation of lipophilic molecules was found favoured [9], however, only moderate lipophilicity will allow the further diffusion through the hydrophilic corneal stroma and partition from the endothelium to reach the aqueous humor [8] [10].

Another major membrane of the anterior segment is the conjunctiva, a mucous transparent and highly vascularized membrane, which surface area is almost 20 times bigger compared to the cornea. Given the much larger contact area and its more permissive epithelium, the parallel absorption by the
conjunctiva will significantly compete with the corneal absorption [11]. Especially for small hydrophilic molecules absorption through the conjunctiva was found significantly faster [12] [13]. Unfortunately, most of the absorption by the conjunctiva is a so called non ocular, conjunctival blood and lymphatic capillaries will distribute the molecules into the systemic circulation; therefore ineffective ocular treatment can be combined with the risk of systemic side-effects [13].

Systemic drug delivery for the treatment of the anterior segment is restrained by the blood-aqueous barrier (iris and ciliary body), similar to the blood-brain barrier, the tight junctions of the capillary endothelium of the iris and on the epithelium of the ciliary body as well as drug transporters avoid the permeation of drug molecules in blood to aqueous humor direction [2] [7].

A possible solution to increase the drug bioavailability to the anterior segment is either formulation based, to increase contact time [14] [15], or in severe cases the direct injection or implantation of the drug formulation into the aqueous humor [16].

1.2 Challenges for posterior segment drug delivery

Most severe and sight threatening diseases involve the posterior segment. This part of the eye globe, however, is much more difficult to access, and given the complexity of barriers to overcome, topically instilled eye drops were found inefficacious in the treatment of posterior segment diseases [10]. A more adapted delivery route for the targeting of the posterior segment is the pericocular administration, for example by subconjunctival or sub-tenon injection [3]. With these techniques, the drug formulation will be in direct contact with the sclera which is composed mainly by collagen fibrils and glycoproteins [17] [18] and can be rather easily overcome by drug molecules and high molecular weight biologicals [19] [20]. This was reported most useful for the treatment of the uvea, but with modest impact on the treatment of the retina. To reach the innermost layer major barriers such as choroidal blood flow and retinal pigmented epithelium have to be overcome. The first with the fenestrated capillaries will lead to a rapid outwash of the drug molecules into the systemic blood flow [1], while further RPE with the
thigh junction will represent a barrier for mostly hydrophilic molecules [21]. In addition, the presence of melanin in the uvea and RPE can influence the drug distribution, given that the binding with melanin can interfere with the drug bioavailability [22, 23].

The systemic administration of drugs to the eye globe once more, like for the anterior segment, is not effective because of the organs isolation from the body’s blood flow. Tight junctions and efflux proteins on the retinal capillaries construct efficient barriers for substances to the retina and vitreous humor (similar to blood brain barrier) [24]; the drug delivery inward form blood to the vitreous as well outward vitreous to blood is strictly limited.

Given these above-mentioned limitations on effective drug delivery to the retina, the direct injection of the active principle into the vitreous humor is performed in clinics. These interventions, however, expose the patients to the risk of severe side effects, low compliance and have to be repeated given the chronicity of most conditions [25].

2. Anatomy of the Skin

The skin is the largest organ of the human body (1.7 m²) with the essential function of protection from the external environment (pathogens, radiations, chemicals and loss of water), the maintenance of body temperature and sensation [26].

The skin barrier is a multi-layered system which is divided into three main regions, the epidermis (~100 μm), followed by the dermis ≥1 mm) and hypodermis, as shown in Figure 3.
Figure 3. Histological cross-section of human facial skin with haematoxylin and eosin staining.

The most profound layer, hypodermis, is mainly constituted by adipocytes and loose connective tissue which attaches the skin to the underlying organs [27]. The above anchored dermis is providing the mechanical strength of the skin with the abundant collagen produced by fibroblasts. Moreover, the dermis contains blood and lymph vessels, numerous cells of the innate immune system (e.g., macrophages, mast cells), nerves, hair follicles, sebaceous and sweat glands[28]. The top layer of the skin is the epidermis, a continuously self-replacing structure were inner cells moving up to the surface, shedding and substituting the outer ones. This dynamic barrier is composed by i) the stratified viable epidermis, with differentiating keratinocytes (95%), melanocytes, mechanoreceptors, and Langerhans cells [29] and ii) the outermost stratum corneum layer, which is composed by corneocytes - the terminal differentiation of keratinocytes [30]. This very thin uppermost layer is providing the main barrier function for limiting body water loss and prevent external penetration of pathogens or chemicals [26]. The corneocytes are filled ~60% with keratin, have lost their nucleus and organelles and are tightly
linked by intercellular boundaries (desmosomes). Between the flat corneocytes a multilamellar lipophilic matrix composed of ceramides, cholesterol and long chain fatty acids [27] [30] is found.

2.1 Challenges topical or transdermal drug delivery

Most convenient drug delivery route is the oral administration; topical or transdermal drug delivery is useful in cases of medicaments with important first pass metabolism or when a chronic administration is needed. Additionally, the topical treatment of a localized skin condition is preferable to limit systemic side effects.

Astonishingly enough, despite the above described complexity of the skin, only the outermost 20 µm thick *stratum corneum* represents the main obstacle for efficient drug delivery [31]. The peculiar arrangement of the interconnected and flattened corneocytes which are embedded in the lamellar disposed lipid matrix results in a thousand fold lower water permeability compared to classical biological membranes [32]. Skin penetration routes can be divided into the transport across the *stratum corneum* (trans- and intercellular) and transappendageal (hair follicles, sweat glands) [33]. The transport via pores and shafts was thought to play a minor role, given the scarce coverage of the total skin surface (0.1%); however, its importance increases according to the area of interest on the body and for molecules which cannot pass otherwise [34]. For the penetration of the *stratum corneum*, two penetration pathways are possible, one crossing the corneocytes (transcellular route) and the other in-between the corneocytes through the lipid matrix (intercellular route) [28]. It is supposed that the preferential pathway depends on the physicochemical properties of the drug, therefore more lipophilic molecules will prefer the intercellular route, whereas the more hydrophilic might partition into the corneocytes and the lipid matrix (transcellular route) [32].

Passive diffusion though the skin at steady state (the amount entering the skin is equal to the amount leaving), can be parametrized using Fick’s first law (*Equation I*).
\[ J_{ss} = k_p * c_v = \left( \frac{D*K}{h} \right) * c_v \]  

Equation I

\( J_{ss} \) is the steady state flux (μg cm\(^{-2}\) s\(^{-1}\)) of the molecule, \( k_p \) the permeability coefficient of the drug across the membrane (cm s\(^{-1}\)) and the \( c_v \) concentration in the vehicle (μg cm\(^{-3}\)). The permeability coefficient is composed by the \( K \) the stratum corneum/vehicle partition coefficient, \( D \) the diffusion coefficient of the compound in the membrane (cm\(^2\) s\(^{-1}\)) and \( h \) the thickness of the membrane (cm) [35]. According to Equation I, to enhance the passive penetration i) the concentration of the donor can be increased (near saturation) ii) the diffusion of the drug in the skin can be enhanced by the addition of excipients able to disturb stratum corneum lipids (penetration enhancers) and finally iii) the partition between the vehicle and the skin be improved (addition of substances to improve solubility of the drug in the skin: e.g. hydration of the skin ) [36]. Such improvement is envisaged in formulation approaches such as for example transdermal patches and nanocarrier formulations (liposome, micelles). Another strategy to overcome the stratum corneum barrier is the transport of molecules though the skin by either depleting the barrier minimally (e.g., laser, microneedles) or the application of electrically assisted techniques (e.g., iontophoresis) [37]

3. Iontophoresis: drug delivery approach for eye and skin

The objective of this thesis focuses on the use of iontophoresis, an active drug delivery technique, to overcome the ocular or skin barrier, for the treatment of intraocular diseases (anterior segment and posterior segment) as well as dermal conditions. The successful combination of the prior chemically modified corticosteroid triamcinolone acetonide into amino acid prodrugs and the application of a mild current will be discussed in detail in the following chapters.
4. References


CHAPTER I

- Basic principles and current status of transcorneal and transscleral iontophoresis
CHAPTER I – Introduction  
Basic principles and current status of transcorneal and transscleral iontophoresis

Basic principles and current status of transcorneal and transscleral iontophoresis

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Abstract

Introduction: Iontophoresis is an active non-invasive drug delivery technique that can increase the transport of charged and neutral molecules into and across biological membranes. Most research to-date has focused on (per) cutaneous iontophoretic drug delivery. However, recent studies illustrate its potential for drug delivery to the eye: corneal iontophoresis may enable targeted topical therapy of intracorneal diseases, whereas transscleral iontophoresis may enable non-invasive intraocular drug delivery.

Areas covered: We describe iontophoretic principles in the context of ocular delivery before providing a summary of recent preclinical studies involving transcorneal and transscleral iontophoresis in vitro and in vivo. Subsequently, an overview of clinical applications with special focus on the transcorneal iontophoresis of riboflavin for corneal cross-linking and transscleral iontophoresis of corticosteroids for the treatment of posterior segment diseases is provided.
Expert opinion: The feasibility of using iontophoresis for ocular drug delivery has been demonstrated. Drug formulation development and the ability to design iontophoretic applicators will now determine its success in the clinic. The specificities of the ocular globe must be taken into account; in particular, its unique morphology, and the smaller surface area available for drug diffusion and the fact that it is more susceptible to irritation and less robust than the skin.

Keywords Cornea; eye; iontophoresis; sclera; transcorneal; transscleral


5. Introduction

Effective targeted topical drug delivery to the eye remains a major challenge. Anterior segment disorders involving the cornea, conjunctiva, and anterior uvea are commonly and, at least in principle, ‘conveniently’ treated by conventional eye drop instillation. However, at least 95% of the active principle in the formulation is washed off by lacrimation and the blinking reflex [1,2]. Thus, uptake by either the conjunctival or corneal epithelia is hindered by the short residence time [3]. Systemic absorption of topically applied formulation through nasolacrimal drainage or by conjunctival blood and lymphatic vessels can sometimes cause undesired side effects in the case of prolonged treatment [4,5]. The resultant poor drug bioavailability means that frequent dosing is needed in order to reach and to maintain therapeutically effective concentrations and this can be compromised by poor patient compliance [6].

Even greater challenges are encountered when the drug must exert its action in the posterior structures of the eye such as the vitreous and/or the retina. Topical instillation is not an efficient means to treat diseases of the deep ocular tissue. Intravitreal injections present the risk of serious potential complications such as traumatic cataract, endophthalmitis, retinal detachment, and vitreous haemorrhage [7–9]. Systemic drug administration entails the administration of significantly higher doses with a concomitant increase in the risk of off-target adverse effects.

Topical delivery kinetics must be improved in order to enhance the efficacy of local administration – the static and dynamic barriers present in the eye mean that simple passive diffusion is not sufficiently rapid. In some cases, it may be possible to benefit from active transport by using drugs that are substrates for protein transporters present in the eye and so facilitate drug delivery; however, this is not universally applicable. Iontophoresis is a method that involves the application of a mild electric field to facilitate a rather different type of ‘active’ transport of drug molecules across biological membranes. The electrical potential gradient introduces a second driving force for molecular transport in addition to the concentration gradient across the membrane [10]. Given that it functions principally by acting on
the molecule rather than impairing the ocular membrane there is less risk of irritation to the eye. Therapeutic concentrations are generally achieved faster than after passive delivery [11] and the drug input kinetics can be modulated via the intensity and duration of the applied current profile [12]. Since its first description in the early 1900s [13], it has been extensively studied for topical and transdermal drug delivery and considerable progress has been made with three prefilled iontophoretic systems having been approved by the US FDA [14–17]. The development of miniaturized application devices has made possible its transposition to other routes of administration, for example, buccal [18,19] and nasal mucosae [20], sclera [21,22], cornea [23], and the nail [24,25]. Among these, ocular iontophoresis seems to be the most advanced and clinical studies have been performed [26-30]. The potential benefits of a non-invasive method such as iontophoresis to improve targeted drug delivery to the eye are obvious. Control of the applied voltage ensures that there are no physiological modifications to the tissue barrier function after treatment. In addition to developing the appropriate applicator systems, the challenge is to determine whether topical iontophoresis can be used for the delivery of therapeutic agents to both the anterior and posterior segments and if so, to what extent can it be used as a surrogate for injection based treatments.

Given the anatomy of the eye, two different modes of ocular iontophoresis have been developed (Figure 1): (i) transcorneal iontophoresis, which targets the anterior segment of the eye and (ii) transscleral iontophoresis that is intended to deliver therapeutics to the posterior segment. In order that a drug reaches the innermost vitreous humor and maintains therapeutic concentrations, both intraocular static barriers to transport – for example, cornea, sclera, choroid, and retinal pigmented epithelium – and dynamic barriers – for example, choroidal and retinal blood flow – must be overcome [2,3].
Here, we provide a concise overview of iontophoretic principles in the context of ocular delivery summarize recent preclinical results \textit{in vitro} and \textit{in vivo} and present case studies showing clinical applications of the technique.

\section*{6. Basic concepts: iontophoretic transport mechanisms}

Molecular transport during iontophoresis can be attributed to three component mechanisms: passive diffusion, electroosmosis (EO) and electromigration (EM) \cite{12}. The total drug flux ($J_{TOT}$) is expressed as the sum of the passive flux ($J_P$) and the fluxes due to EO and EM, $J_{EO}$ and $J_{EM}$, respectively \cite{32}:

\[ J_{TOT} = J_P + J_{EO} + J_{EM} \]

\textit{(Equation I)}

In the case of transdermal delivery, the passive flux ($J_P$) of hydrosoluble, ionized species through the lipid matrix of the \textit{stratum corneum} is usually very small compared to the other two transport mechanisms, which therefore dominate electrotransport. However, ocular membranes do not possess a permeability barrier as effective as the \textit{stratum corneum}. Hence, the passive component may provide a more important contribution to total drug delivery; therefore, it cannot always be neglected. Substitution for the terms describing $J_P$, $J_{EM}$ and $J_{EO}$ at steady state in \textit{Equation I} gives \cite{12, 33}:
Basic principles and current status of transcorneal and transscleral iontophoresis

\[ J_{TOT} = J_p + J_{EO} + J_{EM} = \left[ k_{p,x} + V_w + \left( \frac{i_d}{z_x F} \right) \times \frac{u_x}{\sum_i u_i c_i} \right] \times c_x = \left[ k_{p,x} + V_w \right] \times c_x + \frac{i_d t_x}{z_x F} \]

(Equation II)

where \( i_d \) is the current density (current intensity applied per unit area), and \( u_i \) and \( c_i \) refer to the mobility and concentration of the ions carrying charge across the membrane and \( u_x, z_x, c_x \) and \( t_x \) are the mobility, valence, concentration and transport number of the drug \((x)\) and \( k_{p,x} \) is its permeability coefficient; \( F \) is Faraday’s constant and \( V_w \) is the linear velocity of solvent flow.

The current \((i)\) flowing across a given biological tissue in response to the application of a given potential difference \((\varphi)\), depends on its resistance \((R)\) and is given by Ohm’s law (Equation III):

\[ i = \frac{\varphi}{R} \]

(Equation III)

Thus, at a given voltage, higher currents can be achieved in biological tissues that have a lower resistance. Conversely, a given current can be achieved using a lower voltage; thereby reducing local Joule heating and the risk of irritation or pain. Therefore, although the highest current density that is considered acceptable for transdermal delivery is \(~0.5\) mA/cm\(^2\) (depending on the application area) [34], water rich ocular tissues, with lower electrical resistance, were reported to support much high current densities; e.g. up to 20 mA/cm\(^2\) for 5 min in rabbit cornea [35], and 5.5 mA/cm\(^2\) for 20 min in human sclera [36]. Table 1 presents some examples of current densities tested on cornea and sclera with the observed side effects. Obviously, the duration of current application was also a key factor for the safety of the procedure.
### Table 1. Clinical observations following transcorneal and transscleral iontophoresis at different current intensities and application times

<table>
<thead>
<tr>
<th></th>
<th>Current density (mA/cm²)</th>
<th>Duration (min)</th>
<th>Drug</th>
<th>Clinical observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcorneal iontophoresis</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>In rabbits</strong></td>
<td></td>
<td></td>
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<tr>
<td>0.84</td>
<td>5 &amp; 10</td>
<td>Tobramycin</td>
<td>Reversible epithelium edema (24h) observed with slit lamp. 10 min treatment provoked broad epithelial damage, stoma and endothelium were found intact with electron microscopy</td>
<td>[37]</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>5</td>
<td>Vancomycin</td>
<td>Endothelial cell loss was found for vancomycin and buffer treated eyes, no increase in total corneal thickness was detected. Transient corneal opacity was attributed to drug deposition. Following 5 min application reversibly swollen corneal stroma and epithelial injury detected by fluorescent staining were reported. Scanning electron microscopy of the endothelial layer showed only minimal or negligible damage was claimed by the authors suggesting the upper limit of tolerability.</td>
<td>[38]</td>
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<tr>
<td>20</td>
<td>5</td>
<td>Gentamycin</td>
<td>Following 5 min application reversibly swollen corneal stroma and epithelial injury detected by fluorescent staining were reported. Scanning electron microscopy of the endothelial layer showed only minimal or negligible damage was claimed by the authors suggesting the upper limit of tolerability.</td>
<td>[35]</td>
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<td>25</td>
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<tr>
<td><strong>In humans</strong></td>
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<tr>
<td>1.8</td>
<td>5</td>
<td>Riboflavin</td>
<td>In vitro on human cornea no damage on the Descemet’s membrane and the endothelium was observed.</td>
<td>[39]</td>
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<tr>
<td><strong>Transscleral iontophoresis</strong></td>
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<td><strong>In rabbits</strong></td>
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<tr>
<td>4</td>
<td>5 &amp; 10</td>
<td>Hemisuccinate methyl prednisolone</td>
<td>Transient pitting of the peripheral cornea and conjunctival vessel dilatation was observed after application, no clinical lesions in anterior and posterior segment were reported. Authors suggest current densities inferior to 50 mA/cm² are harmless.</td>
<td>[40]</td>
<td></td>
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<tr>
<td>400</td>
<td>15</td>
<td>Phosphate buffered saline</td>
<td>No histopathological changes in retina (no observable injury) Chorioretinal burns, severity of retinal lesions progressed from moderate (5 min) to severe (15 min)</td>
<td>[41]</td>
<td></td>
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<tr>
<td><strong>In humans</strong></td>
<td></td>
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<td></td>
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<tr>
<td>5.55 &amp; 7.4</td>
<td>20 min</td>
<td>Saline</td>
<td>Only at the highest current density tested (7.4 mA/cm²) some subjects reported transient burning sensation, superficial fluorescein staining was observed, cleared after 22 h. 5.5 mA/cm² was considered as tolerable.</td>
<td>[36]</td>
<td></td>
</tr>
</tbody>
</table>
6.1 Electromigration

For ionized species, EM is the dominant electrotransport mechanism – it can be considered as the ordered movement of ions in the presence of an electric field [10]. Magnetic resonance imaging using Mn²⁺ ions was used to visualize ion accumulation in the vitreous and anterior segment following anodal iontophoresis (10 mA/cm² for 30 min) through sclera and cornea; increased luminescence was observed after iontophoresis as compared to passive application. It was also evidenced that current flow was perpendicular to the applied electrode and that there was minimal lateral loss [42].

The physicochemical characteristics of a drug molecule naturally play a key role in influencing EM since they determine its electric mobility, propensity to interact with the transport pathway and its capacity to carry current in the presence of competing ions (expressed as the transport number, \( t_x \)) \((\text{Equation II})\)[12, 32]. The linear dependence of \( J_{EM} \) on the current density \( i_d \) provides a simple mechanism to control the rate and extent of drug delivery although this can plateau at high current densities [11].

6.2 Electroosmosis

EO can be described as the solvent flow incited by an electric potential applied across an ionized membrane [10, 12]. In the skin, at physiological pH, EO flow is in the anode to cathode direction since the isoelectric point (pI) of the tissue is 4-4.5 [43]. Since corneal and scleral pI were found to be in a similar range, 3.2 [44] and ~ 3.0 to 4.0 [22, 45] respectively, the same phenomenon is suggested to occur and may be expressed as shown in \( \text{Equation II} \) [46]. This transport mechanism favours the electrotransport of cations and opposes that of anions. Neutral molecules can be transported into ocular tissue from the anode. Nicoli and co-workers [22] investigated the electrotransport of acetaminophen (151 Da) and fluorescein isothiocyanate-labelled dextrans (with molecular weights of 4.4 and 120 kDa), through porcine sclera as. They showed that anodal iontophoresis (2.9 mA/cm² for 2 h) increased the transscleral flux of the high molecular weight dextrans by 2-6.5-fold, but it did not significantly increase the transport of acetaminophen [22]. This was surprising, since acetaminophen is commonly used to
report on EO in the skin. The different properties of the tissues mean that, acetaminophen, a neutral polar molecule with poor passive permeation across skin displays significant passive diffusion across ocular tissue – therefore, it cannot be used as a marker for EO in ocular iontophoresis. The enhanced permeation of fluorescently-labelled dextrans by EO was also evidenced in corneal tissue following transcorneal iontophoresis [47]. Another interesting study concerning the EO flow has been performed by investigating the streaming potential of model molecules through porcine and bovine sclera. Given its the dependence on the charges on the membrane, the possible interaction of compounds to the tissue will be detected and can be correlated to the drugs EO [48] A possible concern is whether the solvent flow might affect cornea transparency. Animal and clinical studies are required to assess the safety of the procedure.

7. Transcorneal iontophoresis

7.1 Preclinical studies in vitro and in vivo

Experimental praxis in ocular delivery research is to perform animal studies in vivo in order to determine the effects of both dynamic and static barriers; however, these studies are associated with high costs and ethical concerns. Furthermore, although there are benefits of conducting studies in vivo, the anatomy and physiology of the eye in smaller mammals that are most easily accessible for these studies, may not accurately mimic conditions in the human eye. Therefore, preliminary screening in vitro in more physiologically relevant models that can better approximate the human eye might be advantageous [23]. Franz diffusion cells adapted for use with excised porcine cornea were used to show that corneal barrier function and integrity could be maintained for up to 6 h [23]. It has also been suggested that in addition to obtaining a preliminary evaluation of drug penetration, the risk of membrane impairment due to application time and current density can be monitored in vitro by measuring corneal hydration [49]. Nevertheless, given the difficulties in correlating in vitro results obtained with excised tissue and observations in vivo, direct testing in small animals, preferentially rabbits, remains common [50].
The accumulation of drug in the cornea can create an *in situ* drug depot that enabled a slow sustained release of drug into the aqueous humor and thus eliciting a prolonged therapeutic effect. This was observed following transcorneal iontophoresis of ciprofloxacin for 5 min using isolated porcine cornea or entire eye globes [51]. Increasing the current density from 0.75 mA/cm$^2$ to 6.25 mA/cm$^2$ increased the drug loaded in the cornea by ~six-fold (~20 and 120 ng/mg, respectively) [51].

The transcorneal iontophoresis of antibiotics has been extensively studied as a means for the targeted treatment of infectious corneal keratitis [52]. If not treated efficiently corneal ulceration can cause major vision loss in almost 50% of cases [53]. Although superiority of electrically driven administration of sulfadiazine antibiotics into rabbit corneas over conventional eye drops was shown in 1942 by von Sallmann [54], it was only 40 years later that attention returned to this technique for the delivery of antibiotics such as vancomycin [38], terramycin [55] and gentamicin [35, 56]. An extensive overview of those studies is given by Elijarrat-Binstock and Domb [31].

The delivery of gentamicin was further investigated using applicator systems. First, agar based probes were tested successfully on rabbit and rat corneas [56, 57]. Then, single use applicators were made with the more resistant hydroxyethyl methacrylate (a polymer used for the production of flexible contact lenses) and soaked in 10% w/v gentamicin sulphate solution before application [58]. After incorporation of the probe in a portable iontophoretic system [59], it was shown that transcorneal iontophoresis at a current density of 5.1 mA/cm$^2$ in rabbits for only 1 min, resulted in peak concentrations of $363.1 \pm 127.3 \, \mu g/g$ in the cornea and that after 2 h a concentration of $29.4 \pm 17.4 \, \mu g/ml$ was achieved in the aqueous humor. This was superior to subconjunctival injection of gentamicin and its topical instillation every 5 min for 1 hour; the elimination profile was described by a two compartment pharmacokinetic model and correlated to the experimental data. The possible maintenance of bactericidal gentamicin concentrations in the cornea for at least 8 h was also shown [60]. In a subsequent study, using an experimental rabbit model for infection by *Pseudomonas keratitis*, it was demonstrated that the same system with different conditions (2.6 mA/cm$^2$ for 1 min) was able to
lower the Pseudomonas count in the cornea after incubation for 24 h (the logarithmic values of Pseudomonas colony forming units (CFUs) were 2.96 ± 0.45 and 6.29 ± 0.45, respectively, for the iontophoretic and the control groups, the latter receiving eye drops of 1.4% gentamicin every hour for 8 h [61]. This applicator system was also tested for the delivery of dexamethasone phosphate for the treatment of anterior segment inflammation. Single treatment for 1 min at 5.1 mA/cm$^2$ resulted in a 30-fold increase in drug deposition in the cornea as compared to conventional eye drops (instillation every 5 min for 1 h) [62].

Fungal infections can be another cause of infective keratitis. Fungicidal ketoconazole concentrations were reached in rabbit corneas (27.6 µg/ml) following transcorneal iontophoresis (21.2 mA/cm$^2$ for 15 min) and in aqueous humor (1.4 µg/ml) 1 h after treatment [63]. Despite the very high current density and the relatively long treatment time of the study, the idea was uptaken and the iontophoresis of miconazole was used to treat a 51 year old patient with complex fungal infection using the EyeGate$^\circledR$ Coulomb-controlled iontophoresis system with transcorneal applicator at 2 mA/cm$^2$ for 4 min [64]. Following the treatment, a penetrating keratoplasty was performed and the corneal tissue was incubated over 3 weeks showing negative results for viable fungus and thereby proving that a sufficient amount of miconazole was delivered to control the infection.

Anti-sense oligonucleotides (ODNs) have also been delivered by iontophoresis in proof-of-concept studies with rabbit [65] and rat corneas [66, 67] as well as through “direct ocular iontophoresis” with trans-palpebral iontophoresis on new born mice [68]. The efficiency of “transcorneoscleral” iontophoresis, with an applicator covering the cornea and adjacent scleral limbus, was evidenced by the downregulation of nitrite expression achieved in endotoxin-induced uveitis in rats following iontophoretic delivery of biologically active anti-NOSII oligonucleotides (1 mA/cm$^2$, 4 min) [66]. In order to track the post-iontophoretic distribution in the cornea, fluorescently labelled siRNA and dextrans of up to 70 kDa were visualized by fluorescent stereomicroscopy in mice following iontophoresis for 1 min at 25 mA/cm$^2$. Even though the current density was extremely high, it was
reported that visual examination did not show any corneal damage [47]. More detailed information is provided by Bejjani et al. [69] in an extensive overview on the gene delivery technique and its possible future applications.

The use of ocular iontophoresis may extend beyond the delivery of therapeutic drugs. Recently, the technique has been used in the reverse sense for the extraction of iron in cases of blood stained cornea. Cathodal iontophoresis of a vitamin C (12.6 mg/ml) solution at 6 mA/cm² for 20 min into the corneal epithelium induced a statistically significant increase in the flux of Fe²⁺ ions from the eye. Vitamin C was presumed to be delivered into the cornea via EM, where it was supposed to mobilize iron from ferritin by reducing Fe³⁺ to Fe²⁺ and thereby making its extraction from corneal tissue easier [70].

7.2 Clinical application: Corneal iontophoresis of riboflavin

Transcorneal iontophoresis has been extensively studied for the delivery of riboflavin (vitamin B, a photosensitizing molecule) used in combination with ultraviolet A irradiation (UVA, 370 nm) for so-called corneal cross-linking; a medical procedure performed in order to stiffen the corneal stroma and thereby slow down or halt the progression of keratoconus and iatrogenic corneal ectasia [71]. Riboflavin is a water soluble, negatively charged, small molecule (MW 376.40 Da) and therefore an optimal candidate for electrically assisted delivery.

Animal studies in Lewis rats comparing iontophoretic application of riboflavin-dextran and riboflavin phosphate (2.11 mA/cm² for 4 or 10 min), with or without epithelial debridement, showed that riboflavin phosphate could indeed be successfully delivered under both conditions. As expected, increasing the application time from 4 to 10 min greatly increased the amounts of riboflavin detected in the cornea and aqueous humor. Riboflavin-dextran, which has a molecular weight of 500 kDa, was only able to penetrate the cornea effectively following epithelial removal [72].

The actual depth profile of riboflavin delivered to corneal interior, intermediate and posterior stroma was investigated in vitro in human cadaver samples [73] (Figure 2). All conditions showed a decrease
in riboflavin concentration with increasing depth. Overall stromal concentrations of riboflavin after 20 or 30 min passive application (classical treatment procedure) were $34.1 \pm 7.1 \mu g/g$ and $7.2 \pm 3.7 \mu g/g$ with and without epithelial debridement, respectively. A single iontophoretic treatment of $\sim 1.8 \text{ mA/cm}^2$ for 5 min using a corneal applicator (Iontofor CXL, SOOFT Italia S.p.A) connected to a power supply (I-ON CXL; SOOFT Italia S.p.A) yielded an overall stromal concentration of $15.0 \pm 5.1 \mu g/g$, superior in the deep stromal layers to the traditional treatment with an intact corneal epithelium but with a much shorter application time [73].

![Figure 2](image)

**Figure 2.** a) Iontophoretic applicator set-up using isolated human cornea *in vitro* (Iontofor CXL, SOOFT Italia S.p.A). b) Stromal slices after incision by femtosecond laser following iontophoretic administration of riboflavin. c) Schematic illustration of thickness of lamellae produced by incision with the femtosecond laser and used for regioselective quantification of riboflavin. Reproduced with permission from [39, 73].

These results show the potential of the technique, as current density and application times can be modulated to achieve required concentrations. UVA irradiation effects on the corneal stroma structure following iontophoretic delivery of riboflavin were studied in isolated human corneas and comparable morphological changes were found to classical cross-linking procedures [39]. The same set-up was tested *in vitro* on entire human eye globes [74] with Scheimpflug images of the corneal backscattering [75] and *in vivo* on rabbit corneas; analogous corneal stiffness to the standard treatment was observed even though riboflavin permeated amounts were almost half of those achieved after epithelium debridement followed by passive delivery [76]. Interestingly, the distribution of riboflavin in isolated rabbit cornea was shown traceable via fluorescence microscopy giving a valuable comparison tool for
the different marketed formulations [77]. In the same frame, it has been shown how the optimization of the protocols by variating application time, riboflavin concentration and iontophoretic parameters could affect the depth deposition [78].

Iontophoresis (1.27 mA/cm², 10 min) was used in 19 patients (22 eyes) in with progressive keratoconus to enable transepithelial corneal impregnation with riboflavin before UVA irradiation (370 nm, 3 mW/cm², 30 min) in a clinical trial into the efficacy of the treatment for inducing collagen cross-linking [79]. For this therapy, the anode with an application area of 6 cm², was applied on the neck of the patient (Figure 3a) whereas the cathodal reservoir filled with 0.1% riboflavin solution (10-12 ml, contact area of 0.785 cm²) was applied to the open eye without anaesthesia (Figure 3b). Patient clinical progression was closely monitored during the following year and a significant corneal flattening and stable visual acuity were observed [79]. More recently, a randomized controlled clinical trial, comparing standard epithelial removal cross-linking treatment to iontophoresis assisted cross-linking, was performed applying the same system on a larger patient population (119 patients, 73 eyes: standard cross-linking protocol and 76 eyes: transepithelial iontophoresis assisted cross-linking). Both techniques showed stabilization/regression of the disease during the follow-up period of 24 months; however, the effectiveness of the iontophoretic treatment was shown inferior to the standard protocol, suggesting a more superficial cross-linking of collagen due to insufficient riboflavin accumulation in the deeper tissue layers [30].

In another clinical study, 14 pediatric patients (mean age 13 years) were treated using the above mentioned I-ON XL; SOOFT (5 mA/5 minutes) iontophoretic system followed by 9 min UVA irradiation 10 mW/cm² and corrected distance visual acuity was observed during the following 15 months [80]. Similar outcomes with the same device were published in 2016 on adult and paediatric patients, showing the feasibility of the system as equivalent alternative to the standard protocol in managing the progression of the disease in 6 months [81] and 12 months follow-up studies [82].
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Figure 3. Follow-up of iontophoretic treatment studied by Bikbova et al. [79]. a) and b) application of anode and cathode, respectively. c) and d) filling of the cathodal compartment with riboflavin 0.1% solution and patient position during treatment. Reproduced with permission from [79].

From these studies, it can be concluded that iontophoresis is able to impregnate the stroma with riboflavin and thereby eliminating the need for epithelial debridement, which corresponds to an outstanding gain in patient quality of life given that it is a much less invasive procedure. However, the depth of drug penetration may need to be increased in order to improve its effect in the deeper collagen tissue.
8. Transscleral Iontophoresis

8.1 Preclinical studies in vitro and in vivo

The aim of transscleral iontophoretic drug delivery is to treat posterior segment diseases. Given the structure of the eye, a complex series of barriers has to be overcome by the drug before it can reach the innermost segments such as the retina and vitreous where most diseases are manifest. The dramatic effect of clearance during transscleral iontophoresis was visualized by MRI on rabbits in vivo and postmortem using the contrast ions: Mn$^{2+}$ and MnEDTA$^{2-}$ [83]. A clear reduction of intraocular permeation was shown in vivo when compared to the postmortem model - highlighting the drawback of in vitro models that overestimate drug delivery because of the lack of dynamic elimination pathways (vasculature and lymphatic clearance), although static barriers remain intact for several hours after sacrifice [83, 84]. In order to better predict intraocular drug transport, computational models have been developed to take into account the drug clearance due to the different dynamic barriers [85]. These methods would be a valid option to estimate the drug diffusion from a post-iontophoretic scleral drug depot.

In the case of transscleral iontophoresis in vivo, the rabbit is the preferred model, despite the anatomical and physiological differences to the human eye (half of the scleral thickness and approximately one quarter of vitreous humor volume compared to humans) [86]. Numerous small molecular weight drugs such as antibiotics (e.g. amikacin [87], cefazolin [88], gentamicin [56] ticarcillin [88]), antifungals [63], antimetabolic (5-fluorouracil [89], methotrexate [90]) and antivirals [91] drugs have been tested with success. Once again, the review by Eljarrat-Binstock and Domb provides a very good summary of some of the cited studies [31].

In the last decade, the use of high molecular weight biopharmaceuticals for the treatment of diseases such as age related macular degeneration and diabetic retinopathies has significantly increased [92, 93]. Several in vitro and in vivo studies have been conducted to investigate the transscleral iontophoretic transport of macromolecules with a view to evaluating the technique as a valid alternative to intraocular...
injection. Anodal iontophoresis of cationic cytochrome c (12.4 kDa) was investigated across porcine sclera and choroid-Bruch’s membrane [94]. The trilayer was chosen to simulate the multiple posterior segment static barriers; indeed, passive permeation of cytochrome c after 5 h was 5- to 7-fold lower than that across isolated sclera. Fluorescent dextrans were used to monitor the contribution of EO to protein electrotransport and showed that cytochrome c delivery was due to EM. Transscleral flux increased with current density showing enhancement factors of 6 and 33.2 for the two iontophoresis conditions tested, 1.5 and 5.8 mA/cm$^2$ (2 h), respectively [94].

The relative contribution of EM and EO is strongly influenced by the molecular weight and resultant charge:mass ratio (which affects the electric mobility). Bovine serum albumin (68 kDa, pI ~ 4.7), is a negatively charged protein at physiological pH with a low charge:mass ratio - in this case anodal and not cathodal iontophoresis across isolated human sclera gave the best results [95]. In contrast, for polystyrene sulfonic acid, which has a similar molecular weight of 67 kDa, but a high charge:mass ratio, cathodal iontophoresis significantly increased transport through sclera, evidencing the superiority of EM [95]. Similarly, cathodal iontophoresis (3 mA/cm$^2$ for 2 h) of negatively charged single stranded oligonucleotides (4-11 kDa), enabled their non-invasive delivery through isolated bovine sclera [96]; however, despite the prolonged application, only a fourfold increase in permeability coefficient was found over the passive control. The biodistribution of negatively charged gadolinium conjugated bovine serum albumin (average molecular weight 80 kDa) by MRI in vivo and postmortem through rabbit sclera showed no permeation into the vitreous under passive or iontophoretic conditions [86]. However, major accumulation was found in the sclera and choroid beneath the applicator system after iontophoresis for 20 min at 2 mA (applicator size was not clearly mentioned) whereas MRI images of the passive application did not show any contrast enhancement.

