TRANSVAGINAL DIFFUSION OF SYNTHETIC PEPTIDES

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Topical microbicide peptides are being developed to combat the transfer of HIV, but little is known about the permeation of these compounds through vaginal epithelium. The object of the present study is to investigate the *in vitro* permeation of synthetic transport peptides through vaginal mucosa. The permeation kinetics of three FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 (Mw = 2911.4 Da), MDY-19 (Mw = 2409.5 Da) and PCI (Mw = 2325 Da) across human vaginal mucosa was studied by means of a continuous flow-through diffusion system. Permeability studies were conducted at concentrations of 1 mM, 0.75 mM and 0.5 mM in PBS buffer at 37°C and 20°C, respectively, and over a time period of 24 h, using fluorospectrophotometry as detection method. Effects of a surfactant on MDY-19 permeation and de-epithelialisation of the vaginal mucosa were also studied. Statistical tests used included an ANOVA and Duncan's multiple range test to establish steady state diffusion kinetics. All three peptides readily penetrate vaginal mucosa. Microbicides may be coupled to MDY-19 and PCI to be transported transmucosally. Although increased size of the peptide/microbicides complex may decrease mucosal permeability, this could possibly be overcome by the addition of a permeation enhancer, e.g. a surfactant. Removal of the vaginal epithelium increased the flux rates of the peptides across the mucosa and may have implications for a more rapid uptake of these and other microbicides *in vivo*. Concentration- and temperature- dependency of peptide flux rates must be taken into consideration when performing *in vitro* permeability studies.

Experience with a variety of compounds has demonstrated that the vagina is a safe and highly efficacious site for drug administration (1-2). However, vaginal administration of drugs still remains a relatively unexplored route for drug administration. Advantages of vaginal administration of drugs include: the administration of lower doses, maintenance of steady drug levels, less frequent administration than with e.g. the oral route, avoidance of the first-pass effect and no effect of gastrointestinal (GI) disturbances on the absorption of the drug (1, 3). This route also allows a woman to self-administer medication continuously for prolonged periods of time.

Currently, a resurgence of interest in peptide and protein drugs exists (4). Many of the latter are endogenous compounds regulating endocrine and other physiological processes in the body (5). These amino acid polymers are increasingly used in major research and development programs, especially due to advances in genetic engineering and biotechnology (6). They may act synergistically with each other and with other agents in the host, e.g. magainin 2 shows synergistic antimicrobial effects with the peptide PGLa.

Due to the development of resistant pathogens, it is important to consider new classes of antibiotics,

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such as cationic peptides (5). These small, positively charged peptides are known for their broad-spectrum antimicrobial activity and they are found throughout nature (5). Cationic peptides are produced by most living organisms, from plants and insects to human beings, and form a major part of their immediate defenses against infections. These peptides also have anti-viral and anti-cancer activity as well as the ability to modulate innate immune responses (7).

The unusual physicochemical characteristics of peptides and proteins present considerable challenges to pharmaceutical scientists for their formulation and in selecting a suitable route for their administration. The most commonly used route for protein and peptide delivery has been via parenteral administration due to their lability in the GIT (8). Most proteins and peptides have relatively short half-lives and therefore repeated parenteral administrations (injections) are often required (4). Development of suitable non-parenteral routes for introducing these agents into humans could significantly enhance patient compliance.

Peptides and proteins can be administered topically via mucosal surfaces e.g. vaginal, buccal, nasal and rectal mucosa, thus bypassing first-pass metabolism and making them directly systemically available. Most small peptides, however, do not diffuse readily through mucosal membranes and diffusion enhancers must be added to increase their absorption. Currently much research involves studying the diffusion of small peptide molecules through biological membranes in the presence of chemical permeation enhancers.

This study involves the investigation of diffusion of kinetics of three peptides (MEA-5, MDY-19 and PCI) through human vaginal mucosa. MEA-5 is an antibacterial peptide that binds to cell surfaces, but cannot be internalized. It can act synergistically with other existing microbicides. Both MDY-19 and PCI are transport peptides that bind to cell surfaces and can be internalized. The effects of an absorption enhancer, different permeant concentrations and temperature on the diffusion kinetics of the peptide permeants were also investigated.

