Administration transdermique des peptides par ionophorèse : impact des propriétés moléculaires sur les mécanismes de transport et applications thérapeutiques

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THÈSE

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TABLE DES MATIERES

STRUCTURE DE LA THESE

L'administration transdermique des peptides et des protéines offre de nombreux avantages par rapport aux injections parentérales et implants auxquels on recourt actuellement par manque d'alternatives. Cependant, l'excellente fonction barrière de la peau empêche le passage de ces molécules par simple diffusion passive et conduit au développement de nombreuses stratégies permettant de la surmonter. Parmi elles, la ionophorèse permet d'augmenter la perméation cutanée par le biais d'un faible courant électrique. Le premier chapitre de la présente thèse permet de placer la ionophorèse dans le contexte de l'administration transdermique des molécules de nature peptidique ou protéinique en passant en revue les technologies actuellement disponibles ou en cours de développement, présentant leur principe, les mécanismes impliqués, leurs applications potentielles ainsi que leurs limitations.

Bien que la ionophorèse soit une technique relativement ancienne, son application à l'administration transdermique de molécules telles que les peptides a moins de vingt ans et de nombreux aspects d'ordre mécanistique restent encore à explorer. Dans ce contexte, le chapitre 2 présente l'étude de l'effet de la séquence en acides aminés sur le transport ionophorétique d'une série de tripeptides dérivés de la structure des analogues de la gonadolibérine (LHRH) et de la somatostatine, administrés *in vitro* à travers de la peau d'oreilles de porc. Les contributions relatives des deux mécanismes de transport que sont l'électroosmose et l'électromigration ont été déterminées pour chacun d'eux, ainsi que l'évolution de ces contributions en fonction des conditions expérimentales.

Alors que de nombreuses relations structure - perméation quantitatives ont été établies dans le cas de l'administration transdermique passive (faisant intervenir des paramètres tels que la lipophilie et/ou la capacité à former des liaisons hydrogène), seuls quelques modèles reliant le passage ionophorétique de molécules à des paramètres de taille ont été décrits. Le but du troisième chapitre est donc d'offrir une étude systématique et quantitative portant sur l'effet des propriétés physicochimiques des tripeptides étudiés (propriétés définies par des descripteurs tridimensionnels obtenus par modélisation moléculaire) sur leur transport ainsi que sur l'inhibition de l'électroosmose, phénomène observé lors de l'administration ionophorétique de plusieurs peptides.

Enfin, l'évaluation de la faisabilité de l'administration transdermique par ionophorèse d'un analogue de la somatostatine (vapréotide) ainsi que celle d'un analogue de la LHRH (triptoréline) font l'objet des chapitres 4 et 5, respectivement. Y est étudiée l'influence de différents paramètres tels que la densité du courant appliqué, la concentration du peptide, la présence d'ions compétiteurs ou le type de tissu, sur le passage transcutané ainsi que sur l'électroosmose.

INTRODUCTION

La peau, chef-d'œuvre architectural

D'une surface variant de 1.5 à 2 m^2, la peau est un organe formé de deux tissus superposés, l'épiderme et le derme. La couche la plus superficielle de l'épiderme est la couche cornée. D'une épaisseur de 10 à 20 μm, elle est composée de 10 à 20 strates de cellules mortes (cornéocytes) remplies de filaments de kératine (70%) et de lipides (20%), arrangées à l'intérieur d'une matrice lipidique extracellulaire (ceramides 40%, acides gras libres 20%, cholesterol ~25%) de façon à former un assemblage similaire à un modèle de « briques et ciment » [1;2]. Directement sous la couche cornée, se trouve l'épiderme vivant, épithélium comprenant différents types de cellule (kératinocytes, mélanocytes, cellules de Langerhans et cellules de Merkel). Quant au derme, c'est un tissu conjonctif principalement constitué de fibroblastes, fibres de collagène et fibres élastiques. C'est lui qui contient les vaisseaux sanguins et les récepteurs sensoriels de la peau.

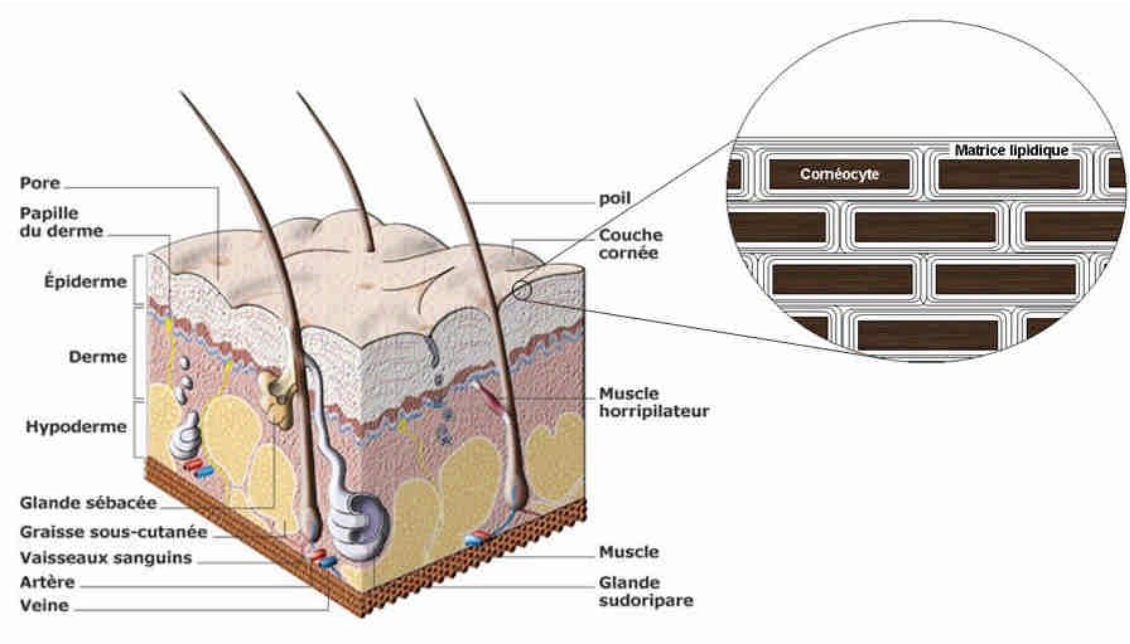

Figure 1. Structures principales de la peau, ses annexes et les tissus sous-cutanés

La peau et ses annexes (glandes sudoripares et sébacées, poils et ongles) remplissent de nombreuses fonctions visant à empêcher des facteurs de l'environnement, tels que les bactéries, l'abrasion, la chaleur, le froid et les substances chimiques, de perturber l'homéostasie de l'organisme. De par sa structure lamellaire unique, c'est la couche cornée qui confère à la peau l'essentiel de sa précieuse fonction barrière.

La peau, voie d'administration

Si de nombreuses préparations pharmaceutiques sont appliquées sur la peau en vue d'une action locale, notamment pour le traitement des différentes dermatoses, d'autres sont destinées à avoir une action sur l'ensemble de l'organisme, *via* la circulation sanguine. Ces systèmes, communément appelés patchs et définis par la Pharmacopée européenne sous le terme « dispositifs transdermiques », permettent de libérer au niveau systémique, des principes actifs qui sont mal absorbés par d'autres voies ou subissent une dégradation trop importante lors d'un premier passage hépatique. Si le marché de cette forme galénique est important (3,9 milliards de dollars en 2002, 6,7 milliards prévus pour 2007) [3], seuls une dizaine de principes actifs sont actuellement commercialisés, conséquence de l'excellente fonction barrière de la peau. En effet, cette dernière limite la gamme des molécules susceptibles d'être administrées par un dispositif transdermique passif. En plus d'être pharmacologiquement puissant, l'agent thérapeutique doit répondre à certains critères d'ordre physicochimique tels qu'un poids moléculaire relativement faible (<500 Daltons), un coefficient de partage octanol-eau modéré ($10<K_{o/w}<1000$), et une solubilité aqueuse raisonnable (>1mg/ml) [4]. Afin d'élargir le champ d'application thérapeutique de la voie transdermique à des molécules plus grandes et plus polaires, de nombreuses stratégies impliquant un transport actif des molécules à travers la peau ont été développées [5]. Parmi elles, la ionophorèse se distingue par son innocuité, son confort d'utilisation et son état de développement avancé [6].

L'ionophorèse, principe

L'ionophorèse utilise un courant électrique de faible intensité (<0.5 mA/cm^2) pour faciliter et contrôler le transport de molécules à travers la peau. Un dispositif ionophorétique consiste en un patch contenant 2 électrodes (une anode et une cathode) que l'on connecte à une source de courant. Le principe actif contenu dans le patch est véhiculé par le biais de différents mécanismes de transport ; en plus de l'électromigration (effet direct du champ électrique appliqué sur les espèces chargées), et de l'augmentation de la perméabilité cutanée induite par le courant, les molécules chargées positivement (placées dans le compartiment anodique) bénéficient d'un troisième mécanisme intitulé électroosmose, un flux de solvant qui résulte de la charge nette négative de la peau à pH physiologique. Ce flux permet également le transport de molécules neutres.

Outre les avantages propres à la voie transdermique, la ionophorèse offre la possibilité, en modulant l'intensité du courant appliqué, d'adapter le profil d'administration aux besoins de chaque patient ou chaque phase de traitement. Cette caractéristique permet également une administration de type pulsatile, particulièrement profitable pour les principes actifs exerçant des effets pharmacologiques différents en fonction du profile d'administration (e.g., la gonadolibérine (LHRH) et ses analogues) ou pour ceux susceptibles d'induire un phénomène de tolérance (e.g., vasopressine).

Aussi, un traitement peut être rapidement stoppé en cas de nécessité par simple arrêt du courant, ce qui présente un avantage précieux par rapport aux implants.

L'ionophorèse, application aux peptides et protéines

Les propriétés physicochimiques des peptides et des protéines ainsi que leur forte susceptibilité à la dégradation, tant chimique qu'enzymatique, rendent leur administration difficile. On recourt actuellement presque essentiellement à la voie parentérale, avec son lot d'inconvénients et la faible compliance qui en résulte. Le développement d'alternatives efficaces et confortables pour le patient fait l'objet de très nombreuses recherches et représente un véritable défi. De par leur taille et leur relative hydrophilie, ces composés ne sont pas à même de diffuser passivement à travers la peau et d'atteindre ainsi la circulation systémique. Cependant, leur caractère souvent chargé fait de ces molécules de bons candidats à la ionophorèse.

L'application de la ionophorèse à l'administration de peptides pharmacologiquement actifs a commencé à la fin des années quatre-vingts et a suscité beaucoup d'intérêt [7]. De nombreux peptides tels que la protiréline (PTH) [8], l'angiotensine [9], l'octréotide [10], la gonadolibérine (LHRH) et ses analogues [11-15], l'arginine-vasopressine [16], la calcitonine [17] et l'hormone parathyroïdienne [18] ont été étudiés et administrés avec succès, tant *in vitro* que *in vivo*. L'extraordinaire potentiel commercial que représente l'insuline conduit inévitablement à une recherche considérable portant sur son administration par cette voie [19]. De par certaines propriétés défavorables, tant physicochimiques (e.g., poids moléculaire ~6000 Da, charge négative) que pharmacodynamiques (doses élevées requises pour effet pharmacologique), son administration par ionophorèse s'avère extrêmement difficile. Cependant, si les protéines de poids moléculaire élevé sortent du spectre d'application de la ionophorèse, l'administration de peptides spécifiques se révèle être un objectif tout à fait réalisable.

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CHAPITRE 1

Stratégies actuelles pour l'administration transdermique des peptides et des protéines

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Résumé

L'administration transdermique est à la pointe de la recherche dans le domaine du développement de méthodes non-invasives pour l'administration systémique des principes actifs de structure peptidique ou protéinique résultant de la révolution biotechnologique. De nombreuses approches ont été proposées pour surmonter l'excellente fonction barrière de la peau; alors que certaines agissent simplement au niveau de la drogue ou en augmentant de façon transitoire la perméabilité de la peau, d'autres sont conçues dans le but d'outrepasser ou même d'enlever la couche superficielle de la peau. Cet article passe en revue les technologies qui font actuellement l'objet d'investigations, en allant de celles encore aux prémices de leur développement, comme l'administration assistée par le laser, aux techniques plus abouties ayant déjà conduit à des produits commercialisés (les systèmes d'injection à pression), en passant par celles dont la faisabilité a déjà été démontrée, comme les microaiguilles. Les principes, les mécanismes impliqués, les applications potentielles, les limitations ainsi que la sécurité d'utilisation sont discutés pour chacune des approches, et les dispositifs les plus avancés sont décrits.

Emerging strategies for the transdermal delivery of peptide and protein drugs

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Abstract

Transdermal delivery has been at the forefront of research addressing the development of non-invasive methods for the systemic administration of peptide and protein therapeutics generated by the biotechnology revolution. Numerous approaches have been suggested for overcoming the skin's formidable barrier function; while certain act simply on the drug formulation or transiently increase the skin permeability, others are designed to by-pass or even remove the outermost skin layer. This article reviews the technologies currently under investigation, ranging from those in their early-stage development, such as laser-assisted delivery to others, where feasibility has already been demonstrated, such as microneedle systems, and finally more mature techniques that have already led to commercialisation (for example, velocity based technologies). The principles, mechanisms involved, potential applications, limitations and safety considerations are discussed for each approach, and the most advanced devices in each field are described.

Keywords: Transdermal drug delivery, peptide and protein delivery, iontophoresis, electroporation, sonophoresis, microneedles, encapsulation, jet-injectors, stratum corneum ablation

1. PEPTIDE AND PROTEIN DELIVERY

Revolutionary advances in biotechnology have given rise to numerous protein and peptide entities with therapeutic potential. One of the major challenges to the successful clinical use of these "biotech" molecules is their efficient and targeted delivery to the site of action. Presently, parenteral delivery is the most routinely employed method for administering polypeptide agents, which are otherwise completely destroyed when given orally. These compounds often have short plasma half-lives, need frequent injections and are, therefore, associated with poor compliance. In recent years, alternative and more patient-friendly modes of drug delivery have been extensively investigated.

2. TRANSDERMAL DELIVERY

Over the past few decades, the skin has generated a great deal of interest as a portal for the systemic delivery of drugs [1]. The potential advantages of this mode of administration have been well documented [2]. The worldwide transdermal market is currently worth more than US$ 4 billion, yet is based on only thirteen drugs. This rather limited number of transdermal drugs is explained by the skin's excellent barrier function, which is accomplished entirely and quite remarkably by the outermost few microns of tissue, the stratum corneum: often referred to as a "brick and mortar" structure [3].

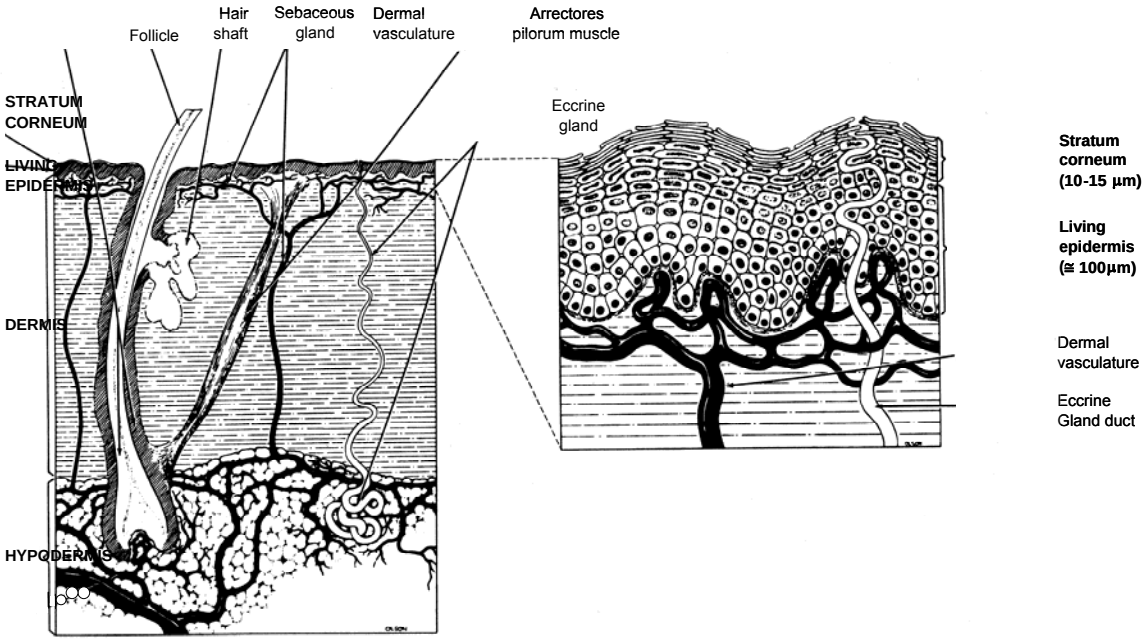


Figure 1. **Major skin structures.**

In addition to being pharmacologically potent, a therapeutic agent must possess a balance of physicochemical properties that render it permeable: a relatively low molecular weight (<500 Daltons), a moderate octanol-water partition coefficient (10 $<K_{o/w}<1000$), reasonable aqueous solubility ($>1 mg.ml^{-1}$) and modest melting point (<200°C) [4].

Most, if not all, peptides and proteins, being large and hydrophilic, do not satisfy these criteria. Yet, the transdermal delivery of these potent therapeutic agents is of particular interest, since

percutaneous administration overcomes many of the problems associated with conventional therapy. Hence, to expand the range of drugs that can be delivered transdermally, and to include peptides and proteins, a number of enhancement technologies are under investigation.

3. FROM SKIN SURFACE TO SYSTEMIC CIRCULATION

Before being taken up by blood vessels in the upper papillary dermis and prior to entering the systemic circulation, substances permeating through the skin must cross the stratum corneum and the viable epidermis. There are three possible pathways leading to the capillary network: across the continuous stratum corneum (SC), through hair follicles and their associated sebaceous glands, or via sweat ducts. Although, the "as-the-crow-flies" diffusion distance across the SC is no more than 10-15 μm, the actual diffusional pathway may be upto fiftyfold greater, depending on the route taken (Fig.1). While crossing the viable skin layers, peptidic drugs can undergo extensive enzymatic degradation [5-7]; numerous proteases (including endopeptidases and exopeptidases) have been detected in the skin, both in the dermis and the epidermis [6-8]. However, as a result of the rapid uptake of penetrants into the general circulation on arrival in the highly vascularized dermal papillary layer, peptide/protein metabolism is most likely to occur during passage through the epidermal layer [9]. Although this phenomenon may appear to mitigate one of the benefits of transdermal delivery (avoidance of gastrointestinal and hepatic enzymatic metabolism), the extent of epidermal catabolism is of course dependent on (a) the application area (e.g., patch size), and (b) the rate of epidermal transport - parameters that are optimized in the design of "active" transdermal systems. In addition, peptide formulations can also be designed to incorporate specific enzyme inhibitors [10, 11]. Parenthetically, certain energy-driven enhancement techniques may also impact on enzyme activity, as reported for ultrasound, which is suggested to deactivate certain skin enzymes [12, 13].

This article reviews the current state of transdermal peptide drug delivery technology. The strategies are classified as a function of the manner in which they overcome the skin barrier (Fig. 2). The approaches range from formulation optimisation, to energy-driven techniques including electrically-assisted transport, through to methods designed to enhance drug delivery by by-passing the barrier or even by removing it.

4. FORMULATION OPTIMISATION

Peptide drugs, which are hydrophilic and often charged molecules, pass through lipophilic membranes such as the SC with some difficulty, if at all. To counteract this, there are two main approaches available to the formulation scientist: (a) the use of chemical enhancers to transiently modify the SC permeability (i.e. render the SC more "leaky" towards hydrophilic molecules), and (b) modification of the therapeutic molecule to render it more hydrophobic and therefore "acceptable" to the membrane. The latter strategy involves either chemical derivatization, or encapsulation within a lipophilic core.

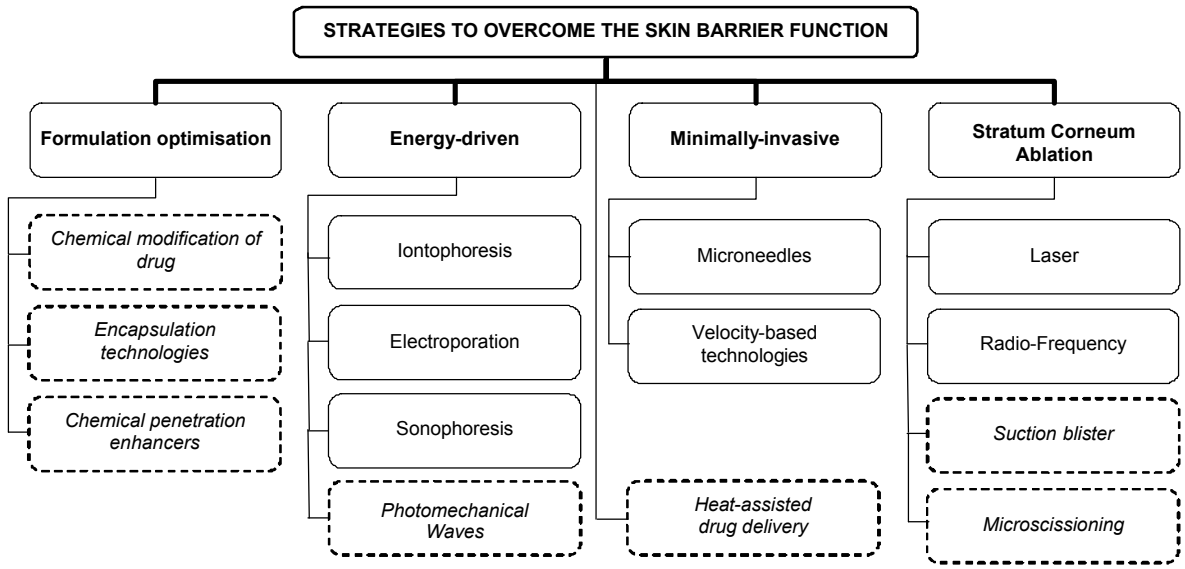


Figure 2. **Current strategies for transdermal drug delivery.**

Italicized legends signify those technologies, which are still in their infancy or are unlikely to find an application in peptide and protein delivery in the near future.

4.1 Chemical penetration enhancers

There exists an extensive literature on penetration enhancers, their mechanisms of action and effects on the skin, and their impact on the permeation of applied molecules [14, 15]. These compounds appear to act by disrupting or altering the ordered lipid structure of the SC. Whereas numerous studies on the passive delivery of peptides and proteins with chemical enhancement were conducted during the 80's and 90's [16], research in this area has somewhat "run out of steam", primarily for two reasons. First, while low MW therapeutics usually tolerate formulation with these permeation enhancers, proteins are less comfortable in the company of these aggressive chemicals. The second and most important reason concerns safety. Unfortunately, for most enhancers, activity is closely correlated with irritation, rendering them clinically unacceptable [17]. Thus, because of the extremely low permeability coefficients of peptide and protein drugs - readily predicted from their hydrophilicity and molecular size - the magnitude of enhancement required to ensure delivery of pharmacologically effective concentrations is expected to be beyond the capability of chemical enhancers tolerated by the skin.

4.2 Chemical modification

Whereas improved intestinal absorption through chemical modification with various fatty acids has been reported for some peptide and protein drugs, the application of this approach to transdermal delivery is quite new. A few research groups have studied the cutaneous delivery of derivatives of peptides such as the vasoactive intestinal peptide [18] and interferon α [19]. The absorption of palmitoyl derivatives of interferon α (p-IFNα) into the viable layers of human breast skin was eightfold greater compared to the parent peptide, suggesting that this approach might find an application in

topical delivery. The enhancement potential in transdermal delivery is less obvious, given that only a two-fold increase in the percutaneous absorption of p-IFNα was observed. These results must also be treated with caution given the surprisingly elevated passive permeability of the parent polypeptide (1.47 ng/cm^2/h).

Finally, one must question whether a modest improvement in delivery is sufficient to warrant the additional regulatory complexities concomitant with the prodrug strategy. During drug development, the chemically-modified derivative will have to undergo – as for the parent molecule - extensive toxicological testing in addition to the suite of developmental investigations.

4.3 Encapsulation technologies

Encapsulation consists of the entrapment of drug within delivery systems such as microspheres, liposomes and nanoparticles. Liposomes, typically consisting of phospholipids and cholesterol, are thermodynamically stable vesicles with an aqueous core and at least one surrounding bilayer. Niosomes, analogues of liposomes, are non-phospholipid vesicles formed by the self-assembly of nonionic surfactants in an aqueous dispersion. Niosomes and classical liposomal systems have been found to be effective in forming drug reservoirs in the upper layers of the skin, for local therapy. The controlled topical delivery of cyclosporin A [20] and interferon α [21] has been studied: ≤ 1µg/cm^2 of liposomally encapsulated interferon α was found in human skin after a 24 hour *in vitro* application. However, the use of liposomes and niosomes for the systemic delivery of macromolecules across the skin has not been successful to-date.

Transfersomes are ultradeformable carriers that are claimed to be driven across the skin by the transdermal hydration gradient [22]. They are suggested to be sufficiently flexible to pass through pores appreciably smaller than their own size (200-300 nm). Insulin-loaded transfersomes were reported to induce a modest 15% decrease of blood glucose concentration in healthy human volunteers [23], a response which is unlikely to provoke a therapeutic effect in diabetic patients.

Ethosomes are phospholipid vesicular systems containing ethanol in relatively high concentrations. According to Touitou et al., ethanol fluidises both the ethosomal lipids and the SC lipid mortar; the soft, malleable vesicles then penetrate through the disorganized lipid bilayers [24]. Several studies have been conducted with non-peptidic drugs such as testosterone [25], trihexyphenidyl hydrochloride (currently used for the treatment of Parkinson's disease) [26] and zidovudine (an antiviral agent) [27]. As is often the case, insulin, because of its huge potential market, was the first peptide studied. The effect of a transdermal insulin formulation on blood glucose levels was investigated *in vivo* in normal and diabetic rats. Despite the significant decrease (≤ 60%) in blood glucose levels achieved in both normal and diabetic rats [28], it is clear that a corresponding effect in humans would require significant scaling-up, which remains to be demonstrated.

Finally, although encapsulation might be considered to offer some protection from cutaneous enzymes, the point at which the drug leaves its 'protective capsule' during its passage through the tissue has yet to be determined. Moreover, any benefits offered are likely to be offset by formulation stability issues.