A proof of concept study in mice in vivo was performed to investigate the possibility of transferring plasmid containing green fluorescent protein (GFP) cDNA as a model for iontophoresis driven gene therapy [97]. Given the fluorescent properties of the protein, actual expression of the transferred gene
could be visualized in the photoreceptor layer after a single iontophoretic application (8.57 mA/cm$^2$ for 15 min); although it should be noted that the current density is a little above the accepted upper limit in humans [36]. Maximal fluorescence was shown in the first 6 days and fluorescence was statistically higher than in untreated control retinas for up to 9 days (Figure 4) [97].

**Figure 4.** Picture of green fluorescent protein expressed in mice retina photoreceptor region (OS) following GFP cDNA plasmid iontophoresis. Reproduced with permission from [97].

As previously discussed, uncharged high molecular weight compounds can be delivered by anodal iontophoresis via EO. For example, transscleral penetration of dextrans was observed through isolated porcine and human sclera [22]. Anodal iontophoresis of fluorescein isothiocyanate (FITC)-bevacizumab (2.5 mg/ml) at 3.8 mA/cm$^2$ for 2 h across excised human sclera *in vitro* resulted in a 7.5-enhancement of flux as compared to passive permeation (37.01 ± 9.37 and 4.92 ± 6.73 µg/cm$^2$ h) [98].

It is important to mention that one of the major limitations of the *in vitro* studies reported here is the long application time (hours), which is unsuitable in clinical practice. Nevertheless, these studies have addressed important issues, that is, the influence of physicochemical properties of the molecules and iontophoretic parameters such as current density, application time on macromolecule penetration and distribution in the vitreous.
8.2 Transscleral iontophoresis of corticosteroids from animal model to clinical trials

Transscleral iontophoresis of corticosteroids has been widely studied since these drugs have multiple uses in the treatment of intraocular inflammatory conditions. This idea was already being explored in 1989 by Lam et al. [99]. In a proof of principle study, it was shown that cathodal iontophoresis of dexamethasone sodium phosphate (415 mA/cm² for 25 min) in rabbits produced an astonishing 7000-fold increase in drug deposition compared to subconjunctival injection. However, the current density used was almost 100-fold greater than the tolerable amount tested in humans (see Table 1) and choriotretinal burns were observed [41].

Since then, dexamethasone phosphate has been the lead molecule in several transscleral iontophoretic studies; its favourable physicochemical properties – two negative charges at physiological pH and high aqueous solubility – make it a good candidate for iontophoresis. It was successfully delivered by transscleral iontophoresis with short application times, on the order of a few minutes, in healthy [62] and endotoxin-induced uveitis animal models [100]. EyeGate Pharmaceuticals Inc. developed an annular transscleral iontophoretic applicator (EyeGate® II delivery system) which is filled with the drug solution and placed on the corneal limbus to avoid any possible current-induced damage to the underlying retina [45]. Efficacy of the system was shown in rabbits following 5 min iontophoresis of dexamethasone phosphate solution (40 mg/ml, EGP-437) at current densities of 2, 4 and 6 mA/cm² [45]. Repeated application (twice weekly over 24 weeks) of the EyeGate® II system filled with EGP-437 solution was investigated in rabbits. Current densities from 3 to 4 mA/cm² applied for 3.5-5 min were shown to be safe. Minor and reversible conjunctival erythema was reported, ascribed to the application of the device with a low negative pressure [21]. Moreover, transient corneal opacity and corneal staining with fluorescein indicator for tissue damage were reversible [21]. Efficacy of the system was further evaluated in clinical trials patients with dry eye [27] and for the treatment of non-infectious uveitis [28]. According to company statements (EyeGate Pharma) clinically relevant improvements were observed. Positive results from a Phase III study were also reported (EyeGate
PHARMA) to show the non-inferiority of the iontophoretic treatment over a 4 week period when compared to the current standard of care (154 drops of prednisolone acetate 1%). In January 2016 a new patient enrolment in a confirmatory Phase III study was initiated.

Transscleral iontophoresis of other corticosteroids has also been investigated; negatively charged methylprednisolone sodium succinate was applied using the EyeGate® applicator in 17 patients with active corneal graft rejection (1.5 mA/cm² for 4 min) [101]. Here, the aim was to induce the lateral diffusion of the corticosteroid into the corneal tissue rather than the posterior segment, and it was shown to be an effective alternative to eye drop instillation. The corneal rejection process was reported to be stopped in 15 out of 17 patients for the mean follow-up period of one year [101]. Intravitreal permeation of methylprednisolone succinate following short duration transscleral cathodal iontophoresis (4 mA/cm² for 2-5 and 15 min) was studied in vitro using porcine sclera [102]. Interestingly, a reduction of iontophoretic transport with increased current was observed. The result was attributed to the high adsorption of the negatively charged drug to the sclera and resultant increase in the EO flow in the opposing anode-to-cathode direction. This finding was consistent with data obtained with dexamethasone phosphate in vivo and highlights the importance of good in vitro models in order to have a better understanding of the iontophoretic transport through ocular tissues [102]. Elijarrat-Binstock et al. studied the cathodal iontophoresis at 2.6 mA/cm² (max. 10 min) of methylprednisolone hemisuccinate in rabbits in vivo, using their in-house hydrogel applicator. Two hours after treatment, the concentrations in the retina, aqueous humor and vitreous were, 178.59 ± 21.63 µg/g, 6.74 ± 2.38 µg/ml, and 2.71 ± 0.57 µg/ml, respectively; significantly, no drug was detected in the same tissues after 2 h following i.v. infusion of 10 mg/kg of methylprednisolone [103].

A common issue for the iontophoretic delivery of corticosteroids is the absence of charge and more importantly their low aqueous solubility; these drawbacks were overcome in the above studies by using the water soluble ester derivatives. Another approach that has been tested is the formulation of drugs in charged polymeric nanoparticles [104], for example negatively charged micelles of egg lecithin and
sodium taurocholate (1:4 molar ratio, 4.4–4.7 nm hydrodynamic diameter) were developed for the transport of dexamethasone. Deposition in human sclera \textit{in vitro} following iontophoretic application at a high current density of 10 mA/cm² for 20 min was higher than that after passive application of the micelles [105]. In a subsequent study, other lipophilic drugs, triamcinolone acetonide and β-estradiol (logP 2.5 and 3.5, respectively), were added to the micellar carrier system [106]. Passive, cathodal and anodal iontophoretic transscleral delivery were evaluated as in the previous study and despite the negative charge of the micellar transport system, anodal iontophoresis was superior to cathodal delivery [106] when compared to the passive delivery indicating that EO was the predominant transport mechanism and suggesting that drug incorporation into charged carriers does not enable EM transport and the use of a charged prodrug, e.g. dexamethasone phosphate might be a better strategy for iontophoresis.

\section{Conclusion}

The feasibility of electrically assisted transcorneal and transscleral drug delivery has been studied \textit{in vitro} and \textit{in vivo}. Both models have their limitations – either the lack of dynamic barriers or the use of animal models such as mice, rats and rabbits with highly different anatomies to the human eye, and the concerns about the ability to extrapolate such data to the human eye. Given the expense and ethical issues inherent to \textit{in vivo} animal testing, preliminary exploratory and mechanistic studies are mostly performed on isolated corneas \textit{in vitro}. In these cases, topical drug deposition can be predicted with greater confidence than transscleral penetration due to the lack of dynamic barriers. Moreover the application times tested are often in the hour range, which is far from realistic for a patient friendly delivery system. Furthermore, the use of excised, isolated tissue can result in overestimation of drug delivery.

\textit{In vivo} delivery data in animals and humans suggest that iontophoresis enables the delivery of therapeutic agents to the anterior or posterior eye segment without the need for any surgical
intervention. Moreover, clinical studies into transcorneal iontophoresis for the administration of riboflavin for corneal crosslinking as well as transscleral iontophoresis of dexamethasone phosphate for the treatment of non-infectious anterior uveitis demonstrate the validity of the technique.

One of the key advantages of ocular iontophoresis treatment is the possibility of a non-invasive short duration treatment performed by healthcare givers which might increase patient compliance. Paediatric and pain intolerant patients could represent potential patients for iontophoretic therapy. Further studies that focus on elucidating iontophoretic transport mechanisms and enable a better understanding of the impact of molecular physicochemical properties and their influence on ocular iontophoretic transport could broaden the range of potential drug candidates and therapeutic possibilities. This could be of particular importance for the eventual development of systems for the ocular iontophoretic delivery of biopharmaceuticals.

10. Expert opinion

Iontophoresis is a delivery technology that has been known for a long time. Pre-filled transdermal iontophoretic patch systems for the delivery of low molecular weight therapeutics have already been approved by the FDA (LidoSite®, Ionsys™ and Zecuity®), demonstrating the safety and potential of the technology. In the last decade the transposition of this technique to other drug delivery routes (e.g. ocular, buccal and ungual) has been the focus of much research. However, the characteristics and properties of an iontophoretic system applied on the skin are substantially different from those expected of an iontophoretic system for ocular delivery. For cutaneous applications the developers seek, among other properties, an ease-of-use that would ideally be similar to a passive transdermal patch and a fully-integrated design and to make it easy to use for the patient. Another challenge is to ensure that the systems are cost-effective. As with all pharmaceutical products, the stability of the drug formulation is obviously crucial and in an iontophoretic delivery system, the stability of the electronic components must also be considered.
For ocular applications, the “user-friendly” concept is less focused on the patient and more on the ophthalmologist, since patients themselves are not expected to have to deal with the device. Ocular iontophoresis is envisaged as a therapy applied in the clinic by a trained health professional. Different models such as a single re-usable power supply could be developed and operated with disposable ocular supports; treatment duration and applied current density could either be modulable or fixed as presets depending on the “intensity” of the treatment. It is evident that clinical studies will determine the tolerable current density and the maximum treatment duration so as to avoid irritation. Hence, for each therapy, it will be necessary to follow an established protocol. Since ocular tissue is a mucous membrane with high water content, the tissue resistance is low and it is capable of supporting high current densities. As mentioned earlier current densities of up to 1.8 mA/cm² for 5 min and 5.5 mA/cm² for 20 min have been safely applied on human cornea and sclera, respectively. Nonetheless, the maximum safe current density still needs to be evaluated and the irritation risk of each molecule under the influence of the electric current needs to determined. For safety reasons, it would be desirable to have built-in safety mechanisms that would stop current flow if the voltage exceeded a threshold value. Another safety concern would be the risk of pH changes in the drug formulation, which can be correlated with treatment duration. Many devices currently in clinical trials, instead of using reversible electrodes such as Ag/AgCl, normally used in research, are integrated with inert electrodes, such as carbon or platinum. In this case, the surface electrochemistry involves the electrolysis of water, which reduces pH medium in the anode and increases it in the cathode. Such pH changes might result in irritation and a significant decrease in delivery due to the generation of hydroxonium or hydroxyl ions. Therefore, the formulations must be buffered even though the addition of these ions may result in competition between charge carriers and a decrease in drug delivery efficiency.

Even with so many technical challenges, ocular iontophoresis has extraordinary clinical potential as it has proven to be as or more effective and less invasive than many procedures already routinely used in clinics, for example. epithelial debridement or intravitreal injection in the cases of riboflavin and
corticosteroid delivery described above. Nevertheless, further long-term studies are obviously necessary to assess the potential risks and the tolerability of frequent/chronic applications before its potential can be fully realized.

11. References

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.


** Clear overview on ocular static and dynamic barriers


** Extensive review on transdermal iontophoresis with detailed description of the transport mechanisms.


* Historic paper bringing the first description of iontophoresis


* One of the first papers investigating the properties of the sclera evidencing differences to skin barrier in the iontophoretic process


* Recent article describing the clinical outcomes of transcorneal iontophoresis for corneal collagen crosslinking


** A less recent but comprehensive review focusing on ocular iontophoresis


* First paper reporting transcorneal iontophoresis


** Very informative review on electrically assisted ocular gene therapy


* Clear overview of the CXL technique and also the use of iontophoresis


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CHAPTER II

- Targeted intracorneal delivery -
  Biodistribution of triamcinolone acetonide following topical iontophoresis of cationic amino acid ester prodrugs
Targeted intracorneal delivery - Biodistribution of triamcinolone acetonide following topical iontophoresis of cationic amino acid ester prodrugs

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Abstract

The aim was to investigate intracorneal iontophoresis of biolabile triamcinolone acetonide (TA) amino acid ester prodrugs (TA-AA). Arginine and lysine esters of TA (TA-Arg and TA-Lys, respectively) were synthesized and characterized; quantification was performed by HPLC-UV and UHPLC–MS/MS. The aqueous solubility of the prodrugs (at pH 5.5) was ~1000-fold greater than TA. Anodal iontophoresis (10 min at 3 mA/cm²) of TA-AA was investigated using isolated porcine cornea. Although no statistically significant difference was observed in total intracorneal delivery of TA (468.25 ± 59.70 and 540.85 ± 79.16 nmol TA/cm², for TA-Arg and TA-Lys, respectively), the different susceptibilities of the prodrugs to hydrolysis influenced intracorneal biodistribution. Quantification of TA in twenty-five 40 mm thick corneal lamellae revealed significantly deeper penetration of TA following TA-Lys iontophoresis. Its superior resistance to hydrolysis enabled sustained electromigration into the deeper cornea suggesting judicious prodrug selection might enable targeted regioselective drug delivery. The intracorneal biodistribution following anodal iontophoresis of TA-Arg (2.3 mM; 10 min, 3 mA/cm²) was visualized by full field optical
coherence tomography providing qualitative confirmation of the extensive intracorneal penetration of TA.

Short duration iontophoresis of TA-AA prodrugs may improve deep corneal bioavailability and efficacy in vivo, constituting a “single-shot” treatment option for corneal allograft rejection.

**Keywords:** corneal graft rejection, triamcinolone acetonide, prodrug, iontophoresis, intracorneal biodistribution, full field optical coherence tomography
CHAPTER II: Targeted intracorneal delivery -
Biodistribution of triamcinolone acetonide following topical iontophoresis of cationic amino acid ester prodrugs

1. Introduction

Corneal graft allotransplantations are the most frequently performed organ transplantation [1, 2]; their success rate is attributed to the low expression of histocompatibility antigens as compared to other organs [3, 4]. However, trauma or viral infection can induce corneal neovascularization [5], which significantly increases the risk of corneal graft failure, with a decade survival rate of approximately 60% [6-8]. Most corneal graft rejections are caused by a T-cell mediated immune response against the major histocompatibility complex antigens of the donor tissue [9]. Leucocyte infiltration and neovascularization were shown to be especially severe when deeper corneal tissues such as stroma and endothelium were involved, leading to graft failure [10]. Topical corticosteroid administration is the standard treatment for the prevention of corneal graft rejection [6]. However, lacrimal drainage and absorption by periocular tissues constitute dynamic barriers that decrease ocular bioavailability and their impact is complemented by the properties of the corneal epithelium, which is a very effective static barrier to molecular transport [11, 12]. Their combined effect means that treatment regimens necessitate hourly drug instillation over several days and this can result in poor patient compliance [13]. Systemic administration of corticosteroids imposes the use of high dosages with the concomitant risk of severe off-target side effects.

Trianclinolone acetonide (TA) is routinely administered by intravitreal injection for the treatment of intraocular inflammatory diseases[14] and sub-conjunctival injections have been employed to suppress corneal graft rejection [15, 16]. However, due to its low aqueous solubility (12 µg/ml[17]) the preparation of an injectable solution remains a challenge; indeed, the available TA formulations are usually drug suspensions, e.g. Kenacort A 40 (Bristol-Myers Squibb AG; Baar, Switzerland). Similarly, topical formulations such as opthalmic ointments also contain TA as a suspension, e.g. Cidermex (UCB Pharma S.A.; Colombes, France) a previously commercialized TA and neomycin combination product. Given the formulation issues, poor bioavailability and the sheer inconvenience of using drug suspensions in the eye, it was decided to develop a series of water soluble biolabile amino acid prodrugs of TA (TA-AA) in order
to facilitate formulation and to enable the use of short duration iontophoresis to improve intracorneal bioavailability of TA.

Iontophoresis is a noninvasive drug delivery technique based on the application of a mild electric potential gradient across a biological barrier, which drives ionized molecules into the membrane [18]. The combined effects of the concentration and potential gradients result in increased molecular transport and hence improve bioavailability, as compared to “simple” passive administration which relies on diffusion along a concentration gradient [19].

At steady-state, the total flux ($J_{TOT}$) can be expressed as the sum of the passive and “active” contributions (namely, electroosmosis (EO) and electromigration (EM)):

$$J_{TOT} = J_p + J_{EO} + J_{EM} = \left[ k_{p,x} + V_w + \left( \frac{i_d}{z_x F} \right) \times \frac{u_x}{\sum_i u_i c_i} \right] \times c_x = \left[ k_{p,x} + V_w \right] c_x + \frac{i_d t_x}{z_x F}$$

Equation I

where $i_d$ is the applied current density, and, $u_i$ and $c_i$ refer to the mobility and concentration of the ions carrying charge across the membrane and $u_x$, $z_x$, $c_x$ and $t_x$ are the mobility, valence, concentration and transport number of species $x$ and $k_{p,x}$ is its permeability coefficient; $F$ is Faraday’s constant and $V_w$ is the linear velocity of solvent flow. Neutral molecules are transported by passive diffusion and via EO; in addition to these two transport processes, cations and anions also benefit from EM which is a far more efficient mechanism and results in superior delivery.

Iontophoresis has been extensively studied for the delivery of water soluble ionizable species into and across the skin for local or systemic action. Given its ability to control drug delivery kinetics and its noninvasiveness, it has also drawn increasing interest in the field of ocular delivery. For example, corticosteroids have been shown to be successfully delivered into the anterior and posterior segments of the eye, using transcorneal and transscleral iontophoresis [20]. Indeed, transscleral iontophoresis of methylprednisolone sodium succinate has been proposed as an alternative treatment for corneal graft
rejection using the annular EyeGate® applicator [21]. Clinical response was seen after 24 h in 10 out of 18 patients although the applicator was not directly placed on the cornea. Lateral diffusion from the scleral limbus into the corneal tissue might be the main driving force for drug penetration.

Given its poor aqueous solubility and the lack of ionizable moieties at physiological pH, TA is a poor candidate for iontophoretic delivery and there are no water-soluble derivatives available. To overcome this, a prodrug approach similar to that used to improve topical iontophoretic delivery of aciclovir, was adopted [22]. It was previously shown that anodal iontophoresis of positively charged amino acid ester prodrugs of aciclovir could be used to increase the bioavailability of aciclovir in the skin – and more specifically, in the basal epidermis, where the virus resides [23]. Hydrolysis of the prodrug generated a non-toxic amino acid side by-product and the active drug moiety was released in the epidermis/dermis by the activity of endogenous enzymes [24].

The chemical modification of antiviral agents to produce amino acid ester prodrugs has previously been shown to facilitate topical corneal penetration via active transport since the prodrugs were substrates of amino acid transporters [25]. The present study focused on a very different type of “active transport” since the delivery of the cationic prodrugs was facilitated by electrotransport upon application of an electrical potential rather than via transporter proteins.

The goals of this study were (i) to synthesize hydrosoluble, biolabile amino acid prodrugs of TA (TA-AA), (ii) to characterize the prodrugs and to evaluate their stability in contact with the cornea and (iii) to investigate the ability of short duration topical iontophoresis to enhance intracorneal TA bioavailability. Iontophoresis with cationic corticosteroid prodrugs would have the advantage of benefiting from both electromigration and electroosmotic transport mechanisms at the diseased tissue (in contrast to anionic phosphate or succinate analogues). Interestingly, TA has also been reported to be a contrast agent in optical coherence tomography (OCT), which is a frequently used technique in ophthalmology [26, 27]. OCT enables the precise visualization of the multilayered corneal epithelium, the collagenous stroma and
endothelium because of the relatively low intrinsic light scattering properties of the tissue [28]. Therefore, in the last part of the study the post-iontophoretic intracorneal biodistribution of TA as a function of corneal depth was visualized by using full field optical coherence tomography (FFOCT) and correlated with the amounts quantified by UHPLC-MS/MS [29].

2. Materials and methods

2.1 Materials.

TA and liquid paraffin were purchased from Haenseler AG (Herisau, Switzerland), N-Boc protected L-arginine and L-lysine (Boc-Arg(Boc)₂-OH and Boc-Lys(Boc)-OH DCHA salts) were sourced from Bachem (Bubendorf, Switzerland). 4-dimethylaminopyridine (4-DMAP), dry dichloromethane (DCM), sodium and potassium chloride, sodium and potassium phosphate were supplied by Sigma-Aldrich (Steinheim, Germany) and N,N’-dicyclohexylcarbodiimide (DCC) and 2-morpholino-ethanesulfonic acid monohydrate (MES) were purchased from Fluka (Buchs, Switzerland). Trifluoroacetic acid (TFA; 99 % extra pure) and glycerol were obtained from Acros Organics (Geel, Belgium). ULC/MS grade formic acid was bought from Brunschwig (Basel, Switzerland). PVC tubing (ID 3.17 mm; OD 4.97mm) used for the saline bridges and O.C.T. mounting medium (PVA, polyvinyl alcohol) were provided by VWR International AG (Nyon, Switzerland). Silver wire and silver chloride (AgCl) for the fabrication of electrodes were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents (acetonitrile (ACN), ethyl acetate (EtOAc), hexane (HEX) and methanol (MeOH)) were HPLC grade (HiPerSolv Chromatonom; Darmstadt, Germany). Deionized water was used to prepare all solutions (Millipore Milli-Q Gard 1 Purification Pack resistivity > 18 MΩ.cm; Zug, Switzerland). Hematoxylin, eosin and Hoechst 33342 used for the histological evaluation of the corneal tissue were bought from Thermo Fisher Scientific (Reinach, Switzerland).

2.2 Synthesis of TA-AA prodrugs.

The TA-AA prodrugs were prepared using a two-step synthesis adapted from the literature (Figure 1) [22, 30]. The first step involved a 4-DMAP catalysed coupling reaction of the N-Boc-protected amino acid...
(arginine or lysine) and TA 21-C primary alcohol. In the second step, N-Boc deprotection was performed by the addition of TFA. TA-AA prodrugs were subsequently recovered by precipitation in diethyl ether. Complete reaction details can be found in the Supplementary material.

![Synthesis of TA-Arg and TA-Lys prodrugs](image)

**Figure 1. Synthesis of TA-Arg and TA-Lys prodrugs**

Final product characterization was performed by high-resolution mass analysis in positive ionization mode with a QSTAR Pulsar (AB/MDS Sciex; Framingham, USA) and by $^1$H and $^{13}$C nuclear magnetic resonance (NMR) analysis at 300 MHz on a Varian GEMINI 300 BB (Varian; Palo Alto, USA). Interpretation of NMR spectra was achieved using MestReNova 8.1.2 software (see Supplementary material). The overall reaction yields were 83% and 85% for TA-Arg and TA-Lys, respectively. Purity of the two TA-AA prodrugs was analyzed by HPLC with UV detection (HPLC-UV; see below) and was superior to 98% for all batches.
2.3 Analytical methods.

2.3.1 Quantification of TA and TA-AA prodrugs by HPLC-UV.

Simultaneous analysis of TA-AA prodrugs and TA was performed using an UltiMate 3000 HPLC-UV system (formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach Switzerland) comprising a HPG-3200SD pump, WPS-3000 auto sampler and VWD-3400 variable wavelength VIS/UV detector. Data collection and integration were achieved using Chromeleon® (version 6.8) software. A reverse phase column LiChrospher 100 RP18 (5 µm particle size; 150 mm × 4.0 mm; BGB Analytik AG; Boeckten, Switzerland) was used for an isocratic separation at a flow rate of 0.8 ml/min and 30°C. The mobile phase comprised 65% water + 0.1% TFA and 35% ACN + 0.1% TFA. Detection was achieved at a wavelength of 240 nm and the injection volume was 50 µl. Retention times, LOD and LOQ for TA, TA-Arg and TA-Lys were: TA 8.3 min, 0.84 nmol/ml and 2.56 nmol/ml; TA-Arg 3.3 min, 0.84 nmol/ml and 2.56 nmol/ml; TA-Lys 2.9 min, 0.75 nmol/ml and 2.27 nmol/ml, respectively. Further details regarding the method validation in accordance with ICH guideline Q2 (R1)[31] can be found in the Supplementary material.

2.3.2 Quantification of TA by UHPLC-MS/MS.

The biodistribution study, i.e. drug quantification in individual 40 µm thick corneal lamellae, required the development of a very sensitive UHPLC-MS/MS method. The Waters Acquity® UPLC® system (Baden-Dättwil, Switzerland) included a binary solvent pump, sample manager and Waters XEVO® TQ-MS detector (Baden-Dättwil, Switzerland). Isocratic separation of corneal extract matrix components and TA was carried out using a Waters XBridge® BEH C18 (50 x 2.1 mm, 2.5 µm) reverse phase column. The column was thermostatted at 45°C and the mobile phase comprised a mixture of ACN and ultrapure water (50:50 v/v) both containing 0.1% formic acid. A flow rate of 0.3 ml/min and an injection volume of 5 µl were used. Mass spectrometric detection was performed by electrospray ionization in positive ion mode (ESI⁺) using multiple reaction monitoring (MRM). The parent ion [M+H⁺] was selected at 435.29 m/z and product ion was chosen at 415.17 m/z following fragmentation and the liberation of HF from the B ring.
Cone and capillary voltages of 35 V and 0.50 kV, respectively were employed; the source temperature was kept at 350°C with a desolvation gas flow of 650 L/h. The collision energy was set at 20 eV and the collision gas flow at 0.15 ml/min. TA retention time was 0.81 min and the LOD and LOQ were 3.5 and 11 pmol/ml, respectively. Additional information regarding precision and accuracy can be found in the Supplementary data.

2.4 Prodrug characterization and stability.

2.4.1 Physicochemical characterization.

The pKa, LogP and LogD of TA and TA-AA prodrugs were predicted by using ACD/Labs software (version 12.01). The aqueous solubility of TA-Arg and TA-Lys was measured experimentally by the addition of excess prodrug to 100 mM MES buffer (pH 5.5) or phosphate-buffered saline (PBS) (pH 7.4), followed by 10 min sonication in an ultrasonic bath (Branson 5510, Branson Ultrasoundics, Danbury, USA) and centrifugation for 15 min at 10000 rpm (Eppendorf Centrifuge 5804, Hamburg, Germany). The supernatant was analyzed by HPLC-UV and the amount of solubilized TA-AA prodrug quantified. This was performed in triplicate. It should be noted that saturation was maintained for a period of only 10 min because of the hydrolysis of the ester linkage in aqueous solution.

2.4.2 Effect of pH on stability.

The effect of pH on TA-AA prodrug ester bond hydrolysis was investigated over 8 h by dissolving TA-Arg and TA-Lys (2.3x10⁻² mM) in pH 5.5 MES buffer (100 mM) or pH 7.4 PBS (100 mM). Samples were withdrawn hourly for HPLC-UV analysis. All experiments were performed in triplicate at 37°C. The first order rate constant of hydrolysis (k<sub>obs</sub>) was deduced from the slope of the regression curve and the half-life (t<sub>1/2</sub>) was calculated (t<sub>1/2</sub>=ln2/k<sub>obs</sub>).
2.4.3 **TA-AA stability in contact with corneal tissue.**

The rate of hydrolysis of the TA-AA prodrugs following exposure to freshly enucleated porcine cornea was evaluated. Isolated porcine corneas were cut into small pieces and introduced into 2 ml PBS (100 mM, pH 7.4) under continuous stirring at 37°C. TA-AA prodrugs (2.3x10^{-2} mM) were added to the mixture. Aliquots were withdrawn at 30 min intervals for HPLC-UV analysis.

2.5 **Ex vivo corneal deposition studies of TA and TA-AA prodrugs.**

2.5.1 **Ocular tissue source.**

Porcine eyes are considered as good models for the human organ because of the size and anatomic similarity [33-36]. Moreover the porcine cornea was recently shown to be a useful surrogate for its more difficult to obtain human analogue, in terms of histology and permeability [37]. Eye globes from adult animals (80-100 kg) were obtained from a local slaughterhouse (Abattoir de Loëx Sarl; Loëx, Switzerland) promptly after sacrifice. The surrounding muscle tissue was removed with a scalpel from the eye bulb which was then used within a few hours of harvesting. Corneal tissue viability over 6 h has been shown previously [38].

2.5.2 **Deposition studies on isolated cornea following passive and iontophoretic delivery.**

In order to compare delivery of TA and the TA-AA prodrugs into the cornea following passive and iontophoretic application, corneal tissue was harvested by excision along the scleral-limbus. The posterior segment was discarded and isolated cornea recovered by gently removing the iris-ciliary body and crystalline using forceps. The isolated cornea was clamped into vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland), with the epithelium facing the donor compartment (permeation area = 0.8 cm²). The donor chamber was filled with 1 ml of TA formulations or TA-AA prodrug solutions (conditions summarized in Table 1). The receiver compartment was filled with PBS (pH 7.4; 12 ml) and kept under continuous stirring. For iontophoretic experiments, the formulation compartment was connected
to the anodal compartment containing a silver anode via a saline bridge (3% agarose in 0.1 M NaCl). The cathode (AgCl) was placed directly into the sampling arm of the receiver compartment. A constant current was applied by a power generator (APH 1000M, Kepco Inc; Flushing NY, USA). At the end of the experiment, a 1 ml aliquot was withdrawn from the receiver compartment. The diffusion cells were dismantled, and the residual formulation was carefully removed from the corneal surface with a PBS impregnated cotton swab. Tissue samples were subsequently cut into small pieces and extracted for 12 h in 2 ml of MeOH:water (50:50) mixture at room temperature. The extraction procedure was validated, and the recovery efficiency of TA was above 95% (see Supplementary data). All samples were centrifuged for 15 min at 10000 rpm and the supernatant analyzed by HPLC-UV.

Table 1. Experimental conditions tested using isolated porcine cornea in vitro.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Passive</th>
<th>Delivery conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3 mM TA ointment in 6,1% liquid paraffin + q.s. vaseline</td>
<td>10 min</td>
<td>-</td>
</tr>
<tr>
<td>TA saturated solution in10 mM MES buffer + 2.2% glycerine, pH 5.7</td>
<td>10 min</td>
<td>-</td>
</tr>
<tr>
<td>2.3 mM TA-Arg in 10 mM MES buffer + 2.2% glycerine, pH 5.7</td>
<td>10 min</td>
<td>3 mA/cm² for 10 min</td>
</tr>
<tr>
<td>2.3 mM TA-Lys in 10 mM MES buffer + 2.2% glycerine, pH 5.7</td>
<td>10 min</td>
<td>0.5, 1.5, 3 mA/cm² for 5 min</td>
</tr>
</tbody>
</table>

2.5.3 Intracorneal TA biodistribution profile.

The intracorneal deposition profiles of TA were analyzed following passive and iontophoretic application of TA-Arg (Table 1). The intracorneal biodistribution of TA was also studied after iontophoresis of TA-Lys at i) 3 mA/cm² for 10 min and ii) 0.5 mA/cm² for 5 min. Once the experiments were terminated, the treated corneal disks were isolated and snap-frozen in isopentane (freezing point -160°C) cooled with liquid
nitrogen for the horizontal slicing procedure. The tissue samples were mounted on a cryotome (Thermo Scientific CryoStar NX70; Walldorf, Germany) sample holder with O.C.T. mounting media exposing the epithelial surface. Twenty-five 40 µm thick lamellae were cut down to a 1000 µm depth, i.e. into the corneal stroma. TA deposited in the cornea was extracted overnight from each individual lamella using 1 ml of MeOH:water (50:50) mixture and analyzed either by HPLC-UV or UHPLC-MS/MS, depending on drug concentration.

2.6 Full field optical coherence tomography imaging of intracorneal TA biodistribution.

2.6.1 Corneal iontophoresis with whole porcine eye globes

In contrast to the preceding experiments, where excised tissue and standard Franz diffusion cells were used, the porcine eye globe was kept intact for the visualization of intracorneal TA distribution with a full field optical coherence tomography (FFOCT) microscope (Light-CT Scanner; LL-Tech, Paris, France). This procedure allowed the preservation of the shape and intraocular pressure to better mimic the conditions in vivo. Custom made vertical diffusion cells (Glass Technology; Geneva, Switzerland) similar to those described by Pescina et al.[39] able to hold entire porcine eye bulbs, were employed (see Supplementary data). The donor compartment with surface area of 0.8 cm² was fixed with a slight pressure on the cornea, whereas the lower part of the system was filled with PBS (pH 7.4, 30 ml). An Ag anode was placed in contact with the donor compartment (2.3 mM TA-Arg in 10 mM MES + 2.2 % glycerine, pH 5.7) through a saline bridge and the return electrode was inserted into the receiver compartment. A constant current of 3 mA/cm² was applied for 10 min either with or without a subsequent 60 min period of passive diffusion. Passive control experiments were conducted in the absence of current, keeping TA-Arg solution in contact with the cornea for 10 min. Histological sections of the corneal epithelium after iontophoresis (3 mA/cm², 10 min) were compared with those obtained after 10 min passive application of buffer solution (10 mM MES + 2.2 % glycerine, pH 5.7) using the above-mentioned set-up. FFOCT images were also compared to those from two other conventional microscopic techniques. Fresh porcine corneal tissue was stained either
with (i) haematoxylin and eosin for visualization under a light microscope (Eclipse 80i, Nikon, Japan) or (ii) Hoechst 33342 (0.25 µg/ml, 30 min, RT) for visualization with the confocal laser scanning microscope (CLSM) (LSM 710, Zeiss, Germany).

2.6.2 **FFOCT sample preparation and imaging.**

Corneal cross-sections were analyzed by FFOCT upon completion of the penetration experiments. The donor compartment was removed, and the formulation cleaned as described above. Subsequently, the anterior side of the eye globe was snap-frozen through immersion in isopentane cooled with liquid nitrogen for 30 s, in order to stop further drug diffusion. Horizontal cross-sections of the entire cornea were taken and placed with the sliced area on top in the Light-CT scanner holder for inspection. TA intracorneal biodistribution was visualized by FFOCT given its well-known brightness enhancing properties [26, 27]. In order to compare the TA-related luminosity and the intrinsic tissue signal, the untreated surrounding cornea, which was not in contact with the donor compartment, was used as the “internal standard”.

2.6.3 **Image processing.**

The images were observed using Light-CT viewer software (version 64) and processed by ImageJ1.47v software. For semi-quantitative analysis of corneal epithelium brightness patterns after passive or iontophoretic application, “optical slices” of 50 x1000 µm were selected with the rectangular tool and integrated for intensity calculation by ImageJ 1.47v software.

2.7 **Data analysis.**

All data are expressed as mean ± standard deviation. Statistical significance of the data was evaluated either by analysis of the variance (ANOVA) or Student’s t-test with the level of significance fixed at α= 0.05.
CHAPTER II: Targeted intracorneal delivery -
Biodistribution of triamcinolone acetonide following topical iontophoresis of cationic amino acid ester prodrugs

3. RESULTS AND DISCUSSION

3.1 Physicochemical properties of TA-AA prodrugs.

The predicted pKa and LogP/LogD values of TA and TA-AA prodrugs, as well as the experimentally determined aqueous solubilities are shown in Table 2. Although TA is neutral at both acidic and physiological pH, the amino acid moieties introduced ionizable groups with predicted pKa values of 7.08, 13.36 for TA-Arg and 6.90, 10.45 for TA-Lys. Consequently, at pH 5.5 the two TA-AA prodrugs were ~90% present as di-cations, whereas at pH 7.4 they were predominantly mono-cationic. The ionized amino groups in the TA-AA prodrugs significantly influenced LogD. In comparison with native TA (Log D = 2.43) Log D values for both TA-AA prodrugs were decreased with LogD_{pH 5.5} of -2.29 and -1.85 and LogD_{pH 7.4} of -1 and -0.81 for TA-Arg and TA-Lys, respectively. Given this, the aqueous solubility was evaluated experimentally only for the TA-AA prodrugs, whereas the solubility of the parent molecule was taken from literature [17]. The aqueous solubilities at pH 5.5 of TA-Arg (25.61 ± 1.23 µmol/ml) and TA-Lys (29.12 ± 1.59 µmol/ml), were ~928- and ~1055-fold greater than the solubility reported for TA (2.76 x 10^{-2} µmol/ml). This was attributed to the newly introduced positively charged amino groups that increased the proton exchange mediated solvation in water [40].