MATERIALS AND METHODS

Human vaginal mucosa

Specimens were obtained from excess tissue removed from 43 postmenopausal patients, ages 40-81 years (mean age 58±11 yr SD), following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

All surgical specimens obtained were immediately placed in a transport fluid and transferred to our laboratory within 1 h. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which an antibiotic and antimycotic were added prior to using it for the transport of mucosal specimens. Excess connective and adipose tissue were trimmed away and all specimens were snap-frozen in liquid nitrogen and stored at -85°C for periods up to 6 months, as previously prescribed (9-13). No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the different specimens. The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study.

Peptides

The three FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 (Mw = 2911.4 Da), MDY-19 (Mw = 2409.5 Da) and PCI (Mw = 2325 Da) were obtained from PEPSCAN, Lelystad, The Netherlands.

Surfactant

The enhancer used was a novel surfactant (prepared in our laboratory). The surfactant suspension contained 13.5 mg/ml DPPC (dipalmitoyl-L-α-phosphatidylcholine) and 1.35mg/ml PG (1,2-dipalmitoyl-L-α-phosphatidylglycerol).

Permeability Experiments

Prior to each permeability experiment, vaginal tissue specimens were thawed at room temperature in phosphate buffered saline (PBS, pH 7.4). The diffusion kinetics of FITC-labelled peptides through thawed frozen vaginal mucosa were then determined. After equilibration of the specimens in PBS, they were carefully cut, so as not to damage the epithelial surfaces, into sections (4 mm in diameter) and then mounted in flow-through diffusion cells (exposed areas 0.039 cm^2) with the epithelial surfaces facing upwards. Permeation studies were performed on 7 tissue replicates for each patient. Prior to commencing each permeability experiment, tissue disks were equilibrated for 10 min with PBS (pH 7.4) at 20°C in both the donor and acceptor compartments of the diffusion cells. Following equilibration, the PBS was removed from the donor compartment and replaced with 0.5 ml of either a 1 mM, 0.75 mM or 0.5 mM solution of FITC-labelled peptide in PBS. PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected, by means of a fraction collector, at 2 h intervals for 24 h. The permeability study was performed under sink conditions,

i.e. at the completion of each run the concentration of permeant in the acceptor chamber never reached 10% of that in the donor compartment. For the detection of FITC peptides, fluorospectrophotometry (emission: 520 nm and excitation: 497 nm) was carried out using a Perkin-Elmer spectrophotometer (Perkin-Elmer, MA, USA). Experiments were also conducted at 37°C.

Mechanical and heat stripping

Mucosal surfaces were de-epithelialised by a heating method, as well as mechanically, to mimic ulceration and its effects on passage of the peptides. De-epithelialisation by means of heat involved the submerging of the vaginal tissue at 80°C water for 30 s, thereafter removing the epithelial layer with tweezers. Mechanical de-epithelialisation was conducted by carefully scraping off the epithelium from the vaginal mucosa with a scalpel, without damaging the underlying connective tissue layer.

Calculation of Flux Values

Flux (J) values of the various chemical compounds across the vaginal membranes were calculated by means of the relationship: J = Q/A x t, where Q = quantity of compound crossing membrane (pmoles), A = membrane area exposed (cm^2) and t = time of exposure (min).

Steady-State Kinetics

Steady state was assumed to have been reached for a particular specimen and chemical compound when no statistically significant differences ($p<0.05$) at the 5 % level (t-test with Welch's correction) between flux values were obtained over at least 2 consecutive time intervals.

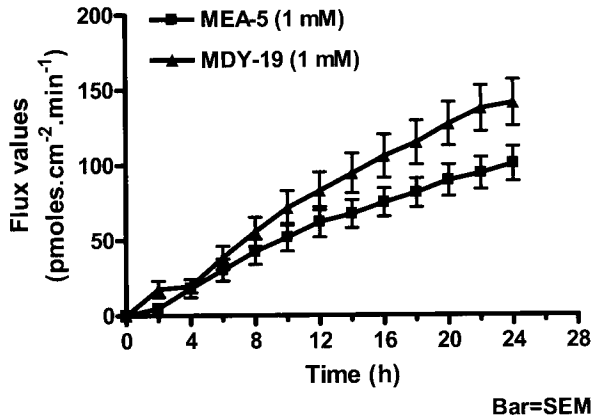

Fig. 1. *The overall mean flux values at 20°C of MEA-5 and MDY-19 versus time across vaginal tissue.*