5. ENERGY DRIVEN METHODS

5.1 Iontophoresis

Iontophoresis is a century-old technique developed to deliver charged molecules through the skin at an enhanced rate via application of a small electric current (≤ 0.5 mA/cm^2). The drug reservoir on the surface of the skin is in contact with an electrode of the same charge as the solute, connected to a grounding electrode and a power supply. In addition to electromigration (direct effect of the applied electric field on the charged species), and current-induced modification of passive skin permeability, positively charged compounds (present in the anodal compartment) benefit from a third transport mechanism called electroosmosis, a convective solvent flow which is a consequence of the skin's net negative charge at physiological pH. This flow also enhances the transport of neutral compounds.

A feature of iontophoresis, which distinguishes it from other enhancement technologies, is that it acts primarily on the molecule itself. The technique does not simply involve passive transport of the drug following barrier disruption: the driving force comes from the applied electric field and is not solely dependent upon the concentration gradient, as in passive delivery. Hence, by modulating the current applied, iontophoresis allows adaptation of the delivery input rate and profile to the needs of each patient or phase of treatment, and offers the possibility of pulsatile drug delivery. Although the advantages of "constant" or "sustained" plasma concentrations have long been endorsed, there are a number of therapies which benefit from the conventional "peak and trough" plasma profiles. Moreover, certain peptides such as human parathyroid hormone (PTH) and luteinizing hormone-releasing hormone (LHRH) have distinct, and often opposing, pharmacological effects depending on their delivery profile. LHRH, for example, must be administered as a bolus every 60-90 min to treat female infertility, but must be given as a continuous infusion in the treatment of certain cancers. Pulsatile delivery is also desirable where downregulation and tolerance may be a concern, as demonstrated for vasopressin [29]. Considerable interest has been and continues to be shown in the iontophoretic delivery of therapeutic peptides and proteins which, given their often charged character, are good candidates for this technology. Numerous peptides including Thyrotropin releasing hormone (TRH) [30], angiotensin [31], octreotide [32], luteinizing hormone releasing hormone (LHRH) and analogues [33-37] arginine-vasopressin [38], calcitonin [39], human parathyroid hormone (1-34) [40] and insulin [41] have been studied. Many of these peptides have also been the subject of mechanistic investigations, and electroosmosis has been proposed to be the predominant mechanism governing the iontophoretic transdermal delivery of large (molecular weight $\geq$ 1000 Da) cationic peptides [42]. However, peptides containing closely juxtapositioned cationic and lipophilic residues are able to inhibit this transport mechanism, and hence their own transport, by altering the permselectivity properties of the skin when iontophoresed [43-48]. This inhibition may be considered as a limitation in the iontophoretic transdermal delivery of certain peptide drugs containing this structural motif. Nevertheless, leuprolide, a synthetic nonapeptide analogue of LHRH containing *D*-Leu-Leu-Arg at position 6-8 (corresponding to the mentioned structural signature) has been successfully delivered *in*

vivo in humans to produce a peak LH response similar to that by subcutaneous injection [49]. Other *in vivo* studies have also compared the delivery profile obtained after iontophoresis, to that measured after conventional needle injections, to demonstrate the potential of this administration route. Such an example is illustrated in Figure 3 for the delivery of the growth hormone releasing factor GRF (1-44), where steady-state plasma GRF levels after iontophoretic delivery are greater and more sustained, relative to those following intravenous and subcutaneous injections [50].

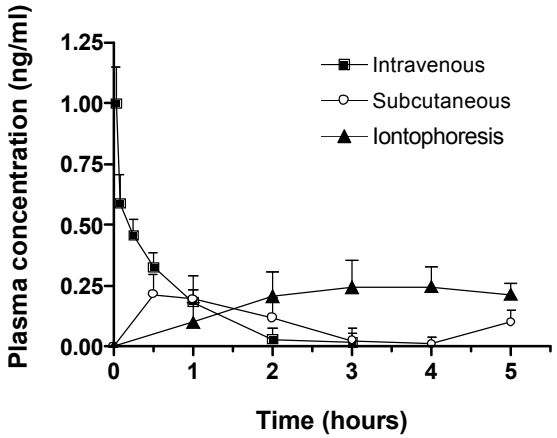


Figure 3. **Plasma levels of growth hormone releasing factor (GRF) in response to transdermal iontophoretic (1 mg/g; 0.17 mA/cm^2; 5 cm^2 patch), intravenous (10 mg/kg; 0.025 mg/ml) and subcutaneous (10 mg/kg; 0.025 mg/ml) GRF administration to the guinea pig.**
Reprinted from Ref. [50], with permission from Elsevier.

Not surprisingly, the iontophoretic delivery of insulin has provoked much attention. However, while the delivery of regular insulin by iontophoresis might be sufficient to treat a small mammals, the best deliveries achieved, even with monomeric insulin analogues [51], are still 1-2 orders of magnitude below those necessary to match the basal secretion level in humans [41].

Owing to the advanced nature of transdermal iontophoretic research and development, these systems are relatively well-characterized and understood. For example, the recently launched LidoSite™ device (Figure 4; Vyteris Inc., NJ, USA) [52] for local anaesthesia and the IONSYS™ system for fentanyl delivery (Alza Corp., CA, USA; currently awating final FDA approval) offer iontophoretic platforms, which can be potentially adapted and customized to the local and systemic iontophoretic administration of peptide drugs.

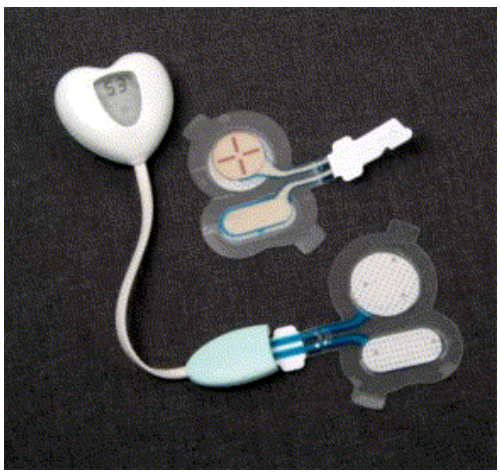


***Figure 4*.** **LidoSite™, an iontophoretic delivery system for lidocaine (Vyteris Inc., NJ, USA).** Reproduced with permission from Ref. [52].

5.2 Electroporation

First used for the introduction of DNA material into cells *in vitro*, the use of electroporation for transdermal delivery was suggested about 10 years ago. Unlike iontophoresis, which employs small

currents (transdermal voltages ≤ 10V) for relatively long periods of time (many minutes to hours), electroporation involves exposure of the skin to relatively high voltages (approx. 100-1000 V) for short times, typically 1 to several hundred milliseconds, which in turn create intense electric fields across the thin stratum corneum. Molecular transport through transiently permeabilized skin is thought to result from a variety of mechanisms: enhanced diffusion through the aqueous pathways produced in the lipid bilayers, electrophoretic movement (for charged species) and, to a small extent, electroosmosis [53].

A study investigating insulin delivery demonstrated that while less than 0.6 μg/cm^2 insulin was transported across porcine epidermis after electroporation (100 V, 1 Hz, 1 ms pulse for 10 min.), around 13 μg/cm^2 (~0.33 U/cm^2) reached the receptor compartment when delivered from a formulation containing phospholipids under the same electroporation regime [54]. The treatment of a diabetic patient (36U, daily) is obviously unfeasible with such an input, but this 20-fold enhancement in transport demonstrates the potential for synergistic combination of electrically-assisted and chemical technologies.

Transdermal heparin transport across human skin *in vitro* was possible at therapeutic rates with electroporation, whereas the low-voltage iontophoretic flux with the same time-averaged current was an order of magnitude lower [55]. Similarly, electroporation significantly enhanced the flux of human parathyroid hormone (1-34) in comparison to iontophoresis [40], suggesting that high-voltage pulsing creates transient changes in skin permeability, which do not occur during iontophoresis.

However, while electrically-assisted skin delivery via iontophoresis has been widely investigated in humans and shown to be safe and well-tolerated, very few human studies have been conducted with electroporation. In electrochemotherapy investigations, where electric pulses greater than 1000 V were applied to the skin of melanoma patients to facilitate the chemotherapeutic treatment of tumour nodules, muscle contractions and mild pain were reported during each pulse in addition to a transient erythema [56]. Although these unwanted side effects subsided after the pulse, further investigation is required to determine whether transdermal drug delivery by electroporation is clinically feasible.

5.3 Sonophoresis

Sonophoresis (or phonophoresis) is defined as the movement of drugs through intact skin and into soft tissue under the influence of an ultrasonic perturbation [57]. Low-frequency ultrasound (frequencies below 100 kHz) has been demonstrated to induce the greatest transdermal transport enhancement [58, 59]. Numerous studies have been devoted to understanding the mechanisms of sonophoresis [60-63]. Acoustic cavitation, the formation and collapse of gaseous cavities, plays the dominant role in low-frequency sonophoresis [64, 65], although significant stratum corneum lipid removal has also been reported [60] and may explain the increased skin permeability observed during, and after, low-frequency ultrasound application.

Mitragotri et al. reported the delivery of insulin (6000 Da), γ-interferon (17000 Da), and erythropoietin (48000 Da) *in vitro*, across human epidermis using ultrasound (20 kHz, 100-ms pulses applied every second for 4 hours). The insulin flux achieved was shown to be sufficient to treat a diabetic subject assuming a transdermal patch area of 40 cm^2 containing insulin at a concentration of 100 U/ml [66, 67]. Yet, despite this optimistic calculation, a sonophoretic insulin patch has not been

developed, highlighting the challenge of extrapolating from *in vitro* studies to clinical scenarios. Similarly, a 50% reduction in blood glucose subsequent to low-frequency insulin sonophoresis in rats (Fig. 5;[68]), while offering a valid "proof-of-concept", does not guarantee that a similar approach will work in an adult diabetic patient.

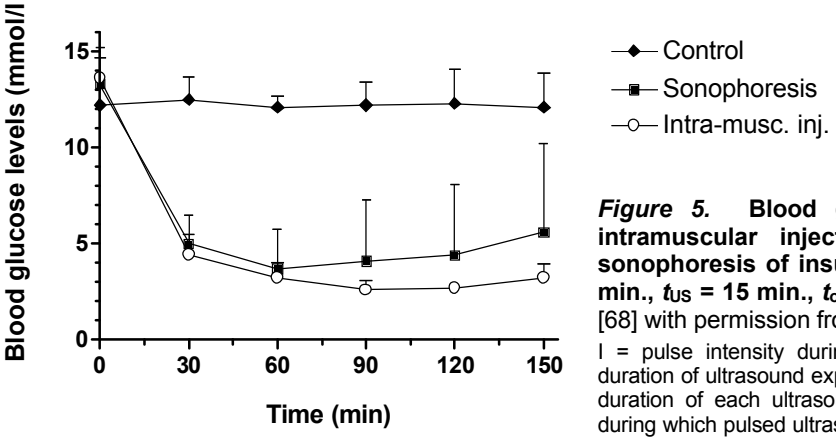


Figure 5. **Blood glucose levels in rats after intramuscular injection of insulin 0.5 U and sonophoresis of insulin (I = 2.5 Watts/cm^2, t_{eff} = 6 min., t_{US} = 15 min., t_{on} = 3.2 s.)**. Reprinted from Ref. [68] with permission from Elsevier.

I = pulse intensity during ultrasound application; t_{eff}: total duration of ultrasound exposure during treatment time, t_{US}; t_{on}: duration of each ultrasound pulse; t_{US}: total treatment time during which pulsed ultrasound was applied.

On the whole, research into peptide and protein delivery using this technique has been limited, although a technological platform for potential applications clearly exists. An ultrasonic skin permeation device (SonoPrep®, Sontra Medical, MA, USA), awaiting FDA-510k clearance, enables rapid delivery of lidocaine and local skin anæsthesia within five minutes after a brief skin pretreatment (55 kHz, ~10 sec pulses) [69]. Nevertheless, the feasibility of such a system at higher intensities and over longer periods needs to be examined. Singer *et al.* [70] noted minimal urticarial reactions after low-intensity ultrasound, but higher-intensity sonophoresis produced significant thermal injuries similar to second-degree burns. Hence, as with other energy-based technologies, sonophoresis exhibits a window of parameters within which safe application can be practiced; whether or not this window encompasses macromolecular delivery remains to be seen.

6. MINIMALLY-INVASIVE SYSTEMS

Numerous "minimally-invasive" strategies for transdermal drug delivery have been described, and recently reviewed by Down and Harvey [71]. Often, these technologies are the subject of patent claims but their therapeutic utility remains unsubstantiated. In this section, we have selected systems, which have been designed, in effect, to "by-pass" the skin barrier without blatant SC removal. Microneedles and velocity-based injectors, because of their ability to breach the SC, offer drug delivery platforms that may be suitable for higher MW drugs.

6.1 Microneedles

Over the last few years, advances in microelectronics have been innovatively applied to a variety of health care-related products – from miniaturized diagnostic tools (for example, biosensors)

to microdevices for therapeutic drug administration. Microneedles (µm dimensions) in various geometries and materials (silicon, metal, and polymer) have been produced using recently developed microfabrication techniques. These microneedle arrays are applied to the skin surface such that they pierce the epidermis (devoid of nociceptors), creating microscopic holes through which molecules can be transported to reach the upper dermal layers. The microneedle arrays penetrate to the dermal microcirculation and allow systemic drug delivery, but are short enough to avoid stimulation of dermal nerves.

Solid microneedles either puncture the skin prior to the application of drug contained in a patch system, or are pre-coated with drug and then inserted into the skin. The first approach implies a two-step application procedure, which might seriously limit its ease-of-use, whereas the second presents the disadvantage of being “surface-limited”: that is, the total amount of drug which can be loaded, and hence delivered, is limited by the total microneedle surface. Microneedles containing a hollow bore provide an alternative to solid structures and offer the possibility of transporting drugs through the interior of well-defined needles by diffusion or, for more rapid rates of delivery, by pressure-driven flow.

Both solid and hollow microneedles have been used to deliver insulin *in vivo* in diabetic rats. McAllister *et al*. demonstrated a 70% reduction in blood glucose 5 hours after a 30-min microinfusion of insulin at a pressure of 14 psi using hollow microneedles [72]. Solid metal microneedles also increased insulin delivery and lowered blood glucose levels by as much as 80% when a 105 microneedle array was inserted into the skin for 10 minutes and removed before the topical application of an insulin solution for 4 hours [73]. Such a reduction of blood glucose in rats corresponds to an insulin dose of 1.6-4.1 mU, a dose significantly lower than the ~36 U of insulin required by a typical diabetic patient each day (12U tds) [67]. Furthermore, the significant lag time between application and therapeutic effect is also of some concern. Recent *in vivo* studies, in hairless guinea pig, investigated the transdermal delivery of desmopressin using the Macroflux® system (Alza Corporation, CA, USA) [74, 75].

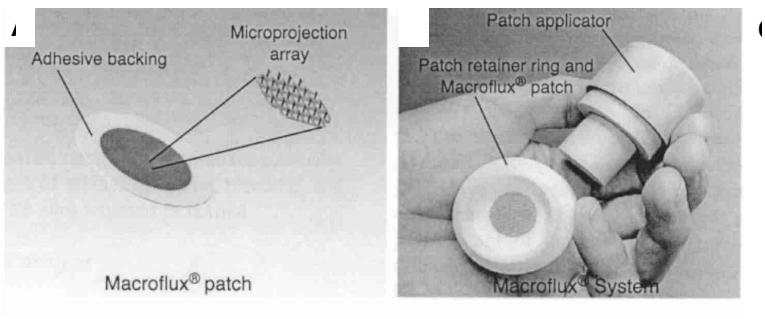


Figure 6. **Macroflux^{Ò} patch technology (Alza Corporation, CA, USA)**

A) The patch comprising the coated microneedle array affixed to an adhesive backing. Reprinted from Ref. [74], with permission from Elsevier.

B) The patch loaded on the disposable retainer ring and the reusable applicator. Reprinted from Ref. [74], with permission from Elsevier.

C) Scanning electron photomicrograph of an array of microprojections (L: 330 µm) and a conventional 25-gauge needle. Reprinted with permission from Ref. [77].

The Macroflux® system (Figure 6), incorporates a 2 cm^2 array of titanium microneedles, which can be coated with drug for rapid bolus administration, or used in combination with a drug reservoir for continuous passive or iontophoretic applications [75]. When coated with desmopressin, these microneedles allowed pharmacologically relevant amounts of this synthetic peptide hormone to be delivered, with a bioavailability as high as 85% (c.f., oral and nasal bioavailability of 0.1% and 3.4%, respectively) in human volunteers (Figure 7; [76]). Average systemic deliveries of desmopressin ranged from 17 to 34 μg after 5 or 15 minute application time, which is significantly more than the 1 to 4 μg recommended daily by subcutaneous, intramuscular or intravenous injection in the management of primary nocturnal enuresis. This device has also demonstrated promising results with vaccines [77].

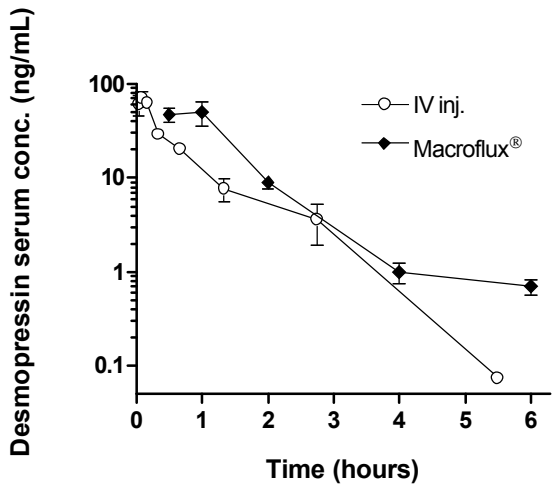


Figure 7. **Comparison of serum desmopressin concentrations following administration by intravenous injection (11 mg), or via a coated microneedle array (82 mg) (microneedle array contact time = 5 min.)**. Reprinted from Ref. [74], with permission from Elsevier.

Microneedles have been described as painless, inducing neither erythema nor edema [78, 79]. In addition, the μm-scale holes produced are significantly smaller than those created by hypodermic needles [80]. However, the reversibility and the consequences of chronic applications of these arrays remain to be studied. The immediate concern with this technology is the possibility of fractured needle fragments remaining in the skin, although it has been reported that the majority of silicon microneedles remained intact after insertion into skin [78]. Nonetheless, the potential risk of residual material in the skin after treatment needs to be examined. In this respect, metal and polymer microneedles offer significant advantages: in addition to being more robust, less expensive and easily scalable for mass production, many metal and polymer (especially biodegradable) materials have established safety records in medical devices, whereas silicon is a new and relatively untested biomaterial [72]. Microneedles, essentially a hybrid of hypodermic needles and transdermal patches, provide an interesting and promising alternative, currently being pursued by several companies and resulting in an impressive number of patents.

6.2 Velocity-based technologies

A jet injector produces a high-velocity jet (>100 m/s) that penetrates the skin and delivers drugs into the epidermis, intradermally, subcutaneously, or intramuscularly by means of a compression spring or compressed air [81]. Most commercial devices produce a single jet for drug delivery through an orifice of around 150 μm in diameter. These systems resemble a pen, as illustrated in Figure 8.

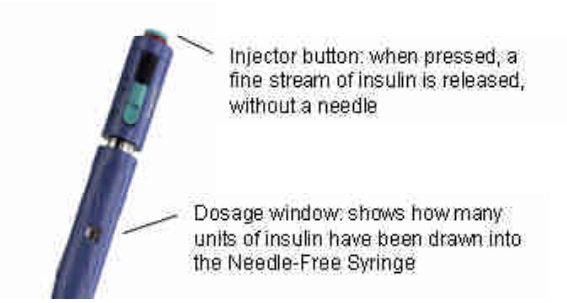


Figure 8. **Medi-Jector Vision® (Antares Pharma Inc., PA, USA), Needle Free Insulin Injection System.**
Reproduced with permission from Antares Pharma Inc.

Interest in these systems as an alternative to the routine use of needles in vaccination has partly stemmed from the increasing incidence of injection-associated, blood-borne pathogen infections (hepatitis B, hepatitis C and human immunodeficiency viruses) in developing countries. An exhaustive, web-based document [82] underlines the attention attracted by this technology.

One category of jet injectors has been developed for the delivery of liquid protein formulations. Figure 9 illustrates the similarity of a profile obtained with one such system to that measured after a conventional needle injection in a human study.

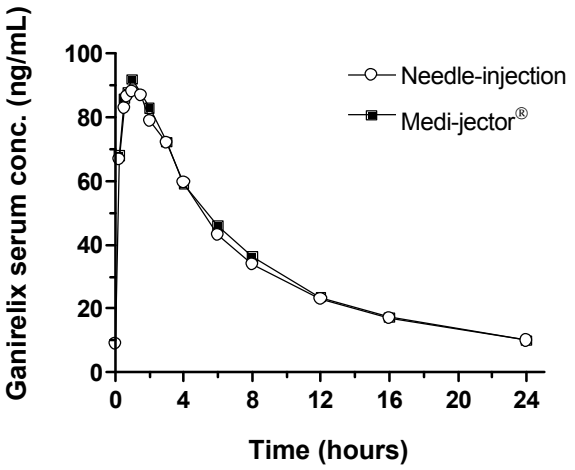
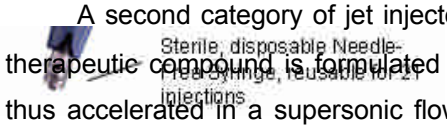


Figure 9. **Comparison of serum ganirelix concentrations following administration of Orgalutran® by subcutaneous injection or via the Medi-Jector® device.**
Adapted from Ref. [117] with permission of Oxford University Press/Human Reproduction.

Numerous devices have been on the market for several years. As shown in Table 1, most of the marketed devices are dedicated to insulin and human growth hormone (hGH). Today, compounds such as pegylated interferon alpha (Pegasys®), erythropoietin and antibodies are also receiving attention for delivery via this system [83].

Table 1. A representative selection of commercially available needle-free jet injectors.

Device tradename	Marketed by	Drug (proprietary name)	References
Vitajet™	Bioject	Insulin	[81]
Biojector®	Bioject	Vaccines and other liquid medications	
Cool.click™	Serono	hGH (Saizen®)	[118]
Serojet™	Serono	hGH (Serostim®)	
Medi-jector®	Antares Pharma	Insulin, hGH, ganirelix (Orgalutran®)	[117, 119]
Zomajet®	Ferring	hGH	[89]
Genotropin ZipTip™	Pfizer	hGH (Genotropin®)	[88]



A second category of jet injectors allows Transdermal Powdered Delivery (TPD), for which the therapeutic compound is formulated as a powder. Fine drug particles (20-100 μm in diameter) are thus accelerated in a supersonic flow of helium gas to penetrate the outer layers of the skin. The powder injection delivery of salmon calcitonin (s-CT) was studied in rabbits *in vivo* using the PowderJect® device (PowderJect Pharmaceuticals plc., recently acquired by Chiron Corporation, CA, USA). The administration of 1 mg of powder containing 40 μg of s-CT using a pressure of 60 bar led to an 11% decrease in serum calcium concentration [84]. When the same system was used to deliver human insulin to rats, blood glucose concentration was found to decrease by approximately 40% [84]. Here again, a corresponding effect in humans would require significant scaling-up, which remains to be demonstrated. This technology also allows targeting of DNA and protein vaccines to the epidermis, a skin layer which is populated by numerous antigen presenting cells, and therefore offers the possibility of needle-free immunization [85].

Although dry powder formulations are better suited to storage than solutions, a significant limitation of the PowderJect® technology is the upper limit on the dose that can be delivered, which is approximately 6 mg. Moreover, the powder must survive the high stress of a gas jet within the device and the ballistic impact with the skin at supersonic velocities. Finally, the dispersed fine particles must then dissolve and diffuse into the skin in order to act locally or to reach the systemic circulation.

With the exception of the PowderJect® system, which was reported as being painless during the course of Phase I and Phase II clinical studies [84], jet injectors are recognized for their propensity to cause pain and bruising [86]. Thus, despite the reported bioequivalence between needle-free devices and needle injection, the superiority of jet injectors in terms of comfort and compliance is, according to several studies, far from obvious [87-92]. Hence, the undisputable advantage of this technology, compared to conventional syringe injection, lies in the absence of a (visible) needle, which may be of benefit to children and needle-phobic patients, and in mass injection programs where the risk of contamination may be an important concern.

Since their introduction to the market eight years ago, jet injectors have not revolutionized the delivery of insulin in diabetic patients, who for the most part, continue to use conventional injection

systems. This suggests that this innovative technology might not be the choice alternative to the parenteral route for delivering peptides and proteins. However, it seems likely to play a key role in tomorrow's vaccination strategies.

7. STRATUM CORNEUM ABLATION

The simplest method for overcoming the barrier imposed by the stratum corneum is to remove it. This can be achieved, for instance, by repeated application of adhesive tape to the skin surface. However, for a number of reasons - including those of convenience, reproducibility, and patient compliance, it is difficult to envisage the routine clinical use of such an approach. Laser-assisted ablation and SC ablation by suctioning are perhaps more realistic approaches but are also likely to be associated with patient compliance issues. Alternative, more recent technologies currently under investigation include microscissioning (see section 9.3, below) and radiofrequency thermal ablation.

7.1 Suction ablation

Suction ablation uses a vacuum to produce a small blister (5-6 mm in diameter), the upper surface of which is excised to reveal a portal for entry of drugs into the dermal circulation [71]. The feasibility of this technique was tested in seven healthy volunteers using the antidiuretic peptide 1-deamino-8-D-arginine vasopressin (DDAVP), in whom the bioavailability was reported to approach 100 % [93]. In a separate study using an oxytocin antagonist, antocin, therapeutic blood levels were measured in healthy volunteers one hour after administration [94]. This technology has resulted in a commercial product, Cellpatch® (Epiport Pain Relief AB, Sweden), which incorporates all the components of the process: suction device, epidermatome (to remove the blister), and a drug reservoir. Clinical studies have tested the feasibility of transepidermal morphine delivery by this methodology in normal healthy volunteers [95] and in postoperative patients [96]. The studies reported an absence of pain (possibly due to the concomitant delivery of morphine?), erythema and scar formation. Regeneration of the epidermis occurred one week after removal of the system. However, the vacuum removal of the epithelium caused pronounced hyperaemia in the de-epithelialised dermis and the sites showed slight, fading pigmentation even 3 months after treatment, suggesting that this procedure may not be appropriate for the treatment of chronic disease. In view of this drawback, and given the absence of published work since 1996, suction ablation is unlikely to be a technology of choice in the near future.