3.2 TA-AA stability.

The chemical hydrolysis of both prodrugs at 37°C was investigated in buffer solutions at pH 5.5 (pH of currently used eye drop formulations) and pH 7.4 (Table 2). An accelerated conversion was observed at pH 7.4; \( t_{1/2}^{pH 7.4} \) was 1.36 ± 0.09 h and 3.41 ± 0.34 h whereas \( t_{1/2}^{pH 5.5} \) was 10.21 ± 0.51 h and 39.21 ± 2.68 h for TA-Arg and TA-Lys, respectively.
Table 2. Experimental and predicted physicochemical properties of TA-AA prodrugs (corresponding values for TA are given for comparison).

<table>
<thead>
<tr>
<th>Property</th>
<th>TA</th>
<th>TA-Arg</th>
<th>TA-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKa&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.87, 13.15</td>
<td>7.08, 13.36</td>
<td>6.90, 10.45</td>
</tr>
<tr>
<td>Log P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43 ± 0.67</td>
<td>1.15 ± 0.80</td>
<td>2.14 ±0.74</td>
</tr>
<tr>
<td>Log D pH 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43</td>
<td>-2.29</td>
<td>-1.85</td>
</tr>
<tr>
<td>Log D pH 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.43</td>
<td>-1</td>
<td>-0.81</td>
</tr>
<tr>
<td>Solubility&lt;sup&gt;b&lt;/sup&gt; (µmol/ml)</td>
<td>(MES, pH 5.5) 25.61 ± 1.23</td>
<td>29.12 ± 1.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(PBS, pH 7.4) 2.76 x10² [17]</td>
<td>1.75 ± 0.09</td>
<td>2.40 ± 0.10</td>
</tr>
<tr>
<td>pH stability (pH 5.5)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>% TA-AA t=8 h 58.72 ± 0.36</td>
<td>86.84 ± 0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k&lt;sub&gt;obs&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;) 0.0680 ± 0.0035</td>
<td>0.0177 ± 0.0012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h) 10.21 ± 0.51</td>
<td>39.21 ± 2.68</td>
<td></td>
</tr>
<tr>
<td>pH stability (pH 7.4)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>% TA-AA t=8 h 0</td>
<td>13.00 ± 0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k&lt;sub&gt;obs&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;) 0.5100 ± 0.0365</td>
<td>0.2046 ± 0.0205</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h) 1.36 ± 0.09</td>
<td>3.41 ± 0.34</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ACD/Labs version 12.01  
<sup>b</sup> Experimental  
<sup>c</sup> Prodrug fractions (%) recovered at t = 8 h at pH 5.5 and 7.4, hydrolysis rate constant k<sub>obs</sub> (h<sup>-1</sup>) and half-life t<sub>1/2</sub> (h). (Mean ± SD, n=3)

The more rapid hydrolysis of TA-Arg was attributed to the formation of a catalytic intramolecular hydrogen bond between the terminal guanidine moiety of the arginine side-chain and the carbonyl bond of the ester group (Figure 2). This polarizes the carbonyl bond further and so enhances the electrophilicity of the carbonyl carbon making it more susceptible to nucleophilic attack. Since at pH 5.5, TA-Arg and TA-Lys had half-lives of ~10 h and ~39 h, respectively, the stability was considered more than sufficient for short duration iontophoresis (5-10 min). The TA-AA prodrug solutions used for the in vitro experiments with porcine cornea were prepared in 10 mM MES buffer at pH 5.7. The pH was chosen to ensure full buffer capacity (buffer range 5.5-6.7, Sigma-Aldrich) and because of the prevalent di-cationic state of the TA-AA prodrugs, which would favour electromigration. The addition of glycerine (2.2 %, w/w) to the final formulation allowed a suitable osmolarity for an ophthalmic application to be maintained [41].
Figure 2. (a) TA-Arg side chain with intramolecular hydrogen bond formation and nucleophilic attack at the electron-deficient carbonyl carbon. (b) TA-Lys side chain is unable to adopt the “pseudo-six membered ring” conformation and so form the hydrogen bond.

The aim of the prodrug synthesis was to obtain biolabile TA-AA prodrugs able to release the active principle in situ in the target tissue; therefore, it was important to determine the rate of bioconversion of the two TA-AA prodrugs in contact with corneal tissue. An increased hydrolysis rate was observed when compared to pure buffer, with $t_{1/2}$ of 18.03 ± 9.86 min and 105.03 ± 9.34 min for TA-Arg and TA-Lys, respectively. Thus, TA-Arg was again found to be the more labile TA-AA prodrug. The increased rate of conversion ($k_{obs}$: 2.83 ± 1.51 h$^{-1}$ for TA-Arg and 0.30 ± 0.16 h$^{-1}$ for TA-Lys) was attributed to enzymatic hydrolysis of the prodrugs by endogenous esterases such as carboxylesterases[42] or cholinesterases which were reported to be present in ocular tissue (data reported from rabbits) [43]. The enzyme abundance was reported to be two-fold higher in the corneal epithelium than in the stroma; therefore, prodrug conversion was expected to take place in the upper layers of the cornea, followed by diffusion of the liberated drug into deeper tissue [44]. Since no esterase was reported in lacrimal fluid, TA-AA prodrugs applied to the eye surface should not be subject to enzymatic degradation in vivo prior to corneal absorption [45].
3.3 Intracorneal deposition of TA and biodistribution profile.

3.3.1 Comparison of TA-Arg and TA-Lys iontophoretic delivery and biodistribution.

There was no statistically significant difference between the total amount of TA delivered following corneal iontophoresis of the two TA-AA prodrugs at 3 mA/cm² for 10 min (468.25 ± 59.70 and 540.85 ± 79.16 nmolTA/cm², for TA-Arg and TA-Lys, respectively). Moreover, the amounts were significantly greater than those observed in the passive “control” experiments, where the same solutions were applied to the cornea surface but in the absence of current (27.15 ± 0.47 and 18.15 ± 1.84 nmolTA/cm², for TA-Arg and TA-Lys, respectively) or TA ointment and TA saturated solution (0.13 ± 0.06 and 0.66 ± 0.24 nmolTA/cm², respectively). Thus, the amounts of TA present in the cornea after iontophoresis of TA-AA prodrugs were >3600-fold greater than after passive application of the TA ointment. In the case of TA-Lys a small amount of TA (0.39 ± 0.21 nmolTA/cm²) was also detected in the receiver compartment following iontophoretic application. Although this was very small compared to the amounts deposited in the cornea, it suggested that perhaps TA-Lys iontophoresis could result in deeper drug penetration into the cornea.

In order to test this hypothesis, the intracorneal biodistribution of TA following iontophoresis of TA-Arg and TA-Lys was investigated. TA deposition was quantified in 25 corneal lamellae, each with a thickness of 40 µm going from the corneal surface to a depth of ~1 mm into the tissue (Figure 3a). Similar TA biodistribution profiles were obtained for both prodrugs to a depth of 600 µm – there was significant TA accumulation in the upper cornea, i.e. the epithelium and upper stoma. However, below 600 µm, there was ~6-fold greater TA deposition following TA-Lys iontophoresis. Thus, the hypothesized deeper penetration of TA-Lys was indeed confirmed.

The different extent of deep corneal penetration observed was attributed to the difference in susceptibility to hydrolysis of TA-Arg and TA-Lys and how this impacted on their ability to exploit electromigration as an electrotransport mechanism. It would appear that entry of the TA-AA into the cornea is determined by their electrotransport properties and more specifically by the transport number – as predicted by theory.
The transport number (\(t_x\)) represents the ability of an ion to carry current as compared to the other ions in the system (Figure 3b). In this regard, TA-Arg and TA-Lys are effectively equivalent (\(t_{TA-Arg} \approx 0.026\) and \(t_{TA-Lys} \approx 0.029\)) and this is evidenced by the statistically equivalent total amounts of TA recovered from the cornea. However, once in the cornea, the ability of the two prodrugs to reach the deeper layers is determined by their susceptibility to hydrolysis – once the amino acid moiety is cleaved, the remaining TA is uncharged and cannot benefit from electromigration. Given the half-lives of \(\sim 18\) min for TA-Arg and \(\sim 105\) min for TA-Lys observed upon contact with excised corneal tissue, it is clear that the superior stability of TA-Lys ensures that it is much better equipped to penetrate down to the deep cornea.

Figure 3. (a) Intracorneal biodistribution of TA following iontophoresis of TA-Arg and TA-Lys at 3 mA/cm\(^2\) for 10 min, (Mean ± SD, n=5). (b) Schematic illustration of the influence of bioconversion during iontophoretic transport in the corneal tissue taking into consideration the rate of hydrolysis of the ester prodrugs. TA-Arg is more readily hydrolysed and the cleavage of the Arg moiety removes its ability to exploit electromigration as an electrotransport mechanism.

In contrast, the more rapidly hydrolysed TA-Arg, releases TA in more superficial corneal layers, after which it can only benefit from electroosmosis which is a much less effective electrotransport mechanism.

Therefore, in this case, TA biodistribution was confined to the upper layers of the cornea. This differential
prodrug stability during iontophoresis might open the possibility of targeting a specific tissue layer. Rapidly hydrolysed prodrugs might be advantageous for targeting superficial conditions in the upper layers of the cornea (e.g. epithelium and upper stroma), whereas deeper layers (e.g. deep stroma and endothelium) might be reached by employing more stable analogues (Figure 3b). The effect of metabolism on passive drug diffusion through biological membranes with concurrent metabolism following topical administration has drawn much attention for percutaneous delivery [46-50] and analogies can be drawn for ocular delivery. It is clear that as the residence time of the prodrug in the membrane increases and exceeds the half-life it will be subject to greater metabolism – this will depend on the relative kinetics of the metabolic and transport processes.

3.3.2 Using iontophoretic current density to modulate intracorneal delivery of TA.

Current densities of 0.5, 1.5 and 3 mA/cm² reported as safe from previous studies in humans[51] and animals[52-54] were applied for 5 min and resulted in TA deposition in isolated porcine cornea of 44.84 ± 10.67, 132.25 ± 32.56 to 290.42 ± 40.41 nmol TA/cm², respectively. Thus, there was a linear relationship between TA deposition and the applied current density ($TA_{dep} = 98.80i_d - 8.83, R^2 = 0.9975, p< 0.05$) (Figure 4a). This provides a convenient means to control the amount of TA delivered to the tissue.

TA intracorneal biodistribution following the mildest iontophoretic condition (0.5 mA/cm² for 5 min) was also investigated (Figure 4b) and at each corneal layer in the stroma the TA deposition was found to be superior to the reported IC₅₀ of the drug (0.7-1 nM) for the inhibition of nuclear factor kappa B and activator protein-1[55], indicating the presence of therapeutically relevant drug levels at the target site. As an example, in the deepest corneal lamella, the TA tissue concentration (0.095 mM) was ~10⁵-fold superior to the reported TA IC₅₀.

The therapeutically relevant TA tissue concentrations point to the feasibility of using short duration, low current density iontophoresis to reduce treatment frequency needed for the management of endothelial and deep stromal graft rejection. The application regimen might be simplified since deep corneal layers can be
reached with single dosing and no additional systemic administration of corticosteroids would be needed [10, 56, 57].

**Figure 4.** (a) Intracorneal deposition of TA following 5 min TA-Lys iontophoresis at 0.5, 1.5 and 3 mA/cm² (grey columns, x₁; y₁) displayed a linear correlation between the intracorneal accumulation of TA and the applied current density (hollow circles; x₂; y₂). (b) Intracorneal biodistribution of TA following iontophoresis of TA-Lys at 0.5 mA/cm² for 5 min. (Mean ± SD, n=5)
3.4 FFOCT corneal imaging.

3.4.1 Visualizing the intracorneal TA biodistribution.

In addition to quantification of the TA biodistribution, it was also decided to visualize the presence of TA in the cornea by using FFOCT. Preliminary experiments compared the images obtained using FFOCT with those of complementary techniques (Figure 5).

Figure 5. Detailed view of (a) Haematoxylin and eosin stained and (b) Hoechst 33342 stained corneal cross sections. FFOCT image of corneal epithelium following (c) Passive application and (d) Iontophoresis (3 mA/cm²) for 10 min of buffer. Bar = 50 µm

The corneal epithelium and the stromal collagen bundles were clearly distinguished by haematoxylin/eosin (Figure 5a) and Hoechst 33342 (Figure 5b) staining and by FFOCT (Figure 5c and 5d). No clear delimitation of the epithelial cell nuclei was visible by FFOCT, but the absence of complicated staining procedures enabled images to be obtained with minimal sample processing. A placebo (10 mM MES buffer + 2.2 % glycerin, pH 5.7), was applied to cornea samples with/or without concomitant current application (3 mA/cm²) for 10 minutes and the samples were observed by FFOCT (Figure 5c and 5d). No change in the epithelium structure, rupture, distortion or modification of thickness was observed in samples that underwent iontophoresis when compared to the passive controls. This was in agreement with previous histological studies: (i) no modification was visible in haematoxylin and eosin stained tissue following
iontophoresis at 6.25 mA/cm² for 5 min using excised porcine cornea[58], (ii) a similar outcome was observed after transcorneal iontophoresis at 2.82 mA/cm² for 10 min in rabbit eyes – no difference was observed in corneal thickness or epithelial cell count [54].

Figure 6. Comparison of FFOCT images of cornea cross-sections following (a) Passive application for 10 min of 2.3 mM TA-Arg solution in 10 mM MES buffer + 2.2% glycerine, pH 5.7 (c) Iontophoresis for 10 min at 3 mA/cm² and (e) Iontophoresis of TA-Arg solution for 10 min at 3 mA/cm² followed by 60 min passive diffusion; Bar = 500 µm. (b), (d) and (f) are the magnifications of the corneal epithelia seen in (a), (c) and (e) Bar = 50 µm. g) Epithelium thickness evaluation for different experimental conditions shown in (Figures 6a and 6b), (Figures 6c and 6d) and (Figures 6e and 6f). * Denotes statistically significant difference (t-test; p<0.05) in between treated and surrounding area. (Mean ± SD, n=5)
Figure 6 shows FFOCT images of the treated cornea cross-sections following passive and iontophoretic (3 mA/cm²) administration of TA-Arg solution. Following passive application, no difference in brightness was detected, in comparison to the surrounding tissue (Figure 6a and 6b). The amount of TA deposited passively in the cornea was apparently insufficient to alter the scattering properties of the tissue. In contrast, a striking increase in brightness was observed following iontophoresis of TA-Arg (Figure 6c and 6d). This was hypothesized to be caused by the 17-fold higher TA deposition quantified in the epithelial and upper stromal layers when compared to passive TA-Arg application. The brightness of the tissue cross-section was quantified with Image J software (integrated density) and a 2.5-fold increase was measured in comparison to the intrinsic luminosity of the cornea. Given the common aromatic steroidal backbone of TA and TA-Arg, it is not possible to attribute unequivocally the luminosity to a given molecular species; however, given the stability data and the measured half-life, it was estimated that ~28% of the signal was due to TA and ~72% from TA-Arg. No visible TA deposition was found in the lateral corneal area (image not shown), indicating a perpendicular current flow and concomitant drug distribution. This is in agreement with previously reported studies of transcorneal iontophoresis of manganese ions (Mn²⁺). Magnetic resonance imaging estimated the lateral distribution of current at less than 10 % [59].

Surprisingly, a statistical significant decrease (t-test, p<0.05, n = 5) of corneal epithelium thickness was evidenced after iontophoresis with TA-Arg (Figure 6c and 6d). In contrast, no epithelium shrinkage was observed after iontophoresis of MES buffer or passive application of TA-Arg solution (Figure 5d and Figure 6a and 6b). This suggests that the phenomenon might be associated to the increased bioavailability of TA rather than the process of iontophoresis itself. Interestingly, it was observed that when the iontophoretic treatment of 10 minutes was followed by 60 min of passive intracorneal TA-Arg diffusion, the epithelial thickness increased – suggesting that the process was reversible even in vitro (Figure 6e, 6f and 6g).
Correlating quantitative and qualitative visual evidence of the intracorneal TA biodistribution.

In order to confirm the correlation between the increased brightness visible in the FFOCT images of the cornea and the effective intracorneal TA deposition, further quantitative biodistribution experiments were performed. Figure 7 shows the intracorneal quantification of TA deposited in each 40 µm lamellae down to a depth of 1 mm, following either a 10 min passive application, a 10 min iontophoretic application or 10 min iontophoretic application followed by a 60 min passive diffusion of TA-Arg. There was a clear relationship between the amount of TA present in each lamella and the luminosity profile in the FFOCT images.

As described in the preceding section, the ~17-fold lower TA deposition following 10 min of passive TA-Arg application (27.15 ± 0.47 nmolTA/cm²) was not sufficient to modify the tissue reflectivity in the FFOCT image. In contrast, following 10 min of iontophoresis, an intense brightness of the corneal epithelium and first stromal layers down to a depth of 440 µm was observed. Indeed 94% of the TA deposition was quantified in these upper tissue layers. Interestingly, when 10 min iontophoresis of TA-Arg was followed by 60 min of passive diffusion TA, increased luminosity was found in the deep stroma. Correspondingly,
a statistically significant increase of TA deposition was detected at a depth of 520 µm to 1000 µm. Passive diffusion of TA can occur following rapid TA-Arg hydrolysis. This highlighted the feasibility of using biolabile prodrug iontophoresis to create a drug depot in the upper tissue layers; TA was able to reach the deeper tissue following diffusion of the lipophilic TA into the stroma.

4. CONCLUSION

The study clearly demonstrated that a combination of chemical modification and short duration iontophoresis can be used to significantly increase intracorneal bioavailability of TA. This was confirmed by quantitative determination of the TA biodistribution by UHPLC-MS/MS and qualitative visual analysis via FFOCT. The impact of prodrug susceptibility/resistance to hydrolysis on iontophoretic transport and its effect on TA biodistribution was also clearly evidenced. The superior stability of TA-Lys ensured more prolonged electromigration resulting in deeper intracorneal penetration and the presence of greater amounts of TA in the stroma. This might provide a mechanism for regioselective drug delivery. The intracorneal deposition of TA following iontophoresis for 5 min from TA-Lys solution displayed a linear dependence over a range of current density from 0.5 to 3 mA/cm². Thus, the TA dose administered to the tissue could be controlled by simply modifying the current density and intracorneal drug distribution imaged via FFOCT directly in the patient. Nevertheless, it is important to specify that in vitro/ex vivo studies described here do not take into account dilution due to lacrimal turnover and loss of the formulation from the eye [60]; thus, these promising results will need to be confirmed in preclinical studies in vivo where the effect of such dynamic barriers must be evaluated. Furthermore, given the labile nature of prodrugs, formulation stability will need to be evaluated and perhaps “dry” systems developed that are only hydrated in situ and so can better ensure the stability of the prodrug molecules.
5. **Supplementary material**

5.1 Synthesis of TA-AA prodrugs

The synthesis of the triamcinolone acetonide amino acid prodrugs (TA-AA) was performed as follows: N-Boc protected arginine or lysine (Boc-Arg(Boc)₂-OH or Boc-Lys(Boc)-OH (present as the free acid) (368.7 or 269.1 mg, 0.78 mmol) and N,N’-dicyclohexylcarbodiimide (DCC, 160 mg, 0.78 mmol) were dissolved in anhydrous dichloromethane (DCM, 0.1 M). Triamcinolone acetonide (TA, 225.5 mg, 0.519 mmol) and a catalytic amount of 4-dimethylaminopyridine (4-DMAP, 6 mg, 0.05 mmol) were then successively added. The reaction mixture was left under agitation overnight at ambient temperature. The crude mixture was purified by flash chromatography on 12 g silica cartridge Reveleris™ Flash System (Grace Davison Discovery Science, Deerfield, USA). A gradient elution was performed with hexane and ethyl acetate (max. 75:25, respectively) and the compounds were recovered after evaporation of the solvent. The N-Boc-TA-AA esters were dissolved in anhydrous DCM (0.1 M) and the N-Boc moieties deprotected by addition of trifluoroacetic acid (TFA, 0.1 M). The mixture was stirred at room temperature for 2 hours. TA-arginine and TA-lysine (TA-Arg and TA-Lys) were obtained in the form of white powders through precipitation in diethyl ether and stored at -20°C and protected from light. NMR, mass spectrum analysis and overall yield of the final TA-AA prodrugs are given below:

**TA-Arg:** Yield: 83%; **1H NMR** (300 MHz, DMSO-d₆) δ 8.51 (s, 3H), 7.74 (s, 1H), 7.31 (d, J=9 Hz, 2H), 7.09 (bs, 3H), 6.25 (d, J=9 Hz, 1H), 6.03 (s, 1H), 5.59 (d, J=3 Hz 1H), 5.32 (d, J=21 Hz, 1H), 4.93 (d, J=21 Hz, 1H), 4.88 (d, J=3 Hz, 1H), 4.25 (bm, 2H), 3.16 (bq, 2H), 2.59 (m, 1H), 2.36 (m, 2H), 2.08 – 1.56 (m, 10H), 1.48 (s, 3H), 1.36 (s, 3H), 1.34 (d, 1H), 1.16 (s, 3H), 0.84 (s, 3H); **13C NMR** (300 MHz, DMSO-d₆) δ 205.55, 185.84, 169.93, 167.15, 157.44, 153.02, 129.74, 124.96, 111.72, 102.83-100.52, 97.73, 81.89, 70.95-70.48, 69.14, 52.12, 48.60-48.23, 45.99, 43.49, 41.60, 36.73, 33.73, 33.28-33.03, 30.75, 28.24, 26.97, 26.02, 24.79, 23.54, 23.46, 16.75; **HRMS** (ESI) calculated for C₃₀H₄₄F₄N₄O₇ [M+H]⁺: 591.3189, found: 591.3183.
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**TA-Lys: Yield:** 85% ; **^1^H NMR** (300 MHz, DMSO-d$_6$) δ 8.49 (s, 3H), 7.73 (s, 3H), 7.32 (d, J = 9 Hz, 1H), 6.25 (d, J= 9,1H), 6.03 (s, 1H), 5.54 (d, J = 3 Hz, 1H), 5.39 (d, J = 18 Hz, 1H), 4.86 (m, 2H), 4.22 (bs, 2H), 2.75 (bs, 2H), 2.60 (m, 1H), 2.37 (m, 2H), 2.08-1.55 (m, 12H), 1.49 (s, 3H), 1.37 (s, 3H), 1.33 (m, 1H), 1.17 (s, 3H), 0.83 (s, 3H); **^1^C NMR** (300 MHz, DMSO-d$_6$) δ 203.59, 185.84, 169.83, 167.18, 153.07, 129.77, 124.98, 111.74, 102.84-100.51, 97.76, 81.92, 71.02-70.54, 69.18, 52.22, 48.54-48.24, 45.91, 43.53, 40.50, 36.65, 34.05, 33.69, 33.22-32.96, 30.77, 30.08, 28.18, 27.12, 26.10, 23.51, 21.83, 16.87; **HRMS** (ESI) calculated for C$_{30}$H$_{43}$FN$_2$O$_7$ [M+H]$^+$:563.3126, found: 563.3127.

The calculated m/z [M+H$^+$] was found to differ by less than 1 ppm with the experimentally obtained m/z. Overall, an efficacious conversion (>80%) of TA to TA-AA prodrugs was achieved, minimizing the loss of the starting material. The presence of the newly formed ester bond was confirmed by $^1$H NMR analysis. The increased chemical shift of the two protons in position 21(Ha and Hb), on the steroid side chain, confirmed the link to the adjacent electron attractive carboxylic ester moiety (TA: H$_{21a}$ 4.53ppm, H$_{21b}$ 4.06ppm; TA-Arg: H$_{21a}$ 5.31ppm, H$_{21b}$ 4.92ppm; TA-Lys: H$_{21a}$ 5.39ppm, H$_{21b}$ 4.87ppm) (**Figure S1**). Moreover, multiplicity of the protons in position 21 changed from doublet of doublets (due to the interaction of the primary alcohol) to simple doublets.

**Figure S1.** a) Magnification on $^1$H NMR spectrum of H$_{21a/b}$ protons of TA. b) Representation of TA-AA ester bond and corresponding $^1$H NMR H$_{21a/b}$ peaks. (300 MHz, DMSO-d$_6$)
5.2 Validation of HPLC-UV method for the quantification of TA

5.2.1 Specificity, linearity, LOD and LOQ

The method specificity and linearity for TA was assessed in a methanol: water mixture (50:50) spiked with corneal extract by using a six point calibration curve over a concentration range from 2.30 to 230.15 nmol/ml. The limit of detection (LOD) and quantification (LOQ) were established according to ICH Q2 (R1) guidelines [31]. No interference or overlapping with the corneal extract components was detected at the retention time of 8.3 min for TA. The method was linear in the investigated concentration range with \( R^2 \) of 0.99. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using the linear regression method and were 0.84 and 2.56 nmol/ml, respectively.

5.2.2 Precision and accuracy

The intra-day precision and accuracy were evaluated by the analysis of standard solutions on the same day, whereas inter-day variability was tested on two different days. Results of accuracy and precision are listed in Table S1 and were found to be included within the acceptance limits.

Table S1. Intra- and inter-day precision and accuracy for TA quantification method  

\(^{a)} \) Precision = \((\text{SD/mean})\times100, \quad ^{b)} \) Accuracy = \((\text{measured concentration/theoretical concentration})\times100

<table>
<thead>
<tr>
<th>Theoretical [TA] (nmol/ml)</th>
<th>Measured [TA] (nmol/ml)</th>
<th>RSD (%) (^{a)}</th>
<th>Recovery (%) (^{b)}</th>
<th>Measured [TA] (nmol/ml)</th>
<th>RSD (%) (^{a)}</th>
<th>Recovery (%) (^{b)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>230.15</td>
<td>229.92 ± 0.68</td>
<td>0.29</td>
<td>99.9</td>
<td>229.61 ± 0.98</td>
<td>0.43</td>
<td>99.8</td>
</tr>
<tr>
<td>115.07</td>
<td>115.84 ± 3.17</td>
<td>2.74</td>
<td>100.7</td>
<td>116.12 ± 0.15</td>
<td>0.13</td>
<td>100.9</td>
</tr>
<tr>
<td>46.03</td>
<td>45.71 ± 0.77</td>
<td>1.68</td>
<td>99.0</td>
<td>46.05 ± 0.83</td>
<td>1.79</td>
<td>100.0</td>
</tr>
<tr>
<td>23.01</td>
<td>22.45 ± 0.28</td>
<td>1.23</td>
<td>97.5</td>
<td>23.32 ± 1.27</td>
<td>5.43</td>
<td>101.3</td>
</tr>
<tr>
<td>11.51</td>
<td>11.85 ± 0.10</td>
<td>0.83</td>
<td>103.8</td>
<td>10.89 ± 0.20</td>
<td>1.70</td>
<td>94.6</td>
</tr>
<tr>
<td>4.60</td>
<td>4.68 ± 0.03</td>
<td>0.57</td>
<td>101.6</td>
<td>4.44 ± 0.20</td>
<td>4.49</td>
<td>96.5</td>
</tr>
</tbody>
</table>
5.3 Validation of HPLC-UV method for the quantification of TA-AA prodrugs

5.3.1 Specificity, linearity, LOD and LOQ

HPLC-UV methods for TA-Arg and TA-Lys were developed for specific quantification of the prodrugs separated from corneal extract and the parent drug TA. Specificity was obtained at retention times of 3.3 min for TA-Arg and 2.9 min for TA-Lys. The chromatographic peak was shown to be distant from TA (8.3 min) and corneal extract (1.38-1.98 min). The LOD and LOQ for TA-Arg and TA-Lys were (0.84 and 2.56 nmol/ml) and (0.75 and 2.27 nmol/ml), respectively.

5.3.2 Precision and accuracy

As previously for TA, intra-day and inter-day variability of the method was tested. At the concentrations tested the method showed precision and accuracy values for both TA-AA prodrugs within the acceptance limits (Table S2, Table S3).

Table S2. Intra- and inter-day precision and accuracy for (a) TA-Arg and (b) TA-Lys quantification methods with HPLC-UV a) Precision = (SD/mean)*100, b) Accuracy = (measured concentration/theoretical concentration)*100

<table>
<thead>
<tr>
<th>(a)</th>
<th>Intra-day (n=3)</th>
<th>Inter-day (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical [TA-Arg] (nmol/ml)</td>
<td>Measured [TA-Arg] (nmol/ml) RSD (%) a</td>
<td>Recovery (%) b</td>
</tr>
<tr>
<td>230.15</td>
<td>230.47 ± 0.49</td>
<td>0.21</td>
</tr>
<tr>
<td>115.07</td>
<td>114.66 ± 0.75</td>
<td>0.66</td>
</tr>
<tr>
<td>46.03</td>
<td>45.82 ± 0.16</td>
<td>0.35</td>
</tr>
<tr>
<td>23.01</td>
<td>22.49 ± 0.59</td>
<td>2.61</td>
</tr>
<tr>
<td>11.51</td>
<td>11.62 ± 0.45</td>
<td>3.86</td>
</tr>
<tr>
<td>4.60</td>
<td>4.84 ± 0.17</td>
<td>3.59</td>
</tr>
</tbody>
</table>
CHAPTER II: Targeted intracorneal delivery -
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5.4 Validation of UHPLC-MS/MS method for TA quantification

5.4.1 Specificity, linearity, LOD and LOQ

A more sensitive UHPLC-MS/MS method was developed to quantify TA in the 40 μm thick corneal lamellae obtained in the biodistribution study. The method was specific and linear in a concentration range of 11.5-230.15 pmol/ml in methanol: water (50:50) mixture spiked with corneal extract (retention time: 0.81 min, \( R^2 \geq 0.98 \)). LOD and LOQ and for TA were 3.5 and 11 pmol/ml, respectively.

5.4.2 Precision and accuracy

Results of intra- and inter-day precision and accuracy were investigated and summarized in Table S3. The obtained values were contained again within the acceptance limits.
### Table S3. Intra-and inter-day precision and accuracy for TA quantification

\(^a\) Precision = (SD/mean)\(^*\)100, \(^b\) Accuracy = (measured concentration/theoretical concentration)\(^*\)100

<table>
<thead>
<tr>
<th>Theoretical [TA] (pmol/ml)</th>
<th>Intra-day (n=3)</th>
<th>Inter-day (n=6)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Measured [TA] (pmol/ml)</td>
<td>RSD (%) (^a)</td>
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<td>230.15</td>
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</tr>
<tr>
<td>115.07</td>
<td>118.11 ± 1.51</td>
<td>1.28</td>
</tr>
<tr>
<td>46.03</td>
<td>44.95 ± 1.17</td>
<td>2.60</td>
</tr>
<tr>
<td>23.01</td>
<td>22.56 ± 1.26</td>
<td>5.58</td>
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<tr>
<td>11.51</td>
<td>11.50 ± 0.48</td>
<td>4.15</td>
</tr>
</tbody>
</table>

### 5.5 Validation of corneal TA extraction procedure

The complete hydrolysis of the TA-AA prodrugs in the extraction mixture methanol: water (50:50) after 12 h at room temperature was proven by HPLC-UV analysis. Therefore, only the extraction of the parent drug (TA) from corneal tissue was validated. Isolated porcine cornea samples of 0.8 cm\(^2\) (n=3) were spiked with TA (288, 575, 2878 nmol\textsubscript{TA}/cm\(^2\)) dissolved in methanol. After evaporation of the solvent the tissue was cut into small pieces and soaked for 12 h in 2 ml of methanol: water (50:50). The extracts were analyzed after centrifugation and dilution in methanol by HPLC-UV. The recovered amount of TA was compared with the amount applied (Table S4).

### Table S4. Validation of TA extraction from corneal samples (Mean ± SD)

<table>
<thead>
<tr>
<th>Theoretical amount (nmol/cm(^2))</th>
<th>Recovered amount (nmol/cm(^2))</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2878</td>
<td>2834.68 ± 247.10</td>
<td>98.53 ± 8.59</td>
</tr>
<tr>
<td>575</td>
<td>551.20 ± 13.04</td>
<td>95.80 ± 2.27</td>
</tr>
<tr>
<td>288</td>
<td>278.24 ± 5.35</td>
<td>96.72 ± 1.86</td>
</tr>
</tbody>
</table>
For all cornea samples an extraction recovery superior to 95% was established and therefore the method was considered appropriate for the in vitro corneal deposition experiments.

5.6 Experimental set-up of corneal iontophoresis experiments using porcine eye globe

Figure S2. a) Scheme of the experimental setup for in vitro corneal iontophoresis on isolated porcine eye globe. b) Photograph of the experimental setup. c) Detailed view of the donor compartment in contact with the cornea.

6. Acknowledgements

We would like to thank the University of Geneva for financial support and for providing teaching assistantships for ML and VS. YNK and SdR would also like to thank the Swiss Commission for Technology and Innovation for financial support (CTI 13933.2) YNK is extremely grateful to Carigest SA for the generous award of a grant to acquire the Light-CT Scanner. VS thanks Dr. Elena Tratta for valuable discussions and advice. We would also like to acknowledge our colleagues Dr. Christoph Bauer and Jerôme Bosset at the Bioimaging Center of the Faculty of Sciences at the University of Geneva for their help with confocal laser scanning microscopy.
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7. REFERENCES


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CHAPTER III

- Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport
Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport

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Abstract

Intracorneal delivery of ten amino acid (alanine, arginine, asparagine, glutamine, glycine, histidine, isoleucine, lysine, methionine and valine) ester prodrugs of triamcinolone acetonide (TA-AA) was investigated in vitro into porcine corneas, using a corneal iontophoresis device (IONTOFOR-CXL; SOOFT Italia S.p.A.) approved for use in humans. Short duration iontophoresis of 1mA for 5 min was performed with all prodrugs and intracorneal deposition of TA was quantified by HPLC-UV and UHPLC-MS/MS. The intracorneal deposition data evidenced the clear advantage of TA-AA prodrug iontophoresis compared to passive delivery. It also revealed unexpected and prodrug dependent deposition profiles. For example, in contrast to the favourable electrical mobility data obtained by capillary zone electrophoresis, the intracorneal delivery of the two double positively charged prodrugs arginine and lysine (TA-Arg and TA-Lys), were not found to outperform the delivery of other prodrugs (alanine: TA-Ala and glycine: TA-Gly). In order to rationalize this behaviour, physicochemical properties of the different TA-AA prodrugs were investigated in silico. In agreement with previously published literature, increasing molecular weight and lipophilicity potential hindered iontophoretic transport. It was considered that the relatively large charge distribution and the lower tendency to interact with the corneal tissue via electrostatic and H-bonds
contributed to the excellent iontophoretic delivery of TA-Ala and TA-Gly. Finally, the biodistribution of TA delivered into porcine cornea from TA-Gly iontophoresis resulted in the delivery of supratherapeutic amounts to the deep corneal stroma, which exceeded the TA IC_{50} for about 10^4-fold. In conclusion, the successful combination of the SOOFT iontophoretic device and the TA-AA prodrugs for corneal iontophoresis was clearly demonstrated.
CHAPTER III: Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport

1. Introduction

The SOOFT iontophoretic system was developed for the current mediated administration of riboflavin (vitamin B2), preceding UV irradiation, in the so called corneal cross linking procedure. This intervention is used to stiffen the cornea and prevent the progression of pathological shape changes known as ectasia e.g. keratoconus [1] [2]. Classical corneal crosslinking procedures include commonly the removal of the corneal epithelium before continuous (each 1 to 5 min) riboflavin eye drop instillation for maximal 30 min and final UV irradiation [3]. The corneal epithelium represents a major barrier for the absorption of hydrophilic molecules; when removed the delivery of riboflavin was found increased [4]. Time and frequency of riboflavin application were chosen to compensate the low bioavailability of vitamin B2 caused by the blinking reflex and lacrimal washout [5]. However, despite all these optimizations, the invasive procedure exposes the patients to the risk of post operational infections, ulcer formation, pain and discomfort [6]. In comparison to the common clinical practice, the SOOFT iontophoresis system however was shown to efficaciously deliver riboflavin into the corneal tissue, with short application time of 5-10 min at low current 1-0.5 mA and no need of the removal of the corneal epithelium [7] [8] [9].

