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Research paper

Establishment and validation of an *ex vivo* human cervical tissue model for local delivery studies

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Abstract

The objective of this study was to establish and validate an *ex vivo* human cervical tissue model appropriate for transport studies of molecular and especially nucleic acid based drugs. For that purpose conditions had to be established for a standardized tissue handling and preparation following hysterectomy to allow an immediate experimental use of fresh tissue samples. Samples of the ectocervical, endocervical and the transition zone representing the entire cervix organ were characterized in Franz diffusion cells by the determination of the *in vitro* permeation of low and high molecular weight markers (propanolol, mannitol, dextran 4000, 10,000, 20,000 and 40,000 Da). Additionally, the permeability of mannitol and dextran 4000 across fresh and frozen cervical tissue was compared. The apparent permeability coefficients (P_{app}) of the various markers demonstrated (i) that with increasing molecular weight the marker permeability decreases, (ii) an upper permeability limit between 10,000 and 20,000 Da, (iii) no significant difference of the permeability across the three cervical tissue zones, (iv) a statistically significant but effectively small variation of the permeability among different patient samples. A continuous difference of approximately two log values between the P_{app} values of mannitol and dextran 4000 makes them suitable as an internal marker control pair for each biopsy. Moreover, the P_{app} values of both markers across fresh and frozen tissue are comparable. According to the presented data we conclude that the human cervical tissue model has been well characterized and is therefore suitable for local delivery and permeation studies.

Keywords: Cervix; Macromolecules; Morphology; Permeability; Uptake

1. Introduction

Cervical cancer is the second most common female tumor entity worldwide. Every year about 230,000 women die from cervical cancer. Cervical intraepithelial dysplasia (CIN) and cervical cancer are caused by the infection with high risk human papilloma viruses (HPV) [1]. Molecular biological screening on cervical cancer tissue samples shows a HPV-DNA prevalence of up to 99% [2]. It has been documented extensively that the expression of the viral oncoproteins E6 and E7 are key factors for neoplastic transformation and tumor growth [3].

Today the only specific therapy applied for advanced CIN is the conization where the affected tissue is excised by means of hot or cold blade surgery. These procedures bear the risk of postoperative complications such as massive bleeding, infection, cervical stenosis and most importantly of preterm birth [4,5]. Given these complications and the fact that most women who must undergo conization are in their reproductive age there is a high medical need for a conservative anti-viral therapy.

A promising new approach lies in a local therapy of the cervix using therapeutic nucleic acids designed to block gene expression of high risk HPV [6]. Especially one class

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of molecular nucleic acid drugs, the antisense oligonucleotides (ASO), is about to gain more and more clinical relevance as a molecular therapeutic [7–9].

ASOs are short single stranded DNA molecules. The proposed molecular mechanism of action consists in their binding to a target sequence within a mRNA and the formation of a DNA/RNA hybrid. This hybrid is the target of cellular RNase H enzymes, which degrade the RNA and thus abrogate gene expression [10].

There are two basic requirements for a successful local treatment of advanced CIN using ASO therapy. First the ASO drug has to penetrate the interior of the cervical cells and second the ASO drug has to reach the lower layers of the virus infected cervical tissue. To address such questions it is important to understand the barrier properties of the target tissue to which the drug will be applied. Therefore a well-characterized cervical tissue model is the basis for reliable transport studies of cervical drugs and the development of locally acting formulations. Only a few attempts to establish such a tissue model using either excised cervical tissue [11,12] or primary cell cultures [13] have been conducted. The main limitation of these studies is that only one marker compound was used to investigate the tissue permeability without addressing a characterization or validation of the *in vitro* model.

The aim of this study was therefore to establish and validate an *ex vivo* human cervical tissue model for local delivery and permeation studies. For that purpose the apparent permeability coefficients of the marker compounds propanolol, mannitol and dextrans with the molecular weights 4000, 10,000, 20,000 and 40,000 Da were determined [14].

Since the human cervix is composed of two different tissue types and three tissue zones (ectocervix, endocervix and the transition zone), the experiments were performed on all three tissue zones.

The permeability studies were conducted to identify the effect of lipophilicity on the permeation across human cervical tissue, the size selectivity and the upper permeability limit of the human cervical tissue, the variability of the P_{app} values among different patients (interindividual variability) and among biopsies of the same patient (intraindividual variability). Furthermore the permeability of fresh versus frozen tissue was compared to explore the option of tissue banking to ensure a continuous tissue availability for extended studies. Finally, the impact of menopausal status and hormone therapy was addressed and appropriate marker compounds for the use as internal controls in drug permeation studies were defined.