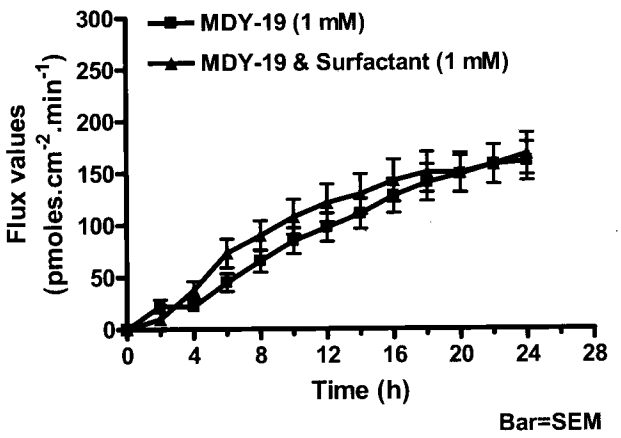

Fig. 2. *The overall mean flux values at 20°C of MDY-19 versus time across vaginal tissue with and without surfactant as a penetration enhancer.*

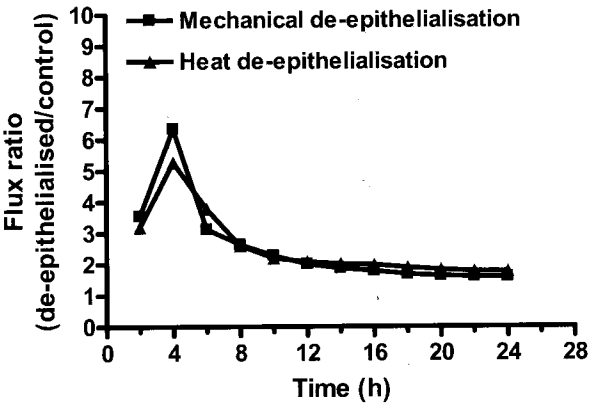

Fig. 3. *Comparison of the flux ratios (de-epithelialised/control) at 20°C obtained for MEA-5 versus time across human vaginal mucosa after heat and mechanical de-epithelialisation.*

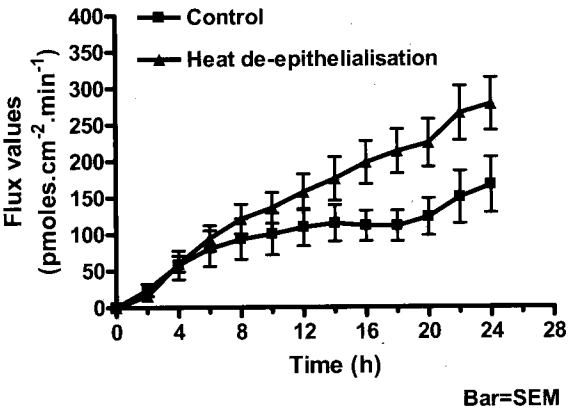

Fig. 4. *Overall mean flux values at 37°C of MDY-19 versus time across intact vaginal mucosa and de-epithelialised mucosa by means of heat.*

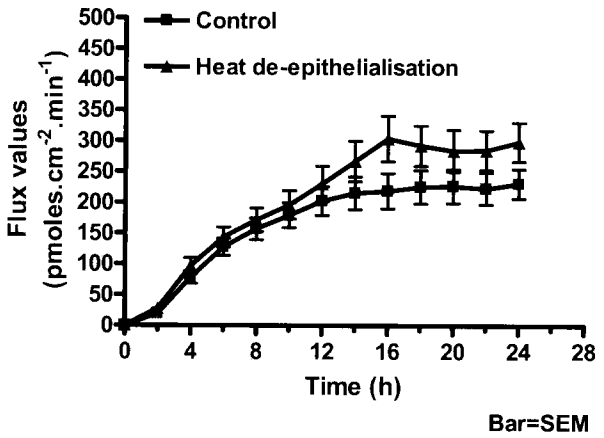

Fig. 5. *Overall mean flux values at 37°C of PCI versus time across intact vaginal mucosa and de-epithelialised mucosa by means of heat.*

Statistical Analysis

Non-linear regression analyses (third order polynomials) were performed using a GraphPad Prism, version 4, 2003 computer programme. An F-test was used to compare entire curves (14). A t-test at steady state was also performed for comparative purposes. A significance level of 5% was used for all tests and comparisons.