Other examples for successful short term corneal iontophoresis (1-10 min) with different applicator systems are the delivery of positively charged antibiotics like gentamicin and tobramycin in vivo on rabbit corneas [10] [11] [12] [13]. The drug amounts delivered reached bactericidal concentrations in the cornea and were superior compared to the standard passive eye drop instillation.

Encouraged by the above described results, in this study an alternative to the classical topical corticosteroid therapy via eye drops for superficial acute inflammatory conditions [14] was investigated by corneal iontophoresis using the SOOFT system -in order to improve the drug bioavailability. The delivery of therapeutically relevant drug concentrations in the deep corneal tissue would be important among others, for the effective treatment of corneal graft rejection, a severe immunological and inflammatory complication of the corneal transplantation [15] [16].
Iontophoresis is an active non-invasive drug delivery technique which relays on the application of a low intensity current which carries the active principles into and through the biological membranes. Ideal drug candidates for the iontophoretic transport were found to be water soluble and charged molecules [17]. These molecules, if applied passively on the cornea would have very low permeability, however if a mild electric current on same charge electrode is applied, an increased driving force on the molecules into the tissue was reported [18]. TA however, itself does not carry any charged moiety and has very low aqueous solubility. Its physicochemical properties: especially high lipophilicity were designed for an optimal passive delivery through biological membranes [19]. This is a very successful strategy in the case of topical skin applications. However unsuited for tolerable aqueous formulations such as eye drop instillation [20] and/or the iontophoretic application. To overcome these limitations, in this study TA was transformed in a suitable candidate for short iontophoretic application by synthetizing water soluble and positively charge amino acid ester prodrugs (TA-AA). As shown in previous studies the iontophoretic delivery of prodrugs enabled successful electrical delivery and prompt liberation of the parent drug via chemical and enzymatic hydrolysis [21] [22] [23] [24].

Ten novel TA-AA prodrugs were tested. Amino acid with ionisable primary amine group were chosen from the different groups of non-polar (alanine (Ala), glycine (Gly), isoleucine (Ile), methionine (Met) and valine (Val)), polar (asparagine (Asn) and glutamine (Gln)) and basic (arginine (Arg), histidine (His) and lysine (Lys)) moieties. The diverse sidechains were selected to cover a broad spectrum of physicochemical properties and in silico modelling was used to understand their influence on the surface characteristics. This was of particular interest since previous investigations reported the dependency of iontophoretic transport kinetics on factors like molecular weight [25] and charge as well as affinity to lipids [26] [27] and tissue binding [28] [29] [30]. By comparison of the TA-AA prodrugs physicochemical characteristics and their iontophoretic delivery into the cornea the features for optimal delivery were commented. Secondly the
delivery of TA-Gly prodrug with the SOOFT system was investigated by the “layer by layer” biodistribution technique to prove the efficacy of iontophoresis for deep corneal drug delivery.

2. Material and methods

2.1 Material

Alanine, arginine, asparagine, glutamine, glycine, histidine, isoleucine, lysine, methionine and valine ester prodrug of TA (TA-Ala, TA-Arg, TA-Asn, TA-Gln, TA-Gly, TA-His, TA-Ile, TA-Lys, TA-Met and TA-Val) were synthesized in house following a previously published procedure [21] (Supplementary data). Buffer salts (sodium and potassium chloride, sodium and potassium phosphate, 2-morpholinoethanesulfonic acid monohydrate (MES)) were purchased from Sigma-Aldrich (Steinheim, Germany). Glycerol was obtained from Acros Organics (Geel, Belgium) and ULC/MS grade formic acid was bought from Brunschwig (Basel, Switzerland). HPLC grade methanol (HiPerSolv Chromatonorm; Darmstadt, Germany) and deionized water was used for extraction and analysis solutions (Millipore Milli-Q Gard 1 Purification Pack resistivity > 18 MΩ.cm; Zug, Switzerland). Power supply (I-ON CXL; SOOFT Italia S.p.A.) and corneal application system (Iontofor CXL, SOOFT Italia S.p.A.) were kindly provided by SOOFT Italia S.p.A.

2.2 Physicochemical properties of the TA-AA prodrugs

2.2.1 Experimental analysis of TA-AA

Solubility

The solubility of the then newly synthesized TA-AA prodrugs was determined in 10 mM MES buffer (pH 5.5) and PBS buffer (pH 7.4) as previously published [21] [22]. The two buffer solutions were chosen to simulate conditions similar to the donor compartment during iontophoresis and the physiological environment in the corneal tissue. In summary, TA-AA prodrugs were added to the buffer solutions up to saturation level, followed by 10 min sonication, centrifugation and immediate analysis of the supernatant
via HPLC-UV. The procedure had to be performed well timed given the instability of the TA-AA prodrugs in aqueous environment. (n=3)

**Half life**

The stability to hydrolysis of TA-AA prodrug ester bond was investigated at 37 °C, pH 5.5 and at pH 7.4 in contact with freshly isolated cornea as previously described [21]. Buffer solution with and without fresh cornea were put in contact with TA-AA prodrugs solution (2.3x10⁻² mM) under agitation. Samples were withdrawn every 10min analysed by HPLC-UV. Based on the slope of the regression curve the first order rate constant of hydrolysis (k\textsubscript{obs}) was retrieved and the half-life (t\textsubscript{1/2}) (t\textsubscript{1/2}=ln2/k\textsubscript{obs}) calculated [21] [22]. (n=3)

**Electrophoretic mobility**

Capillary zone electrophoresis (CZE) experiments were performed to investigate TA-AA prodrugs electrophoretic mobility. The background electrolyte solution of 10 mM MES buffer (pH 5.7) was used as for iontophoretic experiments and fresh solutions of the TA-AA prodrugs (0.23 mM) in the same buffer were prepared. CZE-UV experiments were performed using an HP 3D CE system from Agilent Technologies (Waldbronn, Germany) equipped with an on-capillary diode array detector. Separations were performed using a fused-silica capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 64.5 cm, an effective length of 56 cm and an internal diameter of 50 μm. The positive polarity mode (anode at the inlet) was used with a constant voltage of 30 kV. The capillary was kept at 25 °C. For the calculation of electroosmotic flow, 5% v/v acetone was added into the samples. UV detection was performed at 220 nm.

**2.2.2 In silico investigation of TA-AA**

Physicochemical descriptors of TA-AA prodrugs such as pKa and Log D (pH 5.5 and 7.4) were predicted by ACD/Labs software (version 12.01). 3D surface analysis of the TA-AA prodrugs was performed using the Maestro programme, a tool from the Schrödinger 2013 package (Maestro; Schrödinger, LLC: Portland,
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OR, 2009) and Sybyl 2.1.1 programme (SYBYL-X 2.1.1, Tripos International, St. Louis, Missouri, USA). Energy minimisations were performed simulated water environment on TA and TA-AA prodrug in charged and uncharged state using the OPLS-2005 force-field (10,000 iterations, 0.05 kcal/mol conjugation gradient). Resulting surface properties such as lipophilic surface potential (LP), electrostatic potential (EP), dipolar moment and the hydrogen bond donor/acceptor area were illustrated on Connolly surface in Sybyl 2.1.1 programme and the different prodrugs were compared.

2.3 In vitro intracorneal iontophoresis of TA-AA prodrugs

2.3.1 Tissue preparation

Porcine eyes were reported in literature as anatomically closest surrogate for the human organ [31] [32]. Eye globes from adult animals (80-100 kg) were obtained from a local slaughterhouse (Abattoir de Loëx Sarl; Loëx, Switzerland) and used within few hours after removal of the surrounding muscular tissue.

2.3.2 SOOFT system setup

Power supply (I-ON CXL; SOOFT Italia S.p.A.) and corneal application system (Iontofor CXL, SOOFT Italia S.p.A.) were employed for the intracorneal administration of TA-AA prodrugs. Prior application the polarities of the system were made convertible by inserting wire connectors. In the case of anodal iontophoresis, the cathode, a steel wire, was recovered by the dismantling the patch applicator. On the anodal ending the corneal applicator with the stainless-steel grid electrode was placed (Figure 1a-b). The complete experimental setup is shown in Figure 1c. The porcine eye bulbs were placed with the cornea facing upwards in custom made vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland) and the receiving compartment filled with PBS solution. The SOOFT eye cup was fixed with an annular suction ring on the corneal surface and the stainless-steel wire cathode was inserted in the receiving compartment.
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Figure 1. a) Recovered stainless steel wire from patch electrode applicator system b) in-house modified SOOFT system for anodal iontophoresis c) experimental setup on entire porcine eye globe.

2.3.3 Intracorneal iontophoresis of TA-AA prodrugs

Solutions of 2.3 mM TA-AA prodrugs in 10 mM MES buffer with the addition of 2.2% glycerine pH 5.7, were prepared shortly before iontophoretic experiments. The suitability of the formulation with the stainless steel Iontofor CXL corneal applicator was investigated prior to assure the absence of pH variation due to water electrolysis [17]. SOOFT iontophoretic system (0.8 cm²) was used at 1 mA for 5 min (1.8 mA/cm², 5 min). As soon as the 5 min terminated the donor compartment was emptied and the corneal surface carefully cleaned under running water for 30 sec. Following the corneas were isolated from the eye globe, weighed, cut into small pieces, extracted in 50:50, methanol: water mixture over night and finally analysed by HPLC-UV. Intracorneal “layer by layer” biodistribution study was performed on one of the most promising TA-AA candidates (TA-Gly) in order to investigate the distribution of the active principle in the depth of the tissue down to 1 mm (with slices of 40 µm each) [21].
2.4 Analytical method

2.4.1 HPLC-UV analysis of TA and TA-AA

TA and TA-AA prodrugs were analysed by UltiMate 3000 HPLC-UV system (formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach Switzerland) equipped with HPG-3200SD pump, WPS-3000 autosampler and VWD-3400 VIS/UV detector. Isocratic elution was used with mobile phases consisting of water + 0.1% TFA (A) and acetonitrile + 0.1% TFA (B). Column temperature was kept at 30°C with flow rate of 0.8 ml/min and detection wavelength of 240 nm was employed. Peak readout was integrated using Chromeleon® (version 6.8) software. In Table 1 the respective limit of quantification (LOQ) and limit of detection (LOD) were calculated following the ICH Q2 (R1) guideline [33]. The methods were validated and showed suitable intra-day precision and accuracy (Supplementary material).

Table 1. Analytical method conditions, retention time, LOQ and LOD for TA and TA-AA prodrugs.

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Retention time (min)</th>
<th>Analytical column</th>
<th>Mobile phase (% A: %B)</th>
<th>LOQ (nmol/ml)</th>
<th>LOD (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>8.3</td>
<td>LiChrospher RP18</td>
<td>65:35</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>3.3</td>
<td>LiChrospher RP18</td>
<td>65:35</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td>TA-Lys</td>
<td>2.9</td>
<td>LiChrospher RP18</td>
<td>65:35</td>
<td>2.27</td>
<td>0.84</td>
</tr>
<tr>
<td>TA</td>
<td>4.74</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>2.01</td>
<td>0.66</td>
</tr>
<tr>
<td>TA-Asn</td>
<td>2.6</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>3.35</td>
<td>1.10</td>
</tr>
<tr>
<td>TA-Gln</td>
<td>2.8</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>1.78</td>
<td>0.59</td>
</tr>
<tr>
<td>TA-His</td>
<td>2.2</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>2.58</td>
<td>0.85</td>
</tr>
<tr>
<td>TA-Met</td>
<td>6.49</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>1.80</td>
<td>0.59</td>
</tr>
<tr>
<td>TA</td>
<td>7.4</td>
<td>LiChrospher RP18</td>
<td>60:40</td>
<td>1.58</td>
<td>0.52</td>
</tr>
<tr>
<td>TA-Ala</td>
<td>5.7</td>
<td>LiChrospher RP18</td>
<td>55:45</td>
<td>2.79</td>
<td>0.92</td>
</tr>
<tr>
<td>TA-Gly</td>
<td>5.1</td>
<td>LiChrospher RP18</td>
<td>55:45</td>
<td>3.52</td>
<td>1.16</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>10.8</td>
<td>LiChrospher RP18</td>
<td>55:45</td>
<td>4.06</td>
<td>1.34</td>
</tr>
<tr>
<td>TA-Val</td>
<td>8.4</td>
<td>LiChrospher RP18</td>
<td>55:45</td>
<td>3.08</td>
<td>1.02</td>
</tr>
</tbody>
</table>

a(5 µm particle size; 125 mm × 4.0 mm; BGB Analytik AG; Boeckten, Switzerland)

b(5 µm particle size; 250 mm × 4.0 mm; BGB Analytik AG; Boeckten, Switzerland)
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The analysis of TA delivery in the corneal lamellae (40 µm of height), obtained by vertical slicing of the tissue following iontophoretic application needed a more sensitive analytical method. For this specific study, quantification was performed using a UHPLC-MS/MS system, method validation was previously published [21].

2.5 Statistical analysis

The results were derived from at least triplicates; all the data were expressed as the mean ± standard deviation. Statistical analysis was performed by either ANOVA or Student’s T-test, with the level of significance set at p ≤0.05.

3. Results

3.1 Experimental physicochemical properties

3.1.1 Solubility and stability of TA-AA prodrugs

Solubility at pH 5.5 and 7.4 of the TA-AA prodrugs and the stability of TA-AA ester bonds at pH 5.5 and pH 7.4 in contact with cornea were tested and summarized in Table 2.

Table 2. pKa, LogD and experimentally: solubility and half-life (t ½) (n=3, Mean ± SD).

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Solubility pH 5.5 (µmol/ml)</th>
<th>Solubility pH 7.4 (µmol/ml)</th>
<th>t½ pH 5.5 (h)</th>
<th>t½ pH 7.4 + cornea (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>2.76 x10⁻² [34]</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA-Ala</td>
<td>15.92 ± 0.66</td>
<td>0.24 ± 0.02</td>
<td>31.0 ± 1.8</td>
<td>19.6 ± 2.3</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>25.61 ± 1.23</td>
<td>1.75 ± 0.09</td>
<td>10.2 ± 0.5</td>
<td>18.0 ± 9.9</td>
</tr>
<tr>
<td>TA-Asn</td>
<td>3.78 ± 0.28</td>
<td>0.08 ± 0.02</td>
<td>3.4 ± 0.1</td>
<td>11.5 ± 1.7</td>
</tr>
<tr>
<td>TA-Gln</td>
<td>13.78 ± 3.68</td>
<td>0.40 ± 0.17</td>
<td>24.4 ± 6.5</td>
<td>14.9 ± 1.2</td>
</tr>
<tr>
<td>TA-Gly</td>
<td>23.88 ± 1.2</td>
<td>1.05 ± 0.04</td>
<td>29.34 ± 2.8</td>
<td>24.8 ± 2.2</td>
</tr>
<tr>
<td>TA-His</td>
<td>27.02 ± 5.57</td>
<td>1.09 ± 0.36</td>
<td>22.7 ± 0.1</td>
<td>28.9 ± 2.5</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>5.17 ± 0.13</td>
<td>0.02 ± 0.001</td>
<td>194.6 ± 8.5</td>
<td>106.9 ± 6.6</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>TA-Lys</th>
<th>TA-Met</th>
<th>TA-Val</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29.12 ± 1.59</td>
<td>2.40 ± 0.10</td>
<td>39.2 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>3.03 ± 2.16</td>
<td>0.05 ± 0.02</td>
<td>86.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>5.97 ± 0.40</td>
<td>0.02 ± 0.001</td>
<td>104.2 ± 19.7</td>
</tr>
</tbody>
</table>

Aqueous solubility of TA-AA prodrugs was found increased at pH 5.5 compared to the parent drug TA from 100-fold for TA-Met to 1000-fold for TA-Lys, whereas at physiological pH, due to the reduced percentage of charged prodrugs, solubility was closer or equal to TA. However, importantly for further iontophoretic experiments TA-AA prodrug solubility was found sufficient to enable a minimal concentration of 2.3 mM of the active principle (0.1%) chosen for corneal formulations.

The investigation of TA-AA prodrugs ester bond stability showed the notable influence of the pH and presence of corneal tissue on the hydrolysis rates. At pH 5.5 half-life values were found in the range of several hours whereas at pH 7.4 (data not shown) and pH 7.4 in contact with corneal tissue, half-life was reduced to minutes. Lather was suggested to be caused by chemical and the concomitant enzymatic hydrolysis [22] [21]. The nature of the amino acid sidechain seemed to play a key role in the ester bond stability: in the case of chemical hydrolysis, the most stable prodrugs TA-Ile, TA-Met and TA-Val were also the most lipophilic. A shielding effect of the apolar sidechain on the ester bond was suggested. TA-Asn was found to be the most unstable prodrug in both conditions tested. This was related to the physical proximity of the electron attractive amide on the sidechain to the ester bond, which might lead to an increased electrophilicity of the carboxylic group, facilitating a nucleophilic attack. However, important with this stability tests was to demonstrate: i) that TA-AA prodrug stability in freshly prepared solutions was maintained for the time of application and ii) the liberation of the actual active principle in the biological tissue was assured.
3.1.2 Electrophoretic mobility

The electrophoretic mobility of the TA-AA prodrugs was investigated in conditions equal to the further iontophoretic experiments: 10 MES buffer at pH 5.7. CZE was suggested as useful preliminary screening to identify the most promising candidates for a successful iontophoretic delivery depending on their electrical mobility [35] [36]. Values of electrophoretic mobility were given as $10^{-3}$ cm$^2$ V$^{-1}$ min$^{-1}$. When compared to the charge/mass ratio (considering pH 5.7) a linear correlation for TA-AA prodrugs was found ($R^2 0.91$, $p \leq 0.05$) (Figure 2). Using the molecular weight, a simplification was performed, since electrophoretic mobility is depending on molecules hydrodynamic size which however for small molecules is very closely related. It was clearly seen that the mainly double positively charged TA-AA prodrugs such as TA-Arg and TA-Lys were found with the highest mobility ($10.7 \pm 1.0$ to $12.0 \pm 1.2 \times 10^{-3}$ cm$^2$ V$^{-1}$ min$^{-1}$, respectively). The rest of the Ta-AA prodrugs given their similar single positive charged state didn’t differ consistently in their electrophoretic behaviour ($5.5$ to $7.4 \times 10^{-3}$ cm$^2$ V$^{-1}$ min$^{-1}$).

![Figure 2](image.png)

**Figure 2.** Linear correlation in between electrophoretic mobility determined by CZE analysis and the charge to mass ratio of the TA-AA prodrugs. (n=3)
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Following these results on TA-AA mobility in an electrical field, highest iontophoretic delivery of double charged TA-AA prodrugs such as TA-Lys and TA-Arg was hypothesized, whereas from the other prodrugs a similar delivery would be expected.

3.2 Total iontophoretic delivery of TA-AA prodrugs in porcine cornea

The iontophoretic application of TA-AA prodrugs with the modified SOOFT system, adapted for anodal iontophoresis (1.8 mA/cm² for 5 min), resulted in a total delivery of TA from $69.22 \pm 28.9$ to $729.53 \pm 49.28$ nmolTA/g for TA-Met and TA-Lys, respectively (Figure 3).

Six of the 10 tested TA-AA prodrugs (TA-Met, TA-Ile, TA-Val, TA-Asn, TA-Gln and TA-His) showed a modest TA delivery following iontophoresis within ~ 100-300 nmolTA/g. This TA concentration however was found 80 to 100-fold superior to simple 5 min passive application of TA-AA solutions. Highest TA delivery (< 600 nmolTA/g) was found with four of the prodrugs TA-Ala, TA-Arg, TA-Gly and TA-Lys with no statistically significant difference in between them.

![Figure 3](image_url). Intracorneal deposition of TA following 5 min corneal iontophoresis with in house modified SOOFT iontophoretic system (1.8 mA/cm²) (n=4-6, Mean ±SD).
4. Discussion

The intracorneal deposition of the TA-AA prodrugs showed an unexpected pattern: the iontophoretic delivery was found to not overlap with the experimentally detected electrical mobility of the prodrugs. Double positively charged TA-Arg and TA-Lys were expected to have the highest delivery; however, intracorneal deposition was found comparable to TA-Ala and TA-Gly deposition. Also, in between the remaining single positively charged TA-AA prodrugs, distinct iontophoretic delivery was observed, despite of their similar electrical mobility. This suggested the implication of multiple molecular parameters influencing the iontophoretic transport [37] [28]. In the following paragraphs the physicochemical properties obtained by in silico molecular modelling were related to their effect on iontophoresis.

4.1 2D molecular descriptors vs. intracorneal iontophoresis

Physicochemical properties such as pKa and Log D at pH 5.5 and 7.4 of the tested TA-AA prodrugs were predicted using ACD/Labs software and were summarized in Table 3.

Table 3. TA and TA-AA respective molecular weights, pKa and Log D values at pH 5.5 and pH 7.4 obtained by ACD/Labs software (version 12.01).

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>pKa</th>
<th>Log D_{pH 5.5}</th>
<th>Log D_{pH 7.4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>434.5</td>
<td>12.87, 13.15</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>TA-Ala</td>
<td>505.6</td>
<td>7.47</td>
<td>0.36</td>
<td>1.96</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>590.7</td>
<td>7.08, 13.36</td>
<td>-2.29</td>
<td>-1</td>
</tr>
<tr>
<td>TA-Asn</td>
<td>548.6</td>
<td>5.75</td>
<td>1.03</td>
<td>1.39</td>
</tr>
<tr>
<td>TA-Gln</td>
<td>562.6</td>
<td>6.62</td>
<td>0.2</td>
<td>1.24</td>
</tr>
<tr>
<td>TA-Gly</td>
<td>491.6</td>
<td>7</td>
<td>0.44</td>
<td>1.8</td>
</tr>
<tr>
<td>TA-His</td>
<td>571.7</td>
<td>6.61, 5.11</td>
<td>0.37</td>
<td>1.89</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>547.6</td>
<td>7.58</td>
<td>1.63</td>
<td>3.27</td>
</tr>
<tr>
<td>TA-Lys</td>
<td>562.7</td>
<td>6.90, 10.45</td>
<td>-1.85</td>
<td>-0.81</td>
</tr>
<tr>
<td>TA-Met</td>
<td>565.7</td>
<td>6.56</td>
<td>1.88</td>
<td>2.91</td>
</tr>
<tr>
<td>TA-Val</td>
<td>533.6</td>
<td>7.55</td>
<td>1.15</td>
<td>2.78</td>
</tr>
</tbody>
</table>
**MW**

In the case of transdermal iontophoresis, the inverse relation between iontophoretic delivery and increase of molecular weight of the administered substances was repeatedly evidenced [38] [39] [40]. Even a quantitative relationship in between the transport number vs. molecular weight was established for small cationic molecules with molecular weight up to 223 Da; confirming the tendency of reduced transport with increased size [41]. To compensate this phenomenon for bigger molecules it was shown that the addition of charges was able to overcome the limiting effect of increased MW for the active iontophoretic delivery of peptides [25] as well as for proteins [36] [42].

**LogD**

Another factor reported unfavourable for the iontophoretic transport was the increase of lipophilicity [27]. In several comparative iontophoretic studies of molecules with similar molecular weight and charge (pKa), the lowest delivery was found for those molecules with the highest values of lipophilicity (LogP/D) [43] [44] [45].

In order to rationalize the TA-AA prodrug iontophoretic delivery, deposited TA concentrations following iontophoretic application in the cornea were plotted versus correspondent LogD_{pH 5.5} and MW (Figure 3).
Figure 3. TA intracorneal delivery (nmol/g) by TA-AA iontophoresis (Z-axis) was plotted as a function of Log D at pH 5.5 of the correspondent TA-AA prodrugs (Y axis) and their molecular weight (MW) (X axis). Dotted drop lines to xy plane were added to facilitate the visualization of Log D and MW values.

In Figure 3 groups of TA-AA prodrugs with similar MW, Log D pH 5.5 and intracorneal deposition were evidenced. As example the most successfully deposited prodrugs TA-Ala, TA-Arg, TA-Gly and TA-Lys were clearly divided in two groups. Two (TA-Ala and TA-Gly) were the smallest of all tested prodrugs and with low Log D values (Log D pH 5.5: 0.36 and 0.44, TA-Ala and TA-Gly). The low molecular weight and the high hydrophilicity, might have favoured the transport of these prodrugs. TA-Arg and TA-Lys on the other hand were the biggest prodrugs synthesized (MW: 590.7 and 562.7, TA-Arg and TA-Lys, respectively). However, Log D at pH 5.5 was found to be the lowest since both were 96-97% double positively charged. It could be suggested that the double charge of these TA-AA prodrugs outweigh the increase in molecular weight.

For the TA-AA prodrugs with intermediate molecular weight, lowest delivery was found for the most lipophilic prodrugs (Log D pH 5.5 1.03 to 1.88; TA-Asn, TA-Ile, TA-Met, TA-Val). Followed by the more hydrophilic TA-AA prodrugs TA-Gln and TA-His, which were found to have similar Log D values as TA-
Gly and TA-Ala. Although no direct correlation was found, the hampering effect of elevated Log D and increased molecular weight in iontophoretic delivery was shown.

4.2 Computational modelling of surface properties

For the further understanding and visualization of molecular properties suggested to influence the iontophoretic delivery, TA-AA prodrugs molecular surfaces (Connolly; solvent excluded surface) were created with Sybyl 2.1.1 programme (SYBYL-X 2.1.1, Tripos International, St. Louis, Missouri, USA).

As in the case of the 2D molecular descriptors, no linear relations in between surface properties and the iontophoretic delivery of TA-AA prodrugs were found. However, the closer analysis of the 3D descriptors (numeric and visive) and their relation with literature was used to rationalize the TA-AA prodrugs characteristic iontophoretic delivery.

Lipophilic potential

The lipophilic potential of the TA-AA prodrugs, at their maximal ionization state, was colour coded from brown (lipophilic) to blue (hydrophilic) on correspondent molecular surface (Connolly surface). TA-Ala, TA-Asn, TA-Gln, TA-Gly, TA-Ile, TA-Met and TA-Val were visualized with a single positive charge and TA-Arg, TA-His and TA-Lys with double positive charge. In Figure 4 the prodrugs were divided corresponding to their iontophoretic delivery into the cornea following short iontophoretic application with SOOFT system. The first group is represented by lowest delivery, namely TA-Met, TA-Ile, TA-Val and TA-Asn (~70 to 170 nmol$_{TA}$/g); followed by intermediate delivery of ~300 nmol$_{TA}$/g (TA-Gln and TA-His) and the highest delivery obtained with TA-Arg, TA-Gly, TA-Ala and TA-Lys (~600-700 nmol$_{TA}$/g).
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Figure 4. TA-AA prodrug structures and Connolly surfaces coloured according to the lipophilic potential from brown (most hydrophobic area) to blue (most lipophilic area), obtained with Sybyl 2.1.1 programme. The graph with TA total corneal delivery following iontophoresis was located in the centre of the image to divide the different TA-AA groups accordingly.

In the first line of Figure 4 the TA-AA prodrugs with lowest delivery included the most lipophilic molecules (TA-Met, TA-Ile and TA-Val) and TA-Asn. It seemed surprising to find the latter hydrophilic molecule within the lowest performing prodrugs. The amide sidechain of TA-Asn increased substantially the hydrophilicity of the prodrug, however the proximity of this electron attractive moiety also reduced dramatically the percentage of charged molecules, e.g. at pH 5.7 only ~50% of the molecules were present in charged state (pKa 5.75). This was thought to explain the low delivery rate of this very hydrophilic TA-AA prodrug. As for the remaining TA-AA prodrugs most pronounced hydrophilic areas were found on double positively charged TA-AA prodrugs such as TA-Arg, TA-His and TA-Lys.
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**Charge and electrostatic potential**

As shown above on the example of TA-Asn prodrug the simple consideration of molecules lipophilicity was not exhaustive to rationalize their iontophoretic delivery. The investigation of molecules charge distribution was particularly interesting for the analysis of the superior delivery of TA-Ala and TA-Gly compared to TA-Gln and TA-His. This seemed surprising since the later prodrugs with their polar amino acid moiety seemed excellent iontophoresis candidates. However, TA-Gln and TA-His electron attractive sidechains lowered pKa values on the common amine group compared to the other prodrugs (pKa 6.62 and 6.61 respectively). Approximately 80% of TA-Gln and TA-His were single positively charged. In addition, although TA-His was considered as double positively charged prodrug, with a pKa of 5.11 on the imidazole ring effectively only 20% of the molecules were di-ionized. In comparison, TA-Ala and TA-Gly, which were found as much better charge carriers, 95-99% of the molecules were single positively charged at experimental pH.

Impact of the AA sidechains on the charge surface distribution was visualized by colour coding the electrostatic potential: red for positive charge and purple for negative. It was shown that the positive charge of TA-Ala and TA-Gly occupied 13-15 % of the total surface, compared to the other single positive charged prodrugs where the positive charged area covered approximately 10%. In **Figure 5** TA-Gly\(^+1\) and TA-Gln\(^+1\) are pictured. The notable exposition of the single positive charge of TA-Ala and TA-Gly was supposed to be caused by the lack of shielding sidechains, compared to the bulkier sidechains of TA-Gln and TA-His. The reduced exposure of the charge to the surrounding solution might have contributed to their lower delivery.
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**Figure 5.** Electrostatic potential of TA-Gly and TA-Gln both in monocharged state was displayed on the Connolly molecular surface (Sybyl 2.1.1 programme); increased charge density was displayed from purple to red.

**Electrostatic bond, Dipolar moment and H bond**

In addition to the above described lipophilicity and electrostatic surface parameters, also the possible interactions of the TA-AA prodrugs with the corneal tissue were considered. In contrast to CZE, where the molecules electrical mobility was tested only in buffer, the possible binding with the transport pathway during iontophoretic delivery was reported to hamper the transport of certain molecules [35] [37].

One possible interaction mode with the membrane is the formation of electrostatic bonds. Given the physiological pH 7.39-7.54 [46] of the corneal stroma only TA-Lys and TA-Arg, due to the basic sidechain with pKa above 10, were single positively charged in the tissue. Due to this persistence of charge they were imagined most likely to interact with water soluble negatively charged components of the cornea e.g. glycosaminoglicans, hyaluronic acid [47]. Furthermore, it was specifically reported that the distant location between hydrophobic parts of the molecules and localized charge increased the tendency to bind to tissues [28]. Such is the case for TA-AA prodrugs, where the steroidal part of TA represents the lipophilic side and the amino acid sidechain the charged moiety. One parameter describing the distance of the charges on
the molecular backbone is the dipolar moment; to compare the different TA-AA prodrugs the values for each were listed in Table 4.

Table 4. The dipolar moment (DM) calculated with Sybyl 2.1.1 programme for all ten investigated TA-AA prodrugs at their maximal charged state.

<table>
<thead>
<tr>
<th></th>
<th>TA-Ala⁺¹</th>
<th>TA-Arg⁺²</th>
<th>TA-Asn⁺¹</th>
<th>TA-Gln⁺¹</th>
<th>TA-Gly⁺¹</th>
<th>TA-His⁺²</th>
<th>TA-Ile⁺¹</th>
<th>TA-Lys⁺²</th>
<th>TA-Met⁺¹</th>
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<td>DM</td>
<td>40.92</td>
<td>74.45</td>
<td>33.46</td>
<td>36.28</td>
<td>31.42</td>
<td>51.80</td>
<td>32.83</td>
<td>84.53</td>
<td>35.14</td>
<td>37.80</td>
</tr>
</tbody>
</table>

As can be seen in Table 4, highest dipolar moments were found for the double positively charged prodrugs TA-His and especially TA-Arg and TA-Lys. The distance of the guanidine and the second amine group of TA-Arg and TA-Lys from the steroidal backbone were measured around 12-15Å with Sybyl 2.1.1 programme, whereas the common amino acid amine group was found of a medium distance of 9 Å for all TA-AA prodrugs. This dislocation of the hydrophobic part and the positive charge of TA-Lys and TA-Arg could be one of the factors that eventually decreased the delivery, despite the outstanding electrical mobility detected in CZE, and equalled it to the single positively charged prodrugs such as TA-Ala and TA-Gly.

Another type of interaction which could be imagined with the tissue is the formation of H-bonds. This was thought to be more pronounced for polar sidechain TA-AA prodrugs such as TA-Asn, TA-Gln and TA-His. Given the abundant heteroatoms on their sidechains, the number of H bond acceptors and donors increased. In Figure 6 HB donor surface area of TA-Gly and TA-Gln were compared and found almost doubled for TA-Gln. Accordingly, the hypothesis was that those sidechains if polar (TA-As, TA-Gln and TA-His) or charged (TA-Arg and TA-Lys), were prone to make interactions with the tissue which reduced their delivery.
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Figure 6. H-bond donor surface area displayed on a) TA-Gly and b) TA-Gln Connolly surface with Sybyl 2.1.1 programme.

4.3 TA-Gly depth distribution following intracorneal iontophoresis

Finally, the actual applicability of the novel TA-AA prodrugs with the modified SOOFT iontophoresis system was investigated. Despite the above suggested explanations for the individual TA-AA prodrugs iontophoretic delivery, most importantly was to confirm the utility of the combination with the SOOFT system. The intracorneal distribution of TA-Gly, one of the most successful prodrugs, was investigated to see if effective depth penetration of the active principle into the tissue was achieved. For each lamella the amount of TA deposited was analysed and assuming tissue density equal to 1 expressed as local concentration. In all the corneal lamellae the concentration of TA found was found at least $10^4$ superior to e.g. reported concentration for glucocorticoid receptor binding affinity ($IC_{50} 1.45 \text{nM}$) or glucocorticoid receptor transactivation ($EC_{50} 1.5 \text{nM}$) \cite{48} or also their anti-inflammatory effect by the repression of TNF-\alpha ($IC_{50} 0.76 \text{nM}$) and CD40L ($IC_{50} 0.43 \text{nM}$) induced activation of proinflammatory transcription factors such as nuclear factor-kB \cite{49}, which all were in the nanomolar range. These findings strengthened our initial assumption on the utility of short time iontophoresis with the SOOFT system for the deep treatment of the cornea with the corticosteroid TA via TA-AA prodrugs.
5. Conclusion

The comparison of intracorneal deposition following short term iontophoresis of ten TA-AA prodrugs, which only differentiated by their sidechain, allowed to overview the importance of molecular properties during the iontophoretic transport. Amino acids from the apolar, polar and basic classes were chosen to cover a broad spectrum of physicochemical properties. Influence of the AA charge was immediately seen in the increase of aqueous solubility from 100-1000 folds compared to the parent drug. The added charge enabled prodrug depending delivery into the cornea: for example, lowest delivery was found with the most lipophilic (TA-Met, TA-Ile, TA-Val) and partially ionized prodrug TA-Asn (~70 to 170 nmolTA/g). Concordantly with previously published studies the negative influence of lipophilicity and increased molecular weight on the iontophoretic transport was once more suggested. Double positively charged prodrugs such as TA-Arg and TA-Lys were within the best delivered prodrugs (<600 nmolTA/g), although outstanding delivery was not obtained, which would have been suggested by the preliminary CZE electrical mobility. The key for this was thought to lay in the possible interaction of the molecules with the membrane by electrostatic and H bonds. TA-Ala and TA-Gly, two single positively charged prodrugs were found as most successfully delivered prodrugs (<600 nmolTA/g). Their low tendency to bind with the tissue (H-bond area, low dipolar moment), high percentage of charged molecules (pKa), small molecular weight and most globular structure made them excellent candidates for the iontophoretic transport. These findings showed the synergy of several physicochemical properties playing important role in the iontophoretic delivery of drugs. Even though no linear correlations were established, key factors in the choice of drug candidates were evidenced which should be considered for the selection of a substrate for future experiments.
6. Supplementary data

6.1 TA-AA synthesis

TA-AA synthesis protocol and TA-Arg and TA-Lys structural characterization by proton and carbon nuclear magnetic resonance (NMR) and mass spectrometric (HRMS) analysis were previously reported [21].