2. Materials and methods

2.1. Study design

In fundamental points of establishing the *ex vivo* model, the OECD guidance document for the application of the principles of GLP to *in vitro* studies [15] was followed validating the herein listed relevant parameters. The validation of the *ex vivo* cervical model did not cover all ECVAM recommendations [16], because the principal goal of these experiments was not to establish a tissue model for the replacement of animal studies. The comprehensive aim was to use this *ex vivo* model to support preclinical lead optimization of locally acting substances at the cervical tissue, including the *in vitro* Absorption, Distribution, Metabolism, Elimination and Toxicology (ADMET) characterization of the active substance as well as the characterization of the corresponding formulation in terms of drug release and cytotoxicity.

2.2. Test compounds

The following test compounds were used: [^{14}C]Mannitol was obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). FITC-Dextran 4000, FITC-Dextran 20,000 and 40,000 and [^{3}H]Propranolol were obtained from Sigma (Taufkirchen, Germany). [^{14}C]Dextran 10,000 was obtained from American radiolabelled chemicals (St. Louis, USA).

2.3. Buffer solutions

In Franz cell diffusion studies for the donor (mucosal) compartment HBSS buffer at a pH of 6.0 and for the acceptor (serosal) compartment HBSS buffer at a pH of 7.4 were used. The pH of both buffer solutions was pH-adjusted using NaOH.

HBSS buffer: $MgSO_4$ (0.812 mM), $CaCl_2$ (0.952 mM), NaCl (136.7 mM), KCl (5.36 mM), Na_2HPO_4 (0.385 mM), K_2HPO_4 (0.441 mM), Glucose (25 mM); additionally for the HBSS buffer pH 6.0: MES (10 mM) and for the HBSS buffer pH 7.4: 10 mM Hepes.

2.4. Scintillation and fluorescent analysis of the test compounds

Radioactive samples were analyzed by scintillation counting. Donor samples (20 μl) at the beginning and at the end of the experiments for mass recovery were filled up to 300 μl with HBSS pH 7.4. The samples were mixed with 500 μl of scintillation cocktail and incubated on a shaker (200 rpm) at room temperature for one hour. Samples were measured for 2 min using a Wallac Microbeta (Perkin-Elmer) scintillation counter. The radioactivity was measured by a [^{3}H] or [^{14}C] specific protocol or by a protocol allowing the parallel measurement of both isotopes (for experiments with [^{3}H]Propranolol and [^{14}C]Mannitol). FITC labelled samples were measured in a Wallac Victor (Perkin-Elmer) fluorescent reader.

2.5. Preparations of human cervix

Freshly excised human cervical tissue was obtained from the Knappschaftskrankenhaus Puettlingen (Germany) on the basis of a positive Ethics Committee vote and after

informed consent. For the permeability studies tissue from 34 women undergoing hysterectomy for benign reasons were used. About 21 women were premenopausal with an age range of 31–49 years and 13 were postmenopausal with an age range of 51–83 years. Only tissue was used, which showed demonstrably no tissue abnormalities. Permeation experiments with the cervical tissues were started approximately 2–3 h after surgery.

Immediately after surgery tissue samples representing the ectocervix, endocervix and the transition zone were cut using a biopsy punch (8 mm diameter), transferred into 0.9% NaCl solutions and put on ice. The transport to the laboratory was performed on ice by a local transport service. In the laboratory any visible mucus was carefully removed using the flat end of a tweezer. To cut slices of approximately 3 mm in thickness the samples were fixed on a flexible mat using two needles. The tissues were then cut using a scissor to a thickness of approximately 3 mm taking care that trimmed tissue pieces still contained a small portion of connective tissue. This guaranteed that none of the target tissue was lost. Subsequent measurements using a micrometer confirmed a thickness of approximately 3 mm (±0.3 mm).

2.6. Permeation experiments with human cervix

The permeability studies were conducted using the static Franz cell system. The Franz cell is a diffusion chamber made of glass comprising an upper (donor) and a lower (acceptor) compartment between which the tissues are clamped. The two halves of the cell are held together by means of a ball-and-socket clamp. The prepared samples were mounted in the Franz diffusion cells resulting in a permeation area of 0.125 cm^2. All cervical biopsies were inserted with the mucosal side oriented upwards. The transport experiments were performed at 37 °C applying a pH gradient to simulate the *in vivo* situation. Therefore the donor compartment was filled with 300 µl transport solution pH 6.0 and the acceptor compartment was filled with 5 ml HBSS buffer pH 7.4. The acceptor medium was continuously stirred at a rate of 400 rpm.

The transport solution was defined as HBSS buffer pH 6.0 containing two marker compounds, respectively. A 40 µM concentration of [^{14}C]Mannitol and [^{3}H]Propanolol was used. The radioactive concentrations of the transport solutions were adjusted to 5–8 Million dpm with unlabelled test compounds. [^{14}C]Dextran 10,000 was used at a concentration of 1.5 µg/ml without the addition of unlabelled Dextran 10,000, whereas the FITC-Dextrans were used at a concentration of 40 mg/ml.