RESULTS

The overall mean flux values of MEA-5 and MDY-19 versus time across vaginal tissue (20°C) are shown in Fig. 1. Both MEA-5 and MDY-19 permeated vaginal mucosa well. MDY-19 had a higher flux rate than MEA-5, commensurate with its smaller molecular size (weight). MEA-5 and MDY-19 reached steady state between 10 and 12 hours.

The overall mean flux values of MDY-19 across vaginal tissue (20°C) with and without surfactant as a penetration enhancer are shown in Fig. 2. The surfactant enhanced the flux rate of MDY-19 approximately 1½ times (hours 4-12). The surfactant decreased the lag time of the peptide. The control group reached steady state after 10-12 hours, and the group with the surfactant enhancer had a lag time of 8-10 hours.

Vaginal mucosal surfaces were de-epithelialised by means of mechanical and heat stripping. The comparison of the flux ratios (de-epithelialised/ control) obtained for MEA-5 across human vaginal mucosa after heat and mechanical de-epithelialisation is shown in Fig. 3. Both heat and mechanical de-epithelialisation of mucosa significantly enhanced flux values of MEA-5 (2.5 x) compared with controls (20°C) (Fig. 3), although no marked advantages could be discerned between the two methods. Precipitation of MEA-5 was noted with the initial experiments; therefore further permeability studies were conducted at 37°C. The overall mean flux values of MDY-19 and PCI across intact vaginal tissue (control group) versus de-epithelialised tissue are respectively shown in Figs. 4 and 5. De-epithelialisation of mucosa significantly enhanced flux values of MDY-19 (1.5 x) and PCI (1.2 x) compared with controls (37°C).

The mean steady state flux values of MDY-19 and PCI compared at different concentrations and temperatures (20 °C and 37°C) are shown in Fig. 6 (certain error bars are not visible due to the minimal variations in mean steady state flux values). The mean steady state values of MDY-19 increased with an increase in concentration, but the mean steady state flux values of PCI increased from 0.5 to 0.75 mM and then decreased again as the concentration of PCI was increased to 1 mM. The mean flux at values of 1 mM MDY-19 and 0.75 mM PCI were the highest.

DISCUSSION

HIV is spreading rapidly, especially in sub-Saharan Africa and Southeast-Asia. New prophylactic strategies, e.g. the use of microbicidal vaginal formulations, which have the advantage that women can take control of their own safety, are being investigated (15). Cationic peptides seem to be promising candidates as new therapeutic agents, because they have activity against malaria parasites, and viruses, including HIV, HSV, influenza A virus and vesicular stomatitis virus (7). Where antibiotics only have activity against bacteria, cationic peptides have a wide range of activities against bacteria, fungi, enveloped viruses and eukaryotic parasites.

Both MEA-5 and MDY-19 readily penetrate vaginal mucosa. Novel microbicides may be coupled to the transport peptide MDY-19 to be transported transmucosally or into cells. Although the increased size of a MDY-19/microbicide complex may decrease mucosal permeability, this could possibly be offset by the addition of an appropriate surfactant-containing permeation enhancer. The surfactant used in this study enhanced the flux rate of MDY-19 between 8-12 hr approximately 1 ½-fold (Fig. 2).