TA-Ala, TA-Asn, TA-Gln, TA-Gly, TA-His, TA-Ile, TA-Met and TA-Val carbon and hydrogen NMR analysis as well as HRMS are listed below:

**TA-Ala**

Yield: 92%; $^1\text{H NMR}$ (300 MHz, DMSO-d$_6$) $\delta$ 8.37 (s, 3H), 7.30 (d, $J$ = 9 Hz, 1H), 6.24 (d, $J$ = 9 Hz, 1H), 6.02 (s, 1H), 5.56 (d, $J$ = 6 Hz, 1H), 4.94 (d, $J$ = 18 Hz, 1H), 4.88 (d, $J$ = 6 Hz, 1H), 4.30 (q, $J$ = 3, 1H), 4.20 (bs, 2H), 2.63 (m, 1H), 2.35 (m, 2H), 2.04-1.54 (m, 6H), 1.50 (t, $J$ = 3, 3H), 1.49 (s, 3H), 1.36 (s, 3H), 1.32 (m, 1H), 1.16 (s, 3H), 0.84 (s, 3H); $^{13}\text{C NMR}$ (300 MHz, DMSO-d$_6$) $\delta$ 203.55, 185.85, 170.56, 167.24, 153.09, 129.75, 124.95, 111.71, 102.85-100.52, 97.70, 81.90, 71.03-70.55, 69.06, 48.55-48.25, 48.34, 45.98, 43.52, 36.70, 33.71, 33.19-32.94, 30.76, 28.19, 26.96, 25.99, 23.53, 16.71, 16.75; HRMS (ESI) calculated for C$_{27}$H$_{37}$FNO$_7$ [M+H]$^+$: 506.2549, found: 506.2549.

**TA-Asn**

Yield: 92%; $^1\text{H NMR}$ (600MHz, DMSO-d$_6$) $\delta$ 8.39 (s, 3H), 7.71 (s, 1H), 7.30 (d, $J$ = 12 Hz, 1H), 7.28 (s, 1H), 6.24 (d, $J$ = 12Hz, 1H), 6.02 (s,1H), 5.51 (d, $J$ = 6Hz, 1H), 5.32 (d, $J$ = 18Hz, 1H), 4.88 (d, $J$ = 6Hz, 1H), 4.87 (d, $J$ = 18Hz, 1H), 4.46 (bs, 1H), 4.21 (bs, 1H), 2.82 (m,2H) 2.62-2.35 (m, 3H), 2.06-1.55 (m, 6H), 1.49 (s,3H), 1.36 (s,3H), 1.34 (m,1H), 1.17 (s,3H), 0.84 (s,3H); $^{13}\text{C NMR}$ (600 MHz, DMSO-d$_6$) $\delta$ 203.61, 185.85, 170.56, 167.24, 153.09, 129.75, 124.95, 111.71, 102.85-100.52, 97.70, 81.90, 71.03-70.55, 69.06, 48.55-48.25, 48.34, 45.98, 43.52, 36.70, 33.71, 33.19-32.94, 30.76, 28.19, 26.96, 25.99, 23.53, 16.71, 16.75; HRMS (ESI) calculated for C$_{28}$H$_{38}$FN$_2$O$_8$ [M+H]$^+$: 549.3, found: 549.2.

**TA-Gln**

Yield: 91%; $^1\text{H NMR}$ (600MHz, DMSO-d$_6$) $\delta$ 8.47 (s, 3H), 7.43 (s, 1H), 7.30 (d, $J$ = 12Hz, 1H), 6.98 (s, 1H), 6.25 (d, $J$ = 12Hz, 1H), 6.03 (s, 1H), 5.53 (d, $J$ = 5Hz, 1H), 5.34 (d, $J$ = 18Hz, 1H), 4.90 (d, $J$ = 18Hz, 1H), 4.88 (d, $J$ = 5Hz, 1H), 4.28 (bs, 1H), 4.19 (bs, 1H), 2.60 (m,1H), 2.37 (m, 4H), 2.11-1.55 (m,
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8H), 1.49 (s, 3H), 1.36 (s,3H), 1.34 (m, 1H), 1.17 (s, 3H), 0.84 (s, 3H); $^{13}$C NMR (600 MHz, DMSO-d$_6$) δ 203.79, 186.08, 174.01, 170.12, 167.45, 153.30, 130.00, 125.20, 111.99, 102.50-101.34, 98.00, 82.17, 70.47- 69.43, 65.85, 52.34, 48.72-48.57, 46.23, 43.76, 36.97, 33.96, 33.39-33.26, 31.00, 30.86, 28.38, 27.23, 26.90, 26.33, 23.76-23.72, 17.02; MS (ESI) calculated for C$_{29}$H$_{40}$FN$_2$O$_8$ [M+H]$^+$: 563.3, found: 563.2.

**TA-Gly:** $^1$H NMR (300 MHz, DMSO-d$_6$) δ 8.33 (s, 3H), 7.30 (d, J = 9 Hz, 1H), 6.24 (d, J= 9 Hz, 1H), 6.02 (s, 1H), 5.55 (d, J = 6 Hz, 1H), 5.35 (d, J = 18 Hz, 1H), 4.90 (d, J = 18 Hz, 1H), 4.87, (d, J= 6 Hz, 1H) 4.21 (bs, 1H), 4.03 (s, 2H), 2.64 (m, 1H), 2.33 (m, 2H), 2.08-1.57 (m, 6H), 1.49 (s, 3H), 1.36 (s, 3H), 1.32 (m, 1H), 1.16 (s, 3H), 0.82 (s, 3H); $^{13}$C NMR (300 MHz, DMSO-d$_6$) δ 203.53, 185.86, 168.10, 167.25, 153.14, 129.76, 124.96, 111.71, 102.84-100.51, 97.71, 81.90, 71.04-70.58, 69.02, 48.55-48.25, 45.94, 43.53, 40.56, 36.67, 33.69, 33.21-32.95, 30.76, 28.17, 26.96, 25.99, 23.45, 16.83; HRMS (ESI) calculated for C$_{26}$H$_{35}$FNO$_7$ [M+H]$^+$: 492.2393, found: 492.2392.

**TA-His Yield:** 42%; $^1$H NMR (600MHz, DMSO-d$_6$) δ 14.17 (bs, 1H), 8.56 (bs, 3H), 7.40 (s, 1H), 7.39 (s, 1H), 7.31 (d, J = 12Hz, 1H), 6.25 (d, J= 12Hz, 1H), 6.03 (s, 1H), 5.55 (d, J= 6Hz, 1H), 5.35 (d, J= 18, 1H), 4.93 (d, J= 18, 1H), 4.88 (d, J= 6, 1H), 4.55 (bs, 1H), 4.22 (bs, 1H), 3.23 (m, 2H), 2.61 (m, 1H), 2.34 (m 2H) 2.07-1.56 (m, 6H), 1.49 (s, 3H), 1.36 (s, 3H), 1.35 (m, 1H), 1.14 (s, 3H), 0.83 (s, 3H); $^{13}$C NMR (600 MHz, DMSO-d$_6$) δ 203.70, 186.08, 169.13, 167.40, 158.70, 153.28, 135.75, 134.96, 130.00, 125.22, 112.00, 102.49-101.32, 97.93, 82.15, 71.14-70.90, 65.84, 55.63, 48.71, 46.20, 43.77, 36.95, 33.94, 33.38-33.25, 31.29, 28.42, 27.20, 26.23, 24.64, 23.71, 17.03; MS (ESI) calculated for C$_{30}$H$_{39}$FN$_3$O$_7$ [M+H]$^+$: 572.3, found: 572.2.

**TA-Ile:** $^1$H NMR (300 MHz, DMSO-d$_6$) δ 8.38 (s, 3H), 7.31 (d, J = 12 Hz, 1H), 6.25 (d, J= 12 Hz, 1H), 6.03 (s, 1H), 5.52 (d, J = 6 Hz, 1H), 5.32 (d, J = 18 Hz, 1H), 4.93 (d, J = 18 Hz, 1H), 4.88, (d, J= 6 Hz, 1H) 4.20 (bs, 2H), 2.59 (m, 1H), 2.36(m, 2H), 2.08-1.53 (m, 8H), 1.49 (s, 3H), 1.36 (s, 3H), 1.32 (m, 2H), 1.16 (s, 3H), 1.01 (d, J = 6 Hz, 3H), 0.92 (t, J = 9 Hz, 3H), 0.82 (s, 3H); $^{13}$C NMR (300 MHz, DMSO-d$_6$) δ
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203.63, 185.84, 169.78, 167.24, 153.09, 129.73, 124.95, 111.70, 102.86-100.53, 97.79, 81.88, 71.02-70.53, 69.00, 56.94, 48.56-48.26, 45.92, 43.49, 37.16, 36.68, 33.71, 33.20-32.94, 30.76, 28.21, 26.98, 26.05, 25.47, 23.46, 16.69, 14.71, 12.36; HRMS (ESI) calculated for C_{30}H_{42}FNO_{7} [M+H]^+: 548.3018, found: 548.3017.

**TA-Met Yield:** 52%;^1^H NMR (600MHz, DMSO-d_6) δ 8.47 (s, 3H), 7.30 (d, J= 12Hz, 1H), 6.24 (d, J=12Hz, 1H), 6.02 (s, 1H), 5.54 (d, J= 6Hz, 1H), 5.34 (d, J= 18Hz, 1H), 4.95 (d, J= 18Hz, 1H), 4.89 (d, J=6Hz, 1H), 4.33 (bs, 1H), 4.21 (bs, 1H), 2.67 (m,2H), 2.62 (m,1H), 2.45 (m, 1H), 2.35 (m,1H), 2.14 (m, 2H), 2.07 (s, 3H), 2.05-1.54 (m, 6H), 1.49 (s, 3H), 1.36 (s, 3H), 1.34 (m, 1H), 1.16 (s, 3H), 0.84 (s, 3H);^1^C NMR (600 MHz, DMSO-d_6) δ 203.98, 186.08, 169.99, 167.45, 153.31, 129.99, 125.20, 111.99, 102.50-101.34, 97.99, 82.16, 71.15-70.91, 69.47, 51.72, 48.72-48.57, 46.22, 43.77, 36.94, 33.95, 33.39-33.26, 31.01, 30.77, 29.02, 28.43, 27.22, 26.26, 23.76-23.72, 16.97, 15.16; MS (ESI) calculated for C_{29}H_{41}FNO_{7}S [M+H]^+: 566.3, found: 566.2.

**TA-Val Yield:** 81%;^1^H NMR (300 MHz, DMSO-d_6) δ 8.39 (s, 3H), 7.30 (d, J = 9 Hz, 1H), 6.24 (d, J= 9 Hz, 1H), 6.02 (s, 1H), 5.54 (d, J= 6 Hz, 1H), 5.33 (d, J= 18 Hz, 1H), 4.94 (d, J= 18 Hz, 1H), 4.88, (d, J= 6 Hz, 1H) 4.20 (bs, 2H), 2.63 (m, 1H), 2.34 (m, 3H), 2.08-1.57 (m, 6H), 1.49 (s, 3H), 1.36 (s, 3H), 1.32 (m, 1H), 1.16 (s, 3H), 1.06 (t, 6H), 0.82 (s, 3H);^1^C NMR (300 MHz, DMSO-d_6) δ 203.63, 185.85, 169.40, 167.24, 153.09, 129.74, 124.96, 111.70, 102.87-100.54, 97.75, 81.89, 71.00-70.51, 69.15, 57.65, 48.55-48.25, 45.93, 43.51, 36.68, 33.70, 33.19-32.93, 30.76, 30.25, 28.20, 26.97, 26.00, 23.46, 18.47, 18.09, 16.69; HRMS (ESI) calculated for C_{29}H_{41}FNO_{7} [M+H]^+: 534.3, found: 534.3.
6.2 Validation of HPLC-UV method for the quantification of TA and TA-AA

6.2.1 Specificity, linearity, LOD and LOQ

The method specificity and linearity were established in a six point calibration curve in a concentration range from 1.41 to 230.15 nmol/ml; $R^2$ was found consistently in between 0.98-1. The limit of detection (LOD) and quantification (LOQ) were established according to ICH Q2 (R1) guidelines [33].

**Table S1.** Retention time and calculated LOQ and LOD for TA and TA-AA prodrugs

<table>
<thead>
<tr>
<th></th>
<th>Retention time (min)</th>
<th>LOQ (nmol/ml)</th>
<th>LOD (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>8.3</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>3.3</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td>TA-Lys</td>
<td>2.9</td>
<td>2.27</td>
<td>0.84</td>
</tr>
<tr>
<td>TA</td>
<td>4.74</td>
<td>2.01</td>
<td>0.66</td>
</tr>
<tr>
<td>TA-Asn</td>
<td>2.6</td>
<td>3.35</td>
<td>1.10</td>
</tr>
<tr>
<td>TA-Gln</td>
<td>2.8</td>
<td>1.78</td>
<td>0.59</td>
</tr>
<tr>
<td>TA-His</td>
<td>2.2</td>
<td>2.58</td>
<td>0.85</td>
</tr>
<tr>
<td>TA-Met</td>
<td>6.49</td>
<td>1.80</td>
<td>0.59</td>
</tr>
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<td>TA</td>
<td>7.4</td>
<td>1.58</td>
<td>0.52</td>
</tr>
<tr>
<td>TA-Ala</td>
<td>5.7</td>
<td>2.79</td>
<td>0.92</td>
</tr>
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<td>TA-Gly</td>
<td>5.1</td>
<td>3.52</td>
<td>1.16</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>10.8</td>
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</tr>
<tr>
<td>TA-Val</td>
<td>8.4</td>
<td>3.08</td>
<td>1.02</td>
</tr>
</tbody>
</table>

6.2.2 Precision and accuracy

The intra and inter-day precision and accuracy were evaluated by the repeated analysis of 3 standard solutions. Results of accuracy and precision are listed in **Table S2** and were found to be included within the acceptance limits.

**Table S2.** Intra-and inter-day precision and accuracy for TA and TA-AA quantification method
## CHAPTER III: Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport

<table>
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<th>Theoretical (nmol/ml)</th>
<th>Measured (nmol/ml)</th>
<th>RSD (%) ab</th>
<th>Accuracy (%) bc</th>
<th>Measured (nmol/ml)</th>
<th>RSD (%) ab</th>
<th>Accuracy (%) bc</th>
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<td>TA</td>
<td>230.15</td>
<td>229.59 ± 1.88</td>
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<td>228.92 ± 1.75</td>
<td>0.76</td>
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<td>11.51</td>
<td>11.85 ± 0.10</td>
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<td>230.45 ± 1.24</td>
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<td>45.82 ± 0.16</td>
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<td>152.21 ± 0.39</td>
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<td>30.18</td>
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<td>7.30</td>
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CHAPTER III: Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport

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\( RSD = \frac{SD}{\text{mean}} \times 100 \)

\( \text{Accuracy} = \frac{\text{obtained concentration}}{\text{theoretical concentration}} \times 100 \)

7. Acknowledgments

We would like to thank very warmly our colleagues, Prof. S. Rudaz, Dr. J. Schappler and J. Jacquat of the School of Pharmaceutical Sciences (University of Geneva) for the help with performing the capillary zone electrophoresis studies. V. Santer would like to thank F. Tessaro for the constructive exchange and help with the in silico modelling software. We are extremely grateful to SOOFT Italia S.p.A. company and in specific Dr. D. Rusciano for providing us with their iontophoretic device (power supply (I-ON CXL) and corneal application system (Iontofor CXL)).

8. References


CHAPTER III: Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport


CHAPTER III: Intracorneal delivery of triamcinolone acetonide prodrugs: evaluation of physicochemical parameters governing the iontophoretic transport


CHAPTER IV

- Characterization of triamcinolone acetonide amino acid prodrugs
  iontophoretic transport into the posterior segment of the eye
Characterization of triamcinolone acetonide amino acid prodrugs iontophoretic transport into the posterior segment of the eye

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Abstract

In this study short duration transscleral iontophoresis of four triamcinolone acetonide amino acid ester prodrugs (TA-AA) (alanine, Ala; arginine, Arg; isoleucine, Ile and lysine, Lys) was investigated \textit{in vitro} using whole porcine eyes globes. Post-iontophoretic drug biodistribution was quantified by UHPLC-MS/MS in the different ocular components (cornea, aqueous humor, sclera, ciliary body, choroid and retinal pigmented epithelium (RPE), neural retina and vitreous humor). Transscleral iontophoresis (10 min at 3 mA/cm\textsuperscript{2}) resulted in a significant increase in total drug delivery, 14 to 30-fold compared to passive for the TA-AA prodrugs. However, not all TA-AA prodrugs achieved similar depth distribution; this was related to their distinct physicochemical properties. The deep tissue distribution of the TA-AA prodrugs was hindered by reduced hydrolytic stability (TA-Arg) or lipophilicity (TA-Ile). Intraocular drug distribution was influenced by prodrug binding to melanin (TA-Lys). Interestingly, under conditions of equivalent
charge (6 mA/cm\(^2\) for 5 min vs. 1.5 mA/cm\(^2\) for 20 min, (equivalent to 1.44C), respectively) the longer treatment duration (20 min) enabled delivery of ~6 times more TA into the vitreous humor. Overall the study evidenced the potential of transscleral TA-AA iontophoresis for the non-invasive treatment of posterior segment inflammatory diseases.

**Keywords:** Transscleral iontophoresis, triamcinolone acetonide, prodrugs, eye globe biodistribution
1. Introduction

Intravitreal injections of corticosteroids such as triamcinolone acetonide (TA) have been routinely employed over last 15-20 years for the treatment of major inflammatory and angiogenic posterior eye segment diseases such as exudative age related macular degeneration, diabetic macular edema or retinopathy and uveitis [1] [2]. The serious consequences of those conditions - ranging from major vision impairment to blindness - and the lack of alternatives accounted for the routine use of the invasive procedure despite the elevated risk of severe complications and low patient compliance [3]. Given the complex structure of the eye globe, the organ challenges considerably the conventional approaches for topical or systemic drug administration [4]. Topically instilled formulations are promptly diluted by the lacrimal fluid (1.2 µl/min in humans) and drained by the conjunctiva and nasal mucosa, whereas systemic administration is limited by the large doses needed given the efficacy of the blood retinal barrier which isolates the eye from the systemic blood flow [4, 5].

Transscleral iontophoresis has been intensively studied as an innovative delivery strategy to overcome the ocular barrier system [6] [7]. The sclera is mainly composed of a highly hydrated loose net of collagen fibrils and proteoglycans, as a consequence even the passive permeability of hydrophilic molecules was found to be quite elevated [8, 9]. When combined with iontophoresis, molecular transport across the large and accessible surface was such that, despite the different ocular barriers, it was possible to achieve therapeutically relevant drug concentrations in the posterior segment [10]. This is important since for effective treatment, therapeutic amounts of drug must cross not only the sclera but also the inner ocular static barriers such as the choroid and retinal pigmented epithelium as well as overcome dynamic barriers such as the blood flow [4].

Iontophoresis, an active drug delivery technique, uses the application of a mild current to enhance substantially the transport of charged and uncharged molecules into and across biological tissues [11]. Successful transscleral delivery of dexamethasone phosphate via iontophoresis were shown in clinical
studies for the treatment of dry eye and noninfectious anterior uveitis by the transscleral iontophoretic applicator (EyeGate® II delivery system) developed by EyeGate Pharmaceuticals Inc [12] [13]. However, the drug distribution in the whole eye globe has been rarely studied – there are empiric observations – but few reports presenting the analytical quantification of drug present in the different compartments[14]. Therefore, the aim of this study was to investigate the ocular biodistribution of biolabile prodrugs linking the distribution profile to the molecules physicochemical properties. The iontophoretic delivery of prodrugs in particular amino acid prodrugs was mostly investigated on the skin [15] [16] [17] [18] [19]. There are fewer reports on prodrug iontophoresis in the eye. The prodrug species most frequently encountered in these cases were either succinate [20] [21][22] or phosphate ester [13] [23] prodrugs of methylprednisolone or dexamethasone.

In our group we recently introduced the concept of novel positively charged amino acid prodrugs of triamcinolone acetonide (TA; TA-AA) for corneal iontophoresis [24]. The combination of dramatically increased aqueous solubility and the introduction of ionized groups made these conjugates excellent candidates for iontophoretic delivery. In the present study, four amino acid prodrugs of TA: alanine, arginine, isoleucine and lysine esters (TA-Ala, TA-Arg, TA-Ile and TA-Lys) were synthesized and characterized. Their ocular biodistribution profiles following short duration iontophoresis were determined and correlated to their different physicochemical properties. Finally, the effect of the current application profile was investigated by comparing delivery after iontophoresis at 6 mA/cm² for 5 min and 1.5 mA/cm² for 20 min – i.e. an equal amount of total charge was applied, 30 mA min/cm² (1.44 C), but in the first, higher current for short duration whereas as second a more sustained delivery at a lower current density. In addition to the total amount of TA-Ala delivered, the effect on the biodistribution profile was also investigated.
2. Material and Methods

2.1 Materials

TA-AA prodrugs of L-alanine, L-arginine, L-isoleucine and L-lysine (TA-Ala, TA-Arg, TA-Ile and TA-Lys) were synthesized in-house by adapting previously published protocols \cite{18} \cite{24}. TA, liquid paraffin and sodium carboxymethylcellulose were purchased from Haenseler AG (Herisau, Switzerland). The buffer salts: sodium and potassium chloride (NaCl, KCl, respectively), sodium and potassium phosphate, 2-morpholino-ethanesulfonic acid monohydrate (MES)) as well as Polysorbate 80 were obtained from Fluka (Buchs, Switzerland). Melanin from *Sepia officinalis* and silver chloride (AgCl) used for the construction of the silver electrodes, were acquired from Sigma-Aldrich (Steinheim, Germany). HPLC grade solvents (HiPerSolv Chromatonorm; Darmstadt, Germany) and deionized water were used to prepare all the solutions (Millipore Milli-Q Gard 1 Purification Pack resistivity > 18MΩcm, Zug, Switzerland). Trifluoroacetic acid (TFA; 99 % extra pure) and glycerol were obtained from Acros Organics (Geel, Belgium). ULC/MS grade formic acid was bought from Brunschwig (Basel, Switzerland).

2.2 Analytical methods

2.2.1 *HPLC-UV analysis of TA and TA-AA*

The UltiMate 3000 HPLC-UV system (formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach Switzerland) equipped with HPG-3200SD pump, WPS-3000 auto sampler and VWD-3400 VIS/UV detector, was used to quantify all tested drug entities. Chromatographic separation was achieved with an isocratic elution using water + 0.1% TFA (A) and acetonitrile + 0.1% TFA (B) mobile phase solvents. Compound signal peaks were integrated using Chromeleon® (version 6.8) software. The analytical conditions for the detection and quantification of TA and TA-AA prodrugs are provided in the *Supplementary Material*. The respective limit of quantification (LOQ) and limit of detection (LOD) were calculated following the ICH Q2 (R1) guideline \cite{25}. The methods were validated and showed suitable intra-day precision and accuracy.
2.2.1 *UHPLC MS/MS quantification of TA*

An UHPLC-MS/MS method for the quantification of TA in the different ocular tissues was validated adapting a previously published protocol [24]. The Waters Acquity® UPLC® system (Baden-Dättwil, Switzerland) included a binary solvent pump, sample manager and Waters XEVO® TQ-MS detector (Baden-Dättwil, Switzerland). Calibration curves were prepared in matrix over a concentration range of 11.5 – 2301.5 pmol$_{TA}$/ml and accuracy and precision were evaluated according to the ICH guidelines [25] (Supplementary Material). The LOD and LOQ for TA were 4.1 and 12.4 pmol$_{TA}$/ml, respectively.

2.3 Prodrug characterization

TA-AA prodrugs pKa, and LogD$_{pH5.5}$ were determined by ACD/Labs software (version 12.01). In addition, a surface conformational analysis of TA and TA-AA prodrugs was performed using the Maestro programme, a tool from the Schrödinger 2013 package (Maestro; Schrödinger, LLC: Portland, OR, 2009). The reference structure of TA was used [ZINC3875481]. Energy minimisations were performed in water on TA and TA-AA prodrug in charged (M+H$^+$ or M+2H$^+$) and uncharged state using the OPLS-2005 force-field (10,000 iterations, 0.05 kcal/mol conjugation gradient). Lipophilic potential surface, polar surface area and dipolar moment of TA and TA-AA were measured and visualized using Sybyl 2.1.1 programme (SYBYL-X 2.1.1, Tripos International, St. Louis, Missouri, USA).

2.3.1 Solubility and ester bond stability

Aqueous solubility of the TA-AA prodrugs was established experimentally in conditions similar to the donor compartment composition e.g. 10 mM MES buffer (pH 5.5). An excess of prodrug was added to the buffer solution and kept under sonication for 10 min, followed by the analysis via HPLC-UV of the supernatant.

The stability of the prodrug ester linkage was investigated at 37 °C, at pH 5.5 and at pH 7.4 in contact with freshly isolated ocular tissue (sclera, ciliary body, choroid/retina and vitreous humor). Porcine eye globes.
were collected promptly after slaughter and the different component tissues were separated and harvested within 4 h of receiving the fresh eye globes. Then, an equal weight of the different tissues (~100 mg) was added to 2 ml PBS solution containing the TA-AA prodrugs (23 μM) under agitation. Samples were withdrawn every 10-30 min and immediately analyzed by HPLC-UV. Based on the slope of the regression curve the first order rate constant of hydrolysis (k_{obs}) was estimated and the half-life (t_{1/2}) (t_{1/2} = \ln(2)/k_{obs}) calculated [24] [18]. All experiments were performed in triplicate.

2.3.2 Melanin binding

In vitro binding studies of TA-AA prodrugs to melanin from Sepia officinalis were performed following previously reported protocols [26] [27] [28]. 1.5 mg of melanin was weighed and suspended in citrate buffer pH 5 or PBS pH 7.4 under sonication for 10 min; drug solutions in PBS were added consecutively to a total volume of 1.5 ml. TA-AA concentrations in between 0.0115-0.23 mM for TA-Arg, TA-Ala and TA-Lys and 0.0023-0.023 mM for TA and TA-Ile were tested (in triplicate). The mixture was kept under continuous stirring for 1 h at room temperature. At the end of incubation period, the supernatant containing unbound TA and TA-AA prodrugs was separated by 15 min centrifugation at 10,000 rpm from the melanin-drug complex and analyzed by HPLC-UV or UHPLC-MS/MS.

2.4 Porcine ocular tissue characterization

Given the lack of dynamic barriers in in vitro experimental set-ups, the choice of an adequate animal model is crucial. In the case of this study, freshly excised porcine eye globes from 6-month-old pigs with a medium weight of 100-120 kg were selected. The eye globes were provided by the local slaughterhouse (Abattoir de Loëx Sarl; Loëx, Switzerland). Scleral thickness and dimensions of eye bulbs were measured and compared to reported data on porcine, human and rabbit eye globes. Results were consistent with previous reports and confirmed the comparability to the human eye (Table 1) [29] [30]. Moreover, in terms of anatomical similarity, the rabbit eye globe which currently is the most commonly used, was shown to be anatomically dissimilar and therefore was not considered in this study. Porcine eyes were used for the
experiments within a few hours after receiving the explant to ensure the integrity of static barriers. (e.g. RPE vitality in explanted eye ~ 2 h [31] to 4 h [32], corneal integrity ~ 6h [33]).

Table 1. Summary of experimentally measurements of porcine eye globes compared with literature reported values on porcine, human and rabbit eyes.

<table>
<thead>
<tr>
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<th>Experimental Porcine eye globe</th>
<th>Porcine eye globe</th>
<th>Literature Human eye globe</th>
<th>Rabbit eye globe</th>
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<tr>
<td></td>
<td>Axial length (mm)</td>
<td>24.81 ± 0.47</td>
<td>23.17 to 24 [34] [35] [36] [37]</td>
<td>7 [35] to 16-19 [40] [41]</td>
</tr>
<tr>
<td>Whole eye globe</td>
<td>Horizontal length (mm)</td>
<td>31.27 ± 3.21</td>
<td>23.5 to 24.9 [42]</td>
<td>18-20 [41]</td>
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<tr>
<td></td>
<td>Weight (g)</td>
<td>6.57 ± 0.499</td>
<td>8.01 ± 0.08 [34]</td>
<td>~7 [34]</td>
</tr>
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<td></td>
<td>Limbus (mm)</td>
<td>1.37 ± 0.24</td>
<td>0.91 [45] to 1.12 [46]</td>
<td>0.29 ± 0.02 [48]</td>
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<td>Scleral thickness</td>
<td>Equator (mm)</td>
<td>0.56 ± 0.07</td>
<td>0.58 [45] to 0.86 [46]</td>
<td>0.2-0.25 [46]</td>
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<td>Posterior pole (mm)</td>
<td>1.14± 0.11</td>
<td>0.8-0.9 [46] to 1[45]</td>
<td>0.33 ± 0.02 [48]</td>
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</tbody>
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2.5 TA transscleral delivery in porcine eye globes

2.5.1 Passive delivery experiments

Adhering muscular tissue was carefully removed from the sclera and the entire eye globe was introduced in a custom made vertical Franz diffusion cell. The diffusion cells were designed to embed 1/3 of the eye globe in a hemispheric glass cup attached to a receiving compartment filled with 20 ml PBS. A donor compartment with permeation area of 0.8 cm² was applied on the limbo-equatorial area of the sclera. The position was chosen following the thickness evaluation of porcine sclera, which resulted in this being the most anatomically similar region to its human counterpart. Passive transscleral delivery of TA from formulations made in-house and based on commercial formulations, e.g. Cidermex® (UCB Pharma France S.A) ophthalmic ointment prepared without neomycin sulphate and Kenacort®-A40 (Bristol-Myers Squibb AG; Baar, Switzerland) suspension, was compared to the TA-AA solutions. The compositions of the
formulations are summarized in Table 2. The pH of the TA-AA prodrug solutions was chosen to be within the buffer range of MES (pH 5.5-6.7) and following previously reported transscleral iontophoresis studies in the rabbit in vivo [49].

After an application time of 10 min, the formulations were removed, the permeation area cleaned with cotton swabs and protected by a surgical tissue. The eye globes were individually wrapped and frozen at -80 °C for 24h.

Table 2. Summary of the formulations tested in passive transscleral delivery experiments on entire porcine eye globes.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Active Principle</th>
<th>Concentration (mM)</th>
<th>Excipients</th>
<th>Applied amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA ointment (adapted Cidermex)</td>
<td>TA</td>
<td>2.3</td>
<td>6.1% liquid paraffin + q.s. vaseline</td>
<td>1ml</td>
</tr>
<tr>
<td>TA suspension (adapted Kenacort A 40)</td>
<td>TA</td>
<td>92</td>
<td>0.65% sodium chloride + 0.75% carboxymethylcellulose sodium + 0.04 % polysorbate 80 + q.s. deionized water</td>
<td>1ml</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>TA-Ala</td>
<td>2.3</td>
<td>10 mM MES + 2.2% glycerine (pH 5.7) + q.s deionized water</td>
<td>1ml</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>TA-Lys</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5.2 Transscleral anodal iontophoresis experiments

In the case of iontophoretic delivery of TA-AA prodrugs, conventional silver/silver chloride (Ag/AgCl) electrodes were added to the whole eye globe set-up described above. The Ag anode was connected to the donor compartment via saline bridges (3% agarose + 0.1 M NaCl) to reduce the electromigratory competition from sodium ions. The cathode (AgCl) was directly inserted in the receiving compartment through the sampling arm. Transscleral iontophoresis of TA-Ala, TA-Arg, TA-Ile and TA-Lys using the whole eye globe was carried out for 10 min at a current density of 3 mA/cm² applied by a constant current power generator (APH 1000M, Kepco Inc; Flushing NY, USA). After the iontophoretic treatment, the
formulation was removed and the permeation area was cleaned with cotton swabs. The eyes were either frozen immediately at -80 °C or kept 1h at room temperature before freezing. In addition to these experiments, TA-Ala intraocular biodistribution was also studied following the application of 6 mA/cm² for 5 min and 1.5 mA/cm² for 20 min (equivalent total charge: 1.44 C); both treatments were followed by immediate freezing of the eye globes. All experiments were performed at least in quadruplicate.

2.5.3 Whole eye biodistribution tissue preparation

The treated porcine eye globes were processed after storage for 24 h at – 80°C while still in a frozen state. This procedure has been previously reported to permit the separation of cornea, aqueous humor, ciliary body, sclera, choroid + retinal pigmented epithelium (RPE), neural retina and vitreous humor while limiting the risk of cross-contamination [50]. The isolated tissues were then weighted (average of the tissue weight is shown in the Supplementary Material) and TA was extracted in 50:50 methanol/water solvent mixture over night under agitation. The following day, the samples were centrifuged and filtered through Extrapure® nylon syringe filters with pore size of 0.22 µm (Alys Technologies; Bussigny-près-Lausanne, Switzerland) and analyzed either by HPLC-UV or UHPLC-MS/MS, depending on the concentration. The retrieved TA concentration was calculated assuming a tissue density of 1 g/ml.

2.6 Statistics

Data were expressed as the mean ± SD. Results were evaluated statistically using either an analysis of variance (ANOVA) or Student’s t-test. The level of significance was fixed at α=0.05.

3. Results

3.1 Physical-chemical characterization and stability of TA-AA prodrugs

The influence of the different amino acid attached to TA on pKa, LogDₜ₅.₅ and solubility were investigated either in silico or experimentally (Table 3). It was seen that the presence of ionizable amino functional groups on TA-AA prodrugs induced a net decrease in the LogDₜ₅.₅ as compared to TA 2.43. In the case
of TA-Ile it was decreased to 1.63 for whereas for TA-Arg it fell dramatically to -2.29 (the fall in 4.72 log units corresponding to a > 50 000-fold decrease in lipophilicity. The impact of the amino acid side chain was also seen in the solubility of the prodrugs, which was significantly increased: compared to the parent drug (TA), a 30- to 400-fold increase in solubility was found for TA-Ile and TA-Ala, whereas for TA-Arg and TA-Lys 900-and 1 000-fold increases respectively were measured.

Table 3. Experimentally determined and computational predictions of triamcinolone acetonide (TA) and TA-AA molecular properties and half-lives at pH 5.5 and stability pH 7.4 in contact with fresh ocular tissue. (a ACD/Labs version 12.01, b experimental)

<table>
<thead>
<tr>
<th></th>
<th>TA</th>
<th>TA-Ala</th>
<th>TA-Arg</th>
<th>TA-Ile</th>
<th>TA-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.W.</td>
<td>435.5</td>
<td>505.6</td>
<td>590.7</td>
<td>547.6</td>
<td>562.7</td>
</tr>
<tr>
<td>pKa</td>
<td>12.87, 13.15 a</td>
<td>7.47 a</td>
<td>7.08, 13.36 a</td>
<td>7.58 a</td>
<td>6.90, 10.45 a</td>
</tr>
<tr>
<td>Log D pH 5.5</td>
<td>2.43 a</td>
<td>0.36 a</td>
<td>-2.29 a</td>
<td>1.63 a</td>
<td>-1.85 a</td>
</tr>
<tr>
<td>Solubility(MES, pH 5.5) (µmol/ml)</td>
<td>2.76 x10^{-2} [51]</td>
<td>15.92 ± 0.66 b</td>
<td>25.61 ± 1.23 b</td>
<td>5.17 ± 0.13 b</td>
<td>29.12 ± 1.59 b</td>
</tr>
<tr>
<td>t 1/2 pH 5.5 (h)</td>
<td>-</td>
<td>31.0 ± 1.8 b</td>
<td>10.2 ± 0.5 b</td>
<td>194.6 ± 8.5 b</td>
<td>39.2 ± 2.7 b</td>
</tr>
<tr>
<td>t 1/2 pH 7.4 + sclera (min)</td>
<td>-</td>
<td>64.52 ± 11.46 b</td>
<td>25.7 ± 5.44 b</td>
<td>158.0 ± 19.1 b</td>
<td>103.0 ± 3.46 b</td>
</tr>
<tr>
<td>t 1/2 pH 7.4 + ciliary body (min)</td>
<td>-</td>
<td>14.83 ± 1.22 b</td>
<td>5.25 ± 1.89 b</td>
<td>34.17 ± 2.06 b</td>
<td>95.88 ± 2.79 b</td>
</tr>
<tr>
<td>t 1/2 pH 7.4 + choroid/retina (min)</td>
<td>-</td>
<td>11.35 ± 0.37 b</td>
<td>1.68 ± 0.18 b</td>
<td>33.75 ± 3.15 b</td>
<td>94.52 ± 0.74 b</td>
</tr>
<tr>
<td>t 1/2 pH 7.4 + vitreous humor (min)</td>
<td>-</td>
<td>37.87 ± 6.50 b</td>
<td>7.54 ± 5.72 b</td>
<td>86.99 ± 6.91 b</td>
<td>126.81 ± 1.35 b</td>
</tr>
</tbody>
</table>

The design of the novel amino acid prodrugs was intended to optimize the electromigratory transport of the drug by the addition of positive charge. However, once the TA-AA prodrugs reach the target tissues a rapid hydrolysis to the original active principle was envisaged and therefore a labile ester bond was chosen for the linkage [24].
The stability of the prodrugs in the donor compartment was investigated and the half-lives ranged from 10 h for TA-Arg to 8 days for TA-Ile. In contact with the different ocular tissue homogenates at pH 7.4, the TA-AA prodrug half-lives fell dramatically and were in the range of minutes, indicating a much faster conversion. The fastest rate of hydrolysis for all prodrugs was found when they were in contact with the choroidal/retinal tissue and ciliary body, which is agreement with the literature [52] [53]. The slowest conversion was found in the sclera followed by the vitreous humor, presumably due to the lower enzyme content.