7.2 Laser ablation

In this approach, the high energy of the laser creates pores in the skin that permit the transit of drug through the SC from, for example, a topically applied patch or gel [71]. There are two optimal wavelengths at which skin ablation can be achieved: a wavelength absorbed by tissue proteins (2940 nm) and one absorbed by tissue water (mid-infrared; 2790 nm). During laser irradiation, the energy is

absorbed by the components of the skin in the form of vibrational heating. Water within the irradiated area of the skin quickly reaches its boiling point, and the resulting vapour pressure elicits a microexplosion that results in ablation, as the tissue vaporizes. The rapid loss of energy from the ablated site protects the surrounding skin tissues from heat-induced damage. The level of energy imparted to the skin permits removal of the stratum corneum in a controllable fashion.

The erbium:YAG laser (2940 nm), currently used in plastic surgery for the resurfacing of rhytides, scars, photodamage, and melasma [97] was demonstrated to greatly enhance 5-fluorouracil permeation across mouse skin *in vitro* [98]. The only study performed with a peptide showed a 2.1-fold increase in γ-interferon transport across pig skin using an erbium:YSGG laser (2790 nm) [99].

While laser-assisted drug delivery may be technically feasible, many questions regarding safety remain unanswered [71]; for the moment, the utility of this technology is likely to be limited to niche applications within hospital settings, notably because of the elevated cost of medical lasers.

7.3 Radiofrequency thermal ablation

Radiofrequency (RF) thermal ablation is a well-known and effective technology for electrosurgery and ablation of malignant tissues. A thin electrode is placed directly into the tumor; application of RF energy results in the passage of an alternating frequency current from the tip of the electrode into the surrounding tissue. The movement of ions, which attempt to follow the change in the direction of the alternating current, results in frictional heating of the tissue, producing coagulative necrosis and cell ablation [100]. This technique has only recently been adapted for use as a physical method to enhance drug transport across the skin. A closely spaced array of tiny electrodes is placed against the skin while an alternating current at radio frequency is applied to each of the microelectrodes. This forms microchannels in the outer layer of the skin through the ablation of cells. The ViaDerm™ RF-microchannel generator (Transpharma Ltd, Israel) consists of 140 stainless steel electrodes (length: 100 μm; diameter: 40 μm) spaced 1 mm apart. When a certain pressure is applied to the device in contact with the skin, the RF-generator is activated momentarily, resulting in thermal ablation. Percutaneous penetration studies were performed with granisetron hydrochloride and sodium diclofenac *in vivo* in rats, and *in vitro* using full thickness porcine ear skin. The apparent permeability coefficients obtained for granisetron and diclofenac transport through porcine skin were 7.1 and 3.8 times higher for RF-treated skin compared to intact skin, respectively [100]. Experiments performed on rats *in vivo* with bioactive hGH showed a relative bioavailability of approx. 80% compared to subcutaneous injection [101].

A human safety study on twenty healthy, adult volunteers, reported slight erythema (0.75 out of 8.0) and negligible pain (5 out of 100) as measured by the Draize irritation index and Visual Analogue Scale score, respectively. An RF thermal ablation device has also been developed by Altea Therapeutics (PassPort™ Patch; GA, USA) and is currently in Phase 1 human clinical trials in the U.S.

8. COMBINATION STRATEGIES

An exhaustive inventory of all the possible combinations and implicated synergistic mechanisms with respect to transdermal enhancement offers subject matter for several articles and is clearly outside the scope of this review. The reader is referred to a comprehensive summary of these combination strategies with respect to their efficacy and mechanisms [102]. Since the enhancement effect can be synergistic, this has added to the interest in the field. Nearly all combinations have been tested, at least *in vitro*, and some of these have been applied to peptide drug delivery. These include the combination of iontophoresis with chemical enhancers [103, 104], with electroporation [40, 105], and with jet injection pre-treatment [106]. The Medipad (Elan Pharmaceutical Technologies, Ireland), is a hybrid system coupling iontophoretic delivery with shallow SC puncture [107].

The use of two or more technologies – with different mechanisms of action – permits the same effect to be achieved with safer levels of the 'active driving force'; for example, lower currents, or reduced levels of chemical enhancers. However, paradoxically, this synergy may also result in an increased toxicity: consider the combination of chemical enhancers with iontophoresis or ultrasound. The use of an electrically-assisted method could increase the rate and extent of delivery of the chemical enhancer into the skin, or induce a deeper penetration into the tissues, with concomitantly increased skin irritation. Hence, in addition to the evaluation of the practicality of certain dual technologies, which might lead to relatively complex devices, safety must be validated *in vivo* and in human volunteers before combination technologies can "take off" and be considered as realistic delivery platforms.

9. NOVEL TECHNIQUES IN EARLY DEVELOPMENT

The transdermal enhancement technologies presented below are relatively novel approaches in early development. Although they have yet to be validated with respect to peptide and protein delivery, they are included here because of their ability to overcome the SC barrier function to a greater extent than many conventional methods.

9.1 Photomechanical Waves

Photomechanical waves (PW) are broadband compressive waves generated by intense laser radiation [108]. A PW delivery device consists of a drug reservoir backed with a laser target material (e.g., polystyrene). This system is placed on the skin and the laser is applied to the target. The energy of the laser is strongly absorbed by the target, resulting in the formation of photomechanical waves which are hypothesised to transiently permeabilize the stratum corneum. Drug diffuses passively through the channels momentarily created. The mechanism of channel formation remains to be elucidated, but it is known that the effects of PW are due to mechanical forces [109].

Different molecules and even microspheres have been measured in the skin layers *in vivo* after PW application [110-113], yet, interestingly, this technique does not appear to permeabilize the

stratum corneum*, in vitro*. An isolated report describes successful insulin delivery in rats *in vivo* (~80% decrease in blood glucose level), but after pretreatment with an anionic surfactant [114]. Clearly, this technique is still very much in its infancy and unlikely to be a realistic contender for transdermal peptide delivery.

9.2 Heat-assisted drug delivery

Controlled Heat-Assisted Drug Delivery (CHADD; Zars Inc., UT, USA) is the basis of an innovative patch consisting of a layer containing a heat-generating chemical component and a perforated cover membrane. When the package is opened, air flows at a controlled rate through the holes in the cover membrane into the heating mixture and initiates a chemical reaction that spontaneously produces heat. Heat generated within the patch increases skin temperature and thereby drug penetration rates across the skin. According to the manufacturers, the temperature and duration of the reaction can be controlled by the size and number of holes in the cover membrane and the precise composition and quantity of the chemical components. S-Caine Patch™ (Zars Inc., UT, USA) is a new system for anæsthetic delivery [115], which uses the CHADD technology and which has successfully completed Phase III trials. This technology has not yet been applied to peptide and proteins although systems containing a benzodiazepine and a 5-hydroxytryptamine receptor (5-HT_3) antagonist are currently in preclinical development. One obvious concern with respect to peptides and proteins is their thermal instability, which could severely limit the application of this technology.

9.3 Microscissioning

Microscissioning entails the use of sharp particles to scize defined areas of the skin. Techniques using a combination of momentum transfer and scizing are well known in cosmetic dermatology. The relatively hard, roughened stratum corneum and epidermis resulting from aging processes can be removed by moderate velocity, sharp particles impinging obliquely against the skin surface. However, only one published study reports the application of this technology to transdermal delivery. Aluminium oxide particles (10 to 70 μm) were accelerated in a nitrogen stream under a pressure of 552 kPa, and directed towards the inner wrist of volunteers, after which the site was treated with lidocaine. The experiments, performed on only two adult subjects, demonstrated full anaesthesia around the site within three minutes, whereas topical application without the microconduit required approximately 1.5 hours [116]. This technology is very experimental and requires considerable work, notably in terms of safety. The presence of microparticulate debris in the skin as well as blood in the 200 μm diameter microconduit (although suggested to be useful for the purposes of clinical monitoring of analytes) points to the need for further investigation.

10. CONCLUSIONS

The proliferation of research activity in the field of transdermal drug delivery serves to highlight the pressing need for alternatives to the conventional invasive administration of peptides and proteins via needles and syringes. However, regardless of how attractive any new drug delivery concept may appear to be, it must not only deliver therapeutically realistic levels of drug to the target site, but must also prove its clinical superiority over conventional injections with respect to long-term safety, patient compliance, ease-of-use, impact on protein quality (physical, chemical and biological stability) and of course, commercial viability (high manufacturing costs would result in a non-viable product).

It is perhaps this lack of clinical superiority over injectables, which prevents the velocity-based approach from being the technology of choice in transdermal peptide and protein drug delivery. Iontophoresis, which offers the unique advantage of tight control over the delivery kinetics, is expected to gain momentum now that devices are being commercialised. However, one of the largest sub-classes of the protein-based therapeutics developed by the biotechnology industry is that of monoclonal antibodies, which have been anticipated to represent 30% of pharmaceutical sales by 2007 [83]. Given their size, iontophoresis, which is more suitable to peptide drugs, is certainly not the method of choice to deliver such large macromolecules. Hence, there is an evident need for developing complementary technologies such as microneedles, which represent an excellent example of interdisciplinary research.

With this large range of technologies currently under development, one can hope that in the not too distant future, there will be viable transdermal alternatives for the administration of protein and peptide based therapeutics thus reducing the need to resort to conventional injections.

11. EXPERT OPINION

The oral route is undoubtedly the preferred route of drug administration for most therapeutic agents and ideally, this would also be the case for peptide- and protein-based drugs. However, the gastrointestinal tract has evolved to break down macromolecules, including polypeptides, and facilitate the absorption of their constituents. Moreover, upon absorption, the hepatic portal vein takes these products to the liver, which is the primary site of biotransformation and conversion of these molecules and molecular fragments into products that can be easily eliminated. Approaches such as microemulsions, covalent chemical modification, and carrier-mediated systems, have enabled the oral administration of macromolecules to move forward from the proof-of-concept stage. However, unless a peptide (or protein) can be protected from, or rendered resistant to, the catabolic activity in the gastrointestinal tract, prohibitively large amounts of (expensive) therapeutic agent will have to be delivered to achieve the desired pharmacological effect. One approach is to use peptides that contain D-amino acids and, in general, oral delivery may be more feasible for smaller peptides where there is no significant tertiary structure that must be retained. Based on this logic, it is easier to explain the delivery of desmopressin by the oral route and the recent development of a tablet form for the

treatment of nocturnal enuresis. However, with respect to the oral delivery of larger proteins with more complex structures, the likelihood of an oral formulation on the market place is far from the horizon.

Some peptide drugs, for example, nafarelin, are currently available for administration via the nasal route. However, the bioavailability of therapeutic peptides administered by this route is usually less than 5%, limiting its appeal and subsequent development. Pulmonary delivery has aroused much interest and a great deal of effort has been devoted to developing pulmonary systems for the delivery of insulin. The development of inhalation systems for targeted local drug delivery in the respiratory tract has progressed considerably with the availability of recombinant human deoxyribonuclease for the treatment of cystic fibrosis. Such topical delivery is frequently to be preferred because systemic administration might not achieve the desired drug levels at the disease site, or because high systemic levels of the drugs, e.g., growth factors, that are required for a local effect at the target tissue, often cause unwanted systemic toxicity. In addition to low bioavailability which, as with nasal delivery, can necessitate large and unacceptable drug doses, pulmonary delivery presents paradoxically another dilemma: the large alveolar surface area coupled with the extremely fine nature of the epithelium can result in an extremely rapid absorption with the risk of a large bolus effect. Hence, it might be argued that the pulmonary delivery of proteins may be restricted to therapeutics that do not induce systemic side-effects at high peak serum concentrations or local tissue reactions at the site of absorption.

The application of therapeutic agents to the skin was first realised for the treatment of dermatologic diseases. Since a significant proportion of the population suffers from dermatological disorders, research in this field is expected to steadily continue. Given some of the similarities in formulation strategies, transdermal delivery is expected to benefit from advances in localised dermal delivery. However, the skin is a lipidic barrier membrane and as such, it does not lend itself to the facile delivery of hydrophilic or charged peptides and proteins. Owing to the efficient barrier function of the skin, it is difficult to envisage the delivery of peptides with molecular weights greater than 5-10 kDa at therapeutic input rates by truly non-invasive methods, such as iontophoresis or sonophoresis, which do not set out to puncture the membrane and introduce pores or similar transport channels. It is certainly true that iontophoresis is well-suited to peptide delivery owing to the controlled input kinetics that it alone can provide. For the transdermal delivery of larger macromolecules, we will have to resort to the “minimally-invasive” techniques such as microneedles and other related technologies that abrade or remove the stratum corneum. These methods certainly open the door to the delivery of a much wider range of macromolecules. The numerous jet-injectors also offer a different way into the body and are finding a useful niche for the non-invasive delivery of vaccines and this area, which is also open to exploitation by the microneedle platforms, may prove to be very fruitful. These different delivery technologies provide complementary techniques for the administration of different types of peptides and proteins and there are clearly many opportunities and unmet needs to be addressed. For all of these platforms, the key to success will always be careful selection of the peptide/protein therapeutic agent. Given the advantages of the transdermal route, one can hope that the rational design and synthesis of therapeutics optimised for transdermal delivery with the correct mix of physicochemical properties is not too far off. It is certain that only such close collaboration between medicinal chemists and formulation scientists will enable the successful delivery of tomorrow’s drugs.

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CHAPITRE 2

Effet de la séquence en acides aminés sur le transport des peptides par ionophorèse

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Effet de la séquence en acides aminés sur le transport des peptides par ionophorèse

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Résumé

L'objectif de cette étude était d'évaluer l'effet de la séquence en acides aminés sur l'administration transdermique des peptides par ionophorèse. Une série de tripeptides cationiques de structure similaire basée sur les séquences (i) 6-8 de la LHRH (Ac-X-Leu-Arg-NH_2) et (ii) 3-5 de l'octréotide (Ac-X-*D*Trp-Lys-NH_2) ont été étudiés. L'effet de la concentration en peptide sur le flux ionophorétique a été évalué par des expériences de transport réalisées *in vitro* sur de la peau de porc. L'administration simultanée de paracetamol a permis de séparer les contributions de l'électromigration (EM) et de l'électroosmose (EO) ainsi que de calculer un facteur d'inhibition électroosmotique (IF). Une augmentation de la concentration en peptide dans le compartiment donneur d'un facteur de 2 induit une augmentation du flux ionophorétique de la plupart des peptides ainsi qu'une inhibition de l'électroosmose pour les peptides contenant *D*Nal. L'amélioration du transport ainsi que l'impact sur les composantes EM et EO étaient spécifiques aux peptides. La réduction du nombre de ions compétiteurs dans la formulation augmenta le transport de façon importante, plus spécifiquement la contribution EM; elle augmenta également l'IF des composés susceptibles d'interagir avec la membrane. Le flux se montra indépendant du poids moléculaire ainsi que du ClogP. Le transport ionophorétique des peptides n'a pu être rationalisé en termes ni de poids moléculaire, ni de lipophilie estimée par méthode fragmentale. Les résultats suggèrent qu'une approche tridimensionnelle plus complexe est nécessaire à l'établissement de relations structure-permeation gouvernant l'administration des peptides par ionophorèse.

Effect of amino acid sequence on transdermal iontophoretic peptide delivery

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Abstract

The objective of this study was to investigate the effect of amino acid sequence on the transdermal delivery of peptides by iontophoresis. Structurally-related, cationic tripeptides based on the residues at positions (i) 6-8 in LHRH (Ac-X-Leu-Arg-NH_2) and (ii) 3-5 in octreotide (Ac-X-*D*Trp-Lys-NH_2) were studied. Iontophoretic transport experiments were conducted using porcine skin *in vitro* to investigate the dependence of flux on peptide concentration. Co-iontophoresis of acetaminophen enabled deconvolution of the contributions of electromigration (EM) and electroosmosis (EO) and the calculation of an electroosmotic inhibition factor (IF). A two-fold increase in donor peptide concentration increased iontophoretic flux for most peptides, and electroosmotic inhibition for *D*Nal-containing tripeptides. The improvement in transport and the impact on the EM and EO components were peptide-specific. A reduction in the number of competing ions in the formulation significantly increased transport and, specifically, the EM contribution; it also increased IF of compounds with a propensity to interact with the membrane. No monotonic dependence of flux on either molecular weight or lipophilicity was observed. Iontophoretic peptide transport could not be rationalized in terms of either peptide molecular weight or computational 2D estimates of lipophilicity. Data suggest that a more complex three-dimensional approach is required to develop structure permeation relationships governing iontophoretic peptide delivery.

Key words: Iontophoresis, electroosmosis, electromigration, skin, peptides

INTRODUCTION

Constant current iontophoresis provides an efficient, controlled method of transdermal drug administration since the amount delivered is directly proportional to the quantity of charge passed (i.e., the product of the current intensity and the duration of current application). Modulation of current intensity (and profile) enables the drug input rate to be customized to the needs of each patient, or phase of treatment, and offers the possibility of noninvasive pulsatile delivery.

Iontophoresis may be particularly suited to the controlled transdermal delivery of low molecular weight peptide therapeutics since these are frequently potent, charged and certain among them, e.g., the LHRH analogues, can elicit different pharmacological effects depending on the delivery kinetics [1].

Under physiological conditions, the skin is negatively charged and convective solvent flow in the direction of cation movement complements electromigration and allows the transport of neutral molecules. Hence, the steady-state iontophoretic flux of a cationic peptide (J_{pep}) is the sum of the electromigratory (J_{EM}) and electroosmotic (J_{EO}) contributions (assuming a negligible passive diffusion),

$$J_{pep} = J_{EM,pep} + J_{EO,pep} = \left(\frac{t_{pep} I}{z_{pep} F} \right) + v c_{pep} \tag{1}$$

where z_{pep} and c_{pep} are the concentration and valence, respectively, of the peptide; F is the Faraday constant, and u the electroosmotic flow; t_{pep} is the fraction of the total applied current density (I) carried across the skin by the peptide, its so-called transport number, which can be further defined in terms of the charge, concentration and mobility (u) of all ions (i = 1 to n) in the system:

$$t_{pep} = \frac{z_{pep} u_{pep} c_{pep}}{\sum_{i=1}^{n} z_i u_i c_i} \tag{2}$$

The mobilities and concentrations refer to those in the membrane but, for simplicity, are frequently considered to be either linearly dependent on, or equal to, the values in solution depending on the assumed degree of aqueous character of the transport pathway. From Equation 1, it is clear, given the proportional dependence of iontophoretic flux on both drug concentration and applied current that increasing either parameter should increase the flux. However, the iontophoretic transport of certain LHRH analogues, for example, nafarelin [2] and leuprolide [3], has a distinctly nonlinear dependence on the peptide concentration in the donor compartment. Indeed, in a study examining the iontophoretic delivery of leuprolide to humans *in vivo,* plasma concentrations decreased as the concentration of the peptide in the iontophoretic device was increased [4]. Analogous behaviour was also observed during the iontophoretic delivery of octreotide to rabbits *in vivo*; bioavailability increased with current intensity but decreased with increasing peptide concentration [5].

In contrast to nafarelin and leuprolide, LHRH behaves as predicted by theory even though it only differs from these analogues in the amino acid at position six (a glycine in LHRH; *D*-naphthylalanine in nafarelin; *D*-leucine in leuprolide). Both analogues contain lipophilic residues that, together with the

leucine at position 7, create a bulky lipophilic moiety directly adjacent to a positively charged residue (Arg^8). It has been suggested that the iontophoretic transport pathway contains accessible lipophilic surfaces and anionic sites capable of interacting with lipophilic cations. In general, these interactions could reduce the overall iontophoretic flux for two reasons: (i) the interaction hinders drug transport by reducing the electrical mobility of the molecule, and (ii) strong binding could neutralize the skin's negative charge and decrease EO.

Iontophoresis of a series of oligopeptides, and their effect on mannitol flux, indicated that both lipophilicity and a positive charge were required for EO inhibition to occur [6]. These results validated the hypothesis that the amino acids at positions 6-8 in LHRH and nafarelin were the key to their unique iontophoretic transport behaviour. The inhibitory effect of the tripeptides was related to their proposed lipophilicities [6]. Since the -Phe^3-*D*Trp^4-Lys^5- sequence in octreotide has the same (hydrophobe-hydrophobe-cation) motif identified for the anomalous behavior of leuprolide and nafarelin, the inverse dependence of plasma octreotide levels and the peptide dose might also be attributed to EO inhibition.

To-date there has been no systematic and quantitative study of the incremental effect of molecular weight/lipophilicity on iontophoretic peptide transport. The aim of the present work was to study the effect of amino acid sequence on the iontophoretic behavior of cationic peptides and to identify the role of physicochemical parameters in controlling peptide electrotransport across the skin. The first series of structurally-related oligopeptides, Ac-X-Leu-Arg-NH_2 (where X=Gly, *D*Ala, *D*Leu, *D*Ser, *D*Cit, *D*Phe, *D*Trp, and *D*Nal) was based on the amino acids found at positions 6-8 in LHRH analogues. Substitution of the N-terminal residue enabled a systematic modification of the tripeptide's physicochemical properties. In addition to acetylation of the N-terminal and amidation of the C-terminal, the presence of *D*-amino acids at the N-terminal position further improved resistance to cutaneous metabolism. A second series of tripeptides was based on the sequence at positions 3-5 in octreotide (Ac-X-*D*Trp-Lys-NH_2 (where X = Phe, Ser, and Tyr)). Finally, a series of "hybrid" tripeptides (Ac-Phe-*D*Trp-Arg-NH_2, Ac-*D*Nal-Arg-Leu-NH_2, Ac-Leu-*D*Nal-Arg-NH_2, and Ac-*D*Nal-Leu-Lys-NH_2) containing residues from the "LHRH" and "octreotide" series was also studied. In addition to quantifying peptide transport, the iontophoretic flux of a co-administered EO marker was measured to assess the magnitude of convective solvent flow and to determine the relative contributions of each transport mechanism. Prediction of the relative importance of EM and EO and the identification of molecular properties determining whether, and to what extent, a therapeutic peptide inhibits its own transport is clearly important for formulation design.

MATERIALS AND METHODS

Chemicals

Tripeptides of the form Ac-X-Leu-Arg-NH_2 (where X=Gly, Ala, *D*Ala, *D*Leu, *D*Ser, *D*Phe, *D*Trp, *D*Cit (citrulline), and *D*Nal (2-naphthylalanine), Ac-X-*D*Trp-Lys-NH_2 (where X=Phe, Ser, and Tyr), Ac-Phe-*D*Trp-Arg-NH_2, Ac-*D*Nal-Arg-Leu-NH_2, Ac-Leu-*D*Nal-Arg-NH_2, and Ac-*D*Nal-Leu-Lys-NH_2 were

synthesized either by the Institute of Biochemistry at the University of Lausanne (Lausanne, Switzerland), or by NeoMPS Inc. (Strasbourg, France). For conciseness, single letter codes have been used for the amino acids in the figures. Acetaminophen and ninhydrin were purchased from Fluka (Sigma-Aldrich Chimie Sarl, France). Tris (Tris-(hydroxymethyl) aminomethane), Trizma® hydrochloride and agarose were obtained from Sigma-Aldrich (Sigma-Aldrich Chimie Sarl, France) and 1-heptanesulfonic acid sodium salt from Fisher Chemicals (Fisher Scientific, NJ). De-ionized water (resistivity > 18 Mohm/cm^2) was used to prepare all solutions.

Analytical procedures

Arginine-containing tripeptides were quantified by high-performance liquid chromatography with on-line post-column fluorescence derivatization [7]. Briefly, the method involved detection of the fluorophore derived from condensation of the guanidine moiety of arginine with ninhydrin (contained in the mobile phase) in an alkaline stream. The chromatographic system comprised a Model 600E solvent delivery pump, an in-line degasser, a Model 717plus injector and a reaction system equipped with a mixing tee, a single-piston pulse-dampened pump and a RXN™ 1000 reaction coil (Waters Corporation, MA). The mobile phase consisted of either 22 or 38% acetonitrile depending on the lipophilicity of the peptide. The aqueous phase contained 15 mM sodium heptanesulfonate and 5 mM ninhydrin, adjusted to pH 3.5 with acetic acid. The flow rate was 1 ml/min. After elution through a Zorbax SB-C8 column (4.6 mm i.d., 25 cm long, 5 μm particle size) (Agilent Technologies Inc., CA), the stream was mixed with a 0.5 M sodium hydroxide solution delivered at a flow rate of 0.5 ml/min. Both the column and the reaction coil were maintained at 60°C. A Waters 474 scanning fluorescence detector was used to detect the derivatized fluorophore using an excitation wavelength of 395 nm and an emission wavelength of 500 nm.

The remaining peptides were quantified by high-performance liquid chromatography using the same column maintained at the same temperature, but with UV detection. The mobile phase contained acetonitrile and a triethylaminephosphate buffer solution, pH 2.3. The ratio of organic to aqueous phase was dependent on the compound: 30:70 for Ac-*D*Nal-Leu-Lys-NH_2 (detected at 228 nm), 20:80 for Ac-Phe-*D*Trp-Lys-NH_2, and 12:88 for Ac-Ser-*D*Trp-Lys-NH_2 and Ac-Tyr-*D*Trp-Lys-NH_2. These latter tripeptides were detected at 278 nm.

The RSD of repeatability was less than 1% and the quantification limit was typically between 0.2 and 0.4 μM for all the peptides assayed.

Similarly, acetaminophen was assayed with a mobile phase comprising 92% water and 8% acetonitrile adjusted to pH 3.5 with acetic acid. The flow rate was 1.5 ml/min. Acetaminophen was detected by its UV absorbance at 243 nm. The RSD of the repeatability was less than 1 % and the quantification limit was 0.7 μM.

Skin preparation

Porcine ears were obtained shortly after sacrifice from the local abattoir (SODEXA, Annecy, France) and were cleaned under cold running water. The whole skin was removed carefully from the

outer region of the ear and separated from the underlying cartilage with a scalpel. The tissue was then dermatomed (800 μm), wrapped in Parafilm™ and maintained at –20°C before use for a period no longer than 2 months.