In the marker co-transport studies different concentrations of FITC- and [^{14}C]-labelled dextrans were used. The difference in concentration of [^{14}C]Dextran 10,000 versus the higher concentration of FITC-Dextrans was caused by the different extent of [^{14}C], respectively, FITC-labelling provided by the suppliers. The labelling intensity of FITC-tagged markers was lower than that of the markers with incorporated [^{14}C]. According to the calculation of the apparent coefficient of permeation (see Section 2.7) this coefficient is independent of the applied marker concentrations. Therefore concentrations were used which were appropriate to quantify the markers in each Franz cell compartment.

To allow direct comparison of the different transport experiments performed with fluorescent and radiolabelled test compounds, the permeated marker amounts were expressed as % of the mass of the donor (D_0). The diffusion studies were carried out over a time period of 24 h. During that period and after a preincubation time of 15 min to allow saturation of unspecific binding sites, the acceptor medium was sampled seven times (0.5, 1, 2, 4, 6, 18 and 24 h) in individual volumes of 300 µl and the losses were adjusted with 300 µl HBSS buffer pH 7.4. From these samples 200 µl was immediately transferred into 24-well scintillation dishes for radioactive measurements of [^{3}H]Propanolol, [^{14}C]Mannitol and [^{14}C]Dextran 10,000 and 100 µl in a 96-well plate for fluorescent measurements of the FITC-Dextrans. For experiments with [^{3}H]Propanolol and [^{14}C]Mannitol the entire 300 µl volume was transferred into 24-well scintillation dishes.

2.7. Calculation of permeability

The apparent coefficient of permeation (P_{app}) was calculated according to the formula:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{m_0} \cdot \frac{1}{A} \cdot V_{Donor}$$

where

dQ/dt	is the permeability rate (steady state transport rate) (µg/s). Calculated from the linear regression of at least 3 time points of the concentration vs. time curve
A	is the surface area of exposed tissue (cm^2)
m_0	is the initial mass of test compound in the donor compartment (µg), and
V_{Donor}	is the volume of donor compartment (cm^3)

All calculations were performed using MS Excel 2000. The permeability coefficient in cm/s was calculated only when a linear increase of the acceptor concentrations was observed for at least four time points. Without a linear increase of the acceptor concentrations over time no permeability rates (dQ/dt) and no P_{app} values could be determined.

2.8. Statistical analysis

The statistical analysis of the P_{app} values was based on the log-transformed data, using analysis of variance (ANOVA) and Student's *t*-test. The log-transformed data were closer to a normal distribution than the data on the

original scale. p-Values below 0.05 were considered statistically significant. All statistical calculations were performed with the SAS software Version 8.

3. Results

3.1. Influence of lipophilicity on the permeation across human cervical tissue

To identify the differences in permeation between lipophilic and hydrophilic permeation markers, the transport of the lipophilic [^{3}H]Propanolol ($\log P = 3.6$) and the hydrophilic [^{14}C]Mannitol ($\log P = -3.1$) was studied.

The experiments were performed on 32 biopsies of 8 patients ($n = 2$ for ectocervix and $n = 1$ for both endocervix and transition zone). Both marker compounds were tested simultaneously at the same tissue samples. The representative cumulative transport data of both markers for patient 25/2006 are summarized in Fig. 1 and reveal a continuous increase of [^{14}C]Mannitol in the acceptor medium over the 24-h time course. In contrast, using [^{3}H]Propanolol an intense lag phase was observed. Since low concentrations were found in the acceptor within the first six hours P_{app} values of [^{3}H]Propanolol were calculated only for the steady state period from 6 to 24 h. Nevertheless the hydrophilic [^{14}C]Mannitol and the lipophilic [^{3}H]Propanolol showed a comparable permeation pattern across human cervical tissue (Fig. 1). The permeability of each marker across ectocervical tissue was lower than across endocervical tissue or tissue from the transition zone. These observations are statistically evidenced by the corresponding ANOVA model using the log-transformed P_{app} values of Table 1. The results show no major difference in the P_{app} values between [^{14}C]Mannitol and [^{3}H]Propanolol for all three tissue types ($p > 0.5$). Both markers have significantly smaller P_{app} values on ectocervical tissue ($p < 0.01$). Furthermore the P_{app} values of these markers vary from patient to patient resulting in a significant interindividual variability ($p < 0.01$).