3.2 Melanin binding

The importance of melanin binding in ophthalmic drug delivery was investigated in this study since the presence of melanin was found to impact drug residence time and concentration in tissues such as ciliary body or choroid-RPE [54] [55] [56] [26] [57]. The polyanionic pigment is present as insoluble granules; binding with drug molecules was supposed to be mainly due to electrostatic interactions [58] and accordingly the highest binding affinity was reported with basic molecules [59].

In Figure 1 TA and TA-AA binding to melanin at physiological pH and at pH 5 - the reported pH in non-ocular melanosomes [28] [60], was investigated. TA-Arg and TA-Lys showed the highest binding affinity; approximately 85-86% (pH 7.4) and 95-98% (pH 5) of the available prodrug was found bound to melanin in the tested concentration range. In comparison TA-Ala and TA-Ile binding percentage was between 38-42% for pH 7.4 to 71-74% at pH 5. Only the binding of the parent drug TA was constant (i.e. not statistically different) in the two experimental conditions (4-7%).
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Figure 1. Percentage of TA and TA-AA prodrugs bound to melanin in PBS buffer (pH 7.4) and citrate buffer (pH 5) at room temperature after 1 h. Representation of the average results for the concentration ranges of 0.0115-0.23 mM for TA-Arg, TA-Ala and TA-Lys and 0.0023-0.023 mM for TA and TA-Ile. (Mean ±SD, n=3)

3.3 Intraocular TA biodistribution

3.3.1 Passive transscleral TA delivery

For these experiments, the in-house versions of the commercial formulations and the TA-AA prodrug solutions were applied with contact time of 10 min, although it is clear that in vivo the actual residence time of topical ophthalmic formulations is shorter; blinking, lacrimal drainage were estimated to dilute and rinse the active principle within a few minutes [4].

In Figure 2 the deposition of TA in cornea, sclera and choroid+RPE from TA-ointment (2.3mM), TA-suspension (92 mM) and TA-AA prodrug solutions (2.3mM) are represented. Although the whole eye globe was dissected and the individual tissues extracted, the amount of TA in the aqueous humor, ciliary body, neuro retina and vitreous humor were either below LOQ or even LOD and are therefore not reported.
Figure 2. TA distribution in whole porcine eye globes following 10 min passive application of 1ml TA-ointment, TA-suspension or TA-AA prodrug solution on the sclera. The values, summarized below were given as a concentration by dividing the total amount of TA extracted by the tissue weight (Mean ± SD n ≥4)

<table>
<thead>
<tr>
<th></th>
<th>cornea (nmolTA/g)</th>
<th>sclera (nmolTA/g)</th>
<th>choroid +RPE (nmolTA/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA-ointment</td>
<td>&lt; LOQ</td>
<td>1.24 ± 0.82</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td>TA-suspension</td>
<td>&lt; LOQ</td>
<td>5.24 ± 2.34</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>0.49 ± 0.25</td>
<td>8.26 ± 4.97</td>
<td>0.37 ± 0.41</td>
</tr>
<tr>
<td>TA-Lys</td>
<td>0.62 ± 0.76</td>
<td>18.09 ± 8.20</td>
<td>0.46 ± 0.28</td>
</tr>
<tr>
<td>TA-Ala</td>
<td>0.38 ± 0.21</td>
<td>10.80 ± 0.85</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>0.31 ± 0.31</td>
<td>7.24 ± 2.09</td>
<td>&lt; LOQ</td>
</tr>
</tbody>
</table>

As expected, the highest TA concentration for all formulations was found in the directly exposed scleral tissue. TA-ointment, although equal in drug concentration to the TA-AA prodrug solutions, resulted in the statistically lowest TA delivery (1.24 ± 0.82 nmolTA/g) as compared to all other formulation tested. TA suspension with 40-fold higher TA concentration delivered the second lowest amount, presumably because of the undissolved state of the drug in the formulation; TA water solubility was reported around 0.028 mM [51]. No difference in TA deposition was found between the TA-AA prodrugs. For the most hydrophilic prodrugs, TA-Arg and TA-Lys, a very small drug amount was found in the choroid + RPE although very high standard deviations reduce the meaningfulness of these results, idem for the results obtained in the cornea which might be due to lateral diffusion from the sclera.
3.3.2 Transscleral iontophoresis of TA-AA prodrugs

Prodrug dependent TA distribution profiles in the posterior segment were determined in the ocular tissues following 10 min transscleral iontophoresis. For each TA-AA prodrug, characteristic tissue distribution patterns were observed in eyes frozen immediately after the application and following 1h at RT, allowing the passive diffusion of deposited TA in the tissues (Figure 3 and 4).

![Graph showing ocular biodistribution of TA-AA prodrugs.](image)

**Figure 3.** Comparison of ocular biodistribution of TA-AA prodrugs following anodal transscleral iontophoresis for 10 min at 3 mA/cm². (Mean ± SD, n=4)

As for the passive experiments, highest TA concentrations were found in the sclera following iontophoresis. No statistically significant difference in TA concentration in the sclera was seen in between TA-Arg, TA-Lys and TA-Ala (247.83 ± 24.83, 252.11 ± 16.29 and 276.72 ± 42.99 nmolTA/g, respectively), resulting in a 14- to 30-fold increased drug deposition when compared to passive application of correspondent TA-AA solution. Superiority of the iontophoretic drug delivery was found also for TA-Ile; however, when compared to the other TA-AA prodrugs the TA deposition was significantly lower in all ocular tissues (sclera: 27.18 ± 9.38, choroid + RPM: 0.33 ± 0.18 nmolTA/g) following iontophoresis, except for the cornea.
In the case of TA-Arg, TA-Ala and TA-Lys significant amount of TA could be detected in the posterior eye segments, which are the target tissues for the treatment of uveitis, neovascular age related macular degeneration, and macular edema. Highest TA delivery into the vitreous was found with TA-Lys (0.64 ± 0.14 nmol_{TA}/g) followed by TA-Ala (0.10 ± 0.06 nmol_{TA}/g) and TA-Arg (0.03 ± 0.02 nmol_{TA}/g). The TA deposition in choroid + RPE and neural retina were statistically similar for TA-Lys and TA-Ala whereas TA-Arg delivery was significantly lower.

In order to better understand the TA distribution in the eye globe following the iontophoretic treatment, in a separate experiment eye globes were kept for 1h at RT after TA-AA iontophoresis (Figure 4). The time for passive diffusion was intentionally kept rather short given the perishability of the tissues and the loss of barrier function.

Figure 4. Ocular biodistribution of TA-AA prodrug iontophoresis for 10 min at 3 mA/cm² followed by 1 h of passive distribution before freezing and processing of the eye globes. (Mean ± SD, n ≥ 4)

As a general trend for all four tested prodrugs, redistribution into the surrounding tissues from the sclera was observed. However, once more characteristic distribution profiles for the different TA-AA prodrugs
were found when compared between the species and compared to the previous experiment. The amount of TA delivered into the posterior segment tissues after 10 min iontophoresis and 10 min iontophoresis followed by 1 h passive are shown in Table 4.

Table 4. TA deposition in the posterior segment tissues after iontophoresis for 10 min at 3 mA/cm$^2$ with or without post-iontophoretic passive diffusion for 1 h. (Mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>sclera</th>
<th>choroid +RPE</th>
<th>neural retina</th>
<th>vitreous humor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10min</td>
<td>10min +1h</td>
<td>10min</td>
<td>10min +1h</td>
</tr>
<tr>
<td>TA-Arg</td>
<td>247.83 ± 24.83</td>
<td>223.17 ± 53.97</td>
<td>1.60 ± 0.90</td>
<td>44.13 ± 24.38</td>
</tr>
<tr>
<td>TA-Lys</td>
<td>252.11 ± 16.30</td>
<td>206.18 ± 38.90</td>
<td>20.86 ± 8.81</td>
<td>27.45 ±15.57</td>
</tr>
<tr>
<td>TA-Ala</td>
<td>276.72 ± 42.99</td>
<td>220.79 ±38.44</td>
<td>11.35 ± 5.73</td>
<td>80.17 ± 35.57</td>
</tr>
<tr>
<td>TA-Ile</td>
<td>27.18 ± 9.38</td>
<td>33.89 ± 5.36</td>
<td>0.33 ±0.17</td>
<td>5.55 ±2.69</td>
</tr>
</tbody>
</table>

The deepest penetration and deposition of TA, was observer after iontophoresis of TA-Lys for 10 min at 3 mA/cm$^2$, with TA reaching the vitreous segment; in this case, there was no statistically significant increase in the amount of TA deposited in the posterior segment tissues after the additional 1 h of passive diffusion. Different results were observed for TA-Arg where total TA deposition increased after 1 h of passive diffusion from 0.034% to 1.41%, 0.019% to 0.94% and 0.026% to 0.59% in choroid + RPE, neural retina and vitreous humor, respectively. A similar trend was also found for the other two TA-AA prodrugs (TA-Ala and TA-Ile) where a statistically significant increase in drug deposition was found subsequent to the 1 h of passive TA diffusion. However, the increases observed were less striking than those for TA-Arg.

The impact of iontophoretic application time on the ocular biodistribution of TA following TA-AA iontophoresis was investigated using TA-Ala. TA distribution in the eye was studied by keeping the total charge constant (30 mA min/cm$^2$; 1.44 C) but varying application time and current density: 20 min at 1.5 mA/cm$^2$ and 5 min at 6 mA/cm$^2$. 

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Figure 5. TA concentration in the posterior eye segment following TA-Ala (2.3 mM) iontophoresis at 6 mA/cm² for 5 min and 1.5 mA/cm² for 20 min.

As the total charge was kept constant no statistical significant difference was found in the total TA delivered by the two current intensities (Figure 5). TA concentrations in the sclera were respectively 211.38 ± 37.32 and 202.51 ±35.97 nmolTA/g following 5 min at 6 mA/cm² and 20 min at. However, by comparing the TA concentration in the deep posterior segment tissues, higher drug deposition was seen with the longer application time and lower current density.
4. Discussion

4.1 TA-AA characterization

4.1.1 Influence of charge on solubility and melanin binding

As shown in Table 3, the aqueous solubility of TA-AA prodrugs at pH 5.5, which was similar to the experimental donor conditions, was significantly enhanced compared to the parent molecule due to the addition of the amino acid sidechain. The difference in solvation of the prodrugs was mainly attributed to their total charge. TA-Arg and TA-Lys (pKa 7.08, 13.36 and 6.90, 10.45, respectively) were in a ~96-97 % dicationic at pH 5.5, whereas TA-Ala and TA-Ile (pKa 7.47 and 7.58, respectively) were monocationic. As expected, the solubility of the double positively charged TA-AA prodrugs was greater than that of TA-Ala and TA-Ile (2- to 28-fold higher). Comparison of TA-Ala and TA-Ile clearly demonstrated the impact of sidechain length– for TA-Ile, with four carbons, lipophilicity of the molecule was significantly increased and aqueous solubility correspondingly lower (pH 5.5, Log D: 1.63, solubility: 1.04 ± 0.03 µmol/ml) as compared to the single CH3 in TA-Ala (pH 5.5 Log D: 0.36, solubility: 13.01 ± 0.20 µmol/ml).

Besides influencing the solubility, total charge of the TA-AA prodrugs displayed an impact on the interaction with melanin. Given the abundance of the natural pigment in the eye, its interaction with the drugs was important to study because of its eventual effect on ocular biodistribution and “depth/extent “of prodrug penetration. In vitro melanin binding experiments were conducted at physiological pH and pH 5, which represented the actual acidic composition of melanosomes [28] [60]. At pH 7.4 TA-Arg and TA-Lys were predominantly monocationic, with only 33% and 24 % of species present as dications. In comparison, TA-Ala (pKa 7.5) and TA-Ile (pKa 7.6) were monocations (~55 to 60%) with a significant proportion of prodrug present in unionized form. Lowering the pH to 5, meant that the ionization state of all the cationic prodrugs increased. The relationship between ionization state and the interaction with melanin is clearly seen in Figure 1 – TA-Arg and TA-Lys showed the highest binding affinity to melanin. The interaction of TA-Ala and TA-Ile with melanin changed with pH, binding affinity was found to increase from 30 to 70%
with the increase in the proportion of ionized prodrugs at the lower pH. In contrast to the TA-AA prodrugs, the melanin binding of TA was unaffected by pH variations; this was due to its lack of ionizable functional groups. Therefore, the results confirmed the clear dependence of the interaction with melanin on the total charge of the molecules – in agreement with the literature [61]. However, the result might overestimate the extent of the interaction in vivo, since then the molecules have to enter the melanosomes before coming into contact with melanin [28] [57] [62].

4.1.2 Stability of TA-AA prodrugs

The susceptibility of the TA-AA prodrugs for chemical and enzymatic hydrolysis was tested to ensure the stability and hence feasibility of using them for short term iontophoresis experiments. As reported in Table 3, TA-AA prodrug solutions freshly prepared prior to iontophoresis were shown to allow the administration of mostly unconverted prodrugs, given that even the shortest half-life (for TA-Arg) was 10 h. When TA-AA prodrugs were placed in contact with fresh ocular tissue homogenates, a striking increase in the conversion rate was found. Interestingly, in these conditions the two double positively charged prodrugs behaved very differently – TA-Lys contained the most stable ester bond and TA-Arg, the weakest. The extreme susceptibility of the TA-Arg ester bond to hydrolysis, in buffer solutions and tissue extracts, might be linked to the electroattractive properties of the guanidinium functional group which might render the carboxylic group more prone to nucleophilic attacks. In general, TA-AA hydrolysis in esterase rich tissues was found increased [52] as compared to relatively esterase poor tissues such as vitreous humor.

4.2 TA ocular biodistribution profile

4.2.1 Therapeutic relevance of TA-AA iontophoretic delivery

The ability to achieve therapeutically relevant corticosteroid concentrations in the posterior eye segment is essential for the treatment of diseases such as uveitis, neovascular age related macular degeneration and

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macular edema [1]. In the case of transscleral drug delivery sclera, choroid, RPE and neural retina have to be overcome as static barriers to reach the target area. In addition, the lacrimal washout following topical eye drop instillation reduces significantly drugs bioavailability in the inner eye regions resulting in insufficient drug concentrations for the treatment [63]. Similarly, as shown in this study, 10 min of passive TA formulation application on porcine sclera \textit{in vitro} (Figure 2) resulted in undetectable drug concentrations (i.e. <LOD) in the posterior segment tissues. This problem is partially resolved in the clinic by injection of TA suspension (Kenacort®-A40) into the sub-Tenon space, which thereby prolongs the contact time of the formulation with the sclera and has been reported to be efficient [64] [65] but invasive and difficult to perform. Clinical studies that tracked the amount of TA permeated into patients’ vitreous found a peak concentration of 111.43 ± 138.93 ng\textsubscript{TA}/ml after 1 day and an average concentration between 15-25 ng\textsubscript{TA}/ml over 4 weeks [66] (similar results in [67]). After 10 min iontophoresis of TA-Lys (Figure 3) the TA concentration (277 ng\textsubscript{TA}/ml) in the vitreous was more than 2-fold higher than that found 1 day after sub-Tenon injection. Moreover, the amount of TA delivered by TA-Arg and TA-Ala iontophoresis (equivalent to concentrations of 11.9 and 43.5 ng\textsubscript{TA}/ml) was in the steady-state concentration range observed after sub-Tenon injection. TA concentrations delivered via iontophoresis where 30-640 fold higher than the IC\textsubscript{50} for the trans-repression of the transcription factors AP-1 and NF-kB (1-0.7 nM), which reduces the expression of pro-inflammatory mediators [68]. Moreover, for all of the TA-AA prodrugs, ~99 \% of the administered TA amount was found in the sclera. This TA scleral depot might diffuse with time into the deeper tissues as partially shown in the experiment involving 10 min iontophoresis followed by 1 h passive diffusion (Figure 4).

\subsection*{4.2.2 TA-AA distinct ophthalmic biodistribution profiles}

Taking into account the distinctive biodistribution profiles of the four TA-AA prodrugs studied, the influence of i) various physicochemical properties, ii) stability and iii) tissue interaction rather than simply the total charge of the prodrugs were found to govern the prodrugs’ electromigratory behaviour.


**Influence of stability**

For example, in the case of the double positively charged TA prodrugs (TA-Lys and TA-Arg) their differing susceptibility towards hydrolysis had a demonstrable impact on their electromigration into the ocular tissue. TA amount delivered following 10 min iontophoresis of TA-Lys at 3 mA/cm² resulted in 7- to 21-fold higher statistically significantly deposition in choroid + RPE, neural retina and vitreous humor when compared to TA-Arg. This was surprising given that the molecules have similar structures: both were present essentially as dications (~96-97 %) in the donor compartment and therefore similar electromigratory behavior was expected. However, there was a major difference in their stability, e.g. respective half-lives in sclera tissue extract were: 25.7 ± 5.44 min for TA-Arg and 103.0 ± 3.46 min for TA-Lys. The stability difference was even more pronounced in the deeper posterior segment tissues (Table 3). As it has been suggested previously, the hydrolysis rate of prodrugs influences their depth biodistribution profile by the loss of the charged amino acid sidechain function [24] and this led to their distinct and different allocation in the organ.

**Influence of polar surface area, dipolar moment and charge**

Most unexpected results were obtained by comparing TA-Lys and TA-Ala ocular biodistribution profiles. Despite their net difference in charge no statistically significant difference was found in the amount of TA delivered into the posterior segment tissues, except for the aqueous humor. This was remarkable since the increase in charge was thought to be key factor in improving electromigration [69]. However, similar findings were already reported in literature by Barza *et al.* where no significantly different drug amounts were delivered from mono and double charged antibiotics via equal iontophoretic conditions [70]. It has been described that localized charge centres/uneven distribution of the charge on the molecular surface hinders iontophoretic transport [71][72]. This was attributed to the increased propensity of interaction with negative charges in the tissue (and also melanin – see next paragraph), which would impede electromigration. Indeed, in the case of TA-AA prodrugs, the mainly hydrophobic steroidal backbone is
attached to the more polar amino acid side chain. The dipole moment, which indicates the localization of the positive charge was double in TA-Lys as compared to TA-Ala (84 to 40) (Figure 6). This might also be related to the physical distance of the ε-primary amine group of TA-Lys from the molecule core whereas the primary amine group (on the Cα atom) common for the two prodrugs was less distant.

![Figure 6](image)

**Figure 6.** TA, TA-Ile, TA-Ala TA-Lys and TA-Arg corresponding lipophilic surface potential depicted on the minimized molecular conformations of the charged molecules. The lipophilicity scale ranges from most hydrophilic (-0.09, blue) to most lipophilic (0.07 brown) parts of the surface. LP: minimum/maximum lipophilicity values for each TA entity, PSA: polar surface area and DM: dipole moment, are listed in the table beneath the structures.

<table>
<thead>
<tr>
<th>VAT</th>
<th>TA</th>
<th>TA-Ile</th>
<th>TA-Ala</th>
<th>TA-Lys</th>
<th>TA-Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>-0.028 to 0.042</td>
<td>-0.045 to 0.038</td>
<td>-0.078 to 0.033</td>
<td>-0.087 to 0.024</td>
<td>-0.071 to 0.024</td>
</tr>
<tr>
<td>PSA</td>
<td>149.8</td>
<td>152.5</td>
<td>175.6</td>
<td>252.2</td>
<td>310.7</td>
</tr>
<tr>
<td>DM</td>
<td>4.4</td>
<td>32.8</td>
<td>40.9</td>
<td>84.5</td>
<td>74.5</td>
</tr>
</tbody>
</table>

Interestingly, as previously reported, molecular hydrophobicity had a negative effect on iontophoretic delivery [71]. This was once more confirmed when the TA distribution into the eye globe following transscleral iontophoresis of TA-Ile was compared to the other TA-AA prodrugs. In all tissues a significantly lower TA deposition was evidenced (Figure 3). Indeed, when compared to the other TA-AA prodrugs TA-Ile in its single positively charged state possesses the lowest PSA: 152.52, compared to TA-Ala 175.65 and TA-Lys 252.24, very similar to the uncharged TA parent drug (PSA 149.81).
Correspondingly, the lipophilic surface of TA-Ile was found to most closely resemble the uncharged TA parent drug. In **Figure 6** a clear shielding effect of the lipophilic sidechain was shown, which might be the reason for the decreased electrically driven transport.

**Influence of tissue interaction (melanin binding)**

The dipolar moment was found to correlate also to the melanin binding tendency of TA-AA prodrugs, which was superior for TA-Lys compared to single positively charged prodrugs and TA. As suggested in the previous paragraph, the interaction with the tissues might influence the iontophoretic delivery and intra-tissue distribution. Although porcine sclera has been reported to be structurally very similar to human, it was found to be richer in melanin [26]. This could have contributed to the unforeseen low transport of TA-Lys into the eye globe. In addition to this hypothesis, binding of the TA-AA prodrugs to melanin might help to predict the ocular pharmacokinetics. Binding to the ocular pigment might be associated with a longer residence time of the drug in melanin rich tissues such as choroid and RPE, which might increase effectiveness [53] [56].

Considering the example of TA-Lys, it can be seen in **Figure 3-4** that 10 min iontophoresis delivered superior amounts of TA into deep ocular tissues as compared to other prodrugs; however, after 1 h following the application, the TA tissue concentrations did not increase significantly. In contrast, TA-Ala, TA-Arg and TA-Ile, showed a net diffusion of TA in the surrounding tissues during the 1 h post-iontophoretic passive diffusion period. The distinct behavior of TA-Lys could be attributed to its relative high stability in the sclera (t1/2 103.0 ± 3.46 min) and its high binding affinity to melanin, which might trap the unconverted prodrug in the tissue. However, this effect was suggested to be limited to the first hours following iontophoretic treatment. Given the rather rapid hydrolysis of the ester bonds in contact with the ocular tissues, it is likely that the parent molecule TA will diffuse passively into the deeper tissues. In the case of passive diffusion, the solutes’ physicochemical properties are also crucial in determining the tissue distribution. Limiting factors for choroid and RPE permeation were high molecular weight and increased
CHAPTER IV: Characterization of triamcinolone acetonide amino acid prodrugs iontophoretic transport into the posterior segment of the eye

hydrophilicity [32]. TA itself is highly lipophilic, which in the case of RPE tight junctions might be advantageous for its permeation [73].

4.2.3 TA-Ala transscleral iontophoresis with constant charge

When TA-Ala iontophoretic delivery with varying application time and current density 20 min at 1.5 mA/cm² and 5 min at 6 mA/cm² was investigated, the total delivery was found statistically similar for both conditions. This was unsurprising given that the total charge delivered was kept constant (1.44C). However, the drug distribution in the ocular compartments was seen influenced, i.e. the longer treatment time of 20 min enabled increased TA deposition in the deep ocular tissues (Figure 5). TA delivery in neural retina and vitreous humor was 5- to 6-fold greater after iontophoresis at 1.5 mA/cm² for 20 min than 6 mA/cm² for 5 min. The concentration of TA delivered in the vitreous humor following prolonged treatment was 140- to almost 200-fold above IC₅₀ of TA reported to inhibit the transcription factors AP-1 and NF-kB [68]. This observation might be important for an eventual future application of the system in patients. It was observed that prolonged application might be advantageous for targeting of deeper tissue, allowing the use of lower current intensities, which would improve safety [74]. However, there may be a dissonance with respect to the patient comfort, since shorter treatment duration would be preferred.

5. Conclusions

Transscleral iontophoresis of TA-AA prodrugs was investigated as a non-invasive method for deep ocular delivery and as an alternative to conventional corticosteroid therapy. The addition of readily cleavable amino acids dramatically increased the aqueous solubility of TA: a factor which in addition to the ionizable groups favored electromigration of the TA-AA prodrugs into the posterior segment of the eye. The physicochemical properties of the different amino acid sidechains were found to influence electrotransport and enabled deeper penetration and distribution of some TA-AA prodrugs over others. Shielding of the charged primary amine by lipophilic sidechains – the case of TA-Ile – was found to reduce total transport.
Rapid hydrolysis (implying loss of the ionizable moieties) and tissue interaction e.g. melanin binding, also reduced TA distribution in the intraocular tissues (e.g. for TA-Arg and TA-Lys, respectively). The application of a low current density for a longer period of time seemed to be more efficient with respect to maximizing drug delivery into the vitreous humor and retina than short duration transscleral iontophoresis at high current densities. For example, a moderate current density (1.5 mA/cm²) applied for 20 min was more efficacious than 5 min iontophoresis at 6 mA/cm². Given the fundamental and preliminary character of this study, the direct use on animals would not be justifiable either for ethically or economically reasons. However, we acknowledge the lack of dynamic barriers in the in vitro model used here. In the case of transscleral iontophoresis, the most important would be the conjunctival blood/lymphatic as well as choroidal blood flow \[73\]. Nevertheless, we consider of the understanding gained on the impact of molecular physicochemical properties on transscleral iontophoresis to be of considerable importance, since this can be useful for the optimization of drug candidates to improve drug delivery for the treatment of posterior segment diseases.

### 6. Supplementary material

#### 6.1 Quantification of TA and TA-AA by HPLC-UV

**6.1.1 Specificity, linearity, LOD and LOQ**

The specificity and linearity of the HPLC-UV analytical methods was established with six point calibration curves using a concentration range from 2.30 to 230.15 nmol/ml spiked with whole eye globe extract. The analytical conditions used were summarized in **Table S1**. \(R^2\) was found consistently in between 0.98-1. The limit of detection (LOD) and quantification (LOQ) were established according to ICH Q2 (R1) guidelines.

**Table S1.** Summary of the analytical conditions for the quantification of TA and TA-AA prodrugs, with retention times and respectively calculated LOQ and LOD.

<table>
<thead>
<tr>
<th>Analytical column</th>
<th>Mobile phase (% A:% B)</th>
<th>Flow rate (ml/min)</th>
<th>Detection wavelength (nm)</th>
<th>Column temperature (°C)</th>
<th>Injection volume (µl)</th>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>LOQ (nmol/ml)</th>
<th>LOD (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 6.1.2 Precision and accuracy

The intra and inter-day precision and accuracy were evaluated by the repeated analysis of 3 standard solutions. Results of accuracy and precision were listed in Table S2 and the retrieved data were found included within the acceptance limits.

**Table S2.** Intra-and inter-day precision and accuracy values for TA and TA-AA HPLC-UV quantification method.

<table>
<thead>
<tr>
<th></th>
<th>Theoretical concentration (nmol/ml)</th>
<th>Experimental concentration (nmol/ml)</th>
<th>Precision (%) a)</th>
<th>Accuracy (%) b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TA</strong></td>
<td>230.15</td>
<td>229.59 ± 1.88</td>
<td>0.29</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>45.71 ± 0.77</td>
<td>1.68</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.85 ± 0.10</td>
<td>0.83</td>
<td>103.8</td>
</tr>
<tr>
<td><strong>TA-Arg</strong></td>
<td>230.15</td>
<td>23.47 ± 0.49</td>
<td>0.21</td>
<td>100.1</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>45.82 ± 0.16</td>
<td>0.35</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.62 ± 0.45</td>
<td>3.86</td>
<td>101.0</td>
</tr>
<tr>
<td><strong>TA-Lys</strong></td>
<td>230.15</td>
<td>229.66 ± 4.23</td>
<td>1.84</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>46.21 ± 0.85</td>
<td>1.84</td>
<td>100.4</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.50 ± 0.22</td>
<td>1.96</td>
<td>99.9</td>
</tr>
<tr>
<td><strong>Inter-day (n=3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TA</strong></td>
<td>230.15</td>
<td>229.92 ± 0.68</td>
<td>0.29</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>45.59 ± 0.77</td>
<td>1.68</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.94 ± 0.10</td>
<td>0.83</td>
<td>103.8</td>
</tr>
<tr>
<td><strong>TA-Ala</strong></td>
<td>230.15</td>
<td>230.12 ± 3.19</td>
<td>1.39</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>45.98 ± 0.42</td>
<td>0.90</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.41 ± 0.43</td>
<td>3.73</td>
<td>99.2</td>
</tr>
<tr>
<td><strong>TA-Ile</strong></td>
<td>230.15</td>
<td>227.37 ± 8.83</td>
<td>3.88</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>46.03</td>
<td>44.57 ± 2.99</td>
<td>2.99</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td>11.51</td>
<td>11.33 ± 0.31</td>
<td>2.75</td>
<td>98.5</td>
</tr>
</tbody>
</table>

a) (5 µm particle size; 125 mm × 4.0 mm; BGB Analytik AG; Boeckten, Switzerland)
b) (5 µm particle size; 250 mm × 4.0 mm; BGB Analytik AG; Boeckten, Switzerland)
6.2 Quantification of TA by UHPLC-MS/MS

6.2.1 Specificity, linearity, LOD and LOQ

TA quantification by UHPLCMS/MS in eye extract spiked samples (50:50 methanol: water) was found specific and linear in a concentration range of 11.5-460.3 and 230.2-2301.5 pmol/ml (retention time: 0.81 min, $R^2 \geq 0.98$). LOQ and LOD were calculated according to ICH Q2 (R1) guidelines [25]. and found at 12.4 and 4.1 pmol/ml, respectively.

6.2.2 Precision and accuracy

Results of intra- and inter-day precision and accuracy were reassumed in Table S3.

**Table S3.** Intra-and inter-day precision and accuracy for TA quantification a) Precision= (SD/mean) *100, b) Accuracy = (measured concentration/theoretical concentration) *100
6.3 Scleral extraction validation

The complete hydrolysis of the TA-AA prodrugs in the extraction mixture methanol: water (50:50) after 12 h at room temperature was shown by HPLC-UV analysis. Therefore, only the extraction of the parent drug (TA) from the sclera (the main barrier for trans-scleral drug delivery) was validated. The recovered concentration of TA was compared with the theoretical concentration applied in Table S4 (n=3).

Table S4. Validation of TA extraction from scleral tissue samples (Mean ± SD)

<table>
<thead>
<tr>
<th>Control concentration (nmol/ml)</th>
<th>Sample concentration (nmol/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>46.03</td>
<td>46.37 ± 0.39</td>
<td>100.74 ± 0.84</td>
</tr>
<tr>
<td>115.07</td>
<td>108.0 ± 4.76</td>
<td>93.88 ± 4.14</td>
</tr>
<tr>
<td>230.15</td>
<td>206.63 ± 17.26</td>
<td>89.78 ± 7.50</td>
</tr>
</tbody>
</table>

For the remaining ocular tissues (aqueous humor, choroid+ RPE, neural retina and vitreous humor), TA solution was spiked and recovered 80-100 % in methanol: water (50:50) mixture.

6.4 Porcine eye globe tissue weight

Table S5. Mean weight of separated components of porcine eye globes
CHAPTER IV: Characterization of triamcinolone acetonide amino acid prodrugs iontophoretic transport into the posterior segment of the eye

| Weight (g) | 0.177 ± 0.031 | 0.150 ± 0.031 | 0.499 ± 0.053 | 0.203 ± 0.067 | 1.588 ± 0.194 | 0.087 ± 0.025 | 0.249 ± 0.050 | 3.343 ± 0.174 |

7. Acknowledgments

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8. References


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CHAPTER V

- Iontophoresis for the treatment of hypertrophic and keloid scars;
a study of triamcinolone acetonide prodrugs and verapamil
Iontophoresis for the treatment of hypertrophic and keloid scars; a study of triamcinolone acetonide prodrugs and verapamil.

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Abstract

Hypertrophic and keloid scars, despite not being life-threatening conditions, are a major concern in dermatology given their deforming and emotional load for patients. The common first line treatments are corticosteroid injections, mainly triamcinolone acetonide (TA) (10-40 mg/ml); however, given the painfulness of the procedure and the risk of severe side effects, alternatives would be desirable. In this study the localized iontophoresis of triamcinolone acetonide amino acid (TA-AA) prodrugs synthesized in-house was tested as a potential means for a non-invasive treatment option. Delivery of alanine, arginine, glycine and lysine TA prodrugs (TA-Ala, TA-Arg, TA-Gly and TA-Lys) was quantified in vitro on porcine and human skin, following 20 min anodal iontophoresis at 0.5 mA/cm². In both skin models, 2-fold higher TA deposition following TA-Ala and TA-Gly iontophoresis was found as compared to TA-Arg and TA-Lys. It was supposed that the double positive charged prodrugs (TA-Arg and TA-Lys), given their localized charge and lipophilic backbone, were interacting more with the skin, which reduced iontophoretic delivery. Experiments of the effect of TA-Arg and TA-Lys on the permselectivity of the skin and lidocaine (LID) electromigration reinforced this hypothesis. Nevertheless, when dermal biodistribution profiles were investigated by the horizontal slicing technique, all prodrugs delivered supra-therapeutically amount of TA in the depth of 1 mm. In the second part of the study co-iontophoresis of verapamil (VER) and TA-Arg was
investigated, since synergic effects in the treatment of hypertrophic and keloid scars had been suggested by the combination of the calcium antagonist with the corticosteroid. Co-iontophoresis of TA-Arg (0.5 mM) and VER (9.5 mM) showed therapeutically effective concentrations (VER) in the entire skin specimen of 1 mm, whereas TA concentration was reduced and more superficial. These preliminary results suggest that co-iontophoretic application could be a valid option for effective local treatment minimizing the risk of increased corticosteroid exposure by chronically repeated medication.

**Keywords**: triamcinolone acetonide, prodrugs, verapamil, topical iontophoresis, keloid and hypertrophic scars
1. Introduction

Iontophoresis is a non-invasive drug delivery method, which exploits a low current for the transport of active principles into and through biological membranes [1] [2]. Successful transdermal drug delivery of small molecules [3] [4] [5] and high molecular weight proteins [6] [7] [8] were repeatedly shown in the past. The best suited drugs for the iontophoretic transport are usually water soluble and charged; however only few correspond to these criteria. Thus, one approach to optimize drug molecules physicochemical properties for the iontophoretic transport is to temporarily modify their molecular structure as labile prodrugs (e.g. esterification with amino acid (AA) side chains). As example: the transdermal delivery of dehydroepiandrosterone (DHEA) was found superior to passive via iontophoresis of its glycine ester prodrug; moreover the complete conversion of the AA-prodrug to the parent active molecule was seen in the tissue [9]. In another study glycine, proline, alanine and valine ester prodrugs of a dopamine agonist were synthesized and tested for transdermal iontophoresis in vitro and in vivo. Despite an increase in delivery was observed, transdermal permeation of the active principle was shown delayed most probably because of the prompt hydrolysis of the ester [10]. Therefore, it was concluded that the use of labile prodrugs was especially suited for increased topical delivery. This was shown with the delivery of valaciclovir [11] and other AA prodrugs of aciclovir [12] [13]. The combination of prodrugs to anodal iontophoresis enabled the deposition of supra-therapeutic amounts of aciclovir in the basal epidermis, which is also the main target area for the treatment of topical herpes simplex infections [13].