Iontophoresis procedure

The skin was clamped in three-compartment vertical flow-through diffusion cells (area: 0.726 cm^2), the design of which has been previously described [8]. Tripeptides (2.18 or 4.36 mM) were dissolved in a 154 mM Tris/Trizma® HCl-buffered solution (pH 7.4). In addition to the peptide, the donor solution (1 ml) always contained 15 mM acetaminophen, which was used to report on the convective solvent flow. The cathodal compartment was filled with 1 ml of 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline. The receptor compartment, containing ~6 ml of the same electrolyte solution, was magnetically stirred and perfused at a flow rate of ~3 ml/h.

In a second series of experiments, the anode was isolated from the donor solution via a salt bridge (100 mM Tris/Trizma HCl in 3% agarose) to minimize the effect of competing ions. In this case, the anodal compartment contained a solution of 25 mM Tris/Trizma® HCl normal saline buffered to pH 7.4. The solutions in the donor compartment comprised 4.36 mM of the respective peptide and 15 mM acetaminophen dissolved in 20 mM Tris/Trizma® HCl (pH 7.4).

In all experiments, a constant current (0.5 mA/cm^2) was applied for 8 hours via Ag/AgCl electrodes connected to a power supply (Kepco®, NJ).

All measurements were made in at least triplicate, using skin from different pigs.

Quantifying EO inhibition

Acetaminophen is a neutral hydrophilic compound, with poor passive permeability, that can be iontophoretically driven through the skin by electroosmosis. It was therefore included in the peptide formulation in the donor compartment (15 mM) as a marker for the direction and magnitude of convective flow.

For each experiment, an Inhibition Factor (IF) was calculated using the following equation:

$$IF = [Q_{A\text{-}8h,control}] / [Q_{A\text{-}8h,peptide}] \quad (3)$$

where $Q_{A\text{-}8h,control}$ is the amount of acetaminophen transported into the receptor phase during 8 hours of iontophoresis in the absence of peptide, and $Q_{A\text{-}8h,peptide}$ is the corresponding quantity when a tripeptide was simultaneously iontophoresed.

Determination of EM and EO contributions to iontophoresis

The iontophoretic flux (J) of a charged species is the sum of the component fluxes - J_{EM} and J_{EO} – due to electromigration and electroosmosis (assuming that the passive permeability is negligible):

$$J = J_{EM,pep} + J_{EO,pep} \quad (4)$$

During iontophoresis, the current-induced flow of water (V_w) across the skin can be estimated from [9]:

$$V_w = J_{ace} / C_{ace} \quad (5)$$

where J_{ace} is the acetaminophen flux and C_{ace} is its concentration in the donor compartment. It follows that, if V_w has been quantified in the presence of drug, then the convective component of its iontophoretic transport (J_{EO}) can be calculated by multiplying V_w by the peptide concentration in the donor solution (C_{pep}) [10]:

$$J_{EO} = V_w \cdot C_{pep} \quad (6)$$

This analysis makes three assumptions: (a) that peptide and acetaminophen are transported in a similar fashion by convective solvent flow, (b) that transport of peptide and acetaminophen is independent and there is no interaction between the two species, and (c) that EO transport of the marker molecule is proportional to its concentration in the solvent.

It follows that equations (4)-(6) can be used to estimate the relative contributions of EO and EM to the total iontophoretic flux of the peptide.

Transport number determination

Transport number was calculated for each tripeptide according to the following equation (derived from Equation 1):

$$t_{peptide} = \frac{cumulative\ peptide transport \cdot F}{I \cdot T} \quad (7)$$

where F is the Faraday constant (96485 C/mol), I is the applied current intensity and T the duration of current application.

Peptide accumulation in the skin

The uptake of peptide by the skin during iontophoresis was also quantified. Once the current was stopped, the dermatomed skin sample was washed with water, dried on absorbent paper, and then placed in 4 ml of the mobile phase used in the HPLC analytical method to extract the peptide. After stirring for 14 hours, the solution was filtered (0.45 μm regenerated cellulose syringe filter, Alltech, IL) and the peptide was quantified in the normal way.

Data treatment

The results were derived from at least triplicate experiments conducted with skin samples originating from different pig ears. Outliers, determined using the Grubbs test, were discarded. When two sets of data where compared, Student t tests were performed. The level of statistical significance was fixed at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of peptide concentration

The impact of doubling the donor concentration from 2.18 to 4.36 mM was tested for each tripeptide. The iontophoretic flux for each peptide at the two concentrations is presented graphically in Figure 1.

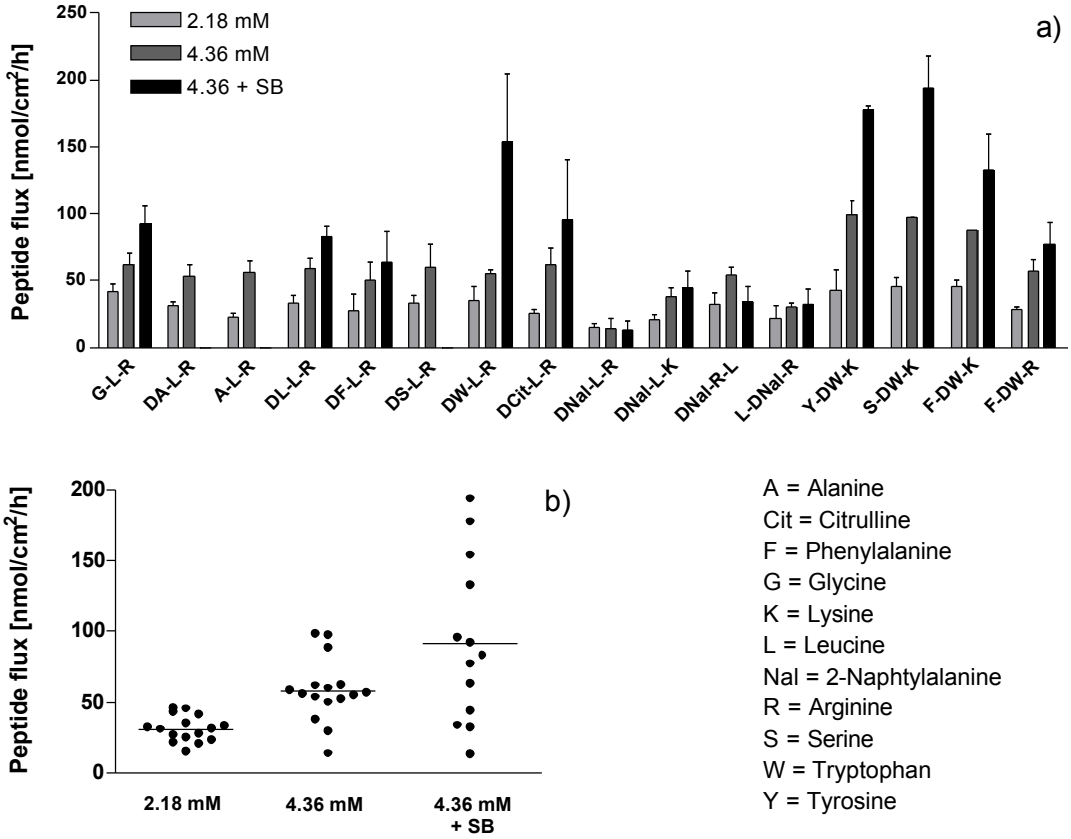

***Figure 1*. The influence of donor [peptide] and [Tris$^+$] on peptide transport across porcine skin *in vitro*.**
The data for each named peptide is shown in the upper panel (a); the spread of data is illustrated in the lower graph (b). SB denotes the use of a salt bridge to decrease electrolyte concentration.

According to Faraday's Law, at low concentrations, and in the absence of peptide-membrane or peptide-peptide interactions, electrotransport should increase proportionally with concentration in the presence of background electrolyte [11]. However, while doubling the donor concentration resulted in significantly increased fluxes for nearly all tripeptides, fluxes of Ac-*D*Nal-Leu-Arg-NH_2 and Ac-Leu-*D*Nal-Arg-NH_2 at 4.36 mM were not statistically different from those measured from a donor concentration of 2.18 mM. This can be partly explained by the suppression of convective solvent flow induced by these peptides. Effectively, these two peptides, which both possess a bulky lipophilic moiety (leucine adjacent to 2-naphthylalanine) were the only molecules to exhibit EO inhibition (IF=2.2; Figure 2). These results are consistent with the previously reported observation that although *D*Nal-Leu-Arg had a significant effect on electroosmosis, there was no inhibition during iontophoresis of Ala-Leu-Arg [6].

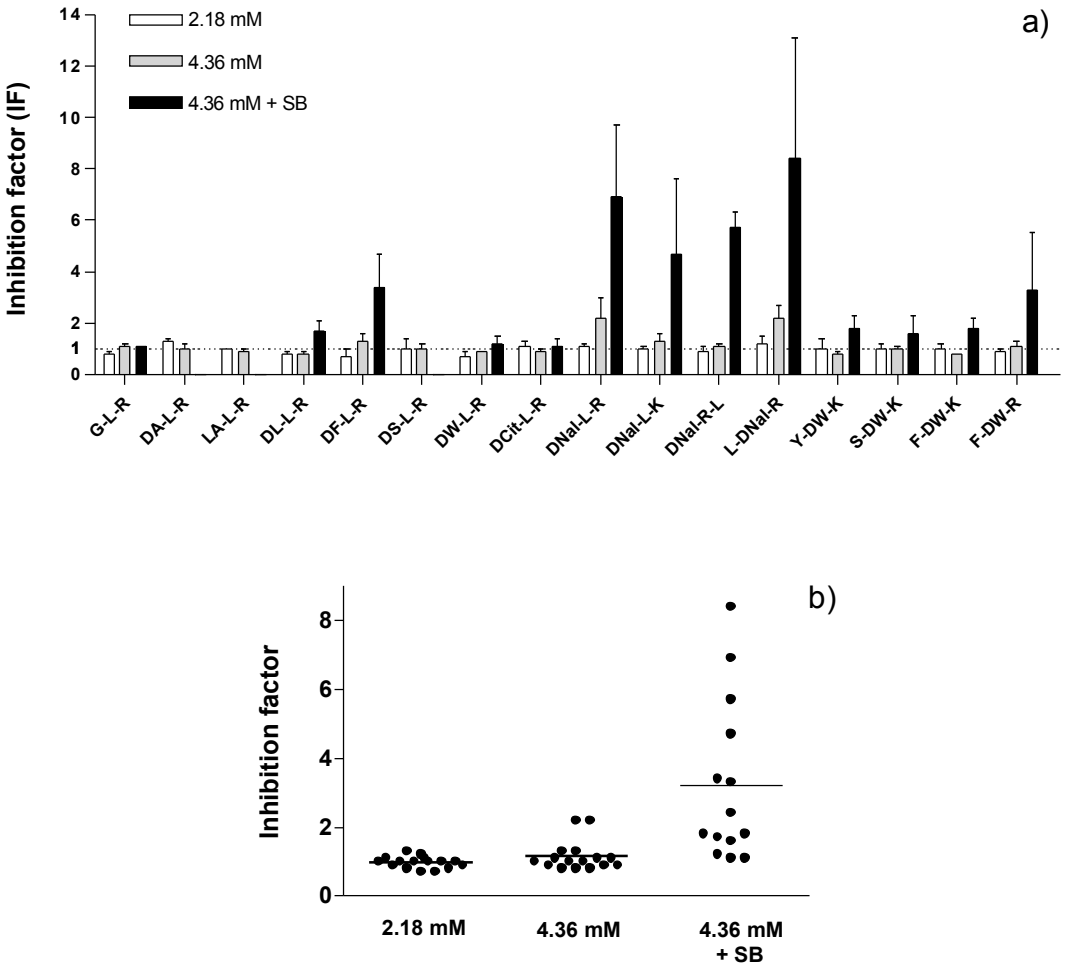

Figure 2. **The influence of donor [peptide] and [Tris$^+$] on electroosmotic inhibition**.

IF = $[Q_{A\text{-}8h,control}] / [Q_{A\text{-}8h,peptide}]$ (see Equation 3 for definitions).
The data for each named peptide is shown in the upper panel (a); the spread of data is illustrated in the lower graph (b).
SB denotes the use of a salt bridge to decrease electrolyte concentration.

Next, we wished to address whether the transport efficacy of the peptides investigated could be correlated to one or several aspects of their physical chemistry (e.g., molecular weight and lipophilicity). However, despite the expansive range of peptides tested - encompassing a spectrum of physicochemical signatures, their iontophoretic transport remained, surprisingly, unaffected. This is illustrated in Figure 1, where the mean J_{ss} for each peptide has been plotted as a function of the donor formulation; while peptide fluxes generally increased at the higher donor concentration, they did not appear to be peptide-specific. Similarly, their impact on EO at the higher peptide concentration was only discernible for two of the peptides tested (Figure 2). That is, the data dispersion was not sufficient to enable discrimination between the peptides on the basis of their physical chemistry.

Since phenomena such as peptide aggregation and EO inhibition, which are dictated by amino acid sequence, and which ultimately affect *total* iontophoretic transport, are generally concentration-dependent, it is possible that the use of higher concentrations would have allowed discrimination

between the peptides. However, due to the limited amounts of peptide available, an alternative approach was employed to increase transport efficacy.

Effect of competing ions on peptide delivery

Ag/AgCl electrodes need chloride ions for anodal electrochemistry (~100mM – for the experiments described here) derived from either the molecule under study or from an external source (e.g., NaCl or buffer); in the latter case the number of competing cations in the anodal formulation is greatly increased. To increase peptide transport efficacy, the number of competitive cations in the donor compartment can be reduced (~20 mM) by using a salt bridge assembly. This is illustrated in Figure 1, which shows the doubling of iontophoretic fluxes upon decreasing donor [$Tris^+$] approximately 5-fold. This increased transport efficacy was similarly reflected by the calculated transport numbers: experiments performed at 4.36 mM without a salt bridge resulted in transport numbers ranging from 0.0004 to 0.003; values as high as 0.0086 were obtained with the reduced levels of donor $Tris^+$. As a consequence, the EM contribution to the total iontophoretic peptide flux was increased when the number of competitive charge carriers was decreased.

In addition to increasing peptide transport, the reduced competition from donor cations also resulted in a more pronounced inhibition of EO for the majority of peptides (Figure 2). For compounds with a propensity to interact with the membrane, the entry of greater quantities of peptide into the skin, leads to greater skin charge neutralization and hence, reduced convective solvent flow.

The total iontophoretic flux is the linear combination of the EM and EO contributions (Eq. (1)). Assuming that these components are independent, it is possible to examine the effect of increasing peptide concentration on each component separately. First, with respect to EM, if the peptide concentration in the donor compartment is increased from $c_{pep,1}$ to $c_{pep,2}$ (assuming that the proportionality constant between the concentrations in solution and skin is unity), then the ratio of the EM fluxes, $J_{EM,2}/J_{EM,1}$, is given by:

$$\frac{J_{EM,2}}{J_{EM,1}} = \frac{c_{pep,2}\left(\sum z_i u_i c_i\right)_1}{c_{pep,1}\left(\sum z_i u_i c_i\right)_2} \tag{9}$$

where $(\Sigma z_i u_i c_i)_n$ refers to the sum of products of the valence, mobility and concentration of the competing charge carriers for the two formulations, respectively. Under the experimental conditions used in this study, the donor compartment contained protonated Tris in addition to the peptide, and the receiver contained Cl^- ions, therefore expansion of Equation 9 yields:

$$\frac{J_{EM,2}}{J_{EM,1}} = \frac{c_{pep,2}\left(u_{TRIS}c_{TRIS} + u_{Cl}c_{Cl} + u_{pep}c_{pep,1}\right)}{c_{pep,1}\left(u_{TRIS}c_{TRIS} + u_{Cl}c_{Cl} + u_{pep}c_{pep,2}\right)} \tag{10}$$

Two limiting conditions are easily identified:

Case 1: [electrolyte] >> [peptide]

$$u_{pep}c_{pep,1} << \; u_{TRIS}c_{TRIS} + u_{Cl}c_{Cl} \; >> u_{pep}c_{pep,2} \Rightarrow \frac{J_{EM,2}}{J_{EM,1}} = \frac{c_{pep,2}}{c_{pep,1}} \quad (11)$$

Case 2: [peptide] >> [electrolyte]

$$u_{pep}c_{pep,1} >> \; u_{TRIS}c_{TRIS} + u_{Cl}c_{Cl} \; << u_{pep}c_{pep,2} \Rightarrow \frac{J_{EM,2}}{J_{EM,1}} = 1 \quad (12)$$

Thus, at low peptide concentrations, the ratio of EM fluxes is equal to the ratio of the concentrations (Case 1). As peptide concentration is increased relative to the electrolyte levels, the EM flux ultimately becomes independent of concentration and the ratio tends to unity (Case 2). Since the background electrolytes in the donor and receiver compartments were in significant excess (either 20 or 154 mM Tris cation in the donor and 154 mM chloride anion in the receiver) compared to the peptide (2.18 or 4.36 mM), CASE 1 was considered to hold for these experiments.

The effect of increasing peptide concentration on EO can be expressed as follows:

$$\frac{J_{EO,2}}{J_{EO,1}} = \frac{vc_{pep,2}}{vc_{pep,1}} = \frac{c_{pep,2}}{c_{pep,1}} \quad (13)$$

Based on Equations 11 and 13, a two-fold increase in peptide concentration is expected to result in a corresponding increase in the EM and EO contributions. The ratio of EM (J_{EM}(4.36/2.18)) and EO (J_{EO}(4.36/2.18)) contributions at the two peptide concentrations are shown in Figure 3a. The J_{EM}(4.36/2.18) and J_{EO}(4.36/2.18) ratios for the Ac-X-*D*Trp-Lys-NH_2 tripeptides were clustered around the predicted value of 2, suggesting an equivalent effect on both transport mechanisms. However, there was more variability associated with the Ac-X-Leu-Arg-NH_2 series.

Upon subsequent reduction of the donor electrolyte concentration, the EM and EO contributions were markedly different (Figure 3b). Under these conditions the relative contribution of EO was significantly reduced as evidenced by J_{EO}(4.36+SB/2.18) < 1 for most peptides. In contrast, J_{EM}(4.36+SB/2.18) was much higher, ranging from 1 to 8. The reduced EO was manifested by those peptides displaying an IF greater than 1 (Figure 2a). Concomitantly, the reduction in the number of competing cations, significantly increased the fraction of current transported by the tripeptides, accounting for an EM contribution of 77 to 93% to the overall transport.

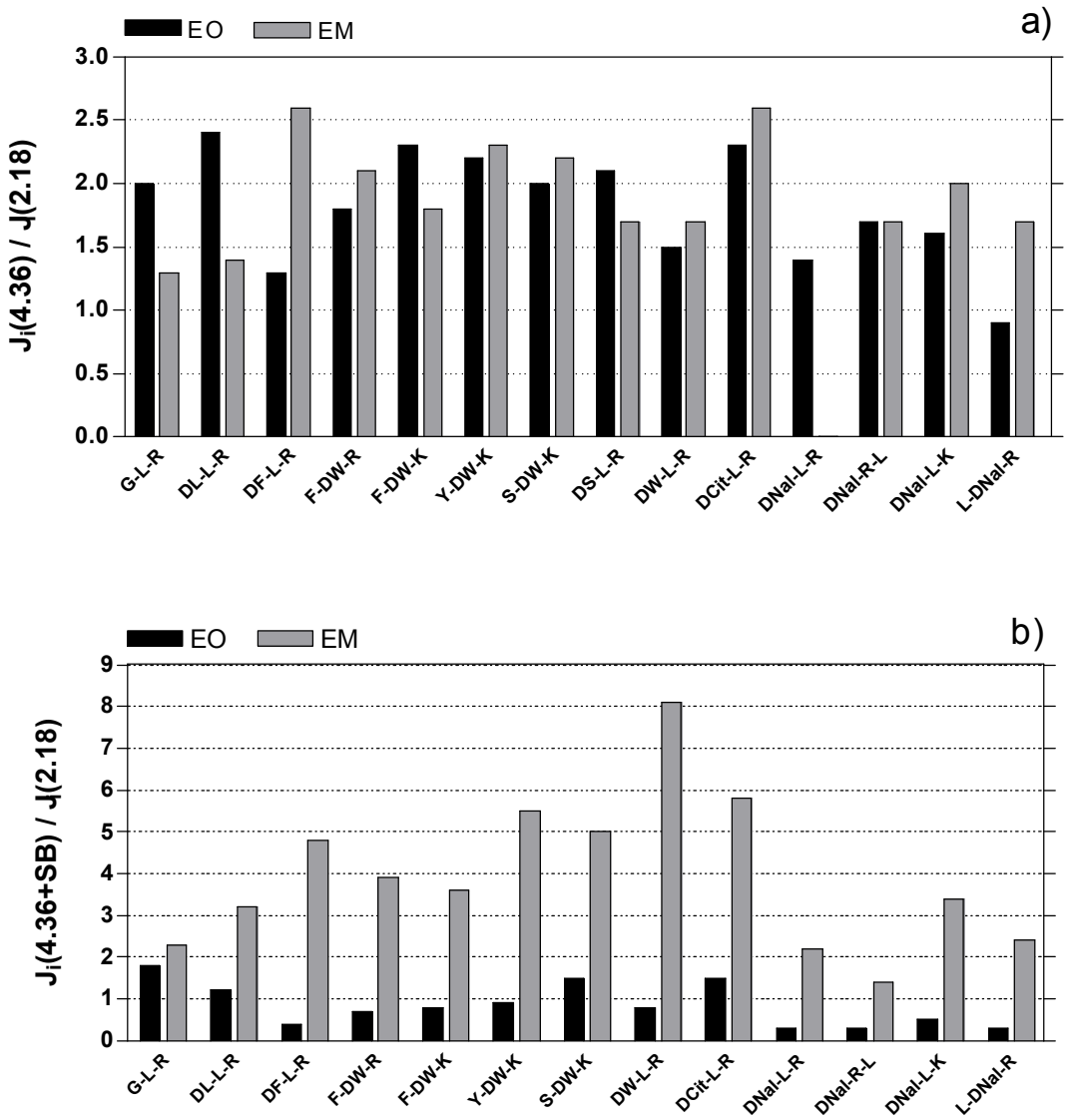

***Figure 3*. The effect of donor peptide concentration (a) and the subsequent impact of reduced donor electrolyte ($Tris^+$) levels (b) on the contributions of EM and EO to iontophoretic transport**.

(**A**) The y-axis, J_i(4.36/2.18), represents the flux ratio of either EM or EO at the two peptide concentrations (2.18 and 4.36 mM).

(B) The y-axis, J_i(4.36+SB/2.18), expresses the additional influence of reduced donor $Tris^+$ levels.

The anomalous behaviour of the Ac-X-Leu-Arg-NH_2 series is further illustrated by Figure 4, which compares the transport behaviour of Ac-Gly-Leu-Arg-NH_2 and Ac-*D*Nal-Leu-Arg-NH_2 under various experimental conditions. Whereas the flux of the former was dependent on donor peptide and electrolyte levels, transport of the latter was indifferent to the experimental conditions employed (Figure 4).

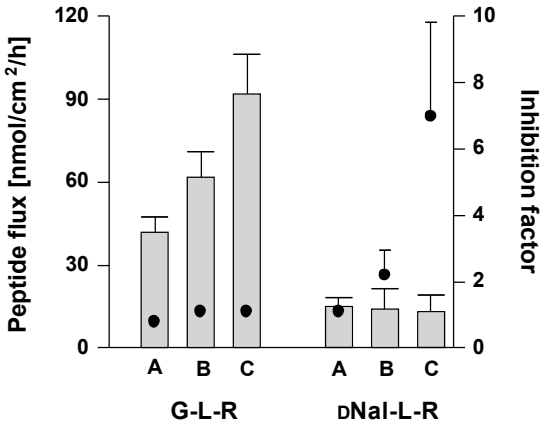

Figure 4. **Iontophoretic fluxes and inhibition factors of Ac-Gly-Leu-Arg-NH_2 and Ac-*D*Nal-Leu-Arg-NH_2 as a function of the experimental conditions**.

A = Donor peptide conc. 2.18 mM and electrolyte conc. 154 mM; B = Donor peptide conc. 4.36 mM and electrolyte conc. 154 mM and C = Donor peptide conc. 4.36 mM and electrolyte conc. ≅20 mM.

Peptide accumulation in the skin does not always imply EO inhibition

At the end of the iontophoretic experiments, bound peptide was extracted from the skin samples and quantified. Generally, greater amounts were recovered for those peptides exhibiting the highest inhibition factors. For example, in the absence of excess Na^+, significantly more Ac-Leu-*D*Nal-Arg-NH_2 (260 ± 30 μg, IF = 8 ± 5) was recovered than Ac-*D*Cit-Leu-Arg-NH_2, (47 ± 16 μg, IF = 1.1 ± 0.3). Conversely, however, the mere accumulation of a cationic peptide in the membrane was not in itself a sufficient condition for significant EO inhibition; as typified by Ac-Phe-*D*Trp-Lys-NH_2, (330 ± 40 μg, IF = 1.8 ± 0.4). This peptide has approximately the same molecular weight as Ac-Leu-*D*Nal-Arg-NH_2; although similar amounts were retrieved from the respective skin samples, the latter is a significantly more powerful EO inhibitor, as evidenced by its IF, which is ~4.7-fold greater.

Effect of peptide physicochemical properties on iontophoretic delivery

Molecular weight and lipophilicity are among physicochemical parameters commonly used to describe variations in passive transport across biological membranes. However, for these model tripeptides, there appeared to be no correlation between their transdermal iontophoretic transport and either molecular weight or calculated lipophilicity (Figure 5).