3.2. Size selectivity and upper permeability limit

To study the size selectivity and the upper permeability limit of human cervical tissue the transport of further hydrophilic markers with increasing molecular weight was tested on all three cervical tissues. The markers were arranged in individual pairs, containing a lower and a higher molecular weight compound. The former one was used during the synchronous testing as a reference substance to allow a better comparability of Franz cell diffusion studies with different marker pairs. The marker pairs used were: [^{14}C]Mannitol/FITC-Dextran 4000, FITC-Dextran 4000/[^{14}C]Dextran 10,000, [^{14}C]Mannitol/FITC-Dextran 20,000 and [^{14}C]Mannitol/FITC-Dextran 40,000. The log-transformed P_{app} values of the markers depicted versus the molecular weight in Fig. 2 show that in principle the permeability of human cervical tissue decreases with increasing molecular weight of the marker substance. This was evidenced by pairwise comparison of the P_{app} values of all used marker molecules using a corresponding ANOVA model ($p < 0.0001$). Furthermore the tissue type showed no influence on the marker permeability when considering all markers simultaneously in the ANOVA model ($p > 0.8$). It became obvious that the human cervical tissue cannot discriminate between 4000 and 10,000 Da since the P_{app} values of the respective dextrans revealed no statistically significant difference (ANOVA; $p > 0.8$). The same findings apply also for the permeability coefficients of FITC-Dextrans 20,000 and 40,000, indicating that the upper permeability limit of human cervical tissue lies around 20,000 Da.

3.3. Interindividual variability

For the determination of the interindividual variability the log-transformed P_{app} values of each marker were

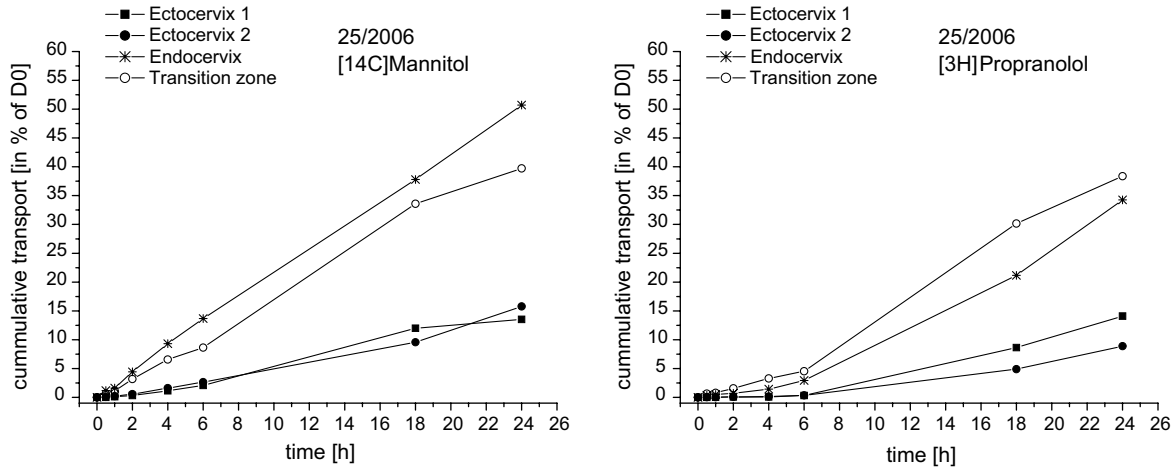

Fig. 1. Representative cumulative transport [% of D_0] of [^{14}C]Mannitol and [^{3}H]Propranolol across cervical tissue of Patient 25/2006 ($n = 1$ for endocervix and transition zone, $n = 2$ for ectocervix).

Table 1
P_{app} values for the transport of [^{14}C]Mannitol and [^{3}H]Propranolol

Patient	Test compound	Ectocervical No. 1	Ectocervical No. 2	Transition zone	Endocervical
P_{app} values (cm/s) $\times 10^{-6}$					
24/2006	[^{14}C]Mannitol	6.58	4.30	6.39	8.35
	[^{3}H]Propranolol	8.19	8.15	8.30	7.98
25/2006	[^{14}C]Mannitol	3.83	3.75	11.00	13.00
	[^{3}H]Propranolol	4.65	2.58	1.21	10.50
29/2006	[^{14}C]Mannitol	10.80	5.94	2.49	10.70
	[^{3}H]Propranolol	5.03	4.57	3.84	9.30
31/2006	[^{14}C]Mannitol	6.51	5.72	8.15	9.09
	[^{3}H]Propranolol	7.25	5.04	8.37	12.50
33/2006	[^{14}C]Mannitol	5.93	7.33	7.64	5.11
	[^{3}H]Propranolol	6.30	11.40	8.82	3.26
35/2006	[^{14}C]Mannitol	8.05	6.89	10.70	13.70
	[^{3}H]Propranolol	8.60	9.13	11.30	10.90
36/2006	[^{14}C]Mannitol	6.29	5.49	8.08	3.61
	[^{3}H]Propranolol	4.61	3.31	5.79	2.16
38/2006	[^{14}C]Mannitol	1.72	1.06	6.25	5.61
	[^{3}H]Propranolol	6.50	5.17	6.13	5.06

P_{app} values for [^{14}C]Mannitol were calculated from 0 to 24 h. P_{app} values for [^{3}H]Propanolol were calculated for the time period from 6 to 24 h only.