In the following study the combination of in house synthesized AA ester prodrugs of triamcinolone acetonide (TA-AA) and anodal iontophoresis was investigated for the topical and non-invasive treatment of hypertrophic and keloid scars. These local conditions are characterized by excessive scar tissue formation which have functional and mostly severe emotional implications for the patients [14] [15]. The underlying process was suggested to be a pathological persistence of wound healing signals which lead to excessive scar formation following trauma in predisposed people [16]. The outcome are protruded, often
hyperpigmented scar tissues, which in case of hypertrophic scars remain confined to the original wound area and eventually regress spontaneously [15] [16] [17]. Keloid scars on the contrary, are more insidious since the scar tissue overgrows the initial area of the trauma and they do not regress spontaneously, additionally the patients suffer from pruritus and pain on the affected sites [14] [18].

The golden standard of treatment are monthly corticosteroid injections, mostly TA suspension (10-40 mg/ml Kenalog) [14] [19]. The effect of corticosteroids is the inhibition of fibroblast proliferation, collagen synthesis and anti-inflammatory, which also reduce the pain and itchiness of the lesion [15] [19]. However, the repeated injections were stated to be very painful for the patients and long term use over a large area can cause severe local damage such as: skin depigmentation, fat atrophy, telangiectasia and ulceration [14] [19] [20] and systemic side effects, such as Cushing syndrome [21] [22]. Also, recurrence of the dermal hyperproliferation is common and therefore corticosteroid therapy is combined with physical approaches such as cryotherapy, silicon pressure therapy or surgical excision. Less common are the combination with other active principles such as immunosuppressant or calcium channel blockers [14] [15].

Calcium channel blockers such as verapamil (VER) were shown to change fibroblasts cell shape, trigger the collagenase production and inhibit the synthesis of extracellular matrix [23] [24] [25]. The treatment of hypertrophic and keloid scars with VER injections was tested with success, although studies were performed on limited number of subjects [26] [27] [28]. The iontophoretic delivery of VER has been already investigated for the treatment of Peyronie’s disease, given the its high aqueous solubility and the positive charge at physiological pH [29] [30] [31]. In the case of hypertrophic and keloid scars the combination[32] [27] or alternation of TA with VER were suggested, given the different mechanism of action of the two principles on the induction of collagenase [15] [20] [33].

In order to propose a non-invasive treatment option for hypertrophic and keloid scars in this study i) the iontophoretic delivery of novel TA-AA prodrugs, ii) influence of AA sidechains, application time and current intensities as well as ii) the co-iontophoretic treatment of TA-AA with VER were investigated.
2. Materials and Methods

2.1 Material

TA was purchased from Haenseler AG (Herisau, Switzerland) and TA-AA prodrugs (TA-Ala, TA-Arg, TA-Gly, TA-Lys) were in-house synthesized following a previously published protocol [34]. Silver wire and silver chloride (AgCl) for the fabrication of electrodes were purchased from Sigma-Aldrich (Steinheim, Germany). Buffer salts (sodium and potassium chloride, sodium and potassium phosphate, 2-morpholinoethanesulfonic acid monohydrate (MES) and sodium chloride (NaCl)); as well as verapamil hydrochloride salt (VER), lidocaine hydrochloride (LID) and acetaminophen (ACE) were acquired by Sigma-Aldrich (Steinheim, Germany). HPLC grade solvents (HiPerSolv Chromatonorm; Darmstadt, Germany) and deionized water (Millipore Milli-Q Gard 1 Purification Pack resistivity > 18MΩcm, Zug, Switzerland) were used. Trifluoroacetic acid (TFA; 99 % extra pure) were obtained from Acros Organics (Geel, Belgium) and ULC/MS grade formic acid was bought from Brunschwig (Basel, Switzerland).

2.2 Characterization of TA-AA prodrugs

TA-AA prodrugs physicochemical properties such as molecular weight, pKa, LogD and solubility at pH 5.5 and pH 7.4, listed in the Supplementary data (Table S1), were either predicted in silico using ACD/Labs software (version 12.01) or experimentally retrieved following published protocols [34]. The stability of the TA-AA prodrugs ester bond was investigated in MES buffer pH 5.5 and PBS buffer pH 7.4 with addition of thawed homogenized porcine skin pieces [34].

2.3 In vitro dermal deposition studies of TA-AA prodrugs

2.3.1 Porcine skin preparation

In vitro penetration experiments were performed on porcine skin because of its largely reported use as valuable research model for human skin [35] [36] [37] [38]. Pig ears were kindly provided by the local slaughterer (CARRE; Rolle, Switzerland); after cleaning under cold water, full thickness (1.2-1.5 mm) skin pieces were separated from the underlying cartilage by scalpel and circles (32 mm diameter) were collected.
with a manual skin puncher (Berg & Schmid, HK 500, Remseck, Germany). The skin was stored at -20°C and thawed for 15 min preceding the experiments in a 0.9% NaCl solution.

2.3.2 Human skin preparation

Human skin samples were retrieved from abdominal aesthetic surgeries performed at the Geneva University Hospital (HUG). Underlying fat tissue was removed carefully to obtain full thickness samples (1.5–2 mm) which were stored, wrapped in Parafilm™, at -20°C in a biobank until use. Permission for the study on human tissues was given by the Central Committee for Ethics in Research (CER: 08-150 (NAC08-051); HUG).

2.3.3 TA skin deposition following TA-AA passive and iontophoretic delivery

The delivery of TA into the porcine and human skin following passive and iontophoretic application of the TA-AA prodrugs was investigated to define the influence of prodrug characteristics on the intradermal drug deposition. In vitro permeation experiments were performed on vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland), with the stratum corneum facing the donor compartment (permeation area = 2 cm²). The donor chamber was filled with 1 ml TA-AA prodrug solutions (10 mM TA-Ala,-Arg,-Gly,-Lys solution in 10 mM MES buffer pH 5.5). In the receiver compartment 10 ml of PBS (pH 7.4) were kept under continuous stirring at 32 °C. For iontophoretic experiments, a constant current was applied by a power generator (APH 1000M, Kepco Inc; Flushing NY, USA) connecting the silver anode via a saline bridge (3% agarose in 0.1 M NaCl) to the donor compartment; the cathode (AgCl) was inserted in the sampling arm of the receiver compartment. The different experimental settings performed were listed in Table 1.

Table 1. Summary of the experimental conditions employed for the passive and iontophoretic delivery of TA-AA prodrugs in porcine and human skin.

<table>
<thead>
<tr>
<th>TA-AA prodrug formulation</th>
<th>delivery conditions</th>
<th>skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>passive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>iontophoresis</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER V: Iontophoresis for the treatment of hypertrophic and keloid scars; a study of triamcinolone acetonide prodrugs and verapamil

|                | 20 min | 20 min | 0.5, 0.25 and 0.1 mA/cm² |  
|----------------|--------|--------|--------------------------|--------
| TA-Ala [10 mM] in 10 mM MES buffer pH 5.5 |        |        |                          | porcine |
|                |        |        |                          | porcine |
| TA-Arg [10 mM] in 10 mM MES buffer pH 5.5 | 20 min | 20 min | 0.5 mA/cm²               | human   |
| TA-Gly [10 mM] in 10 mM MES buffer pH 5.5 | 20 min | 20 min | 0.5 mA/cm²               | human   |
| TA-Lys [10 mM] in 10 mM MES buffer pH 5.5 | 20 min | 20 min | 0.5 mA/cm²               | human   |

At the end of each application period the diffusion cells were dismantled, and the skin surface carefully cleaned from residual formulation. The permeation area was cut in small pieces for extraction of total deposition or snap-frozen for investigation of TA biodistribution profile. Lather was performed via horizontal slicing (50 µm) from the stratum corneum down to a 1000 µm depth with a cryotome (Thermo Scientific CryoStar NX70; Walldorf, Germany) [13, 39]. All samples were extracted overnight in methanol: water (50:50) mixture (1 ml and 10 ml, respectively) and analysed either by HPLC-UV or UHPLC-MS/MS.

2.3.4 Acetaminophen co-iontophoresis (investigation of TA-AA effect on electroosmosis)

ACE is a neutral hydrophilic molecule with negligible passive penetration through the skin. Its main transport via anodal iontophoresis was proven to be via electroosmosis. Therefore, if added to the donor compartment, it can be used as indicator of the systems solvent flow and the calculation of the inhibition factor (IF) which describes the effect of molecules (e.g. TA-AA prodrugs) on skin permselectivity [40]. Lather was relevant for the comparison of mono and double charged TA-AA prodrugs (TA-Ala/TA-Gly and TA-Arg/TA-Lys, respectively) and calculated according to the following equation (Eq1.) [8]:

\[
IF = \frac{J_{ACE \, control}}{J_{ACE,TA - AA}}
\]

Eq.1
J_{ACE, control} and J_{ACE, TA-AA} represent the steady state flux of permeated ACE (15 mM) obtained within 10h of anodal iontophoresis (0.5mA/cm²) (hourly sampling or the receiver compartment and analysis via HPLC-UV), in the absence and presence of TA-AA prodrug (10 mM) (TA-Ala, Arg, Gly and Lys) in the donor compartment, respectively. (n ≤ 5)

2.3.5 Lidocaine pre-iontophoresis (investigation of TA-AA effect on electromigration)

LID was widely used in iontophoretic studies, given its favourable transport via electromigration. In the frame of this study it was used as indicator for the influence of different TA-AA prodrugs on the skin barrier preceding the electromigratory transport of LID [41].

20 min iontophoretic pre-treatment (0.5 mA/cm²) of the skin samples was performed either with simple MES buffer (10 mM, pH 5.5) (control condition) or 10 mM TA-Ala, TA-Arg and TA-Lys loaded MES buffer (10 mM, pH 5.5). Following the pre-treatment, the current was stopped, and the sample skin surface was carefully cleaned. 40 mM LID (in 10 mM MES buffer, pH 5.5) were applied in the donor compartments and administered via iontophoresis for either 20 min or 5 h at 0.5 mA/cm². Samples of the receiver compartment were taken after 20 min and each hour, deposited LID in the skin was extracted in 10 ml methanol: water mixture (70:30) at the end of the experiment and all samples were quantified by HPLC-UV.

2.3.6 TA-Arg and VER co-iontophoresis studies

The TA-Arg and VER co-iontophoresis experiments were performed given the reported efficacy of both active principles for the treatment on hypertrophic and keloid scars [27] and the suggested efficacy of alternate or combined treatment [26] [20]. The previous described iontophoretic setup was used with porcine skin and experiment length was kept for 20 min. Five formulations (A-E), composed by TA-Arg and VER with respectively varying concentrations (total drug amount 10 mM) were investigated as listed in the following Table 2. The extraction procedure in methanol: water (50:50) was found suitable also for
the extraction of VER from the skin, and total drug delivery as well as biodistribution profiles (procedure described above) were quantified for both drugs either by HPLC-UV or UHPLC-MS/MS.

**Table 2.** List of investigated TA-Arg and VER donor solutions with constant total drug amount (10 mM) for co-iontophoretic experiments (20 min, 0.5 mA/cm$^2$). (n=5)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>TA-Arg (mM)</th>
<th>VER (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>2.5</td>
<td>7.5</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>9.5</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

### 2.4 Analytical methods

#### 2.4.1 *ACE, LID, TA, TA-AA and VER quantification by HPLC-UV*

UltiMate 3000 HPLC-UV system (formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach Switzerland) comprising a HPG-3200SD pump, WPS-3000 auto sampler and VWD-3400 variable wavelength detector VIS/UV was used to quantify all used drug entities. Data collection and processing were achieved using Chromeleon® (version 6.8) software. In **Table 3** the chromatographic conditions employed for the quantification are reassumed and respective limit of detection (LOD) and limit of quantification (LOQ) were listed. All methods were validated according to ICH Q2 (R1) guideline [42] (Supplementary data).

**Table 3.** Summary of the analytical conditions for the quantification of TA, TA-AA prodrugs, ACE, LID and VER. The mobile phase solvents used were A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA, C: methanol and D: 10 mM phosphate buffer pH 7.4. All analyses were performed in isocratic mode.

<table>
<thead>
<tr>
<th>Analytical column</th>
<th>Mobile phase</th>
<th>Flow rate (ml/min)</th>
<th>Detection wavelength (nm)</th>
<th>Column temperature (°C)</th>
<th>Injection volume (µl)</th>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>LOQ (nmol/ml)</th>
<th>LOD (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiChrospher RP18 (125 mm)$^2$</td>
<td>A 65%; B 35%</td>
<td>0.8</td>
<td>240</td>
<td>30</td>
<td>50</td>
<td>TA</td>
<td>8.3</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TA-Arg</td>
<td>3.3</td>
<td>2.56</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TA-Lys</td>
<td>2.9</td>
<td>2.27</td>
<td>0.84</td>
</tr>
</tbody>
</table>
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2.4.1 TA and VER quantification by UHPLC-MS/MS

The Waters Acquity® UPLC® system (Baden-Dättwil, Switzerland) included a binary solvent pump, sample manager and Waters XEVO® TQ-MS detector (Baden-Dättwil, Switzerland). Waters XBridge® BEH C18 (50x2.1 mm, 2.5 µm) reverse phase column was used at 40°C with a mobile phase mixture of ACN and ultrapure water (70:30 v/v, isocratic) both containing 0.1% formic acid. The flow rate was set at 0.3 ml/min and an injection volume at 5 µl. The retention time for TA was found at 2.55 min and VER 2.95 min, with total runtime of the method of 3.6 min. Mass spectrometric detection was performed by electrospray ionization in positive ion mode (ESI+) using multiple reaction monitoring (MRM), in the following Table 4 the detection settings were specified.

Table 4. MS/MS settings for TA and VER detection

<table>
<thead>
<tr>
<th></th>
<th>TA</th>
<th>VER</th>
</tr>
</thead>
<tbody>
<tr>
<td>parent ion [M+H]^+ (m/z)</td>
<td>435.29</td>
<td>455.25</td>
</tr>
<tr>
<td>daughter ion (m/z)</td>
<td>415.17</td>
<td>303.12</td>
</tr>
<tr>
<td>collision energy (eV)</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>cone voltage (V)</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>capillary voltage (kV)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>capillary temperature (°C)</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>
The daughter ions following fragmentation were the liberation of HF from the B ring for TA [43], whereas for VER it derives from the split of C-C bond reported by Chytil et al. [44]. LOD and LOQ were established in accordance with ICH Q2 (R1) guideline [42] at 2.51 and 7.61 for TA, 5.06 and 15.33 pmol/ml for VER respectively.

2.5 Data treatment
The results were obtained from at least triplicate experiments with skin samples from different animals; all the data were expressed as the mean ± standard deviation. Statistical analysis was performed by either ANOVA or Student’s T-test, with the level of significance set at p=0.05.

3. Results
3.1 TA-AA prodrugs stability
TA-AA prodrugs hydrolytic stability of the ester bond was investigated in order to demonstrate the effective liberation of the active principle TA. As a general tendency TA-AA prodrugs hydrolysis rates were found increased at higher pH (7.4) (data not shown) when compared to pH 5.5 of the donor compartment [12]. The stability of TA-AA prodrugs in the donor compartment medium (pH 5.5) were found in between \( t_{1/2} \) 10-40h; suitable for a short term iontophoretic application (~\( t_{1/2} \): Ta-Arg 10 h, TA-Gly 29 h, TA-Ala 31h and TA-Lys 39 h).

Similarly to the previous studies [34] an increased hydrolysis rate was observed when the TA-AA prodrugs were exposed to the tissue extracts [11]. Indeed, also in this study for all TA-AA prodrugs hydrolysis rates were found intensively accelerated (~\( t_{1/2} \): Ta-Arg 8.52 min, TA-Gly 16.99min, TA-Ala 17.45 min and TA-Lys 41.16 min) once the TA-AA prodrugs were put in contact with the skin homogenate. This was linked

<table>
<thead>
<tr>
<th>desolvation gas flow (L/h)</th>
<th>650</th>
<th>650</th>
</tr>
</thead>
<tbody>
<tr>
<td>collision gas flow (ml/min)</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>
to the vast presence of esterase in the *stratum corneum* [45], viable epidermis [46] and also dermis, yet decreased by 10-folds compared to the upper viable epidermis [11]. Nevertheless, the storage conditions of the skin at -20°C were reported to reduce the esterase activity, therefore the conversion *in vivo* is suspected to be even higher [47] and favourable for a prompt release of the active principle.

### 3.2 Intradermal deposition of TA-AA prodrugs

#### 3.2.1 Total drug delivery following passive and iontophoretic TA-AA application on porcine and human skin

The deposition of TA following passive (20 min) and iontophoretic delivery (20 min at 0.5 mA/cm²) of TA-AA prodrugs in human and porcine skin was illustrated in **Figure 1**. According to TA-AA prodrugs charge state in the donor solution (pH 5.5) they were grouped in two categories. The double positively prodrugs: TA-Arg and TA-Lys and the single positively charged: TA-Ala and TA-Gly. The behaviour of the molecules within the two groups was found similar for passive and iontophoretic delivery to the skin. Focusing first on the passive TA delivery, the resulting TA deposition from the monocharged TA-AA prodrugs (43.03 ± 10.91 and 38.07 ± 15.30 nmolTA/cm² for TA-Ala and TA-Gly, respectively) was roughly 4 times higher than from double charged prodrugs (9.10 ± 3.22 and 10.32 ± 3.72 nmolTA/cm² for TA-Arg and TA-Lys, respectively) (data from porcine skin). Anodal iontophoresis, regardless TA-AA different behaviours, substantially augmented TA deposition; 14 to 11-folds increase for TA-Arg and TA-Lys whereas 5.5 and 6-folds for TA-Ala and TA-Gly, when compared to correspondent passive controls. Comparing the amount of TA delivered by iontophoresis from the different prodrugs it was interesting to observe that the monocharged TA-AA prodrugs outperformed the double positively charged prodrugs (TA-Arg and Lys) which were thought from the experience in literature be more favourable for the electromigratory transport [48]. These findings were found repeated for active and passive delivery in human skin, with no statistically significant difference (T-test, p<0.05); confirming once more porcine skin to be a valuable representative model for the human tissue.
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**Figure 1.** Total delivery of TA in human and porcine skin following passive (Pass) and iontophoretic (Ionto) (0.5 mA/cm$^2$) application of TA-AA prodrugs (10 mM, pH 5.5) for 20 min was shown. TA-AA prodrugs were divided in double positively charged TA-AA$^{+2}$ (with 96% of charged entities) and monocharged TA-AA$^{+1}$ (>98% of charged prodrugs); correspondent Log D values at pH 5.5 were listed above the graph. (n=5)

3.2.2 **TA-Arg, TA-Lys and TA-Ala biodistribution profiles**

The overproduction of fibrous tissue in the dermis and subcutaneous space, characterizing the formation of keloid and hypertrophic scars, represents a thick diseased tissue specimen to treat. As stated in literature, topical corticosteroid treatment failed to deliver sufficient drug through the intact stratum corneum into the dermis [19]. With the idea to substitute intraregional TA injections with the iontophoresis of TA-AA prodrugs, significant deep distribution of the active principle into the dermis was needed.

Therefore, intradermal biodistribution profiles for three of the TA-AA prodrugs with substantial physicochemical differences were investigated. TA-Arg and TA-Lys were chosen, although both carry double positive charge on the molecule backbone, because of their previously reported stability difference [34]. TA-Ala was selected as representative molecule for the monocharged prodrugs since its comparable physicochemical properties, stability and drug deposition to TA-Gly. In Figure 2 the biodistribution
profiles of TA into porcine skin following 20 min of 0.5 mA/cm² iontophoretic application of 10 mM TA-AA prodrug solution were shown.

In correspondence to the previous paragraph (Figure 1), the relative superior TA deposition following TA-Ala iontophoresis was also observed in the biodistribution profile. In 80 to100% of the lamellae the amount of deposited TA form TA-Ala iontophoresis was statistically significantly (p<0.05) higher than compared to TA-Lys and Arg iontophoresis. Nevertheless, all concentrations obtained at maximum depth of 950-1000 µm by the application of the TA-AA prodrugs were found ~1000-5000 fold superior to the calculated concentration in the plasma following the oral administration of 5 mg TA, which was reported to have a pharmacological action [49].

Figure 2. Depth biodistribution profiles of TA in 50 µm thick skin slices following TA-Ala, TA-Arg and TA-Lys (10 mM in 10 mM MES buffer pH 5.5) iontophoretic application (20 min, 0.5 mA/cm²). TA deposition in deep layers from 450 µm to 1000µm were shown additionally in magnification. (n=5)
3.2.3 \textit{TA-Ala: current and time dependent iontophoretic delivery}

Given the above shown superiority of monocharged TA-AA prodrugs iontophoresis into the skin, the dependency of TA-Ala derived intradermal TA deposition on current density and application time were investigated. This was found especially important in prospective of a use on patients, when the regulation of the system with reduced current or application time could ameliorate the treatment and compliance.

The effect of current density was investigated by reducing from 0.5 to 0.2 and 0.1 mA/cm\(^2\) the density while keeping the application time fixed (20 min) (Figure 3a). In the second experiment the current density was kept constant at 0.5 mA/cm\(^2\) and the application time shortened from 20 min to 5 min as shown in Figure 3b. A similar linear dependency of TA deposition with the changing current density and time were found. This was not surprising given that similar amounts of charge were applied in both cases: For example, 0.24 coulomb were applied in 0.1 mA/cm\(^2\) for 20 min whereas for 0.5 mA/cm\(^2\) for 5 min 0.3 coulomb were delivered. However, when biodistribution profiles of the two lowest conditions were tested (Figure 3c), it could be seen that below a depth of 600 µm the longer TA-Ala iontophoretic application for 20 min at 0.1 mA/cm\(^2\) leaded to a statistically higher TA delivery in 75\% of the lamellae (p<0.05). This might suggest that a prolonged application time favours the deep distribution of the active principle; moreover, the use of a lower current density might also reduce the risk of skin irritations. However, the patient compliance might be decreased since the treatment times would be prolonged.
Figure 3.  a) TA total delivery following 20 min of iontophoretic application of TA-Ala in porcine skin with varying current densities (0.1, 0.25 and 0.5 mA/cm²)  b) TA total delivery into the skin following constant current application of 0.5 mA/cm² of TA-Ala and varying application time (5, 10, 20 min)  c) depth biodistribution profile comparing TA deposition in 50µm lamellae down to 1 mm of depth following TA-Ala iontophoresis for either 5 min at 0.5 mA/cm² or 20 min at 0.1 mA/cm². (n=5)

3.3 VER and TA-Arg co-iontophoresis

For a safer alternative with reduced corticosteroid exposure the co-iontophoretic delivery of TA with the calcium antagonist VER was investigated. Although VER was reported to be less effective compared to TA in the treatment of keloids recurrence, its safety profile was found suitable for protracted treatments [50]. Moreover, an alternant switch between the two active principles might be a valid substitute to conventional monotherapy [20] [26] [32]. In addition to the feasibility study the secondary aim was to better understand mechanistic peculiarities of biolabile TA-AA prodrugs when compared/combined to a monocharged stable molecule. The short application time of 20 min was maintained.

Table 5. Comparison of TA-Arg and VER physicochemical properties such as pKa, LogP/LogD and aqueous solubility.
### Table 5

<table>
<thead>
<tr>
<th>Property</th>
<th>TA-Arg</th>
<th>VER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (MW)</td>
<td>590.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>454.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pKa</td>
<td>13.36, 7.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.97&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log P</td>
<td>1.15 ± 0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.02 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log D pH 5.5</td>
<td>-2.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log D pH 7.4</td>
<td>-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Solubility µmol/ml (MES 10 mM, pH 5.5)</td>
<td>25.61 ± 1.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>169.02 [51]</td>
</tr>
<tr>
<td>Solubility µmol/ml (PBS, pH 7.4)</td>
<td>1.75 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> free type  
<sup>b</sup> ACD/Labs version 12.01  
<sup>c</sup> experimental

In Table 5 the physicochemical properties of TA-Arg and VER were listed. The two molecules designated for the co-iontophoresis experiments were shown to have comparable molecular weights (TA-Arg 590.3 and VER 454.6), however in the donor compartment at pH 5.5 TA-Arg was 96% double positively charged, whereas VER 99.9% single positively charged. An additional difference was observed in relation to the molecules stability in contact with skin; whereas TA-Arg rapid hydrolysis to TA was shown, no degradation was established for VER in the experimental conditions.

TA-Arg and VER different concentration (formulation A-E) were tested at 0.5 mA/cm<sup>2</sup> current density for 20 min on porcine skin and total drug depositions was reported in Figure 4. Focusing on formulation A and E where the two drug candidates were applied to the skin singularly (10mM), TA deposited in the skin was found 14-fold (for TA-Arg: 128.92 nmol<sub>TA</sub>/cm<sup>2</sup>) and 6-fold (VER: 254.70 nmol<sub>VER</sub>/cm<sup>2</sup>) increased compared to the passive application (TA 9.10 ± 3.22 nmol<sub>TA</sub>/cm<sup>2</sup> and VER 38.86 ± 7.07 nmol<sub>VER</sub>/cm<sup>2</sup>, data...
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not shown in the graph). This first proved the both molecules as suitable candidates for iontophoresis and the superiority of the active drug delivery over simple passive diffusion. However, it was noticeable that again, as for the case of the single positively charged TA-AA prodrugs, VER outperformed TA-Arg iontophoretic delivery.

When the drug delivery from formulation B, C and D of TA and VER were analysed, a linear increase in VER delivery was found following the concentration profile from 5, 7.5 to 9.5 mM ($R^2$ 9.77). However, it was found surprising that at equal drug amounts in the formulation B (5 mM for TA-Arg and VER) the delivery of TA was higher than VER (115.82 ± 23.67 nmol$_{TA}$/cm$^2$ and 53.52 ± 10.28 nmol$_{VER}$/cm$^2$, respectively). This was unexpected given that in the formulations A and E, were TA-Arg or VER were delivered singularly, VER outperformed TA. It seemed if as TA-Arg if present up to 25% of the total concentration it had the ability to reduce the total drug delivery compared to the single delivery of VER.

**Figure 4.** Total drug deposition (nmol/cm$^2$) following TA-Arg and VER co-iontophoresis for 20 min at 0.5 mA/cm$^2$ were illustrated. The composition of the donor solutions were A: 10mM TA-Arg and 0mM VER, B: 5 mM TA-Arg and 5 mM VER, C: 2.5 mM TA-Arg and 7.5 mM VER, D: 0.5 mM TA-Arg and 9.5 mM VER and E: 0 mM TA-Arg and10 mM VER). (n=5)

The skin biodistribution profiles of TA and VER in 50 µm thick lamellae were established as described in the previous paragraphs by cutting horizontal slices of the skin samples from the stratum corneum to the deep dermis with a cryotome. **Figure 5a** shows the depth profile of TA and VER following the separate
iontophoretic application of TA-Arg (10 mM, formulation A) and VER (10 mM, formulation E). As it was shown in the previous Figure 4, single compound iontophoresis of VER and TA-Arg resulted in doubled delivered amount of VER compared to TA. This superior iontophoretic delivery was further confirmed in the biodistribution profile, significantly higher (p < 0.05) amount of VER was found compared to TA in 90% of the slices. However, when the two drugs were co-administered in equal concentrations (Formulation B), TA-Arg iontophoretic behaviour seems to reduce significantly VER delivery and depth distribution. This finding was interesting from a mechanistic point of view on the exploration of TA-AA iontophoretic delivery, however undesired for the purpose of this co-iontophoretic study. The final goal was to propose a treatment alternative for keloid and hypertrophic scars with reduced risk of corticosteroid systemic delivery and therewith derived side effects. In this sense, a superficial delivery of TA in the upper skin layers and a sustained VER delivery through the lesion site were envisaged. To achieve this distribution profile the amount of TA-Arg was reduced drastically in the formulation to only 5%. In Figure 5c) the desired biodistribution profiles were illustrate, VER delivery was optimized and significantly detectable TA concentration was limited to the upper 800 µm of the skin.
Figure 5. Skin biodistribution profiles following the iontophoretic delivery (20 min 0.5 mA/cm²) of a) formulation A (10 mM TA-Arg) and E (10 mM VER), b) formulation B (5mM TA-Arg and 5 mM VER) and c) formulation D (0.5 mM TA-Arg and 9.5 mM VER). The amount of molecules deposited in the depth of 450 until 1000 µm are shown additionally in the magnification window, for better visual analysis. (n=5)

4. Discussion

4.1 TA-AA topical delivery

4.1.1 Passive TA deposition from TA-AA solution

TA deposition following 20 min passive application of TA-Ala and TA-Gly solution was found 4-folds increased when compared to TA-Arg and TA-Lys. The difference in delivery was thought to be related to the charge state of the prodrugs. In the donor solution at pH 5.5, TA-Arg and TA-Lys were double positively charged whereas TA-Ala and TA-Gly only single positively charged. The corresponding lipophilicity potential for the monocharged prodrugs was found higher (Log D of 0.36 and 0.44, respectively), and this was thought to favour the partition of the molecules into the lipophilic stratum corneum [52, 53] [40] increasing their passive penetration.
4.1.2  Iontophoresis of TA-AA prodrugs into the skin

The deposition profiles retrieved following iontophoretic delivery of TA-AA prodrugs were found in contrast to predictions made based on previous published work [48]. The two double positively charged prodrugs (TA-Arg and TA-Lys) were estimated as more favourable for the iontophoretic transport given their increased charge ratio. However, in our case they were outperformed by both monocharged prodrugs Ta-Ala and TA-Gly (Figure 1). Double increase in correspondent transport numbers (tₙ) for the TA-AA prodrugs in the tested conditions (20 min, 0.5 mA/cm²) confirmed the superiority of monocharged TA-AA prodrugs t₉-TA-Ala: 0.0253, t₉-TA-Gly: 0.0258 compared to t₉-TA-Lys: 0.0112, t₉-TA-Arg: 0.0139. However, in the literature it was already suggested that despite favourable charge ratio, the presence of a cationic residue adjacent to a lipophilic moiety might reduce the electrical mobility of a molecule because of interactions with the skin [54]. Such contradictory behaviour has been seen as example in large molecules such as lysozyme, where the higher amount of charge and electrophoretic mobility, compared to similar proteins, was not reflected in increased iontophoretic transport into the skin [41]. In another study, charge and lipophilic surface distribution, of small tripeptides were shown to influence the iontophoretic transport and skin interaction [55]. In silico 3D surface modelling of the tripeptides revealed the negative influence of surface descriptors like lipophilicity and isolated charges, on the iontophoretic transport of the molecules into the skin [40].

TA-AA prodrugs certainly enter in this category of molecules, with a localized positive charge opposite to a very lipophilic corticosteroid ring system. Moreover, all prodrugs have in common one amine group, however TA-Arg and Lys possess an additional basic functional group with pKa 13.15 and 10.45, respectively. The skin was reported to have a pH gradient from stratum corneum (~pH 5) to pH 7 in the upper viable epidermis [56]. Imagining the penetration of the intact TA-AA prodrugs into the skin (pH 7.4), the common amine group (pKa in between 6.9-7.57) will get partially neutralized on the contrary to the Lys and Arg sidechain amine. This persistent cationic sidechain was supposed to increase interaction with
the negatively charged components of the skin, changing the skin properties or hinder following molecules (analysed in following sections).

The depth biodistribution profiles of the three representative TA-AA prodrugs provided a further insight on their distinct iontophoretic behaviours [34]. In addition to the increased TA delivery following TA-Ala iontophoresis, it was seen that the transport was influenced by the susceptibility of the prodrugs to hydrolysis [57] [58] [12]. Considering only the two double positively charged prodrugs, TA-Arg and TA-Lys, a superior TA delivery in a depth of 600 µm was found following TA-Lys application (75%). TA-Lys was thought to have a prolonged depth transport via electromigration compared to TA-Arg which was losing its charged pro-moiety much earlier. However, this differences were less striking compared to previously published work [34], probably due to the use of prior frozen skin samples, where enzyme activity has been shown to be substantially reduced [47]. It could be that in fresh skin or in vivo the distribution profiles would differentiate even more due to the acerbation of the hydrolysis and with it the depletory effect on the iontophoretic transport.

4.1.3 TA-AA effect on acetaminophen electroosmosis and lidocaine electromigration

In order to deeper investigate the results obtained with the TA-AA prodrugs, their influence on i) electroosmotic (EO) transport of ACE and ii) on the electromigratory (EM) transport of LID were investigated experimentally and detailed in the following sections.

Acetaminophen

Co-iontophoresis with ACE was performed to determine the effect of TA-AA prodrugs on skin permselectivity, since the interaction with the tissue and certain cationic molecules [54] were found to decrease the current induced solvent flow from anode to cathode. This phenomenon is called EO inhibition. ACE, being a neutral molecule with low passive skin permeation will be only transported by this solvent flow and was therefore used as indicator molecule. ACE permeation through the skin following the
application of 0.5 mA/cm² constant current density was tested with and without the presence of the TA-AA prodrugs. The resulting inhibition factors (IF) were found: 6.49 and 3.46 for the double positively charged TA-AA prodrugs TA-Lys and TA-Arg, and 1.73 and 2.36 for TA-Ala and TA-Gly. Interestingly the inhibition factor of both double positively charged prodrugs was found superior to the monocharged prodrugs, suggesting a stronger interaction with the skin molecules. As reported in literature, the mere accumulation of molecules was not shown to necessarily correlate with the more or less powerful EO inhibition [40]. So as much, despite the inferior TA deposition from double charged prodrugs (TA-Arg and TA-Lys) an increased interference with the skin permselectivity and reduced ACE transport was found.

However, the reduction of the EO flow by the di-charged TA-AA prodrugs, was supposed to not be the only effect on the iontophoretic transport mechanism, given its minor importance for charged molecules [1].

Lidocaine

In order to better understand the iontophoretic transport of mono- and di-charged TA-AA prodrugs, their effect on LID iontophoretic delivery was investigated. The hypothesis was made that the prodrugs might interact differently with the skin and more or less obstacle the EM of further LID molecules, influencing the total delivery [59] [41].

Comparable to previous experiments the application time of TA-AA prodrugs was kept for 20 min at 0.5 mA/cm². The iontophoretic pre-treatment with TA-Ala, TA-Arg or simple MES buffer were followed by 20 min of LID iontophoresis at 0.5 mA/cm². The deposited amount of LID in the skin was extracted and resulted significantly decreased (p< 0.05) when the delivery followed TA-Arg (644.31 ± 57.91 nmol_{LID}/cm²) iontophoretic pre-treatment compared to blank buffer pre-treatment (1074.37 ± 95.27 nmol_{LID}/cm²) (Figure 6a). However, TA-Ala iontophoretic pre-treatment did not result in the same effect (972.83 ± 104.80 nmol_{LID}/cm²). A similar inhibitory behaviour as TA-Arg was found with TA-Lys when 20 min iontophoretic pre-treatment preceded 5h of LID iontophoresis (0.5 mA/cm²) and samples of
permeated LID were collected each hour from the receiver compartment. As shown in Figure 6b, in the first hour permeated LID amount was found significantly decreased following TA-Lys pre-treatment (81.69 ± 25.28 nmol LID/cm²) compared to MES buffer pre-treatment (221.69 ± 86.16 nmol LID/cm²). Given these results, the hypothesis was made that double positively charged prodrugs, in addition to their effect of decreasing skin permselectivity, also hinder EM. Herewith the reduced total TA delivery following TA-Arg and TA-Lys iontophoresis could be suggested caused by their hindering effect of their own iontophoretic transport.

**Figure 6.** a) Total LID skin deposition after 20 min iontophoretic pre-treatment with 10 mM TA-Arg, TA-Ala or MES buffer and following 20 min LID iontophoresis at 0.5 mA/cm² of (40 mM). b) LID permeation profiles following 20 min pre-treatment with TA-Lys or MES buffer and sequent 5h of LID iontophoretic delivery at 0.5 mA/cm² (40 mM). (n=5) * Denotes statistically significant difference (t-test; p<0.05)

4.2 TA-Arg and VER co-iontophoresis

Repeated corticosteroid injections were found mostly effective for the treatment of keloid or hypertrophic scars; however depending on the area and frequency of the treatment, especially in paediatric patients an elevated risk of developing Cushing syndrome was described [21] [22] [60] [61]. VER injections on the
other hand, were reported to flatten and soften the scar tissue without provoking any reported local or systemic side effects [26]. Given these findings, a co-iontophoretic treatment with TA and VER was tested to ensure a profound drug delivery to the entire scar tissue with VER while limiting TA systemic exposure. To achieve this, TA-Arg was chosen because of its low hydrolytic stability, which was supposed to provoke the loss of the EM driving force in the upper skin layers [34]. VER on the contrary was supposed to penetrate more profoundly into the scar tissue, given its stability and constant charge.