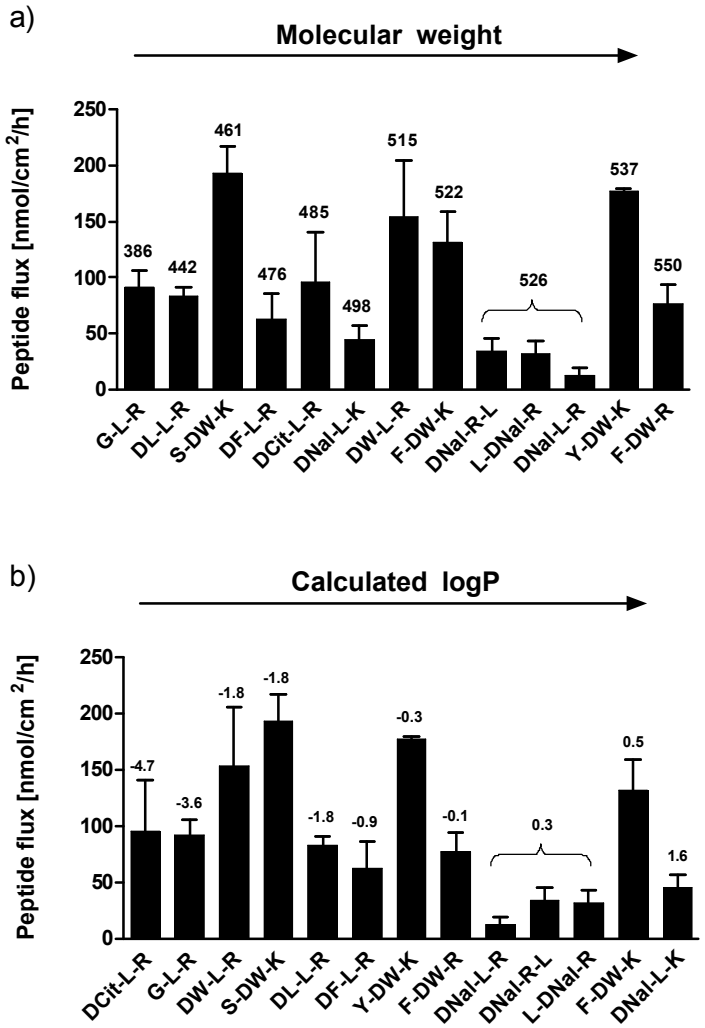

Figure 5. **Peptide fluxes as a function of a) Molecular weight and b) logP values calculated using Daylight ClogP** (Daylight Chemical Information Systems, Inc., Mission Viejo, CA (www.daylight.com)).

Donor peptide conc. 4.36 mM and electrolyte conc. ≅ 20 mM.

For example, the flux of Ac-*D*Nal-Leu-Arg-NH_2 (MW = 526 Da), was ~10-fold lower than that of Ac-*D*Trp-Leu-Arg-NH_2, a peptide of comparable molecular weight (MW = 515 Da). More striking was the lack of correlation between peptide flux and lipophilicity over values of calculated P spanning 5 orders of magnitude. The flux of Ac-*D*Nal-Leu-Arg-NH_2 (ClogP = 0.26) was more than 5-fold lower than that of Ac-Phe-*D*Trp-Arg-NH_2, a peptide only slightly less lipophilic (ClogP of -0.15). This strongly suggests that 1D- and 2D-descriptors cannot adequately explain iontophoretic transport in terms of peptide physicochemical properties, and that a computational study involving the 3D-spatial distribution of these properties over the entire molecular surface is necessary to explain the observed transport kinetics.

CONCLUSIONS

The electrically-assisted delivery of structurally related tripeptides derived from the amino acid sequences at positions 6-8 in LHRH (Ac-X-Leu-Arg-NH_2) and 3-5 in octreotide (Ac-X-*D*Trp-Lys-NH_2**)** was investigated to identify the effect of single amino acid substitutions on iontophoretic transport. Co-iontophoresis of acetaminophen enabled deconvolution of the EM and EO contributions. Although

increasing donor peptide concentration generally produced an increase in peptide flux, the relative effects on EM and EO were peptide-dependent. Transport was further increased when electrolyte levels were reduced and under these conditions, the dominance of electromigration was more pronounced. Nevertheless, certain tripeptides, where the first two residues created a large hydrophobic surface in proximity to the positive charge at the C-terminal position were able to shut down convective solvent flow. Qualitatively, lipophilic cations appeared to have poorer transport; however there was no direct dependence of transport on either molecular weight or calculated log P. It is possible that peptide flux depends on the spatial distribution of lipophilicity and other physicochemical parameters. The increased data dispersion obtained with reduced levels of donor electrolyte should prove useful for ongoing computational studies aimed at identifying (i) structure-permeation relationships governing peptide iontophoresis and (ii) the impact of specific physicochemical properties and their spatial distribution on delivery.

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CHAPITRE 3

Relations structure-perméation pour l'administration transdermique des peptides par ionophorèse

CHAPITRE 3

Relations structure-perméation pour l'administration transdermique des peptides par ionophorèse

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Résumé

Cette étude a pour objectifs (i) d'évaluer l'importance de la séquence en acides amines, (ii) d'étudier comment la distribution spatiale des propriétés physicochimiques des peptides influence leur électrotransport, (iii) de développer un modèle quantitatif permettant de prédire le passage des peptides. Les résultats expérimentaux ont montré que la séquence en acides aminés, qui détermine la distribution des propriétés moléculaires au sein du peptide, exerce un effet important sur l'administration par ionophorèse : différents arrangements des même résidus résultent en des transports différents. Des études computationnelles ont permis de générer des relations structure-perméation quantitatives tridimensionnelles (3D-QSPR) basées sur des descripteurs 3D. Le modèle prédit que la ionophorèse est favorisée par l'hydrophilie des peptides alors qu'elle est entravée par une hydrophobie volumineuse et localisée. Les caractéristiques moléculaires qui favorisent l'électrotransport à travers la peau sont contraires à celles requises pour une diffusion passive. Les résultats présentent la première analyse de l'électrotransport de peptides en termes de distribution spatiale des propriétés moléculaires et offrent un premier pas vers la prédiction *ab initio* de l'administration transdermique des peptides par ionophorèse

Structure-permeation relationships for the transdermal delivery of peptides by iontophoresis

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Abstract

The aims of this study were (i) to evaluate the importance of amino acid sequence, (ii) to investigate how the spatial distribution of peptide physicochemical properties influence their electrotransport, and (iii) to develop a quantitative model with which to predict peptide transport rates. Experimental results showed that amino acid sequence, which determines the distribution of molecular properties over the peptide surface, significantly affected iontophoretic delivery: different arrangements of the same residues resulted in different transport behavior. Computational studies generated three-dimensional *Q*uantitative-*S*tructure-*P*ermeation-*R*elationships (3D-QSPR) based on 3D descriptors. The model predicted that iontophoresis was favored by peptide hydrophilicity but hindered by voluminous, localized hydrophobicity. The molecular characteristics that favor electrotransport are the converse of those required for passive diffusion across the skin. The data represent the first analysis of peptide electrotransport in terms of the spatial distribution of molecular properties and provide insight into the *ab initio* prediction of transdermal iontophoretic peptide delivery.

Keywords: transdermal iontophoresis; electroosmosis; skin; peptide; QSPR

The skin represents an easily accessible, relatively large site for drug administration. However, the barrier properties of the skin restrict the number of systemically-acting drugs that can be passively delivered by this route to a select group of low molecular weight, potent compounds with the appropriate balance of physicochemical properties. Transdermal delivery of potent, therapeutic peptides is precluded by their polar (or charged) nature, which limits their transport across the stratum corneum, the lipidic rate-limiting barrier to percutaneous transit. Moreover, attainment of delivery rates required to achieve and to maintain therapeutic levels, by simple passive diffusion, would probably require large (and unrealistic) patch areas as well as the use of aggressive chemical enhancers to reduce skin barrier function (an approach that is likely to be unacceptable for chronic application because of local irritation).

Iontophoresis is an electrically-assisted drug delivery technology that enables non-invasive, controlled administration of low molecular weight peptide therapeutics. A small electric potential is used to drive ions into the body; increased drug mobility enables greater amounts to be delivered in shorter times. Drug input rate is controlled by the applied current intensity enabling individualized therapy according to patient needs and disease progression. Moreover, modulation of the current allows complex drug delivery profiles to be achieved. For example, continuous delivery of the LHRH analog, leuprolide, used in the treatment of prostate cancer, suppressed testosterone levels and resulted in biochemical castration in human volunteers [1]. In contrast, pulsatile administration of LHRH, again in humans, was able to mimic the body's natural release profile and promoted secretion of LH [2].

Electrotransport occurs through two principal mechanisms: electromigration (direct effect of the applied electric field on the charged species) and electroosmosis (convective solvent flow in the anode-to-cathode direction, as a result of the skin's net negative charge at physiological pH). Although theory suggests, and experiments typically confirm, that electrotransport generally increases proportionally with current density and concentration [3], the transport of certain cationic compounds, including several peptides, shows the opposite behavior, a phenomenon attributed to an inhibition of electroosmosis [4-7]. Subsequent studies, involving measurement of mannitol flux, a marker for electroosmosis, during the iontophoresis of a series of oligopeptides, suggested that the presence of a bulky hydrophobic moiety adjacent to a positively charged residue, enabled the peptides to bind to the negatively charged skin membrane, thereby eliminating the membrane's cationic permselectivity, and shutting down electroosmosis [8].

To-date there has been no systematic, quantitative study into the key molecular properties that govern peptide electrotransport. The objectives of the present investigation were to identify these parameters and to demonstrate that 1- and 2-dimensional descriptors are inadequate measures to account for the complex behavior observed: even for small, dynamic oligopeptides, transport can only be interpreted accurately in terms of the 3-dimensional spatial distribution of these properties over the molecular surface. Thus, different arrangements of the same amino acids within a given peptide significantly alter transport behavior. The longer-term goal was to use experimentally determined iontophoretic tripeptide fluxes to develop a quantitative structure-permeation relationship (QSPR) capable of *ab initio* prediction of electrotransport. While several models have been proposed to

predict passive skin permeation [9], none employing molecular properties (beyond molecular weight) have been developed to explain “active” delivery by iontophoresis.

RESULTS

Molecular weight and 2-dimensional parameters are inadequate descriptors of peptide transport

Because of their greater mobilities in an electric field, low molecular weight cations display much higher iontophoretic permeabilities than high molecular weight proteins; but molecular weight or size is not a sufficiently discriminating parameter to explain subtle differences in transport.

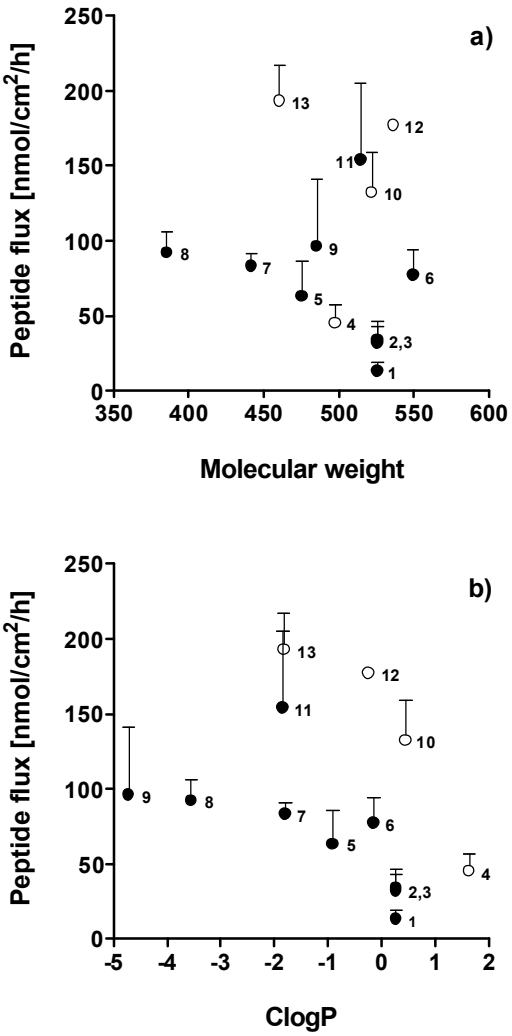


Figure 1. **Absence of correlation between iontophoretic peptide flux and either (a) molecular weight (Daltons), or (b) calculated octanol-water partition coefficient (ClogP).** Open circles correspond to lysine-containing peptides

Figure 1a clearly shows that this parameter cannot explain the differences observed between the peptides studied here. For example, Ac-*D*Trp-Leu-Arg-NH_2 (n°11) and Ac-*D*Nal-Leu-Arg-NH_2 (n°1) have similar molecular weights (515 and 526 Da, respectively), but the flux of the former is ~12-fold greater (150 ± 50 *versus* 13 ± 6 nmol/cm^2/h). Similarly, since iontophoretic peptide transport [8] has

been correlated with lipophilicity, octanol-water partition coefficients (ClogP) were calculated for all compounds. Although it is generally preferable to use log D to denote lipophilicity of ionizable compounds, log P was used here as the ionization state of all the peptides studied was assumed to be identical since the experimental pH was always 3 units below the pK_a of the basic residue. Thus the effect of ionization on lipophilicity was assumed to be constant in this homologous series of tripeptides originated from either arginine or lysine residue. Figure 1b shows that ClogP is poorly correlated with measured peptide flux. For instance, Ac-Phe-*D*Trp-Lys-NH_2, which is only slightly more hydrophilic than Ac-*D*Nal-Leu-Arg-NH_2 (calculated ClogP values of 0.263 and 0.449, respectively), had a 10-fold higher flux (130 ± 30 *versus* 13 ± 6 nmol/cm^2/h). Furthermore, multilinear regression analysis failed to establish a satisfactory correlation between peptide flux and conventional two-dimensional (2D) parameters (e.g., solvent accessible surface area, molecular flexibility, topological indices) included in the DRAGON software package [10] (data not shown). This suggested that 2D descriptors were poorly adapted to the task of interpreting electrotransport of these peptides across the skin. In light of these results, a three-dimensional approach was adopted.

Quantifying physicochemical properties over the 3D molecular surface

An iterative variable selection allowed a large decrease of the VolSurf molecular descriptors derived from the WATER probe, the DRY probe, MLP and MHBPs. Indeed starting from the 148 original parameters, only the 24 most pertinent descriptors were retained to describe the iontophoretic flux and led to a two-component PLS model, which accounted for 85% of the total variance of the matrix (Figure 2).

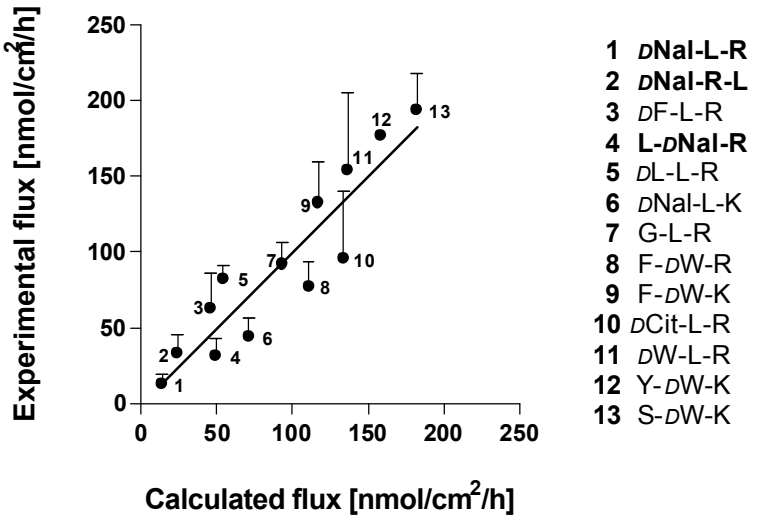
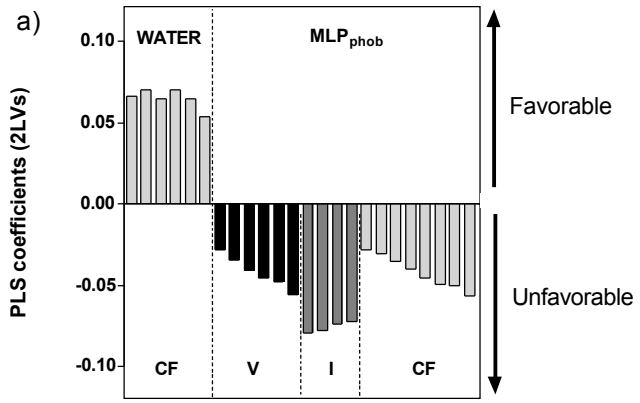


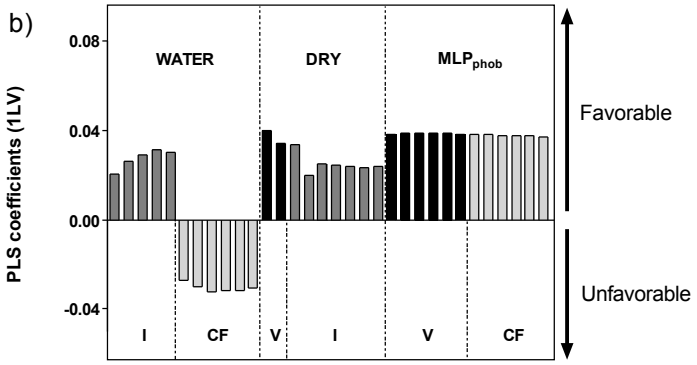
Figure 2. **Relationship between experimental and calculated peptide iontophoretic fluxes using the two-component PLS model** (13 compounds, 24 descriptors, $r^2 = 0.85$, $q^2 = 0.78$).

The coefficient plot of this model (Figure 3a) shows the contribution of the retained 24 VolSurf descriptors. The vertical bars represent the contribution of each individual descriptor, the height of the

columns reflecting the weight of the contribution. Positive coefficients correspond to descriptors that favor peptide transport.



(13 compounds, 24 descriptors, r^2 = 0.85, q^2 = 0.78)



(13 compounds, 32 descriptors, r^2 = 0.77, q^2 = 0.70)

Figure 3. **PLS coefficient plots correlating VolSurf descriptors with (a) peptide iontophoretic flux, and (b) electroosmosis inhibition.**

Volume (**V**) = isoenergetic contours of hydrophobic interaction regions; capacity factors (**CF**) = volume parameters ($V_{hydrophilic}$ or $V_{hydrophobic}$), divided by the total molecular surface; integy moments (**I**) = resultant (or sum) of the vectors pointing from the centre of mass of the molecule to the centres of the hydrophobic regions.

According to this plot, a good iontophoretic transport is favored by molecular hydrophilicity, as indicated by the positive Capacity factors derived from the WATER probe (CF_{Water}). In contrast, the descriptors of hydrophobic interactions ($V_{MLPphob}$, $I_{MLPphob}$ and $CF_{MLPphob}$) are inversely correlated with the measured transdermal fluxes, demonstrating the negative effect of hydrophobicity on peptide iontophoretic delivery. The strongly negative contributions of the integy moments ($I_{MLPphob}$, resultant (or sum) of the vectors pointing from the centre of mass of the molecule to the centres of the hydrophobic regions) reveal a major influence of the hydrophobicity distribution on transport; localized hydrophobicity, asymmetrically distributed over the molecular surface creates potential interaction sites with the membrane that may impede transport.

As in the case of the peptide flux, an iterative variable selection procedure allowed the identification of the most relevant VolSurf descriptors encoding for properties describing

electroosmosis inhibition. Although less successful, the model was nevertheless able to establish a correlation between IF and these molecular descriptors. The coefficient plot (Figure 3b) shows that electroosmotic inhibition, and hence decreased transport, is induced by strong hydrophobic interactions (positive $V_{MLPphob}$ and $CF_{MLPphob}$), confirming the above findings that these properties hinder peptide transport (Figure 3a). Moreover, the spatial distribution of the physicochemical properties over the molecular surface was again shown to play a role. Neutralization of skin charge and concomitant abolition of electroosmosis was favored by localized hydrophilicity, as evidenced by the magnitude of the hydrophilic integy moment contribution (positive I_{Water}). In physical terms, localized hydrophilicity (corresponding to positive charge centers) creates a site capable of strong electrostatic interaction with the fixed negative charges in the skin.

Predicting iontophoretic peptide transport

The experimental data showed that significantly different fluxes were observed for two tripeptides containing different arrangements of the same amino acids, specifically Ac-*D*Nal-Leu-Arg-NH_2 (13 ± 6 nmol/cm^2/h) and Ac-Leu-*D*Nal-Arg-NH_2 (32 ± 11 nmol/cm^2/h). The PLS model was also sensitive to the amino acid arrangement (Figure 2). The predictive power of the model was, therefore, tested by delivering an additional peptide, Ac-Leu-Gly-Arg-NH_2, comprising the same amino acids as Ac-Gly-Leu-Arg-NH_2 - a member of the original (training) set - but with the amino acids arranged in a different order. The predicted iontophoretic flux of Ac-Leu-Gly-Arg-NH_2 (149 ± 10 nmol/cm^2/h) was significantly higher than that of Ac-Gly-Leu-Arg-NH_2 (93 ± 1 nmol/cm^2/h; calculated). This result was in very good agreement with experiments since the iontophoretic flux of Ac-Leu-Gly-Arg-NH_2 (150 ± 30 nmol/cm^2/h) was measured higher than that of Ac-Gly-Leu-Arg-NH_2 (92 ± 14; experimental).

DISCUSSION

Passive diffusion *versus* iontophoretic delivery

Accurate *in silico* prediction of skin permeability to drug substances and other chemical agents present in the environment would have considerable utility for both transdermal drug delivery and toxicology fields. This has led to the development of several mathematical models that attempt to relate passive skin permeability to the penetrant's physicochemical properties [9]. Many of these models have identified lipophilicity as a key property, which positively contributes to skin permeation [11-14]. A recent study using the same 3D molecular interaction fields as used here related the physicochemical properties of 79 compounds to their passive permeation across human epidermis *in vitro* (Bajot F., Geinoz S., Rey S., Cruciani G., Guy R. H., Carrupt P.-A., The Volsurf approach in structure-permeation relationships: molecular interaction fields focused on specific intermolecular interactions, manuscript in preparation). It was found that good passive skin permeation was favored by bulky, localized hydrophobic regions, and that large hydrophilic interaction volumes were unfavorable. In the present study, we have demonstrated that transdermal peptide iontophoresis is favored by increased hydrophilicity (as measured by the hydrophilic capacity factor, CF_{Water}) but

hindered by strong, localized hydrophobic interactions. These results emphasize the differences between passive and electrically-assisted transport across the skin, and corroborate the idea that different transport mechanisms and pathways are involved in passive and electrically-assisted transport. Whereas passive solute permeation occurs mainly via the intercellular, lipidic matrix of the stratum corneum [15], it seems likely that ionic shunts [16], essentially aqueous pathways, are predominantly involved in iontophoresis.

External prediction of flux

Although additional peptides must be tested to determine the real predictive power of the model, the similarity between the predicted and experimental iontophoretic flux of Ac-Leu-Gly-Arg-NH2 constitutes a promising first step towards the validation of the predictive capacity of the model.

Impact of amino acid sequence

The experimental iontophoretic flux of Ac-Leu-Gly-Arg-NH_2 (150 ± 30 nmol/cm^2/h) and Ac-Gly-Leu-Arg-NH_2 (92 ± 14 nmol/cm^2/h) confirmed that amino acid sequence influences peptide transport. In addition to higher hydrophilic capacity factors, Ac-Leu-Gly-Arg-NH_2 has significantly smaller and less localized hydrophobic regions thereby (according to the PLS model) favoring iontophoretic peptide transport. Figure 4 illustrates the bulkier hydrophobic surfaces in Ac-Gly-Leu-Arg-NH_2 compared to Ac-Leu-Gly-Arg-NH_2.

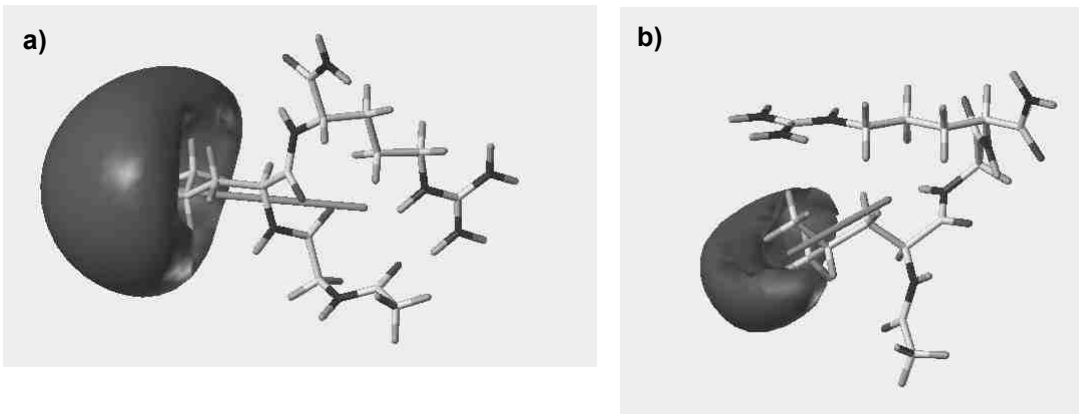


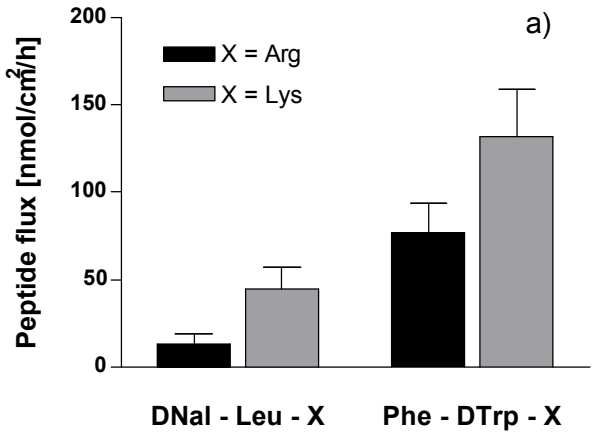
Figure 4. **Hydrophobic interactions calculated using the MLP field for (a) Ac-Gly-Leu-Arg-NH_2, and (b) Ac-Leu-Gly-Arg-NH_2 at 0.34 kcal/mol.** Integy moments (resultant (or sum) of the vectors pointing from the centre of mass of the molecule to the centres of the hydrophobic regions) are shown as grey bars.

The favored 3D configuration of Ac-Leu-Gly-Arg-NH_2 places the leucine side chain adjacent to arginine, reducing the “apparent hydrophobicity” of the former and hindering its participation in hydrophobic interactions. Moreover, the carbonyl group in the peptide bond between the Leu and Arg residues, provides a polar interaction site, further reducing the lipophilicity of leucine. It can be seen that the hydrophobic interaction region in Ac-Gly-Leu-Arg-NH_2 is both larger and more localized and accessible. According to the model, therefore, this localization will influence interactions with the skin

transport pathways, and have an impact on the overall transdermal flux. This effect of amino acid arrangement on transdermal iontophoretic delivery is worth further investigation.