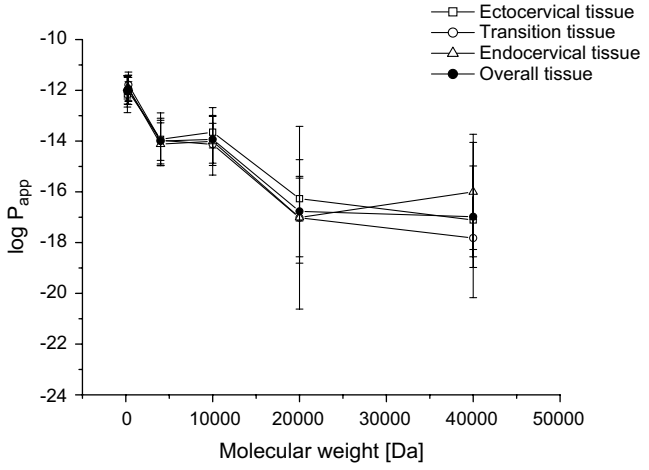

Fig. 2. Log-transformed P_{app} values of [^{14}C]Mannitol, [^{3}H]Propanolol, FITC-Dextran 4000, [^{14}C]Dextran 10,000, FITC-Dextran 20,000 and FITC-Dextran 40,000 versus molecular size. Ectocervical tissue/transition zone/endocervical tissue/overall were based on $n = 40/24/24/88$ biopsies for [^{14}C]Mannitol, $n = 16/8/8/32$ for [^{3}H]Propanolol, $n = 24/16/16/56$ for FITC-Dextran 4000, $n = 6/6/6/18$ for [^{14}C]Dextran 10,000, $n = 3/3/3/9$ for FITC-Dextran 20,000 and $n = 3/3/3/9$ for FITC-Dextran 40,000.

summarized and the descriptive statistics [mean, standard deviation (STD) and absolute coefficient of variation (abs. CV)] were calculated. The data of Table 2 show that a low interindividual variability was detected for each marker size and tissue type. All absolute coefficients of variation are within a low variability range (up to 15%), with increasing values from 3.84% for [^{3}H]Propanolol to 12.36% for FITC-Dextran 40,000. This also reveals that the interindividual variability increases as the marker molecule becomes larger.

Table 2
Coefficient of variation for log-transformed P_{app} values of [^{14}C]Mannitol, [^{3}H]Propanolol, FITC-Dextran 4000, [^{14}C]Dextran 10,000, FITC-Dextran 20,000, FITC-Dextran 40,000

Marker	*N*	Mean	STD	abs. CV
Mannitol	88	−12.0	0.62	5.13
Propanolol	32	−12.0	0.46	3.84
Dextran 4000	56	−14.0	0.90	6.45
Dextran 10,000	18	−13.9	0.94	6.72
Dextran 20,000	9	−16.8	2.04	12.17
Dextran 40,000	9	−17.0	2.10	12.36

3.4. Intraindividual variability

Since two ectocervical biopsies were obtained from 20 patients we were able to assess the intraindividual variability by the difference of the log-transformed P_{app} values of each ectocervical tissue pair. The corresponding P_{app} values were gained in the co-transport studies of [^{3}H]Propanolol/[^{14}C]Mannitol (8 patients with 8 ectocervical tissue pairs) and [^{14}C]Mannitol/FITC-Dextran 4000 (12 patients with 12 ectocervical tissue pairs). The overall mean difference was compared to zero by means of a two-sided *t*-test. Differences near zero are indicative of a good repeatability and a low intraindividual variability. This test revealed no statistically significant differences in the P_{app} values derived

from both cervical samples of each patient ($p > 0.6$), demonstrating a low intraindividual variability.

3.5. Tissue storage – fresh versus frozen

The effect of freezing on the permeability of the co-transported [^{14}C]Mannitol and FITC-Dextran 4000 was tested on tissue samples from four patients comprising all tissue types including two ectocervical biopsies per patient. After a 6 h transport using fresh biopsies, the transport solution from the donor compartment and the acceptor solution from the acceptor compartment were removed. The tissue samples still mounted in the Franz diffusion cells were then frozen at −80 °C. On the following day the tissue was defrosted at 37 °C for one hour. Fresh transport solution containing [^{14}C]Mannitol and FITC-Dextran 4000 was added to the donor compartment and fresh HBSS buffer to the acceptor compartment and the sampling was repeated after 6 h. The descriptive statistics for the log-transformed P_{app} values (Table 3) showed no difference in the permeability of both markers across frozen and fresh tissue. This observation is confirmed by the results of a corresponding ANOVA model ($p > 0.3$).