When the two molecules were applied separately via iontophoresis, similar to the above described findings with the different TA-AA prodrugs, the total delivery from TA-Arg iontophoresis (formulation A) resulted 2-folds lower than VER delivery (formulation E). Again, the iontophoretic transport of a monocharged drug (VER) was seen more successful. When the two molecules were combined the limiting effect of TA-Arg on the iontophoretic transport mechanism was furthermore evidenced. At equal concentrations of TA-Arg and VER (formulation B), the resulting total delivery of both molecules ($169.34 \pm 33.95 \text{ nmol}_{\text{TA+VER}}/\text{cm}^2$) remained statistically lower than single VER delivery ($254.70 \pm 38.50 \text{ nmol}_{\text{VER}}/\text{cm}^2$). Moreover, the TA-AA prodrug seemed to be the preferred charge carrier, therefore higher TA amounts were deposited ($115.82 \pm 23.67 \text{ nmol}_{\text{TA}}/\text{cm}^2$) compared to VER ($55.52 \pm 10.28 \text{ nmol}_{\text{VER}}/\text{cm}^2$) (formulation B). It could be hypothesized that TA-Arg once in contact with the skin blocked the passage for coming molecules, reducing herewith the total delivery of the drugs.

In the biodistribution profiles of formulation D and E, effective concentrations of VER (100 $\mu$mol/ml) were delivered down to 1 mm into the skin. With this drug concentration an effective alteration of fibroblast cell shape, pro-collagenase synthesis and concomitant degradation of collagen in the extracellular matrix were observed [23] [24] [62]. Moreover, in the upper half of the layers VER concentrations were 6 to100-fold superior to the effective concentration. This drug depot was thought to further sustain the treatment by the release VER via passive diffusion also after the iontophoretic application.
For the co-iontophoresis treatment the biodistribution profiles revealed unfortunately a less localized corticosteroid repartition than hoped, which was envisaged in the beginning to limit the systemic exposure. Although the very low concentration of TA-Arg in formulation D (0.5 mM), TA deposition was significantly quantified down to 800 µm, with an \(\approx 500\)-fold superior effective concentration, compared to plasma concentrations following oral administration [49]. However, it was suggested that \textit{in vivo} conditions esterase activity might be much increased and this could lead to a more confined distribution of TA in the uppermost layers [47]. Additionally, the total amount of TA delivered locally is inferior to 5 µg, 1000-fold lower than the oral dose, therefore even if absorbed, toxicity profile was supposed to be limited.

5. Conclusion

Four novel TA-AA prodrugs were tested on porcine and human skin for short term dermal iontophoresis as treatment option for hypertrophic and keloid scars. In addition to the successful delivery of TA into deep dermal regions (1mm), distinct iontophoretic behaviour of the prodrugs was evidenced. Against all expectations, TA delivery from double positively charged TA-AA prodrugs (TA-Arg and TA-Lys), with the most favourable charge mass ratio, was found inferior compared to monocharged TA-Ala and Gly prodrugs. This astonishing finding adds to the various reports in literature describing molecules which despite their promising physicochemical properties achieved only suboptimal delivery. In case of TA-Arg and TA-Lys, with their localized positive charge on a hydrophilic backbone, a strong interaction with the skin was supposed. This interaction was found not only to influence the skin permselectivity, by reducing the EO transport of ACE, but also significantly reduce EM which was shown via the iontophoresis of LID as marker. The inhibitory effect on EM was furthermore shown in the co-iontophoretic experiments with VER, where only the reduction of TA-Arg to less than 25% in the formulation enabled the efficacious delivery of VER. This combination therapy was suggested as interesting alternative to gold standard corticosteroid therapy, especially in the maintenance phase to avoid any relapse in scar formation. Furthermore, in forecast to an application on patients, TA delivery was found to be linearly correlated to
time and current density and therefore drug delivery would be adaptable to the severity of the scar and patient needs, evidencing the advantage of non-invasive iontophoretic treatment.

6. **Supplementary data**

6.1 **TA-AA prodrug physicochemical properties**

Table S1. Summary of TA and TA-AA molecular weight (MW), pKa, LogP/D and experimental solubility values.

<table>
<thead>
<tr>
<th></th>
<th>TA</th>
<th>TA-Arg</th>
<th>TA-Lys</th>
<th>TA-Ala</th>
<th>TA-Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>434.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>590.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>562.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>505.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>491.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pKa</td>
<td>13.15, 12.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.36, 7.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.45, 6.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log D&lt;sub&gt;pH 5.5&lt;/sub&gt;</td>
<td>2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log D&lt;sub&gt;pH 7.4&lt;/sub&gt;</td>
<td>2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Solubility in µmol/ml (MES 10 mM, pH 5.5)</td>
<td>25.61 ± 1.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.12 ± 1.59</td>
<td>15.92 ± 0.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.88 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.76 x10&lt;sup&gt;-2&lt;/sup&gt; [63]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubility in µmol/ml (PBS, pH 7.4)</td>
<td>1.75 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.40 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.24 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.05 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> free type  
<sup>b</sup> ACD/Labs version 12.01  
<sup>c</sup> experimental

6.2 **TA and VER: validation of skin extraction**

The extraction of TA and VER in 10 ml methanol: water mixture (50:50) overnight were evaluated by solvent evaporation method on porcine skin. Three concentrations of each molecule were tested in triplicates (100, 50 and 10 nmol/cm<sup>2</sup> for TA and VER). Briefly: drug solutions were applied on the skin samples, solvent evaporated and the skin samples extracted. The extraction samples were analysed by HPLC-UV and drug amount summarized in Table S2. Overall the resulting total recovery rate of 85-98% was found acceptable for the further skin deposition studies.

Table S2. Validation of TA and VER extraction from porcine skin samples (Mean ± SD)
CHAPTER V: Iontophoresis for the treatment of hypertrophic and keloid scars; a study of triamcinolone acetonide prodrugs and verapamil

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Theoretical amount (nmol/cm²)</th>
<th>Recovered amount (nmol/cm²)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>100</td>
<td>96.79 ± 1.13</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.23 ± 0.70</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.29 ± 0.49</td>
<td>93</td>
</tr>
<tr>
<td>VER</td>
<td>100</td>
<td>96.60 ± 20.78</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>42.83 ± 4.14</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.50 ± 1.01</td>
<td>85</td>
</tr>
</tbody>
</table>

6.3 Analytical method validation of HPLC-UV quantification of TA, VER, ACE and LID

The analytical method validation was performed following the ICH Q2 (R1) guidelines [42].

6.3.1 Linearity and specificity

The calibration curves in the concentration range of: 6.62 to 661.53 nmol/ml ACE, 3.69 to 369.28 nmol/ml LID, 2.30 to 230.15 nmol/ml TA and 2.04 to 203.64 nmol/ml VER were found linear and specific for the analytical conditions described in the main text.

6.3.2 Precision and accuracy

Results of intra- and inter-day precision and accuracy were reassumed in Table S4; all values were found within the acceptance limits.

Table S4. Intra- and inter-day precision and accuracy for ACE, LID, TA and VER quantification

<table>
<thead>
<tr>
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<th>Intra-day</th>
<th>Inter-day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical (nmol/ml)</td>
<td>Measured (nmol/ml)</td>
</tr>
<tr>
<td>ACE</td>
<td>661.54</td>
<td>661.07 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>132.31</td>
<td>130.08 ± 5.64</td>
</tr>
</tbody>
</table>
CHAPTER V: Iontophoresis for the treatment of hypertrophic and keloid scars; a study of triamcinolone acetonide prodrugs and verapamil

6.4 UHPLC-MS/MS analytical method for TA and VER quantification

6.4.1 Specificity, linearity, LOD and LOQ

The method was found specific and linear in a concentration range of 11.51-230.15 and 230.15-2301.50 pmol/ml for TA; 20.36-203.64 and 203.64-2036.4 pmol/ml for VER. LOQ and LOD for TA were found at 7.61 and 2.5 pmol/ml, for VER 15.33 and 5.06 pmol/ml, respectively.

6.4.2 Precision and accuracy

Good intra and inter-day accuracy and precision were found for the simultaneous TA and VER quantification method (Table S5).

Table S5. Intra-and inter-day precision and accuracy for TA quantification a) Precision= (SD/mean)*100, b) Accuracy= (measured concentration/theoretical concentration)*100

<table>
<thead>
<tr>
<th>Theoretical (pmol/ml)</th>
<th>Measured (pmol/ml)</th>
<th>RSD (%) a)</th>
<th>Recovery (%) b)</th>
<th>Measured (pmol/ml)</th>
<th>RSD (%) a)</th>
<th>Recovery (%) b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2301.50</td>
<td>2277.50 ± 90.76</td>
<td>3.99</td>
<td>99.0</td>
<td>2296.06 ± 164.28</td>
<td>7.15</td>
<td>99.8</td>
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<tr>
<td>460.30</td>
<td>471.73 ± 33.32</td>
<td>7.06</td>
<td>102.5</td>
<td>455.06 ± 12.33</td>
<td>2.71</td>
<td>98.9</td>
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<tr>
<td>230.15</td>
<td>231.22 ± 4.64</td>
<td>2.01</td>
<td>100.8</td>
<td>232.46 ± 8.39</td>
<td>3.61</td>
<td>101.0</td>
</tr>
<tr>
<td>46.03</td>
<td>44.55 ± 4.14</td>
<td>9.29</td>
<td>95.6</td>
<td>45.69 ± 1.29</td>
<td>2.82</td>
<td>99.3</td>
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<tr>
<td>11.51</td>
<td>12.39 ± 0.23</td>
<td>1.83</td>
<td>108.6</td>
<td>12.73 ± 0.02</td>
<td>0.16</td>
<td>110.6</td>
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<tr>
<td>LID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>369.28</td>
<td>369.60 ± 5.07</td>
<td>1.37</td>
<td>99.6</td>
<td>367.69 ± 3.55</td>
<td>0.96</td>
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<td>73.86</td>
<td>75.90 ± 2.36</td>
<td>3.11</td>
<td>102.3</td>
<td>72.97 ± 6.56</td>
<td>5.07</td>
<td>101.1</td>
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<td>18.46</td>
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<td>101.0</td>
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<tr>
<td>230.15</td>
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<td>247.92 ± 0.96</td>
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<td>107.7</td>
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<tr>
<td>TA</td>
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<td>45.33 ± 0.45</td>
<td>1.00</td>
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<tr>
<td>11.51</td>
<td>44.68 ± 0.33</td>
<td>2.79</td>
<td>100.1</td>
<td>11.38 ± 0.61</td>
<td>5.33</td>
<td>98.9</td>
</tr>
<tr>
<td>VER</td>
<td>203.64</td>
<td>203.29 ± 8.24</td>
<td>4.06</td>
<td>97.8</td>
<td>202.75 ± 5.88</td>
<td>2.90</td>
</tr>
<tr>
<td>40.73</td>
<td>40.59 ± 0.94</td>
<td>2.30</td>
<td>98.6</td>
<td>39.19 ± 1.31</td>
<td>3.33</td>
<td>96.2</td>
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<tr>
<td>10.18</td>
<td>10.10 ± 0.28</td>
<td>2.77</td>
<td>98.2</td>
<td>10.76 ± 0.72</td>
<td>6.72</td>
<td>105.7</td>
</tr>
</tbody>
</table>

LID: Lidothene; TA: triamcinolone acetonide; VER: verapamil.
7. Acknowledgements

We would like to thank the University of Geneva for the financial support and Dr. Brigitte Pittet-Cuenod, Department of Plastic, Aesthetic and Reconstructive Surgery, Geneva University Hospital for providing human skin samples. YNK would like to thank the University of Geneva, the Fondation Ernst and Lucie Schmidheiny and the Société Académique de Genève for providing financial support to enable the acquisition of the Waters Xevo® TQ-MS detector.

8. References


CHAPTER V: Iontophoresis for the treatment of hypertrophic and keloid scars; a study of triamcinolone acetonide prodrugs and verapamil


CONCLUSIONS
In this study, the ocular and dermal delivery via iontophoresis of novel synthesized triamcinolone acetonide (TA-AA) amino acid ester prodrugs was investigated. The physicochemical properties of the TA-AA prodrugs were characterized, and the correspondent aqueous solubility was found increased up to 1000-fold compared to the parent drug TA. The added charge on the molecular backbone was found not only to raise the solvation of the compounds but enabled a superior TA delivery via iontophoresis when compared to passive. Net enhancement was obtained with short application times of 5 to 20 min \textit{in vitro} onto porcine cornea and whole eye globes as well as porcine and human skin (0.5-3 mA/cm$^2$ on cornea, 1.5-6 mA/cm$^2$ on sclera and 0.1-0.5 mA/cm$^2$ on skin). The reconversion of the TA-AA prodrugs to the parent compound (TA) in contact with the ocular tissue and the skin was shown within minutes due to chemical and enzymatic hydrolysis, confirming the prompt liberation of the active species in the target tissues. Interestingly it was established by depth biodistribution profiles of cornea as well as in the whole eye globe, that the hydrolytic stability of the TA-AA prodrugs influenced the tissue distribution. Therapeutically relevant TA concentrations were found in the target regions such as corneal stroma, humor vitreous; however, higher TA amounts in the deep corneal stroma and posterior segment tissues (retina and cornea), were found delivered by the hydrolytically more stable prodrugs (e.g. TA-Lys). This was suggested to be linked to the prolonged effect of the current on the intact prodrug which carries the positive charge essential for the efficient current induced transport. In comparison to the hydrolytic stability, the relation in between the charge on the TA-AA backbone and the iontophoretic delivery was found less straightforward. For example: despite the two-fold increased electrical mobility established by capillary zone electrophoresis of double positively charged TA-AA prodrugs (TA-Arg and TA-Lys) compared to the remaining prodrugs, this superiority was not reflected in the correspondent iontophoretic delivery. Equal (cornea and sclera) to inferior (skin) delivery of TA from the double positively charged TA-AA prodrugs was found when compared to single positively charged TA-AA prodrugs like TA-Ala and TA-Gly. This unexpected behaviour was related to the possible interaction of the TA-AA prodrugs with the different biological membranes. Only basic amino acid prodrugs will remain principally ionized throughout the tissues (pH 7.4), therefore electrostatic
interaction in between the negatively charged components present in the biological membranes (e.g. proteoglycans, melanin) were supposed, like reported in the literature, to diminish the transport of the molecules. This inhibitory effect of the double positively charged prodrugs was clearly evidenced in the co-iontophoretic experiments, when TA-Arg was added in the donor compartment with verapamil for iontophoretic delivery into the skin. In conclusion, the results obtained in this study illustrate the importance of preliminary \textit{in vitro} experiments for the selection of candidates for iontophoresis, by evidencing the difficulty to predict drugs iontophoretic transport in presence of the complex biological membranes. However, especially for the case of intraocular drug delivery, the absence of dynamic barriers represents the major drawback of the \textit{in vitro} system. It must be acknowledged that the drug delivery results obtained are almost certain an overestimation of the \textit{in vivo} situation and therefore main information is given on the overcoming of the static physical barriers.
Appendix I

- Hyaluronic acid after subcutaneous injection - an objective assessment
Hyaluronic acid after subcutaneous injection - an objective assessment

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Submitted to: Dermatologic Surgery

Abstract

BACKGROUND Hyaluronic acid (HA) fillers are the preferred injectable products for aesthetic correction of skin depressions and restoration of facial volume.

OBJECTIVE To investigate the subcutaneous distribution of three, biophysically distinct, CE marked and FDA approved HA fillers.

MATERIALS AND METHODS Belotero Balance (BELB), Juvederm Voluma with lidocaine (JUVV) and Restylane Lidocaine (RESL) were injected ex vivo in porcine and human skin. Immediately after
injection, the skin samples were snap-frozen, cross-sectioned and visualized using stereo microscopy and full field optical coherence tomography. Images were compared with histological sections after haematoxylin and eosin staining.

RESULTS HA fillers were distributed as homogeneous bolus in the ex vivo skin. The injection bulks were found to preserve the fibrous trabecular network, shift the fat lobules and displace the adjacent adipocytes layers independently of the formulation injected.

CONCLUSION For the first time the subcutaneous injection of three HA fillers with markedly different biophysical properties was systematically investigated by complementary visualization techniques. Despite their different properties, no difference in distribution was found following subcutaneous injection. The global preservation of the hypodermis structure observed was consistent with the good tolerability seen in clinical practice after implantation of the HA fillers in the subcutaneous skin layer.
1. **Introduction**

The biocompatibility, versatility and unique biophysical properties of hyaluronic acid (HA) fillers have made them increasingly popular products for soft tissue correction and volume restoring procedures [1]. Statistics from the American Society for Aesthetic Plastic Surgery (ASAPS), show that injection of HA fillers was the second most practiced non-surgical procedure in the USA, with more than 2.4 million interventions in 2016 [2].

HA fillers are designed by manufacturers for injection either into the dermal layer, in the case of the superficial products, or into the subcutaneous skin layer and the supraperiostic zone for the so-called “volumizer products”. The behavior of HA gel fillers following intradermal injections has been extensively reported [3-7] evidencing the high tolerability of the HA fillers with preservation of the dermal cells and the extra-cellular matrix. The intradermal distribution of the HA fillers was found to depend on their biophysical properties, i.e. their visco-elastic properties [8,9] and their cohesivity levels [10]. HA fillers with a high cohesivity and low visco-elasticity showed a rather homogeneous integration in the dermis while HA fillers with a poor cohesivity and a high visco-elasticity revealed a more heterogeneous dermal integration [3, 4]. However, although reports have demonstrated the feasibility of intradermal injection of HA fillers by expert injectors (e.g. corroboration via the “blanching” technique developed by Micheels et al. by ultrasound imaging), [5, 11] clinical experience, observation of current injection techniques and physical constrains (e.g. dimensions of a 30G needle) suggest that the vast majority of injections are directly into the subcutaneous skin layer and not into the dermis- despite the product claims and indications [12]. In this regard, Arlette et al. showed that the predominant localization of HA fillers injected for the treatment of nasolabial folds was indeed within the subcutaneous skin. Given that excellent cosmetic results were nevertheless obtained, it was concluded that the dermal localization of the HA filler products was not a condition *sine qua non* for the treatment of this common indication [13]. In contrast to the reports on the distribution of HA fillers in the dermis, little is known about the behavior of HA fillers in subcutaneous fat; this is paradoxical.
since this is where they are most likely to be found after injection. A recent preliminary investigation described the subcutaneous injection of two volumizer HA fillers into a female subject scheduled for abdominoplasty and showed that the product with the highest cohesivity appeared to better maintain gel integrity and homogeneity in the hypodermis, which was consistent with results obtained in the dermis [14].

Given the scarcity of literature on the behavior of HA fillers in the subcutaneous space, the aim of the present study was to make a rigorous and systematic assessment of the subcutaneous distribution of three CE marked and FDA approved HA fillers: Belotero Balance (BELb), Juvéderm Voluma with lidocaine (JUVv) and Restylane Lidocaine (RESL). We would like to emphasize that although not all of these products are indicated for subcutaneous implantation, they were chosen in order to cover a broad spectrum of biophysical characteristics (Table 1) [8, 10, 15].

**Table 1. Summary of the main biophysical properties of the HA fillers investigated in this study.**

<table>
<thead>
<tr>
<th>Properties</th>
<th>BELb</th>
<th>JUVv</th>
<th>RESL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-linking technology</td>
<td>CMP</td>
<td>Vycross</td>
<td>NASHA</td>
</tr>
<tr>
<td>Cross-linker</td>
<td>BDDE</td>
<td>BDDE</td>
<td>BDDE</td>
</tr>
<tr>
<td>HA content (mg/ml) [15]</td>
<td>22.5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Indication based on CE marking</td>
<td>Indicated to fill moderate facial wrinkles and folds</td>
<td>Indicated to restore facial volume</td>
<td>Indicated for the correction of wrinkles</td>
</tr>
<tr>
<td>Implantation depth based on CE marking</td>
<td>Dermis</td>
<td>Subcutis and/or supraperiosteal zone</td>
<td>Dermis</td>
</tr>
<tr>
<td>Cohesivity level according to the Gavard-Sundaram cohesivity scale [10]</td>
<td>Cohesivity score = 5 (=fully cohesive)</td>
<td>Cohesivity score = 2 (=dispersed)</td>
<td>Cohesivity score = 1 (=fully dispersed)</td>
</tr>
<tr>
<td>Elasticity G’ (Pa) at 0.7 Hz (shear-stress conditions) [8]</td>
<td>63 ± 3</td>
<td>314 ± 5</td>
<td>677 ± 13</td>
</tr>
<tr>
<td>Elasticity E’ (Pa) at 0.7 Hz (compression conditions) [8]</td>
<td>31457 ± 1132</td>
<td>41747 ± 947</td>
<td>8456 ± 256</td>
</tr>
<tr>
<td>Normal force FN (N) at 1.5 mm</td>
<td>0.51 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>
Appendix I - Hyaluronic acid after subcutaneous injection - an objective assessment

<table>
<thead>
<tr>
<th>HA gel macrostructure</th>
<th>“Spider-web” like</th>
<th>Particulate</th>
<th>Particulate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(optical microscope)</td>
<td>[15-17]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA gel microstructure</td>
<td>Fibrous network</td>
<td>Fibrous network</td>
<td>Fibrous network</td>
</tr>
<tr>
<td>(cryo-SEM) [15, 18]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As can be clearly seen in Table 1, BEL$_B$ and RES$_L$ represented the extreme opposites in terms of cohesivity levels, rheological properties (elasticity in shear stress conditions and static compression) and HA gel structures. BEL$_B$ was reported as a highly cohesive and low visco-elastic HA filler, whereas RES$_L$ as a poorly cohesive and highly visco-elastic HA filler; JUV$_V$ was selected as a HA filler with intermediate biophysical properties in comparison to BEL$_B$ and RES$_L$.

The focus of the study was on the elucidation of the subcutaneous distribution of these commercially available gels at time zero, i.e. immediately following subcutaneous implantation in three different $ex$ $vivo$ skin models: i) porcine ear skin, ii) human skin samples from abdominoplasty and iii) human skin samples from facial lifting. HA distribution in the hypodermis was visualized with the help of three complementary imaging techniques: macroscopic observation (using a stereo microscope), full field optical coherence tomography and histological analysis of the injection area in order to identify eventual characteristic distribution patterns of the HA fillers under investigation.

2. Materials and Methods

Three CE marked and FDA approved HA fillers were purchased from commercial sources: Belotero Balance (BEL$_B$) manufactured by Merz Pharma (Geneva, Switzerland), Juvederm Voluma with lidocaine (JUV$_V$) manufactured by Allergan (Pringy, France) and Restylane Lidocaine (RES$_L$) manufactured by Galderma (Uppsala, Sweden). Isopentane and formaldehyde for the snap-freezing and conservation of the skin samples were bought from Sigma-Aldrich (Steinheim, Germany).

The porcine ears from 6-month old pigs (100-120kg) were obtained from a local slaughter house (CARRE, Rolle, Switzerland). Human skin samples from facial liftings (5 subjects) and
abdominoplasties (5 subjects) were kindly provided by the private Hôpital de La Tour (Geneva, Switzerland). The use of the human tissues was approved by the ethical committee for clinical and ambulatory research (Association des Médecins du canton de Genève; AMG, protocol 10-25).

2.1 EX VIVO subcutaneous injections and visualization

2.1.1 Porcine ear skin vs. human skin

Porcine ears were chosen for this study given the anatomical and structural similarity to human skin. A further rationale for choosing this model was the possibility to inject the investigated HA fillers into the hypodermis of the intact porcine ear; this enabled the physiological tension of the tissue to be maintained. This was not possible for the human skin samples, which were harvested following abdominal and facial interventions and therefore lacked basal and lateral fixation.

Obviously, the facial skin samples were the closest to reality given that in clinical practice most HA gel injections are administered to the face. However, the small sizes (i.e. area and volume) of the sample specimens hampered the execution of the subcutaneous injections. Skin samples from the temple skin area were used because of the presence of significant fat tissue, accepting the presence of thick cranial hair shafts in the images. In contrast abdominal skin samples did not have these limitations – samples were larger and subcutaneous fat was abundant, moreover only villous hairs were found.

2.1.2 Subcutaneous injections and visualization techniques

With the aim to use conditions as close as possible to actual treatment: 0.1 ml of each commercial HA filler was injected ex vivo through the skin into the subcutaneous fat, by the same expert injector. Needles were used as provided in the commercial product (BELb, 30G½; JUVv, 27G½and RESl, 29G½). The injection angle was directed in between 20 and 45° depending on the tissue samples, to ensure that the product was deposited in the hypodermis. For the human abdominal skin, the needle penetration depth was measured to be approx. 6 mm whereas for porcine skin – as well as the human facial skin samples – more superficial injections with a depth of 2 mm were performed.
Following the injection, each sample was isolated and snap-frozen in isopentane chilled with liquid nitrogen (−196 °C). In the frozen state, the injection areas were cross-sectioned and immediately observed with a full field optical coherence tomography (FFOCT) microscope (Light-CT Scanner; LL-Tech, Paris, France) and a stereo microscope (LEICA S6D, Heerbrugg, Switzerland).

FFOCT tissue imaging was of particular interest given the rapidity of sample preparation and the 1 µm resolution of the images [19]. Skin cross-sections were inserted in the FFOCT holder and observed straight away with no need for complex preparation such as staining that could alter the aspect of the skin. The subcutaneously injected HA gel was not visible directly given its lack of light backscattering properties, whereas the surrounding fat tissue and the upper dermis were clearly defined. Observation of the injection site under the stereo microscope was used to confirm the presence of the gel in the injection cavity. All samples were then fixed in formaldehyde and submitted for histological slicing in paraffin and staining with haematoxylin and eosin.

3. Results

3.1 Porcine ear skin

The injections of the three HA fillers were performed in the porcine ear skin in order to visualize the effect of the injection bulk on the surrounding subcutaneous structures and vice versa. Given the tissue attachment present in this skin model, e.g. on the cartilage and/or the muscle, one interesting question was whether this tension would influence the diffusion properties of the HA gels. Injection area cross-sections were immediately observed by FFOCT, the minimal sample preparation enabled the imaging of the skin in an almost native state.
Appendix I - Hyaluronic acid after subcutaneous injection - an objective assessment

**Figure 1.** Subcutaneous injection sites of HA fillers (RESL and JUVv) in porcine ear skin: a,c) FFOCT images and b,d) corresponding stereo microscope images. For purpose of clarity HA filler (*) and subcutaneous trabeculae (->) are indicated. Scale bar = 500 µm

As shown in Figure 1, minimal deformation of the hypodermis was observed following the HA gel injections. In FFOCT the direct visualization of HA fillers was not possible given the absence of contrast of the HA gel; however, complementary observation of the injection area with the stereo microscope revealed its presence.

**Figure 2.** FFOCT images of BELb injections into porcine ear hypodermis: a) cross-section, b) zoom on upper injection site with focus on the trabeculae fibers and adipocytes. Scale bar = 500 µm
From the FFOCT images it was clear that the fat lobules were displaced – thus, the adipocytes in the first layers of cells adjacent to the HA gel were shifted but not structurally altered and this was systematically observed in all of the samples (Figure 2). Moreover, the interlobular network of trabeculae seemed to surround recurrently the injection site and partially divide the HA gel bolus. As images of the three biophysically distinct HA fillers highlighted (Figure 1 and 2), no distinguishable difference between the effects of the gels could be observed. Furthermore, when the injection site was compared with the human skin ex vivo (see Figure 3), no influence of the tissue tension present could be identified, i.e. no distinct HA gel distribution was observed.

3.2 Human abdominal skin

The subcutaneous injection of the HA fillers in human abdominal skin showed similar behavior to the results in porcine ear skin. All three HA fillers, injected into the abdominal subcutaneous fat formed a mainly homogeneous bolus.

Figure 3. Cross-section macro images following subcutaneous injection into human abdominal hypodermis of a) BEL₉, b) JUV₉ and c) RES₉. For purpose of clarity HA filler (*) and subcutaneous trabeculae (->) are indicated. Scale bar = 1000 µm

Figure 3 shows representative images taken with the stereo microscope of the cross-sections of BEL₉, JUV₉ and RES₉ in the abdominal skin fat tissue (n=5 replicates for each product). Interestingly,
comparison of the images shows that the horizontal distribution of the HA gels appeared to be delimited by the interlobular trabeculae (fibrous septa) that cross the fat tissue and link the dermis to the underlying fasciae [20, 21].

The subcutaneous trabecular structure was very clearly delineated after haematoxylin and eosin staining of the samples; macro images and histology slices of the corresponding skin samples are shown in **Figure 4**. It is important to mention that in the histological cross-sections only very few traces of the HA gels were visible. It was noticed that during the slicing procedure in paraffin – required for preparation of the histological samples – HA gels were eliminated from the lamellae; it appeared that the soft fat tissue was unable to retain the HA gels during processing. This is why it was essential to observe the samples immediately following injection and cross-sectioning with the stereo microscope, which confirmed the presence of the gels with their “jelly-like” aspect. Comparison of the images of the same area using the different visualization techniques confirmed that the void spaces seen in the histological slices corresponded to the imprint of the HA gel distribution in the subcutaneous skin layer.
Figure 4. Human abdominal skin subcutaneous injection. Side by side panels show the macro and the corresponding histological images of a,b) BEL, c,d) JUV and e,f) RES. *Indicates the areas corresponding to the HA gel injection sites – during slicing and haematoxylin and eosin staining most of the gel was eliminated from the samples. Scale bar = 1000 µm
3.3 Human facial skin

Comparable to the results with human abdominal skin and porcine ear skin, the HA fillers were again distributed in the facial subcutaneous skin mostly as homogeneous boluses that were occasionally confined by the interlobular fibrous septa (trabeculae).

![Figure 5](image)

**Figure 5.** Facial skin subcutaneous injection area of BELB: a) stereo image b) FFOCT image with focus on displaced adipocyte layers surrounding the HA filler and the trabecular filament. Air bubbles can clearly be seen in the HA gel in the stereo image. Scale bar = 1000 µm

It was important to note that the fibrous structures of the hypodermis seemed unaltered by the injected HA fillers, only the fat lobules were displaced by the HA gels and the first layers of adipocytes adjacent to the gel were shifted. This is evidenced in **Figure 5** which shows the macro image of BELB injection in the temporal subcutaneous fat layer. The detailed image with the FFOCT microscope evidences once more the adipocytes and the proximate trabeculae surrounding the HA gel.

In comparison to abdominal skin, subcutaneous fat was less present and the connective tissue network more abundant in facial skin samples. Comparative macro images of injection areas with the corresponding histology images are shown in **Figure 6**.
Figure 6. Facial skin images after subcutaneous injections of \( \text{BEL}_b \), \( \text{JUV}_v \) and \( \text{RES}_L \). Images \( \text{a,c,e)} \) show injection site cross-sections under the stereo microscope, whereas images \( \text{b,d,f)} \) present the corresponding histological slices with haematoxylin and eosin staining. *Denotes the empty areas in the histological images of the HA injection bulk, parts of the HA gels were still visible as purple scale like structures. Scale bar = 500 µm

4. Discussion

In this article, for the first time three complementary visualization techniques, stereomicroscopy, FFOCT and histology, were employed to investigate the subcutaneous distribution of three commercially available HA filler formulations. The lower elasticity of the subcutaneous skin as compared to the dermis meant that it did not significantly influence the HA gel distribution. Therefore, the distinct biophysical characteristics of the HA fillers chosen for this study (\( \text{BEL}_b \), \( \text{JUV}_v \) and \( \text{RES}_L \)), did not lead to characteristic “product-specific” distribution patterns.
All HA gels seemed to distribute as homogeneous boluses in between the fibrillary network displacing the fat lobules. The subcutaneous trabeculae network was seen to form a resistant envelope, which contains and constrains the HA filler, limiting its movement. On the other hand, HA gel contoured by the quite rigid and undamaged collagenous structures was found to shift and displace the adjacent adipocyte layers.

It is also important to note that no alteration of the trabecular network and/or compression of the fat lobules were evidenced in any of the *ex vivo* models used. Histological images obtained by haematoxylin-eosin staining revealed the morphological preservation of the cutaneous structure. Although experiments were not designed to detect inflammatory responses (due to the *ex vivo* nature of the study), the structural integrity of the subcutaneous tissue that was observed, was consistent with the excellent tolerability of HA fillers in clinical practice.

Acknowledging these findings, it is however important to consider the limitations of the present *ex vivo* study. First, it was, of course not *in vivo*, the experiments were performed on small excised human skin samples without lateral anchorage. Second, the injected volume of HA filler was 0,1 ml, which is much lower than the total volume that is used in clinical practice. Moreover, due to the immediate processing of the skin samples after injection, HA filler distribution was only observed at time zero; there is no insight into time dependent effects, in particular the impact of tissue dynamics and movement that might modify HA filler behavior over time *in vivo*. Further investigations, with an increased patient and injector number, are obviously required for a better understanding of the behavior of HA filler *in vivo* over time and when exposed to dynamic conditions and degradation in the skin. Nonetheless, the results presented here constitute a valuable and important first step. Translating these *ex vivo* data to the “bedside” and what they mean for the clinician: (i) the biomechanical properties of the HA fillers did not seem to affect their distribution in the subcutaneous tissue, which is, more often than not, where they arrive after injection (with the caveat of the lack of time-dependent and/or dynamic effects) (ii)
this systematic imaging study provides an additional “biophysical” validation of the excellent safety profile of clinical procedures involving injection of HA filler into the subcutaneous skin layer

5. Conclusion

Images from this preliminary ex vivo study showed no difference in the subcutaneous integration of BEL₈, JUVᵥ and RES₉ in porcine ear and human skin samples following injection. This was in contrast to their previously reported behavior in the dermis and the very distinct biophysical properties of the investigated HA fillers. The difference in the resistance of skin layers was thought to be key given the high elasticity of the dermis in comparison to the “relative inelasticity” of the subcutaneous fat.

For all of the investigated HA fillers, the visualization of the injection areas showed the preservation of the hypodermal structure. The fibrous trabecular network was unaltered and the intercommunicating collagenous trabecular structures appeared to contain and constrain HA filler distribution and limit the movement of the product. The displacement and compression of the soft fat lobules provided space for HA filler integration into the subcutaneous fat – independent of the formulation injected. The results provide further corroboration of clinical observations concerning the high tolerability of HA filler injections into the hypodermis.

Nevertheless, these findings correspond to static ex vivo conditions, which neglect the dynamic mechanical constraints that are constantly applied to an implanted HA filler (e.g. changing facial expression). Further studies to observe the dynamic behavior of HA filler products as a function of time in the subcutaneous skin layer under physiological conditions would be useful to complete the understanding of HA distribution in the hypodermis from injection to product degradation.
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7. References


Conference communications/ oral presentations


**Poster presentation:** Santer V, Lapteva M, Mondon K, Möller M, and Kalia YN. Visualizing skin penetration of a fluorescent ciclosporin A analogue following delivery from polymeric MPEG-dihexPLA micelles. *Skin Forum 14th Annual Meeting Percutaneous penetration – measurement, modulation and modelling, Prague, Czech Republic, September 4-5, 2014.*

**Podium presentation:** Santer V, Kalia YN. Visualization and quantification of intra-corneal triamcinolone acetonide biodistribution following topical iontophoresis: a new approach to treat corneal graft rejection. *The 12th International Symposium on Ocular Pharmacology and Therapeutics, Berlin, Germany, July 9-12, 2015.*

**Poster presentation:** Santer V, del Río Sancho S and Kalia YN. Iontophoresis of triamcinolone acetonide amino acid ester prodrugs for increased intracorneal drug biodistribution. *14th European Symposium on Controlled Drug Delivery, Egmond aan Zee, The Netherlands, April, 13-15, 2016.*


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