Impact of charge type

Peptides derived from the somatostatin analogues (Ac-X-*D*Trp-Lys-NH_2), with a C-terminal lysine residue, exhibited significantly higher fluxes than their counterparts derived from LHRH (Ac-X-Leu-Arg-NH_2) in which the positive charge originates from the C-terminal arginine residue. To determine whether charge type was implicated in this difference, "hybrid" peptides were synthesized in which the arginine in Ac-*D*Nal-Leu-Arg-NH_2 (from nafarelin) was replaced by lysine, and the lysine in Ac-Phe-*D*Trp-Lys-NH_2 (from octreotide) was replaced by arginine. Figure 5a shows that, whereas the replacement of arginine by lysine increased the "nafarelin-type" peptide flux from 13 ± 6 to 40 ± 12 nmol/cm^2/h, the reverse transformation decreased the "octreotide-type" peptide flux from 130 ± 30 to 80 ± 17 nmol/cm^2/h.



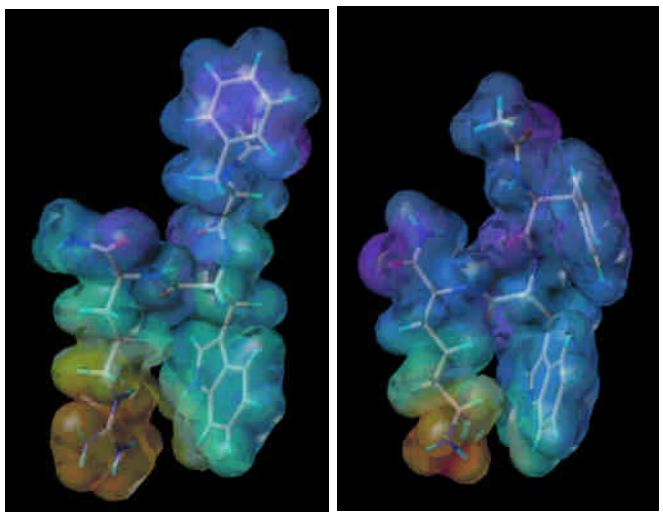


Figure 5. **Comparing arginine and lysine positive charges.**

(a) Effect of charge type on the anodal iontophoretic flux of four cationic peptides. (b) Electrostatic potential distributions on the Van der Waals molecular surface of Ac-Phe-*D*Trp-Arg-NH_2 (left) and Ac-Phe-*D*Trp-Lys-NH_2 (right), calculated using the SYBYL 6.9 molecular modelling package (Tripos Associates, MO). Increasing charge density is colour-coded from blue to red.

Charge type certainly appeared to play a significant role, therefore, in the iontophoretic transport of cationic oligopeptides - with the primary amine in the lysine side-chain functioning as a more favorable positive charge centre than the more delocalized guanidinium group present in arginine.

Visualization of the spatial distribution of the electrostatic potential, around the respective charge centres on the Van der Waals molecular surface, confirmed the more localized charge, and greater charge density, for the primary amine group in lysine (Figure 5b).

CONCLUSIONS

The current study provides the first systematic, quantitative analysis of iontophoretic peptide transport. It was shown that conventional 2D descriptors could not be correlated to the measured fluxes and that it was essential to consider the 3D environment, in particular, the spatial distribution of molecular descriptors. A PLS model was developed and demonstrated that increasing hydrophilicity favored peptide transport, whereas bulky and localized hydrophobic regions were a hindrance. Comparison with the preferred physicochemical characteristics for passive diffusion emphasized that iontophoretic and passive diffusional transport pathways must be different. Moreover, the arrangement of the constituent amino acids, even for dynamic tripeptide systems, governs electrotransport. This finding merits further investigation as the development of iontophoretic drug delivery systems, especially those incorporating peptides, is pursued.

METHODS

Log P calculation. Log P values were calculated using Daylight ClogP version 4.73 (Daylight Chemical Information Systems, Inc., Mission Viejo, CA (www.daylight.com))

Overview of the 3D approach. The procedure consisted of the following major steps:

1. Modelling 3D peptide molecular structures.
2. Computation of the Molecular Lipophilicity Potential (MLP), Molecular hydrogen-bonding potentials (MHBPs) and GRID 3D molecular fields.
3. Generation of the VolSurf descriptors.
4. Statistical analysis using partial least squares discriminant analysis (PLS).

Dataset. Iontophoretic fluxes of 13 tripeptides derived from LHRH and somatostatin analogs were measured *in vitro* across porcine ear skin, using vertical diffusion cells with a salt bridge system (full experimental conditions and a complete description of the effect of formulation conditions on peptide transport are given in Y.B.S., A.N., R.H.G. and Y.N.K., Effect of amino acid sequence on transdermal iontophoretic peptide delivery, submitted to European Journal of Pharmaceutical Sciences). All tripeptides were blocked both at the N-terminal (by acetylation) and the C-terminal (by amidation) so that the charge was only provided by the constitutive amino acid side chains. For conciseness, single letter codes have been used for amino acids in the graphics. (Cit = Citrulline and Nal = 2-Naphthylalanine). Acetaminophen, a neutral hydrophilic compound with poor passive permeability, that can be driven through the skin only by electroosmosis, was incorporated in the donor

compartment as a marker of the magnitude of convective flow. For each peptide, an Inhibition Factor (IF) was calculated according to the following equation:

$$IF = [Q_{A\text{-}8h,control}] / [Q_{A\text{-}8h,peptide}] \quad (1)$$

where $Q_{A\text{-}8h,control}$ is the amount of acetaminophen transported into the receptor phase during 8 hours of iontophoresis when no peptide was present in the donor solution, and $Q_{A\text{-}8h,peptide}$ is the corresponding quantity when the tripeptide was iontophoresed simultaneously.
Permeation and inhibition data are collated in Figure 6.

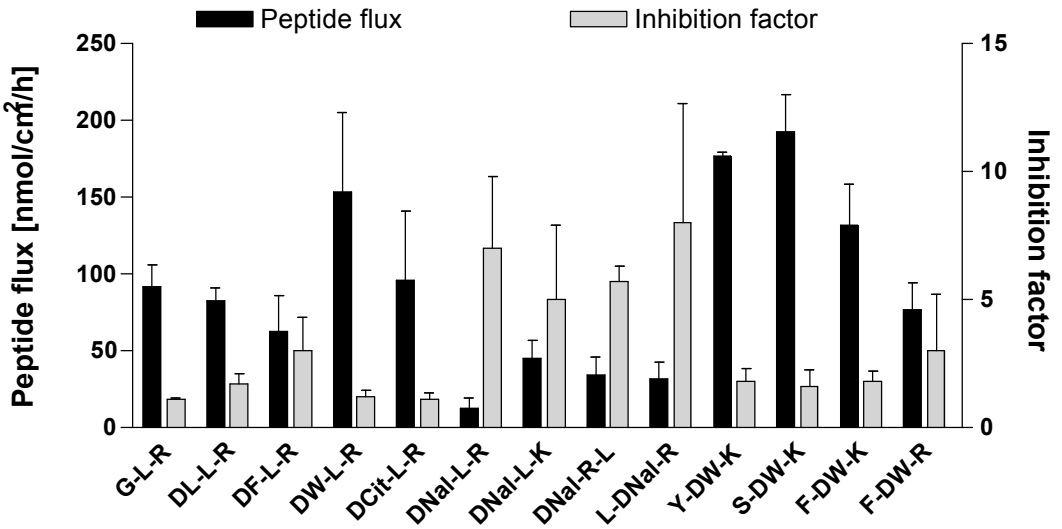


Figure 6. **Fluxes and inhibition factors of peptides iontophoresed across porcine ear skin *in vitro*.**

Modelling of 3D molecular structures. Tripeptides were drawn in their charged state using the biopolymer building tool of Sybyl 6.9 (Tripos Associates, MO, 1995), and energy-optimized using the Tripos force field (Tripos Associates, MO, 1995). For each tripeptide, a conformational search was performed using the Sybyl Genetic Algorithm protocol. The lowest energy structure was retained and optimized using the Tripos force field. Minimizations were performed in vacuo ($\varepsilon = 1$).

Computation of 3D molecular fields (GRID, MLP and MHBPs). The GRID field is one of the most widely used computational tools to map molecular surfaces of drugs and macromolecules [17]. It uses a potential based on the total energy of interaction (the sum of Lennard-Jones, H-bonding and electrostatic terms) between a target molecule and a probe, which can either be a single atom or a group of atoms. By moving probes over the surface of the target molecule, GRID yields a property distribution of attractive and repulsive forces between the probe and the target molecule. The molecular interaction fields were calculated using the WATER and the DRY probes available in the GRID program [18]. The WATER probe simulates hydrophilic interactions, whereas the DRY probe encodes for polarizability.
At a given point in space, the MLP represents the result of the intermolecular interactions encoded by the lipophilicity of all fragments in the molecule [19]. Thus, the MLP represents the relative affinity of a

solute for water and octanol. The MLP spreads out molecular lipophilicity on the solvent-accessible-surface of a molecule, allowing a quantitative visualization by color coding. Negative MLP values correspond to "hydrophilic" parts of the molecule, whereas positive MLP values (MLP_{phob}) quantify pure hydrophobic interactions. Molecular hydrogen-bonding potentials (MHBPs) allow the exploration of three-dimensional H-bonding properties. The development of this tool is based on a stepwise procedure comparable to that used to calculate the MLP. Two MHBPs are generated, able to distinguish between donor ($MHBP_{do}$) and acceptor ($MHBP_{ac}$) H-bonding properties [20].

Generation of VolSurf descriptors. VolSurf allows the extraction of one-dimensional numerical descriptors from 3D-isoenergetic (isopotential) contours [17]. The most important descriptors, calculated at different energy levels, are:

- Volumes (**V**), corresponding to the space enclosed by the isoenergetic contours of hydrophilic (hydrophobic) interaction regions
- Integy moments (**I**), which are resultants (or sums) of the vectors pointing from the centre of mass of the molecule to the centres of the hydrophilic (hydrophobic) regions
- Capacity factors (**CF**), which are the volume parameters (V), divided by the total molecular surface

Statistical analysis. PLS analysis was performed with SIMCA-P 6.1 (Umetrics AB, Sweden, 1996) using default settings. A "leave-one-out" cross-validation procedure was used to quantify the predictive power of the PLS model, yielding the predictive correlation coefficient (q^2) [21,22]. Descriptors were discarded if their associated coefficient possessed a confidence interval (derived from jack knifing) larger than the coefficient itself. An iterative procedure was used to progressively refine the model and retain the most relevant descriptors.

ACKNOWLEDGMENTS

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CHAPITRE 4

Administration transdermique du vapréotide acétate par ionophorèse

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Administration transdermique du vapréotide acétate par ionophorèse

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Résumé

But. Evaluer la faisabilité de l'administration transdermique du vapréotide, analogue de la somatostatine, par ionophorèse.

Méthode. Des expériences *in vitro* ont été réalisées avec de la peau d'oreille de porc soit dermatomisée, soit traitée à la chaleur pour l'obtention de l'épiderme. En plus de la quantification du transport du vapréotide dans et à travers le tissu cutané, l'effet de l'administration du peptide sur la permsélectivité de la peau a également été mesuré. L'influence (i) de la densité de courant appliqué (ii) du traitement de la peau avant et après ionophorèse, (iii) des ions compétiteurs et (iv) de l'inclusion d'albumine dans le récepteur sur l'administration du vapréotide ont été étudiés.

Résultats. L'épiderme s'est révélé être un meilleur modèle que la peau dermatomisée pour l'étude du transport du vapréotide. Malgré sa susceptibilité à la dégradation enzymatique, un flux de 1.7 $\mu g/cm^2/h$ a pu être atteint après 7 heures d'ionophorèse à courant constant ($0.15 mA/cm^2$). L'extraction après ionophorèse a révélé qu'en fonction des conditions expérimentales, 80 à 300 μg de peptide étaient fixés dans la peau. Le vapreotide interagit avec la peau et exerce une inhibition de l'électroosmose dépendante du courant. Cependant, ni les stratégies de pré-traitement pour saturer les sites de fixation, ni les protocoles suivis après ionophorèse dans le but de déplacer le peptide lié se sont avérés efficaces.

Conclusions. Sur la base du flux de vapréotide mesuré et de ses propriétés pharmacocinétiques, il apparaît que des concentrations thérapeutiques sont atteignables avec un patch de 15 cm^2.

Transdermal iontophoretic delivery of vapreotide acetate

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Pharmaceutical Research 2005 **22** *(8):1305-1312*

Abstract

Purpose. To evaluate the feasibility of delivering vapreotide, a somatostatin analogue, by transdermal iontophoresis.

Methods. *In vitro* experiments were conducted using dermatomed porcine ear skin and heat-separated epidermis. In addition to quantifying vapreotide transport into and across the skin, the effect of peptide delivery on skin permselectivity was also measured. The influence of (i) current density, (ii) pre- and post-treatment of the skin, (iii) competitive ions, and (iv) inclusion of albumin in the receptor on vapreotide delivery were investigated.

Results. Epidermis proved to be a better model than dermatomed skin for vapreotide transport studies. Despite the susceptibility of vapreotide to enzymatic degradation, a flux of 1.7 μg/cm^2/h was achieved after 7 hours of constant current iontophoresis (0.15 mA/cm^2). Post-iontophoretic extraction revealed that, depending on the experimental conditions, 80-300 μg of peptide were bound to the skin. Vapreotide was found to interact with the skin and displayed a current-dependent inhibition of electroosmosis. However, neither the pre-treatment strategies to saturate the putative binding sites, nor the post-treatment protocols to displace the bound peptide were effective.

Conclusion. Based on the observed transport rate of vapreotide and its known pharmacokinetics, therapeutic concentrations should be achievable using a 15 cm^2 patch.

Keywords: transdermal iontophoresis; vapreotide; electroosmosis; skin barrier

INTRODUCTION

Vapreotide is one of several potent long-acting analogues of somatostatin synthesized for clinical use in the treatment of acromegaly and endocrine tumors of the gastroenteropancreatic system. It has additionally demonstrated success in the early treatment of oesophageal variceal bleeding and has been awarded orphan drug status in the US [1]. These analogues require subcutaneous or intramuscular administration. In an attempt to avoid the parenteral route, more convenient "patient-friendly" routes of drug delivery have been investigated. The results have been mixed: orally administered octreotide (an other somatostatin analogue commercialized as Sandostatin®) is hampered by a relatively low bioavailability [2] and because of poor local tolerability, nasal administration is also inconvenient [3]. The transdermal route is an attractive alternative to deliver therapeutic drugs [4]. The physicochemical properties of peptides (charged; high molecular weight) render them inappropriate for passive transdermal delivery. In contrast, iontophoresis, an electrically-assisted drug delivery technology, offers a controlled and non-invasive means of administration [5]. The two main transport mechanisms during iontophoresis are electromigration (direct effect of the applied electric field on the charged species) and electroosmosis (convective solvent flow in the anode-to-cathode direction, as a consequence of the skin's net negative charge at physiological pH). Since electrical mobility decreases with molecular weight, it is hypothesised that electroosmosis is the major transport mechanism for peptides [6]. However, it is also known that peptides containing adjacent cationic and lipophilic residues can inhibit electroosmosis, and their own transport, by altering skin permselectivity [7-12]. The association of these lipophilic cations with the membrane neutralizes, to varying extents, the intrinsic negative charge of the skin causing a significant reduction in the normal anode-to-cathode electroosmotic flow. Since the degree of inhibition depends on the number of molecules in the skin, it is usually more pronounced at higher applied current densities and at increased peptide concentrations.

The iontophoretic delivery of octreotide has been successfully achieved in rabbits *in vivo* [13]. An investigation of the effect of current density and drug concentration on plasma levels of octreotide revealed a decrease in the amount of peptide delivered, with increasing concentration. This might be attributed to an inhibition of electroosmosis, since the -Phe^{3}-*D*-Trp^{4}-Lys^{5}- sequence in octreotide corresponds to the structural moiety thought to be a pre-requisite for this phenomenon [10].

The aim of the current study was to evaluate the feasibility of delivering vapreotide by transdermal iontophoresis. In view of the structural similarity of vapreotide to octreotide, the iontophoretic delivery of vapreotide was expected to interact with the skin and affect electroosmotic solvent flow. Hence, in addition to quantifying peptide transport, the magnitude of electroosmotic flow was also determined under the different experimental conditions employed. Experiments were also conducted in the presence of a salt bridge; this allowed the use of lower electrolyte concentrations in the donor compartment, decreasing the number of competing charge carriers and, in theory, increasing peptide delivery efficiency (5). The impact of current density and salt bridge use on both peptide transport and electroosmotic inhibition was measured. Different approaches to modulate the interactions between the peptide and the skin were investigated, including co-iontophoresis with cetrimide, pre- and post-iontophoretic treatment of the skin with Ca^{2+} and propranolol - substances

capable of interacting with the biomembrane and occupying potential “peptide” binding sites. Vapreotide transport across heat-separated epidermis and dermatomed skin was compared.

MATERIALS AND METHODS

Chemicals

Vapreotide (MW = 1131.4) *D*-Phe-Cys-Tyr-*D*-Trp-Lys-Val-Cys-Trp-NH_2, in the form of its acetate salt was a generous gift from Debiopharm (Debiopharm Galenic Unit, Switzerland). Acetaminophen was purchased from Fluka (Sigma-Aldrich Chimie Sarl, France). Tris (Tris-(hydroxymethyl) aminomethane), Trizma® hydrochloride, agarose, DL-propranolol and cetrimide were obtained from Sigma-Aldrich (Sigma-Aldrich Chimie Sarl, France) and bovine serum albumin was purchased from Fluka BioChemika (Sigma-Aldrich Chimie Sarl, France). De-ionized water (resistivity > 18 MOhm/cm^2) was used to prepare all solutions.

Analytical procedures

Vapreotide extracted from skin samples was quantified using a high-performance liquid chromatography system equipped with a variable wavelength UV detector (Waters Corporation, MA, USA). The mobile phase, comprising 25% acetonitrile and 75% triethylaminephosphate buffer solution (pH = 2.3), was passed through a C18 PartiSphere column (4.6 mm i.d., 12.5 cm long, 5 μm particle size) (Whatman Inc., NJ, USA) maintained at 40°C, at a flow rate of 1 mL/min. The peptide was detected at 280 nm. The RSD of the repeatability was less than 1 % and the limit of quantification was 115 ng/ml. Direct competitive enzyme immunoassay (EIA), as a result of its superior sensitivity, was used to quantify the presence of vapreotide in the receptor compartment. This competitive binding assay is based on the relative affinity of (i) the vapreotide (in the sample) and (ii) an enzymatic tracer prepared by covalent coupling of vapreotide to an enzyme (acetylcholinesterase), to anti-vapreotide antibodies. The quantification limit corresponded to 100 pg/ml.

Acetaminophen was assayed by high-performance liquid chromatography using a Hypersil BDS C8 column (150 mm x 4.6 mm, Supelco®, France) maintained at 40°C. The mobile phase (delivered at a flow rate of 1 mL/min) consisted of 92% water and 8% acetonitrile adjusted to pH 3.5 with acetic acid. Acetaminophen was detected by its UV-absorbance at 243 nm. The RSD of the repeatability was less than 1 % and the quantification limit was 22 ng/ml.

Skin preparation

Porcine ears, obtained from a local abbatoir (Société d’Exploitation d’Abbatage, Annecy, France) within a few hours post-mortem, were cleaned under cold running water. The whole skin was removed carefully from the outer region of the ear and separated from the underlying cartilage with a scalpel. The tissue was then either dermatomed (800 μm) or heat-treated to separate the epidermis [14]: pieces of fresh full-thickness skin were immersed in water at 58°C for 2 minutes after which the

epidermis was carefully separated from the dermis with a spatula. Pieces of epidermis and dermatomed skin were wrapped individually in Parafilm™ and maintained at –20°C until use and were stored for no longer than a period of 2 months.

Iontophoresis procedure

The skin was clamped in three-compartment vertical diffusion cells (area: 0.73 cm^2), the design of which has been previously described [15]. Unless otherwise stated, vapreotide was dissolved in a 100 mM NaCl solution that was adjusted to pH = 5.5 with HCl to produce a 3 mM solution of the peptide; 1 mL of this solution was placed in the donor (anodal) compartment. In addition to vapreotide, the donor compartment always contained 15 mM acetaminophen. The cathodal compartment was filled with 1 mL of 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline. The receptor compartment (~6 mL) was filled with the same electrolyte solution, containing in addition, when stated, 44 g/L bovine serum albumin (BSA; to mimic physiological conditions) and was stirred magnetically throughout the permeation experiments.

Constant current iontophoresis was used throughout the study. The current, ranging from 0.05 to 0.5 mA/cm^2 was applied for 4 to 24 hours via Ag/AgCl electrodes connected to a power supply (Kepco, NJ, USA). When specified, the anode was isolated from the donor solution via a salt bridge (100 mM Tris/Trizma HCl in 3% agarose) to minimize the competition between the peptide and the electrolytes necessary for the anodal reaction. Under these conditions, the anodal compartment contained a solution of 25 mM Tris/Trizma® HCl normal saline buffered to pH 7.4 and the vapreotide in the donor compartment was dissolved in 20mM Tris/Trizma® HCl (pH 7.4). Schematic representations of these two different experimental set-ups are provided in Figure 1.

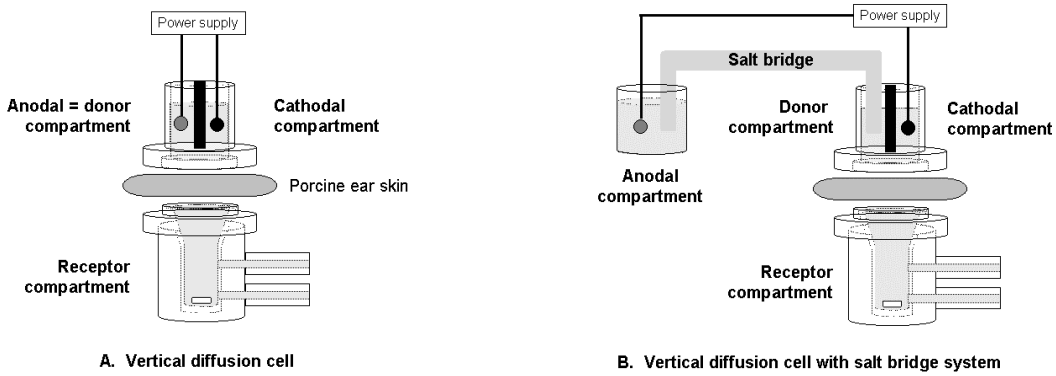

Figure 1. **Schematic representations of the iontophoretic diffusion cell assemblies used in this study**.

Quantifying electroosmotic inhibition

Acetaminophen is a neutral hydrophilic compound, with poor passive permeability (~2 nmol/cm^2/h); under the influence of an electrical current, this uncharged but polar molecule is driven through the skin predominantly by electroosmosis. Hence, in these studies it was incorporated into the donor formulation (15 mM) to report on convective solvent flow.

For each experiment, an Inhibition Factor (IF) was calculated using the following equation:

$$IF = [Q_{A\text{-}8h,control}] / [Q_{A\text{-}8h,peptide}] \quad (1)$$

where $Q_{A\text{-}8h,control}$ is the amount of acetaminophen transported into the receptor phase during 8 hours of iontophoresis when no peptide was present in the donor solution, and $Q_{A\text{-}8h,peptide}$ is the corresponding quantity when vapreotide was iontophoresed.

Effect of current density

To evaluate the effect of current density on electroosmotic inhibition by vapreotide, current densities of 0.15, 0.3 and 0.5 mA/cm^2 were applied for 8 hours across dermatomed skin. A solution of 3 mM vapreotide in 20 mM Tris/Trizma® HCl buffered to pH 7.4 was placed in the donor compartment.

Additional experiments were also performed using heat-separated epidermis to assess the effect of current density on the amount of vapreotide transported. Again, a 3mM vapreotide donor solution was iontophoresed for 8 hours.

Vapreotide accumulation in the skin

The uptake of vapreotide into dermatomed skin during iontophoresis was also investigated. Upon terminating current application, the skin sample was rinsed with water, dried on absorbent paper, and then placed in 4 mL of a mixture acetonitrile/water (25/75). After agitation for 14 hours, the solution was filtered (0.45 μm regenerated cellulose syringe filter, Alltech, IL, USA) and the amount of peptide quantified.

Skin metabolism experiments

The influence of porcine ear skin on the stability of vapreotide was evaluated by filling the receptor compartment with a 5 μM vapreotide solution in 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline. Both dermatomed and epidermal skin samples were separately evaluated. The anodal and cathodal compartments were filled with 1 mL of 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline. A current of 0.15 mA/cm^2 was applied for eight hours and the percentage of intact peptide was calculated as follows:

$$\textit{Intact peptide (\%)} = \frac{Final\ amount\ of\ peptide}{Initial amount\ of\ peptide} x\,100\% \quad (2)$$

Modifying interactions between vapreotide and the skin

Co-iontophoresis: 1 mL of 4.4 mM vapreotide in 20 mM Tris/Trizma® HCl (pH 7.4) containing either 0.1% or 1% (2.7 or 27 mM) cetrimide was placed in the anodal compartment and a current of 0.15 mA/cm^2 was applied for either 8 or 24 hours.

Pre-treatment: Vapreotide iontophoresis was preceded by the iontophoretic delivery of either propranolol or cetrimide:

A: A solution of 40 mM propranolol or 0.1% cetrimide in 25 mM Tris/Trizma® HCl (pH 7.4) normal saline was placed in the anodal compartment and a current of 0.3 mA/cm^2 was applied for 8 hours.

B: Subsequently, 1 mL of 4.4 mM vapreotide in 20 mM Tris/Trizma® HCl (pH 7.4) was placed in the donor compartment and a current of 0.15 mA/cm^2 was applied for 24 hours using a salt-bridge.

Post-treatment: Solutions of $CaCl_2$, NaCl, propranolol and cetrimide were tested with respect to their capacity to release vapreotide bound to, and accumulated within, dermatomed skin. These experiments also comprised two parts:

A: 1 mL of vapreotide (3 mM) in 20 mM Tris/Trizma® HCl (pH 7.4) was placed in the donor compartment and a current of 0.15 mA/cm^2 was applied for 24 hours using a salt-bridge.