3.6. Impact of menopausal status and hormone therapy

From the 34 patients tested 21 were pre- and 13 were postmenopausal. To determine the influence of the menopausal status the P_{app} values of the experimental groups [^{3}H]Propranolol/[^{14}C]Mannitol, [^{14}C]Mannitol/FITC-Dextran 4000 and FITC-Dextran 4000/[^{14}C]Dextran 10,000 were statistically evaluated. These groups showed a suitable distribution of patients of both menopausal statuses. The results of an ANOVA model in which the factor patient was replaced by the menopausal status showed a highly significant impact on the permeability for [^{3}H]Propranolol/[^{14}C]Mannitol ($p < 0.001$), a borderline impact for FITC-Dextran 4000/[^{14}C]Dextran 10,000 ($p = 0.0672$) and no impact for [^{14}C]Mannitol/FITC-Dextran 4000 ($p > 0.5$).

Since the menopausal status had a highly significant influence on the permeation of [^{3}H]Propranolol and [^{14}C]Mannitol a more detailed evaluation was conducted on the three tissue types. The data of Table 4 show that the permeability coefficients gained on premenopausal tissue are significantly smaller for both markers ($p < 0.05$) and for all three tissue types ($p < 0.05$) indicating a slower permeation across premenopausal than across postmenopausal tissue. These differences are most pronounced in the ectocervical tissue. Additionally, on basis of the P_{app} values gained in the co-transports of [^{3}H]Propranolol/[^{14}C]Mannitol and FITC-Dextran 4000/[^{14}C]Dextran 10,000 the influence of hormone therapy on the marker permeability was assessed. In the former group two and in the latter group one patient received hormonal therapy. The corresponding ANOVA model revealed that the hormonal therapy had no impact on the permeability of the marker pair [^{3}H]Propanolol/[^{14}C]Mannitol ($p > 0.05$), while the p-value of the marker pair FITC-Dextran 4000/[^{14}C]Dextran 10,000 was close to significance ($p = 0.0625$).

3.7. Marker compounds for internal control

The transport data of [^{14}C]Mannitol and FITC-Dextran 4000 gained during the comparison of fresh versus frozen tissue already indicated a good repeatability. Therefore cumulative transport studies were conducted on tissue of 10 patients using all tissue types to examine the suitability of both markers to act as internal controls for permeation studies. From eight patients pairs of ectocervical samples were available.

The transport data reveal a higher permeability for [^{14}C]Mannitol than for FITC-Dextran 4000 (Table 5). The very large p-value for the tissue types ($p > 0.8$) demonstrates that the tissue type has no impact on the permeability coefficients, demonstrating a very low intraindividual variability. For both markers a continuous difference in the P_{app} values of approximately two log values could be assessed (see Table 5), independently if the data were gained on tissue samples of different patients or on different tissue types of the same patient. Because of this consistency

Table 3
Fresh versus frozen tissue. Descriptive statistics for log-transformed P_{app} values

Tissue	Marker	N	Mean	STD	Min	Med	Max
Ectocervical	Fresh: Mannitol	8	−12.10	0.59	−13.0	−12.1	−11.2
	Frozen: Mannitol	8	−11.88	0.58	−12.8	−11.9	−11.0
	Fresh: Dextran 4000	8	−15.49	1.10	−17.2	−15.3	−14.2
	Frozen: Dextran 4000	8	−14.97	0.73	−16.0	−15.0	−13.8
Transition	Fresh: Mannitol	4	−12.43	0.12	−12.6	−12.4	−12.3
	Frozen: Mannitol	4	−12.11	0.12	−12.3	−12.1	−12.0
	Fresh: Dextran 4000	4	−16.37	0.64	−17.1	−16.3	−15.7
	Frozen: Dextran 4000	4	−15.46	0.33	−15.9	−15.4	−15.2
Endocervical	Fresh: Mannitol	4	−11.94	0.62	−12.7	−12.0	−11.2
	Frozen: Mannitol	4	−11.70	0.44	−12.3	−11.7	−11.2
	Fresh: Dextran 4000	4	−15.42	1.53	−17.2	−15.5	−13.5
	Frozen: Dextran 4000	4	−15.07	0.85	−16.0	−15.2	−13.9

Table 4
Impact of menopausal status (Premenopausal/Postmenopausal)