De-epithelialisation mimics the situation when integrity of vaginal epithelium is compromised due

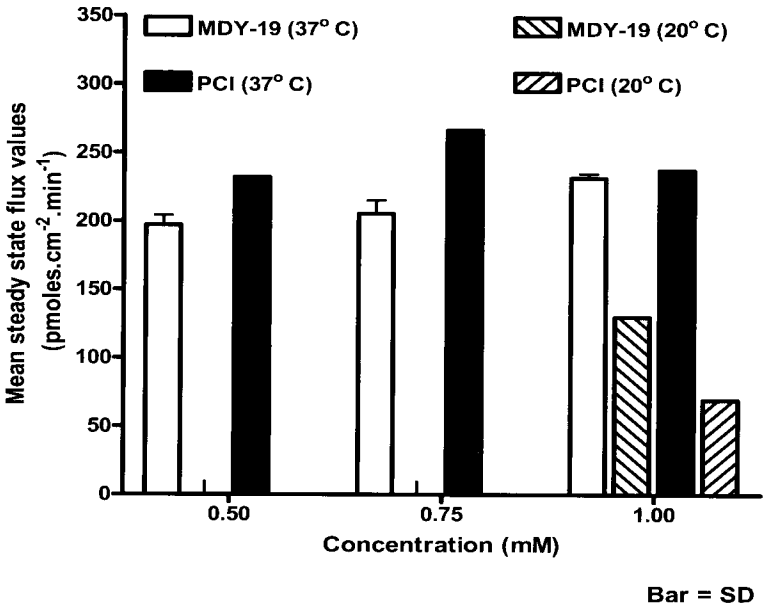

Fig. 6. *The mean steady state flux values obtained for various concentrations of MDY-19 and PCI across intact vaginal mucosa at 20°C and 37°C.*

to disease or local trauma and significantly increased the permeability of MEA-5, MDY-19 and PCI over controls by approximately 2.5x, 1.5x and 1.2x, respectively. This supports the premise that the main barrier is located in the epithelium and it may have possible implications for increased *in vivo* uptake of microbicides as well as HIV by traumatised vaginal mucosa. It is in keeping with the finding that other sexually transmitted diseases e.g. genital herpes, chlamydial infection, trichomoniasis (ulcerative and non-ulcerative) are known to cause disruption of the vaginal epithelium and can increase HIV transmission 3- to 10-fold (16-17). Initially two methods e.g. mechanical and heat de-epithelialisation were used to establish the most effective and convenient method to remove epithelium from the vaginal mucosa. The MEA-5 peptide was used to evaluate these two de-epithelialisation methods. Because de-epithelialisation by heat appeared to be the more convenient method, it was therefore used for mucosal de-epithelialisation for the experiments using the peptides MDY-19 and PCI.

Precipitation of the peptides MDY-19 and PCI on the vaginal mucosa during the permeability experiments at room temperature (20°C) and at a concentration of 1 mM occurred. For this reason, permeability studies of MDY-19 and PCI were repeated at concentrations of 1 mM as well as lower concentrations of 0.75 mM and 0.5 mM, in PBS buffer, at a temperature of 37°C. The overall mean flux values of 1 mM MDY-19 and PCI at 37°C at steady state were higher than the overall mean flux values at 20°C. At temperatures of 37°C the mean flux values at steady state of MDY-19 increased with concentration according to well-established diffusion theory. However, the flux values of PCI increased from 0.5 to 0.75 mM and then decreased again as the concentration of PCI was increased to 1 mM (Fig. 6). This may indicate a decrease in solubility of the latter peptide. PCI has a less overall positive charge than MDY-19 since it contains less of the basic amino acid, arginine. At high concentrations (1 mM) precipitation of PCI may occur, because it is less water-soluble than MDY-19 and therefore the flux values of the former peptide across vaginal tissue decrease. At room temperature, the mean steady state flux values for MDY-19 are 1.9x higher, and at 37°C it is 1.03x higher than the corresponding values found for PCI. This may be explained by a higher water solubility of PCI at a higher temperatures, leading to less precipitation on the vaginal tissue, which in turn yields higher flux values.

The average flux values at steady state of 1 mM MDY-19 and 0.75 mM PCI were the highest, and

it would therefore seem that these concentrations are the most suitable for conducting permeability experiments.

In conclusion, we have demonstrated that the de-epithelialisation of the vaginal mucosa increased the permeability of all three peptides tested in the study. This supports the premise that the main epithelial barrier is located in the epithelium and it may have possible implications for increased microbicide as well as HIV uptake *in vivo*. Furthermore, the results of the study have demonstrated the concentration- and temperature dependency of flux rates of peptides across vaginal mucosa. This should be taken into consideration for determining optimum diffusion conditions for *in vitro* permeability studies.

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