B: Solutions of either 100 mM $CaCl_2$, 100 mM NaCl, 40 mM propranolol or 0.1% cetrimide in 25 mM Tris/Trizma® HCl (pH 7.4) normal saline were added to the anodal compartment and a current of 0.3 mA/cm^2 was applied for 8 hours.

For each pre- and post-treatment experiment, the receptor compartment was replaced between phase A and phase B.

Data treatment

The results were derived from at least triplicate experiments conducted with skin samples originating from different pig ears. Outliers, determined using the Grubbs test, were discarded. When two sets of data where compared, Student *t* tests were performed. The level of statistical significance was fixed at $P < 0.05$.

RESULTS AND DISCUSSION

Skin metabolism of vapreotide

Cutaneous metabolism has been widely investigated [16-18] and several studies into transdermal peptide delivery have reported the presence of proteolytic activity in the skin [9;19-21]. With the concentration tested, only 39 ± 18% of the vapreotide in contact with the dermal face of dermatomed skin samples was found to be intact after 8 hours of iontophoresis. Similarly, only 32 ± 5% of the peptide remained intact after exposure to the interior epidermal surface. The levels of degradation observed following exposure to either dermatomed skin or isolated epidermis were not significantly different.

Although the presence of proteolytic enzymes in the skin is generally accepted, their exact location and distribution in the distinct tissue layers of the skin remains unknown. Although the epidermis is claimed to be the major site of drug degradation, activity has also been ascribed to the

dermis [17]. Skin contact studies with vapreotide suggest that both epidermal and dermal tissues possess some form of proteolytic activity. Moreover, the results also demonstrate that the enzymes located in the epidermis are resistant to the heat separation treatment used to isolate the epidermal membrane.

Nevertheless, it is difficult to extrapolate these *in vitro* results to the eventual situation *in vivo* (particularly in humans), notably due to major interspecies differences in the structure and function of such enzymes. In addition, the residence time of the peptide in the enzymatic barrier is likely to be significantly reduced *in vivo* as molecules reaching the epidermal-dermal junction are taken up rapidly by the skin's microcirculation. Although enzymatic activity is expected to be higher in viable tissues, it can be argued that *ex vivo* skin preparation procedures might result in increased release of intracellular enzymes. However, the skin tissue used in these permeation experiments was frozen prior to use, a process reported to reduce metabolic activity of skin samples [22]. Thus, *in vitro*/*in vivo* correlations for labile peptides are complicated by the interplay of these opposing factors.

Vapreotide transport

Under the same iontophoretic conditions, significantly more (~10-fold) vapreotide was transported across the epidermis (~10 μg) than across dermatomed skin (~1 μg) when using the experimental set-up employing a salt-bridge (Figure 2).

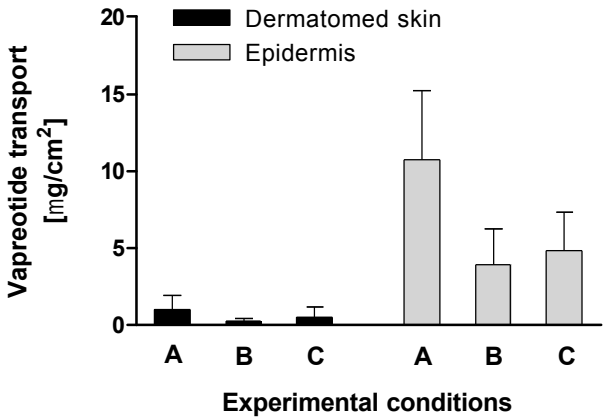

Figure 2. **Cumulative amount of vapreotide measured in the receptor compartment after 8 hours of iontophoresis at 0.15 mA/cm^2 across epidermis and dermatomed skin as a function of the experimental set-up.** (A: With Salt bridge, B: Without salt bridge, C: Without salt bridge; with BSA).
There were statistically significant differences between epidermis and dermatomed skin in each corresponding experimental condition ($p < 0.05$).

It is generally accepted that the remarkable barrier function of the skin is primarily located in the outermost layer of the epidermis, the stratum corneum, and that, *in vivo,* substances are taken up by the capillary network adjacent to the epidermal-dermal junction, after diffusing on the order of 100 μm through the membrane. In separate studies (data not shown), there was no difference between the amount of vapreotide transported across tape-stripped and intact dermatomed skin, suggesting that the stratum corneum was not the rate-limiting barrier to transport at least under *in vitro* conditions. The use of dermatomed skin (~800 μm in thickness) in these *in vitro* experiments introduces an additional mass of dermal tissue, which acts as a "pseudo-receptor" compartment, notably for compounds such as vapreotide, which display a high skin affinity. Therefore, the presence of dermis provides an artifactual reservoir for the drug that has already crossed the epidermis, and which, *in*

vivo, would be taken up by the capillaries just below the epidermal-dermal junction. Thus, epidermis, which is a well-established model for transdermal drug delivery, appears to be more appropriate than dermatomed skin for transdermal vapreotide transport studies. The fate of a peptide reservoir *in vivo*, if indeed it exists, remains to be studied.

Vapreotide accumulation in the skin

Despite the inefficient transfer of vapreotide across dermatomed skin, significant amounts of the peptide (80-300 μg) were recovered from the skin samples after iontophoresis (Table I). These peptide accumulation data further attest to the suspected strong association between molecules possessing a positive charge, in close proximity to a lipophilic surface, and the skin. In addition to these specific interactions between the peptide and structures present in the skin, the accumulation may also be the result of peptide-peptide interactions, which result in vapreotide aggregation and eventually in peptide deposition in the transport pathways.

Table I. **Vapreotide accumulation in the skin after iontophoretic current application for 8-24h using formulations containing 1.5-4.4 mM vapreotide**. Experiments were conducted using the experimental set-up shown in Figure 1b.

Experimental condition	Amount of vapreotide in the skin [μg]
24h iontophoresis at 0.15 mA/cm^2, 4.4 mM donor concentration	210 ± 10
8h iontophoresis at 0.15 mA/cm^2, 3 mM donor concentration	80 ± 20
18h iontophoresis at 0.5 mA/cm^2, 1.5 mM donor concentration	300 ± 60

Effect of vapreotide transport on skin permselectivity

Previous studies investigating iontophoretic transport across hairless mouse, rabbit, porcine and human skin have confirmed the existence of skin permselectivity, a phenomenon which gives rise to electroosmosis and contributes to the iontophoretic transport of neutral and cationic species [23-25]. Moreover, the ability of certain lipophilic, peptidic and non-peptidic cations to inhibit the convective solvent flow has also been established [10;11;26]. The magnitude and significance of this inhibition depends on the skin type and the physicochemical properties of the molecule. The effect of current density on the inhibition of electroosmosis by vapreotide is illustrated in Figure 3. The inhibitory capacity of vapreotide is strongly dependent on the applied current density, increasing the inhibition factor by almost 50-fold at 0.5 mA/cm^2. As current density is increased, more charge has to be transported across the skin; this is partly carried by the peptide, which is driven into the membrane in greater amounts, leading to a more extensive neutralization of the skin's negative charge and a more pronounced inhibition of electroosmosis.

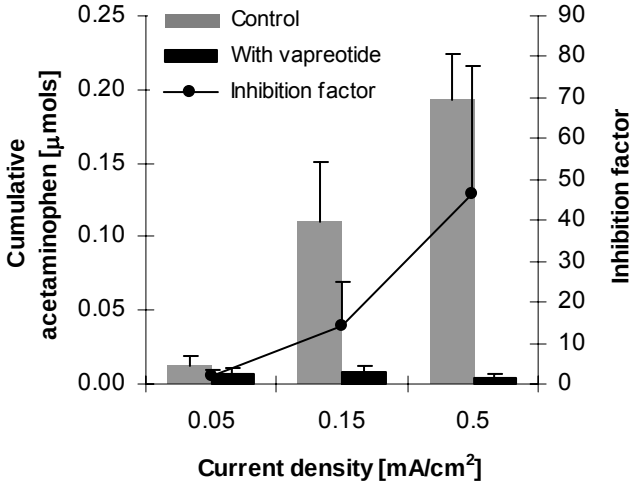

Figure 3. **Cumulative amounts of acetaminophen delivered across porcine ear skin during 8 hours of transdermal iontophoresis at 0.05, 0.15, and 0.5 mA/cm^2 in the presence and absence of vapreotide.** The inhibition factor is calculated according to Equation 1.

Effect of reducing competing ions in the formulation

Because Ag/AgCl electrodes need chloride ions for anodal electrochemistry, the anodal compartment must contain a supply of such ions derived from either the active agent (e.g., hydrochloride salts) or from an external source, e.g., NaCl, in which case the total number of cations in the anodal formulation is greatly increased. These cations compete with the positively-charged drug as charge carriers. The use of a salt bridge enables fewer competing cations (~20 mM) to be incorporated in the donor compartment, increasing vapreotide transport efficacy, as indicated in Figure 2. To confirm that the ionic strength and not the donor pH, which also varied between the two experimental set-ups, was responsible for the modified transport of vapreotide, the effect of pH was also investigated. A control experiment using the 'salt bridge' set-up was performed at pH 5.5 and revealed that there was no significant difference in the quantity of vapreotide transported (11.9 ± 3.8 μg/cm^2 at pH 5.5 *versus* 10.7 ± 4.5 μg/cm^2 at pH 7.4).

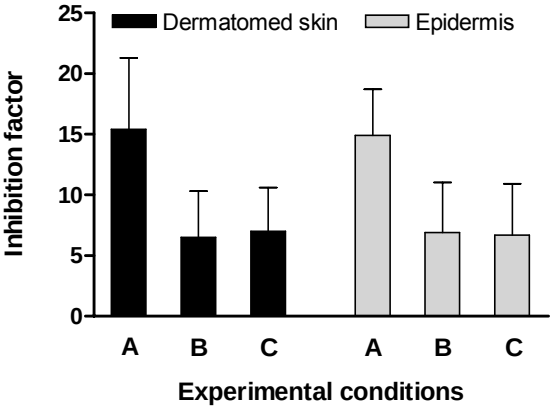

Figure 4. **The electroosmotic inhibition factor after 8 hours of iontophoresis at 0.15 mA/cm^2 across epidermis and dermatomed skin as a function of the experimental set-up.** (A: With Salt bridge, B: Without salt bridge, C: Without salt bridge; with BSA).
There were statistically significant differences between the IFs observed with and without salt bridges for both epidermal and dermatomed skin samples ($p < 0.05$).

Figure 4 indicates that the inhibition factor is increased two-fold in the presence of the salt bridge. As discussed above, vapreotide transport increases with use of a salt bridge; hence, more peptide reaches the skin membrane, resulting in a more complete neutralization of skin charge and, as a result, greater reduction in electroosmotic flow. However, this increased inhibition is not sufficient to outweigh the increased transport resulting from the reduced ion competition (Figure 2). Finally, the

degree of electroosmotic inhibition was also independent of the skin preparation and experimental conditions employed (Figure 4).

In addition to increasing the proportion of charge carried by the peptide, salt bridges allow the isolation of the peptide from the electrode compartment and have the advantage of preventing interactions between the electrode and the drug. Patch-based iontophoretic systems employing the same principles have been developed where the electrode is separated from the drug reservoir by a size exclusion membrane [27].

Modifying interactions between vapreotide and the skin

Given the substantial amounts of peptide measured in the skin, the liberation and subsequent transport of even a small fraction of this bound peptide would result in the delivery of significant amounts of drug. A number of different approaches, as shown in Figure 5, were employed to modulate the impact of peptide-skin interactions on vapreotide transport.

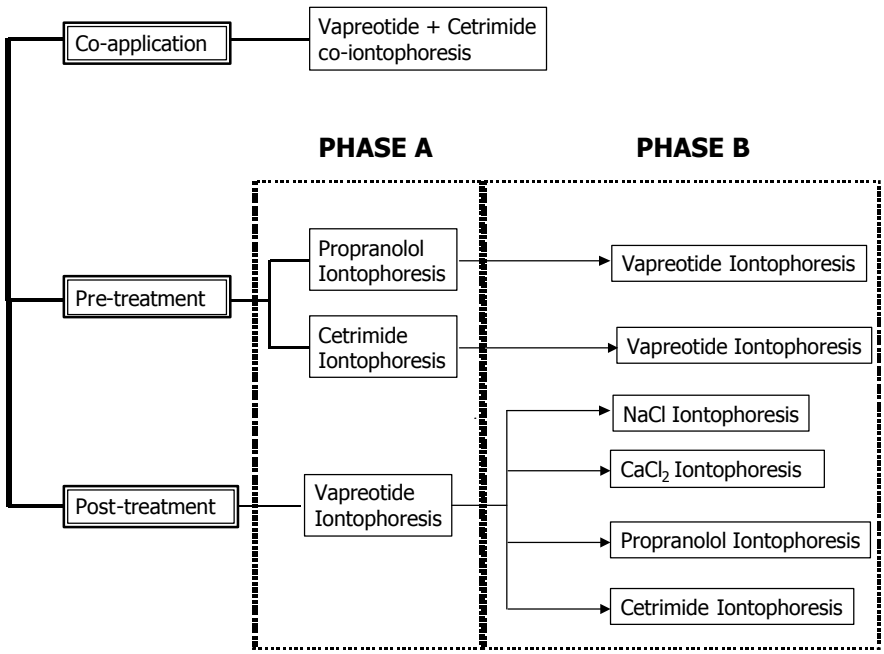

Figure 5. **Experimental strategies investigated to reduce the degree of vapreotide fixation to the skin**.

The first approach involved co-iontophoresis of the cationic surfactant, cetrimide. The results in Figure 6a show that co-iontophoresis of 0.1% cetrimide led to a two-fold reduction in the amount of peptide recovered from the skin. As shown in Figure 6b, increasing the cetrimide concentration from 0.1% to 1% produced a greater effect. Although it is tempting to attribute this to an interaction of the cationic cetrimide with putative binding sites, the observation that peptide transport *through* the membrane was not increased, suggests that the surfactant itself acted as a competing charge carrier.

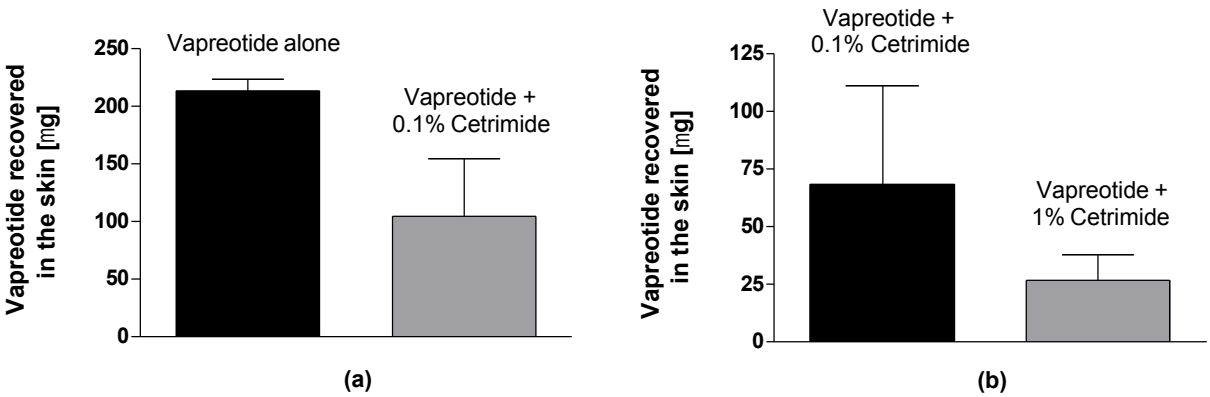

Figure 6. Effect of co-iontophoresis of cetrimide on the amount of vapreotide bound to the skin. (a) Co-iontophoresis of 0.1% surfactant with 4.4 mM vapreotide for 24 hours at 0.15 mA/cm^2. (b) Co-iontophoresis of 0.1 and 1% surfactant with 4.4 mM vapreotide for 8 hours at 0.15 mA/cm^2.

To exclude the risk of competition, subsequent studies employed pre-and post-treatment of the skin wherein cetrimide and other species known to bind to the membrane, e.g., Ca^{2+} and propranolol were iontophoresed either prior to or after vapreotide iontophoresis. It has been proposed that the lower transport number of Ca^{2+} as compared to Na^{+} is due to its superior interaction with negatively-charged binding sites in the skin [28]. Likewise, the iontophoresis of propranolol (40 mM) has been shown to reduce the electroosmotic transport of mannitol across porcine ear skin [26]. Pre-iontophoretic treatment with either cetrimide or propranolol was thus attempted to saturate the putative binding sites, and hence prevent the interaction of vapreotide with the skin. Vapreotide transport following these pre-treatments was not statistically different from the control ($p < 0.05$). Previous reports showed that cetrimide pretreatment reduced iontophoretic delivery of propranolol and this was attributed to a neutralisation of skin charge (29). Based on the acetaminophen flux measured in our studies, inclusion of cetrimide in the peptide formulation produced a ~two-fold increase in EO inhibition as compared to iontophoresis of vapreotide alone; furthermore, it may also accumulate in the transport pathways impeding vapreotide passage. Post-iontophoretic treatment methods were investigated with the aim of displacing bound peptide from the skin. However, almost negligible amounts of the peptide (<20 ng/cm^2) were released after 8 hours post-iontophoretic treatment with the different cationic species tested.

Taken together, these results indicate that the strategies employed to modify skin-vapreotide interactions in order to improve peptide delivery were unsuccessful.

Can therapeutic amounts of vapreotide be delivered?

To compare our *in vitro* vapreotide transport studies with the earlier *in vivo* investigation conducted by Lau *et al*. into the transdermal iontophoresis of octreotide [13], the final set of permeation experiments were conducted without a salt bridge and with BSA in the receptor compartment. The amount of vapreotide reaching the receptor compartment after 4, 6 and 8 hours of iontophoretic current application (0.15 mA/cm^2) are shown in Figure 7.

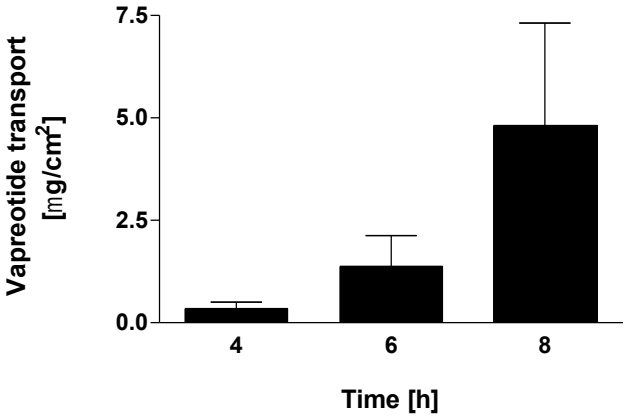

Figure 7. Cumulative amount of vapreotide present in the receptor compartment after 4, 6, and 8 hours of iontophoretic delivery at 0.15 mA/cm^2 across porcine epidermis.

These cumulative quantities were used to estimate the vapreotide flux at 7 hours (1.7 μg/cm^2/h). Since the experimental conditions chosen for the *in vitro* study were similar to those used by Lau *et al*. for the iontophoretic delivery of octreotide acetate in the rabbit, a comparison of the transport of these two somatostatin analogues is feasible. After 8 hours of iontophoresis at 0.15 mA/cm^2, the plasma concentrations of octreotide were 0.85 and 1.7 ng/mL with donor concentrations of 2.5 and 5 mg/mL, respectively [13]. Based on our vapreotide data (donor concentration of 3 mM ≅ 3.9 mg/mL) and the relationship equating flux to plasma concentration (Steady state flux · Area = Clearance · [plasma]), it is possible to estimate the plasma concentration of vapreotide if it were delivered to the rabbit under similar conditions (assuming the same clearance of 2.3 L/h); the estimated value is 0.7 ng/mL, suggesting a slightly lower transport for vapreotide than for octreotide. Aside from the subtle differences in amino acid sequence, this might be due in part to (i) the different skin models used in the two studies (rabbit skin is recognised as being more permeable than porcine skin) and (ii) the above-mentioned differences in metabolic activities (both inter-species and *in vitro/in vivo*).

Given a total vapreotide clearance of 16.7 L/h in humans (personal communication), an input rate of ~25 μg/h must be achieved in order to maintain a therapeutic level of 1.5 ng/mL. In view of the experimental *in vitro* flux (1.7 μg/cm^2/h), it follows that a patch of ~15 cm^2 could achieve the desired delivery kinetics. Moreover, given the modest experimental conditions used in this preliminary study, the enhancement of vapreotide delivery by further fine-tuning of the formulation and current profile is a realizable goal. For example, the results obtained with a salt bridge system clearly demonstrate that vapreotide transport can be enhanced by reducing the number of extraneous ions. Hence, based on the data presented here, the transdermal iontophoretic delivery of therapeutic amounts of vapreotide certainly appears to be feasible.

CONCLUSIONS

As with the LHRH analogues, nafarelin and leuprolide, and other non-peptidic compounds that possess a structural motif characterized by the close proximity of charge and lipophilicity (e.g., propranolol and quinine), vapreotide has been shown to interact with the skin membrane. The degree

of electroosmotic inhibition increased with increasing current density. As a consequence of this interaction, and perhaps the aggregation of the peptide, large quantities of vapreotide were found in the skin. The pre-treatment strategies to saturate the skin binding sites and the post-treatment methods to displace bound vapreotide proved ineffective. It was also shown that significantly greater amounts of peptide were transported across heat-separated epidermis than across dermatomed skin. Vapreotide was susceptible to a significant degree of metabolism (~60-70%) when placed in contact with the interior surface of heat-separated epidermis or dermatomed skin. Given its susceptibility to enzymatic degradation, and its capacity to shut down electroosmotic solvent flow, vapreotide may not appear as an ideal candidate for transdermal iontophoresis. Nevertheless, analysis of the cumulative amounts permeated across the epidermis and the human pharmacokinetics, suggests that iontophoretic delivery might be an effective alternative to the conventional parenteral administration of vapreotide.

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CHAPITRE 5

Administration transdermique de la triptoréline (Decapeptyl®) par ionophorèse

CHAPITRE 5

Administration transdermique de la triptoréline (Decapeptyl®) par ionophorèse

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Résumé

La faisabilité de l'administration transdermique de la triptoréline ([*D*-Trp[6]]LHRH) par ionophorèse a été évaluée *in vitro*. Le transport du peptide a été mesuré à travers la peau d'oreille de porc avec différentes concentrations du compartiment donneur, à différentes densités de courant. L'administration concomitante d'un marqueur de l'électroosmose a permis de déterminer les contributions respectives de l'électromigration et de l'électroosmose au transport ionophorétique. A une concentration donnée (3mM), une augmentation de la densité de courant d'un facteur de trois produit une augmentation correspondante de la quantité de peptide présente dans le compartiment récepteur. Inversement, le doublement de la concentration à 6 mM produit une réduction de la quantité de peptide administré, en partie en raison d'une inhibition concentration - dépendante de l'électroosmose. L'électromigration se révéla être le mécanisme de transport prédominant, comptant pour 80% de l'administration totale. Finalement, en dépit de l'inhibition de l'électroosmose, les résultats indiquent que l'application d'un courant ionophorétique de 0.8 mA sur une surface relativement petite (4 cm^2) permettrait d'atteindre un taux d'administration excédant 35 μg/h, largement supérieur aux exigences thérapeutiques.

Transdermal iontophoretic delivery of Triptorelin (Decapeptyl®)

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Abstract

The feasibility of delivering triptorelin ([*D*-Trp^6]LHRH) by transdermal iontophoresis was evaluated *in vitro*. Peptide electrotransport at different current densities and donor concentrations was measured across porcine ear skin. The concomitant delivery of an electroosmotic marker enabled calculation of the respective contributions of electromigration and electroosmosis to iontophoretic delivery. At a given concentration (3 mM), a three-fold increase in current density produced a corresponding increase in the cumulative amount of peptide present in the receptor compartment. Conversely, doubling the concentration to 6 mM produced a two-fold reduction in the amount of peptide delivered, partly due to a concentration-dependent inhibition of electroosmosis. Electromigration was revealed to be the predominant transport mechanism, accounting for 80% of overall delivery. Finally, despite the inhibition of electroosmosis, the results indicate that application of an iontophoretic current of 0.8 mA over a relatively small contact area (4 cm^2) would provide a delivery rate exceeding 35 μg/h, largely sufficient for therapeutic requirements.

Keywords: transdermal iontophoresis, triptorelin, electroosmosis

INTRODUCTION

Transdermal delivery has been proposed as a route for administering peptide and protein therapeutics on the grounds that it offers a viable alternative to the conventional, and inconvenient, administration by parenteral injection. However, given that peptides are often charged and of high molecular weight, their passive transdermal delivery is not feasible. Hence, different strategies have been developed to overcome the skin's excellent barrier properties in a transient and reversible fashion [1;2]. Iontophoresis offers the advantage of providing a controlled and non-invasive delivery method that has been extensively investigated [3]. The two main transport mechanisms during iontophoresis are electromigration (EM; direct effect of the applied electric field on the charged species) and electroosmosis (EO; convective solvent flow in the anode-to-cathode direction, as a consequence of the skin's net negative charge at physiological pH).

A distinguishing feature of iontophoresis is that, in contrast to other enhancement technologies, it acts primarily on the molecule itself. That is, enhanced delivery is *not* due to increased passive drug transport subsequent to barrier disruption: the driving force is supplied by the applied electric field. Furthermore, iontophoresis enables customized therapy: the drug input rate can be adapted to the individual needs of each patient, or to the phase of treatment in response to disease progression, by modulating the intensity of the applied current. In addition, different permutations of the current profile enable continuous or pulsatile delivery and other more complex drug input profiles, e.g., drug input at a basal rate followed by an "on-demand" bolus as in patient-controlled analgesia.

Control over the input profile is particularly valuable for drugs that have different pharmacological effects depending on their input rate, such as luteinising hormone-releasing hormone (LHRH) and human parathyroid hormone (PTH). For example, pulsatile administration of LHRH, every 60-90 minutes, is used in the treatment of female infertility due to hypothalamic hypogonadism [4], in order to stimulate gonadotrophin release. In contrast, prolonged continuous application of LHRH, and its analogues, suppresses gonadotrophin secretion and is the underlying mechanism in the therapy of hormone-dependent cancers [5].