Marker	Tissue	Menopause	*N*	Mean	STD	Min	Med	Max
Mannitol	Ectocervical	Pre	8	−12.44	0.75	−13.8	−12.3	−11.4
		Post	8	−11.97	0.19	−12.4	−11.9	−11.7
	Transition	Pre	4	−12.01	0.64	−12.9	−11.9	−11.4
		Post	4	−11.73	0.21	−12.0	−11.7	−11.4
	Endocervical	Pre	4	−11.83	0.59	−12.5	−11.8	−11.3
		Post	4	−11.67	0.40	−12.2	−11.7	−11.2
Propanolol	Ectocervical	Pre	8	−12.33	0.29	−12.9	−12.3	−11.9
		Post	8	−11.76	0.25	−12.2	−11.7	−11.4
	Transition	Pre	4	−11.96	0.48	−12.5	−12.0	−11.3
		Post	4	−11.60	0.15	−11.7	−11.7	−11.4
	Endocervical	Pre	4	−12.07	0.72	−13.0	−11.9	−11.5
		Post	4	−11.77	0.60	−12.6	−11.6	−11.3

Descriptive statistics for log-transformed P_{app} values for [^{14}C]Mannitol and [^{3}H]Propanolol.

Table 5
Descriptive statistics for log-transformed P_{app} values for [^{14}C]Mannitol and FITC-Dextran 4000

Tissue	Marker	*N*	Mean	STD	Min	Med	Max
Ectocervical	Mannitol	18	−12.01	0.78	−13.4	−12.0	−9.6
	Dextran 4000	18	−13.97	0.99	−15.2	−14.0	−11.1
Transition	Mannitol	10	−12.01	0.56	−13.2	−12.1	−11.2
	Dextran 4000	10	−13.72	0.63	−15.0	−13.7	−12.9
Endocervical	Mannitol	10	−11.99	0.38	−12.7	−11.9	−11.5
	Dextran 4000	10	−13.91	0.50	−14.7	−13.9	−13.3

[^{14}C]Mannitol and FITC-Dextran 4000 are suitable internal marker molecules.

4. Discussion

The human cervix is an important infection site for pathogens causing sexually transmitted diseases (STDs) like human immunodeficiency virus (HIV) and high risk types of human papilloma virus (HPV). It could be shown that the cervical mucosa is more accessible to HIV than the vaginal tissue, because of the presence of HIV receptor sites [17]. A widely recognized strategy to prevent infection of both viruses is the local application of microbicides to kill virus particles entering the vaginal tract [18–20]. In the case of HPV a local therapeutic strategy is aspired, because the cervical portio represents the viral entry site as well as the area where cervical dysplasia and eventually cervical cancer will develop. Additional arguments for a local therapy are the limited area to be treated and the good accessibility of the target organ. Pilot studies had been done to investigate the penetration characteristics of the chemotherapeutic 5-fluorouracil across cervical tissue released from bioadhesive patches [12]. Topical application of retinoid acid has been reported to reverse CIN II lesions [21].

The fact, that the progression from cervical dysplasia to cervical cancer is closely related to the action of viral oncogene products E6/E7, favors a target specific anti-viral therapy [22]. Nucleic acid based molecular drugs like small interfering RNA (siRNA) and ASOs revealed their potential that the expression of the E6/E7 proteins of high risk and low risk HPV types could be blocked in mice [23,24]. The problem of developing a nucleic acid based therapeutic for the treatment of advanced CIN is that the drug has to enter the virus infected mucosal cells in order to exert its curative effect. In particular polynucleotides are known for their ineffective cellular uptake because of their large size and their hydrophilic and polyanionic character. Furthermore nucleic acids are susceptible to a rapid enzymatic degradation. While the stability of siRNA and ASOs can be improved by modifying the molecule itself, the intracellular localization has to be mediated by a locally acting formulation [25,26]. There are various auxiliary agents having permeation enhancing potential, which could be part of a suitable formulation. But some of them are known to be cytotoxic, especially at higher concentrations [27].

For these reasons we established and validated an *ex vivo* human cervical tissue model to allow the selection of suitable formulations with low cytotoxicity supporting the intracellular uptake of nucleic acid based drugs.

A major concern was to conserve *in vivo* properties of the excised tissue. For that purpose conditions for tissue preparation and tissue transport were adapted to minimize tissue injury and to allow an experimental use in Franz cell diffusion studies within 3 h after surgery. Furthermore in the Franz cell diffusion studies different pH values in the donor and acceptor compartment were used to correspond to the native pH gradient between the vaginal milieu and the interior of the cervical cells.

The human cervical tissue model has been characterized by determination of the *in vitro* permeation of lipophilic and various hydrophilic, low/high molecular marker compounds. This has been done on biopsies of different patients and on biopsies of all cervical tissue types representing the ectocervix, endocervix and the transition zone.