Several studies investigating the iontophoretic transdermal transport of LHRH and its more potent and longer-acting analogues have been conducted [6-11]. Some of the most relevant results were obtained with [*D*-Leu^6,Pro^9-NHET]LHRH (leuprolide, Lupron™), which was successfully delivered *in vivo* in humans: a peak LH response similar to that obtained with subcutaneous injection was measured after iontophoretic delivery of leuprolide [12]. Interestingly, these results were accompanied by some unexpected behaviour, not consistent with theory: namely, that increasing the dose did not result in enhanced delivery [8;13]. Similar findings have been described for other LHRH analogues and have been attributed to the association of the lipophilic cations with the membrane, neutralizing the intrinsic negative charge of the skin and leading to a significant reduction in the electroosmotic transport of the peptide [10;14].

The aim of this study was to evaluate the feasibility of delivering triptorelin ([*D*-Trp^6]LHRH, Decapeptyl®) by transdermal iontophoresis and to investigate the transport mechanisms involved. Co-iontophoresis of acetaminophen was used to deconvolve the contributions of EO and EM and to report on the impact of triptorelin iontophoresis on skin permselectivity. The effect of current density and

peptide donor concentration on delivery was assessed. Triptorelin transport was also compared to that of a tripeptide (Ac-*D*-Trp-Leu-Arg-NH_2) corresponding to its sequence at positions 6-8.

MATERIALS AND METHODS

Chemicals

Triptorelin (MW = 1311.5) *p*Glu-His-Trp-Ser-Tyr-*D*-Trp-Leu-Arg-Pro-Gly-NH_2, in the form of the acetate salt was a generous gift (Debiopharm Galenic Unit, Switzerland). Ac-Tyr-(*D*-Trp)-Lys-NH_2 and Ac-(*D*-Trp)-Leu-Arg-NH_2 were custom-synthesised (NeoMPS SA, France). De-ionized water (resistivity > 18 MOhm/cm^2) was used to prepare all solutions.

Analytical procedures

Triptorelin was quantified by high-performance liquid chromatography. The HPLC system comprised a pump (Waters 600E System Controller, Waters Corporation, MA), dual wavelength UV detector (Waters 2487 Dual λ Absorbance Detector), autoinjector (Waters 717plus Autosampler) and was equipped with a C18 PartiSphere column (4.6 mm i.d., 12.5 cm long, 5 μm particle size) (Whatman Inc., NJ) maintained at 40°C. The mobile phase (25% acetonitrile and 75% triethylaminephosphate buffer solution pH 2.3) delivered at a flow rate of 1 mL/min was degassed in-line (Waters In-Line Degasser AF). Triptorelin was detected at 280 nm. The repeatability was less than 1 % and the quantification limit was 130 ng/ml. The tripeptides were analysed using the same conditions but with a mobile phase consisting of acetonitrile: triethylaminephosphate buffer, pH 2.3 (12:88). The repeatability was less than 1 % and the quantification limit was 55 ng/ml.

Acetaminophen was assayed by high-performance liquid chromatography using a Hypersil BDS C8 column (150 mm x 4.6 mm, Supelco®, Sigma-Aldrich Chimie Sarl, France) maintained at 40°C. The mobile phase (92% water and 8% acetonitrile adjusted to pH 3.5 with acetic acid) was delivered at a flow rate of 1 mL/min. Acetaminophen was detected by its UV-absorbance at 243 nm. The repeatability was less than 1 % and the quantification limit was 22 ng/ml.

Skin preparation

Porcine ears were obtained from the local abattoir shortly after sacrifice. After cleaning under cold running water, the whole skin was removed carefully from the outer region of the ear and separated from the underlying cartilage with a scalpel. Given that epidermis is a well-established model for transdermal drug delivery, and that dermatomed skin might act as an artifactual reservoir and binding site [15-17], all experiments with triptorelin were performed using heat-separated epidermis [18]. Pieces of fresh full-thickness skin were immersed in water at 58°C for 2 minutes after which the epidermis was carefully separated from the dermis, wrapped in Parafilm™ and maintained at –20°C for no longer than a period of 2 months before use.

Stability experiments

The susceptibility of triptorelin to degradation by porcine skin enzymes was assessed in the following manner. Epidermal sections were mounted in diffusion cells and the receptor compartment was filled with a 5 μM triptorelin solution, in 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline; both the anodal and cathodal compartments were filled with 1 mL of 25 mM Tris/Trizma® HCl-buffered (pH 7.4) normal saline. The concentration of intact triptorelin in the receptor compartment was determined after application of a 0.15 mA/cm^2 current for eight hours.

Iontophoresis

The skin was mounted in three-compartment vertical diffusion cells (area: 0.73 cm^2), the design of which has been described in detail elsewhere [19]. Except in the case of the stability experiments, the anode was isolated from the donor solution via a salt bridge (100 mM Tris/Trizma HCl in 3% agarose) to minimize competition between the peptide and the electrolytes necessary for the anodal reaction. Anodal, cathodal and receptor compartments contained a solution of 25 mM Tris/Trizma® HCl normal saline buffered to pH 7.4. Triptorelin (3 mM unless otherwise stated) was solubilized in 20 mM Tris/Trizma® HCl (pH 7.4). In addition to the peptide, the donor compartment (1 mL) always contained 15 mM acetaminophen.

Constant current iontophoresis was used in all the experiments. The current, ranging from 0.15 to 0.5 mA/cm^2 was applied for 4 to 8 hours via Ag/AgCl electrodes connected to a power supply (Kepco, NJ).

Quantification of electroosmotic solvent flow

Acetaminophen is a neutral hydrophilic compound, which is primarily transported through the skin by electroosmosis. It was therefore included in the donor compartment formulation (15 mM) as a marker for the magnitude of convective flow (15 mM). For each experiment, an Inhibition Factor (IF) was calculated according to the following equation:

$$IF = [Q_{A\text{-}8h,control}] / [Q_{A\text{-}8h,peptide}] \quad (1)$$

where $Q_{A\text{-}8h,control}$ is the amount of acetominaphen transported into the receptor phase during 8 hours of iontophoresis when no peptide was present in the donor solution, and $Q_{A\text{-}8h,peptide}$ is the corresponding quantity when triptorelin was iontophoresed..

Effect of current density and triptorelin concentration

To evaluate the effect of current density on both triptorelin transport and inhibition of electroosmosis, current densities of 0.15, 0.3 and 0.5 mA/cm^2 were applied for 8 hours using a donor peptide concentration of 3 mM.

The iontophoretic delivery of a higher concentration (6 mM) at 0.5 mA/cm^2 for 8 hours was also investigated to determine the impact of the donor concentration on the electroosmotic flow and overall peptide transport.

Data treatment

All measurements were performed in at least triplicate, using skin samples originating from different pig ears. Outliers, determined using the Grubbs test, were discarded.

RESULTS AND DISCUSSION

Triptorelin stability in the presence of skin

Investigation of the *in vitro* stability of triptorelin in the presence of epidermis revealed a degradation of 9 ± 2% after 8 hours, suggesting that it was quite resistant to proteolysis. Peptide susceptibility to enzymatic degradation is obviously dependent on the amino acid sequence: under the same conditions, again using porcine epidermis, the somatostatin analogue, vapreotide, suffered significantly greater metabolism (68 ± 11%) [17]. Amidation of the C-termini ensures that both peptides are protected against carboxypeptidases. The cyclic *p*Glu and *D*-Phe residues at the N-termini protect triptorelin and vapreotide, respectively, against aminopeptidase activity. Thus, endopeptidases or reductases targeting the disulphide bridge, present in vapreotide, but not in triptorelin, might also be active in the skin.

Triptorelin delivery and the effect of current density

The quantities of triptorelin transported across porcine epidermis from a 3 mM donor solution after 4, 6 and 8 hours of iontophoresis (at a current density of 0.5 mA/cm^2) are depicted in Figure 1.

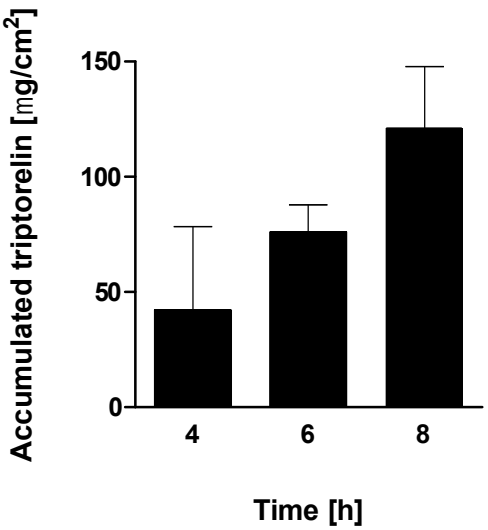

Figure 1. Cumulative transport of triptorelin across porcine skin *in vitro* following iontophoretic current application at 0.5 mA/cm^2 for 4, 6 and 8 hours, respectively. The formulation in the donor compartment comprised 3 mM triptorelin in 20 mM Tris/Trizma at pH 7.4.

After 8 hours of iontophoresis, 120 ± 30 μg/cm^2 of triptorelin was detected in the receptor compartment, with an estimated flux of 22 μg/cm^2/hr at 7 hours. The iontophoretic transport rate of [*D*-Trp^6,Pro^9-NHET]LHRH, which has almost complete sequence homology with triptorelin ([*D*-Trp^6]LHRH), across hairless mouse skin *in vitro* was very similar (17 μg/cm^2/hr) at the same current density (0.5 mA/cm^2) and donor concentration (3 mM) [7]. Although iontophoretic delivery of triptorelin across human and rabbit skins has been reported [11], quantitative comparison is rendered difficult by the differences in experimental conditions and the variability of the data.

The iontophoretic flux (J) of a charged species is the sum of two transport mechanisms - electromigration (J_{EM}) and electroosmosis (J_{EO}), assuming that passive diffusion is negligible:

$$J = J_{EM} + J_{EO} \tag{2}$$

$$J_{EM} = \frac{t_{\#} \cdot I}{Z \cdot F} = \frac{Z \cdot u \cdot c}{\sum_{i=1}^{n} z_i \cdot u_i \cdot c_i} \left(\frac{I}{Z \cdot F} \right) \tag{3}$$

According to Faraday's law, J_{EM} is related to the applied current density (I), to the transport number ($t_{\#}$), electrical mobility (u), charge (Z), and concentration (c) of the drug, and to the concentrations, mobilities and charges of the other ions present in the system (c_i, u_i, z_i). Since the electroosmotic flow, from anode-to-cathode under physiological conditions, increases with applied current density [20], both transport mechanisms, and hence the total iontophoretic flux, are related to the applied current. This is illustrated by the data in Table 1 which show that a three-fold increase in current density (0.15 to 0.5 mA/cm^2) resulted in a corresponding increase in the cumulative amount of triptorelin in the receiver compartment (35 ± 9 to 120 ± 30 μg/cm^2).

Table 1. Effect of current density on the iontophoretic transport and electroosmosis inhibition of triptorelin (3 mM).

Current density [mA/cm^2]	Triptorelin transport [μg/cm^2]	Inhibition factor
0.15	35 ± 9	1.0 ± 0.8
0.3	60 ± 30	3.6 ± 1.8
0.5	120 ± 30	6 ± 3

In contrast, the increase in current produced a sharp decrease in acetaminophen transport; a six-fold reduction was observed at 0.5 mA/cm^2, indicative of considerable EO inhibition.

Linear correlations between flux and current density have been reported for non-peptidic compounds as well as for small peptides (e.g., TRH [21] and Threo-Lys-Pro [22]). For larger peptides, although an increased current density usually results in increased permeation, straightforward linear correlations are not always observed. For example, a poor correlation was observed between DGAVP (9-desglycinamide, 8-arginine-vasopressin) flux and applied current; a more than six-fold increment in current density did not even double the flux [23]. However, it was shown that increasing the applied current density from 0.1 to 0.5 mA/cm^2 produced an almost 3-fold increase in the flux of the structurally-related peptide [*D*-Trp^6,Pro^9-NHET]LHRH [7].

Contributions of electromigration and electroosmosis to triptorelin transport

Since acetaminophen is a neutral hydrophilic molecule with negligible passive skin permeability, its iontophoretic transport is almost exclusively due to electrically-induced convective solvent flow and, as such, its transport can be used to report on electroosmosis. During iontophoresis, the velocity (V_w) of the current-induced water flow (units of cm/h, equivalent to a permeability coefficient) across the skin can be estimated using equation 4 [24]:

$$V_w = J_{ace}/C_{ace} \quad (4)$$

where J_{ace} and C_{ace} are the flux and donor concentration of acetaminophen, respectively. It follows that a measurement of J_{ace} at known C_{ace} allows V_w to be determined. It is then possible to calculate the EO contribution to the flux of the peptide by multiplying V_w by its concentration in the donor solution ($C_{peptide}$) [25]:

$$J_{EO} = V_w \cdot C_{peptide} \quad (5)$$

Two assumptions are implicit in this analysis: (a) that drug and acetaminophen are transported in a similar fashion by convective solvent flow, and (b) that electroosmotic transport of the marker molecule is proportional to its concentration in the solvent.

Co-iontophoresis of acetaminophen (15 mM) with triptorelin (3 mM) at 0.5 mA/cm^2, for 8 hours, resulted in an acetaminophen flux of 18 nmol/cm^2/h; this was used to calculate V_w using Equation 4. The quantity of triptorelin transported by electroosmosis could then be estimated by substitution of V_w (1.2×10^{-3} cm/h) into Equation 5. Given the measured *total* flux of triptorelin under the same conditions (17 nmol/cm^2/h), Equation 2 allows assignment of the relative contributions of EO and EM to the total iontophoretic flux. The analysis reveals that EM is the dominant transport mechanism for triptorelin, accounting for ~ 80% of overall transport (J_{EO} = 3.6 nmol/cm^2/h; J_{EM} = 13.4 nmol/cm^2/h). It is worth noting that in the absence of any inhibition, the maximum theoretical EO contribution ($J_{EO,max}$, that is, assuming V_w ~ 4.8×10^{-3} cm/h) to the iontophoretic delivery of triptorelin (3mM at 0.5 mA/cm^2) would only be ~14 nmol/cm^2/h; implying that for this decapeptide (MW ~1311), EM would still account for ~50% of iontophoretic transport.

Acetaminophen transport in the presence of triptorelin clearly demonstrated that the latter inhibited EO (IF of 6 $\pm$ 3), albeit to a much lesser extent than certain other peptides. For example, vapreotide iontophoresis under equivalent conditions resulted in an IF of 50 $\pm$ 30 [17]. Vapreotide is doubly charged (due to the lysine side chain and the free N-terminal) and this probably favours the interaction with negatively charged sites in the skin and accounts for its greater propensity to inhibit EO. Furthermore, the presence of a disulphide bridge in vapreotide probably contributes to a more compact three-dimensional structure than triptorelin; the preferred conformations may orient the key moieties so as to favour interaction with the skin's binding sites, and hence facilitate EO inhibition.

Are peptide fragments useful predictors of transport ?

Nafarelin ([*D*-Nal(2)^6]LHRH) and the tripeptide (*D*-Nal(2))-Leu-Arg (representing the amino acids at positions 6 to 8) were demonstrated to be equipotent EO inhibitors [14]. In contrast, while triptorelin

caused EO inhibition (IF = 6 ± 3), co-iontophoresis of its "peptide motif" Ac-(*D*-Trp)-Leu-Arg-NH_2 with acetaminophen failed to result in a corresponding effect (IF = 1.1 ± 0.2) (Figure 2).

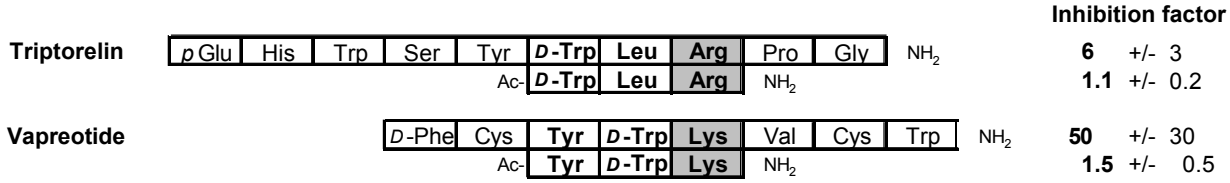

Figure 2. Inhibition factors of triptorelin, vapreotide and their constituent tripeptides after iontophoretic current application at 0.5 mA/cm^2 for 8 hours. The formulation in the donor compartment comprised 3 mM peptide in 20 mM Tris/Trizma at pH 7.4.

Iontophoresis of the tripeptide Ac-Tyr-(*D*-Trp)-Lys-NH_2, derived from the residues at positions 3 to 5 in vapreotide, under the same experimental conditions, also produced almost no inhibition of EO (IF = 1.5 ± 0.5) compared to the parent peptide (IF = 50 ± 30) [17]. Hence, the occurrence and extent of this phenomenon cannot, as a rule, be accurately predicted from the behaviour of the structural motif presumed responsible for the skin interaction. It should be noted that interspecies differences (the triptorelin and vapreotide studies were conducted with porcine skin whereas nafarelin and (*D*-Nal(2))-Leu-Arg data were obtained using hairless mouse skin) may also play a role in the interpretation of mechanistic data. This is further illustrated by the observation that the dependence of iontophoretic propranolol delivery on donor concentration (in the presence of competing ions), across these two membranes, is different [25].

Effect of increasing triptorelin concentration on peptide transport and electroosmosis

The impact of drug concentration on iontophoretic flux is a commonly studied experimental parameter. According to Equation 3, an increase in the formulation's drug-load should result in an increase in the EM component and hence in the total drug flux (with the assumption that the formulation concentration is equivalent to that present in the supposed aqueous transport pathways within the membrane). Indeed, when the donor formulation contains background electrolyte, a source of competing ions, the initial linear dependence that is observed between flux and drug concentration dwindles as concentration increases: once the product of the drug concentration and mobility (see Equation 3) is in sufficient excess of the corresponding values for the competing ions, the flux becomes independent of drug concentration. Triptorelin contains the (*D*-Trp-Leu-Arg) sequence at positions 6 to 8 in its primary structure; this sequence corresponds to the oligopeptide motif (hydrophobe-hydrophobe-cation) hypothesized to be responsible for the "anomalous" iontophoretic behaviour observed with nafarelin, leuprolide and octreotide upon increasing the donor concentration. In a second series of transport experiments with twice the triptorelin concentration (6 mM) in the donor compartment, only 60 ± 40 μg/cm^2 of triptorelin was measured in the receptor compartment, compared to 120 ± 30 μg/cm^2 with the lower donor concentration (3 mM); a two-fold increase of peptide in the formulation produced a two-fold *decrease* in delivery. The simultaneous administration of acetaminophen as an electroosmotic marker, allowed the inhibition factor to be calculated. The IF

values at 3 and 6 mM triptorelin concentrations were 6 ± 3 and 10 ± 5, respectively, substantiating the inhibitory effect of this peptide on the convective solvent flow. However, given that EO accounted for only ~20% of iontophoretic transport at a donor concentration of 3 mM, the increased EO inhibition cannot alone explain the two-fold reduction in the cumulative amount delivered: the increase in donor concentration must also impact on EM. Hence, it seems likely that other interactions, perhaps involving the formation of triptorelin aggregates, which would hinder peptide delivery, must occur within the transport pathways.

The separate effects of aggregation and inhibition could be represented by modification of Equation 2:

$$J_T^{EXP} = (1-a)J_{EM}^{PRED} + (1-b)J_{EO}^{NO\ INHIB} \quad (6)$$

where α and β represent the degree of aggregation and EO inhibition, respectively. Increasing aggregation would reduce drug mobility and predominantly affect EM; β, which is proportional to IF, would negatively impact upon convective solvent flow. Thus, peptide accumulation could occur without affecting EO. At the same time, peptides could strongly inhibit EO and still have that as the major transport mechanism, if they also exhibit a high degree of aggregation.

Can therapeutic amounts of triptorelin be delivered by transdermal iontophoresis?

The “bottom line” of any feasibility study with a therapeutic molecule is to determine whether sufficient drug can be delivered to achieve the desired pharmacological effect. For triptorelin, the plasma concentration required for biochemical castration is ~1.7 nmol/L (equivalent to ~2.2 ng/mL) [26]. Given that the total body clearance in healthy individuals is ~200 mL/min [27], the target input rate that must be achieved to maintain the steady state triptorelin concentration necessary for durable biochemical castration is ~26 μg/h. The measured triptorelin flux of 22 μg/cm^2/h (at 7 hrs) signifies that, at the current density used in these experiments (0.5 mA/cm^2), it would be entirely feasible to deliver therapeutic levels of triptorelin with a patch application area of less than 2 cm^2. As reported above, triptorelin delivery increases essentially linearly with current; hence, the measured peptide flux can be normalized by the applied current density to obtain a measure of mass transport per unit current per unit time; the “current-normalized” flux for triptorelin is 45 μg/mA/h. A current density of 0.5 mA/cm^2 is at the upper limit of the range generally considered acceptable for human use, and is probably unsuitable for prolonged application. The “current-normalized” flux can be used to calculate the application area necessary to deliver therapeutic amounts of triptorelin at lower, and better tolerated, iontophoretic current densities. For example, the application of a 0.8 mA iontophoretic current over a 4 cm^2 contact area, equivalent to a far more acceptable current density of 0.2 mA/cm^2, would be sufficient to provide a delivery rate exceeding 35 μg/h.

CONCLUSIONS

These preliminary *in vitro* studies illustrate the contrasting effects of current density and triptorelin concentration on delivery. At a given concentration (3 mM), a three-fold increase in current density produced a corresponding increasing in the cumulative amount of peptide transported across porcine epidermis. Conversely, doubling the concentration to 6 mM produced a two-fold reduction in the amount of peptide delivered following iontophoresis for 8 hours at 0.5 mA/cm^2. Although theory would suggest equivalent effects upon increasing current or concentration, the experimental results reveal that this is not always the case. Thus, formulation parameters must be carefully selected to optimize the delivery of complex molecules. Quantification of acetaminophen transport in the presence of triptorelin revealed that EM was the predominant transport mechanism, accounting for ~80% of overall delivery. The acetaminophen data also revealed that triptorelin was capable of a concentration-dependent EO inhibition. Nevertheless, the degree of inhibition was insufficient to explain the inverse dependence of transport on peptide concentration, suggesting the involvement of peptide aggregation. Despite the EO inhibition and putative aggregation phenomena, the cumulative amount of triptorelin delivered and the estimated iontophoretic flux suggest that drug input rates sufficient to achieve plasma levels capable of ensuring prolonged biochemical castration may be attainable using transdermal iontophoresis.

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CONCLUSIONS ET PERSPECTIVES

L'étude portant sur l'administration du vapréotide illustre la fonction barrière métabolique qu'exerce la peau en plus de sa fonction barrière physique plus évidente. La forte susceptibilité de cet analogue de la somatostatine vis-à-vis de la dégradation enzymatique ainsi que son importante capacité à inhiber l'électroosmose, et par là même son propre transport, font de ce peptide un candidat non idéal à l'administration transdermique par ionophorèse. Si les résultats obtenus indiquent qu'un patch de 15 cm^2 pourrait tout de même permettre d'atteindre des concentrations thérapeutiques, les flux mesurés pour la triptoréline sont nettement plus importants, notamment en raison de sa faible dégradation enzymatique. Cet analogue de la LHRH s'avère donc être un bien meilleur candidat et son administration par ionophorèse semble à même d'offrir une alternative aux formes médicamenteuses actuellement disponibles. Des expériences conduites *in vivo* avec ces deux peptides fourniraient des informations supplémentaires permettant de mieux anticiper les corrélations *in vitro - in vivo* dans le cas de molécules comme celles-ci, notamment en termes de dégradation enzymatique. Elles pourraient valider les flux estimés et ainsi constituer un pas décisif vers l'administration transdermique de peptides thérapeutiques par ionophorèse.

Alors que ces deux peptides ont montré leur capacité à inhiber l'électroosmose (inhibition suspectée par leur structure présentant un acide aminé chargé près d'un groupement lipophile), l'étude du comportement ionophorétique des tripeptides correspondant à cette séquence clé révéla que l'inhibition induite par un peptide ne peut être prédite sur la base de l'inhibition exercée par le tripeptide présentant la juxtaposition « charge et lipophilie ». Ceci démontre bien la complexité de l'interaction avec la membrane et met en évidence le manque de connaissance actuel sur les éléments structuraux influençant les mécanismes de transport en ionophorèse.

L'étude systématique menée sur une série de tripeptides dont la séquence est dérivée de celle des analogues de la LHRH et de la somatostatine, a permis de mettre en évidence des propriétés moléculaires-clé intervenant dans le passage transdermique de ces composés par ionophorèse. L'hydrophobie est apparue comme paramètre déterminant, s'opposant au passage des peptides. Si il a été montré qu'elle favorise l'inhibition de l'électroosmose, elle semble impliquée dans d'autres interactions ayant pour conséquence de restreindre le transport. En montrant que non seulement le volume des régions hydrophobes mais également leur distribution au sein de la molécule ont un impact sur le transport, cette approche computationnelle a démontré qu'il est essentiel de prendre en considération l'environnement tridimensionnel des composés. Elle a ainsi permis de mettre en évidence la différence de lipophilie pouvant exister entre deux tripeptides constitués des mêmes acides aminés mais arrangés selon un autre ordre, différence qui s'est manifestée au niveau de leur transport ionophorétique. Des interactions telles que celles existant entre les chaînes latérales

des acides aminés sont susceptibles d'influencer la conformation tridimensionnelle, modulant ainsi la lipophilie du peptide, ce qui ne peut être détecté par une méthode fragmentale bi-dimensionnelle telle que le ClogP. Cet effet de la séquence en acides aminés sur le transport ionophorétique mérite d'être plus amplement étudié. La ionophorèse de peptides supplémentaires ainsi que la mise en évidence expérimentale de cette différence de lipophilie par des mesures de voltamétrie cyclique peut en effet être envisagée. Il serait par ailleurs intéressant de confirmer l'effet du type de charge observé lors de la comparaison du transport des peptides contenant la lysine à ceux dont la charge est fournie par l'arginine.

Enfin, il serait particulièrement profitable d'étayer le modèle établi puis de l'élargir à d'autres molécules, ce qui permettrait notamment d'approfondir les connaissances actuelles des mécanismes de transport impliqués dans l'administration transdermique par ionophorèse. Aussi, on pourrait imaginer qu'une telle approche permette un jour d'évaluer la faisabilité de l'administration d'un candidat médicament par cette technologie, sans expérimentation *in vitro*, voire même avant la synthèse du composé.

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