These studies demonstrated that the human cervical tissue is to a large extent size selective, because the marker permeability decreased with increasing molecular weight, with the exception of the dextrans 4000 and 10,000. There was no difference between the diffusibility of both permeants. The same observation was made for porcine buccal mucosa measuring the cumulative flux rates of 4- and 10-kDa dextrans [28]. In contrast to these observations Bijl and Eyk [29] reported an *in vitro* size selectivity for human vaginal and buccal tissue for FITC- dextrans 4.4 kDa and 12 kDa, but similar flux rates for permeants below 4.4 kDa. These controversial results pushed us to establish our own *ex vivo* model and not to rely on other *in vitro* models like vaginal or buccal tissue [30].

The results of the here reported transport studies show that the markers can be arranged in the following series according to a decreasing permeability: [^{3}H]Propanolol $\approx$ [^{14}C]Mannitol $>$ FITC-Dextran 4000 $\approx$ [^{14}C]Dextran 10,000 $>$ FITC-Dextran 20,000 $\approx$ FITC-Dextran 40,000. Additionally, the upper permeability limit can be estimated around 20,000 Da, since no significant statistical difference in the permeabilities for FITC-Dextrans 20,000 and 40,000 was observed. Molecular weight markers higher than 40 kDa were not investigated in the present study, but may exhibit P_{app} values comparable to those of the Dextrans 20,000 and 40,000. This is in concordance with transport studies across porcine buccal mucosa, where the passage of FITC-dextrans was found to be restricted to test compounds lower than 20 kDa [28]. The permeability of all marker substances used in this study was comparable across all biopsies of the three tissue zones. This is remarkable in the light of a significant but low interindividual variability, because the biopsies of one patient can be considered as one tissue type, thus allowing to conduct a higher number of parallel experiments in drug permeation studies. The lack of an intraindividual variability is also confirmed by the fact that the marker transports on the ectocervical sample pairs of 20 patients were highly comparable.

To identify the effect of lipophilicity on the tissue penetrability, the permeation of the lipophilic propanolol and the hydrophilic test compound mannitol was tested. In *in vitro* experiments on gastrointestinal tract tissues propanolol served as a transcellular marker and mannitol as a paracellular marker [31]. Bijl and Eyk [32] compared additional markers on human *in vitro* colon and intestinal tissues versus vaginal tissue and found a more pronounced permeability of the vaginal in comparison to the intestinal tissues. In tissues that form only very few aqueous pores the permeability of mannitol will be always much lower than the permeability of propanolol, since mannitol unlike propanolol cannot permeate through the cells due to its hydrophilic character. However, in our experiments the permeability of mannitol was not lower than the permeability of propanolol. This demonstrates that human cervical tissue samples have a high number of aqueous pores. Moreover propanolol was found to have an extensive lag phase, meaning that in comparison to mannitol propanolol could be detected only after six hours in the acceptor compartment. That difference may be explained by the different solubility of the test compounds in the tissues. Because of the lipophilic nature propanolol might be adsorbed by the tissue and thus a linear increase in the acceptor compartment will be observed only after the tissue is saturated with propanolol. In contrast, mannitol was not bound by the tissue and therefore did not show a lag phase. The experiments with propanolol and mannitol indicate that differences in hydrophilicity/lipophilicity of markers with similar molecular weight have no impact on the transport rates in our *ex vivo* model. Therefore FITC-labelling of dextrans should not alter their diffusibility in our model. This is supported by the fact that the $\log P$ value of FITC-Dextran 4.4 kDa (-2.0) and the $\log P$ value of glucose (-2.82) representing the monomer of unlabelled dextrans are in the same order of magnitude [33,34].

The portio cervicalis has only a small size. This limits the number of tissue samples, which can be obtained from one patient. For larger test series specimens of different patients have to be included. To make sure that experimental results based on different patient biopsies were comparable with each other, an important aim of our *in vitro* permeation studies was to find marker substances showing reproducible transport characteristics. The comparison of the P_{app} values of [^{14}C]Mannitol gained on 88 biopsies and of FITC-Dextran 4000 gained on 56 biopsies showed a remarkable reproducibility with a CV of 5.1% for mannitol and a CV of 6.4% for dextran 4000. Furthermore a constant P_{app} value difference of approximately 2log values could be detected independently if the transport studies were conducted on samples of different patients or on biopsies of the same patient. This 2log difference was evidenced also in transport studies, where both markers had been used simultaneously. Because of this consistency and the fact that both compounds revealed no lag phase we conclude that [^{14}C]Mannitol and FITC-Dextran 4000 are suitable internal marker molecules.

The influence of the menopausal status on the marker permeability was difficult to assess precisely. The corresponding ANOVA results indicate that there is a highly significant impact on the P_{app} values for [^{3}H]Propanolol and [^{14}C]Mannitol, a borderline impact for FITC-Dextran 4000 and [^{14}C]Dextran 10,000 and no impact for [^{14}C]Mannitol and FITC-Dextran 4000. A more detailed statistical evaluation revealed smaller P_{app} values for [^{3}H]Propanolol and [^{14}C]Mannitol in premenopausal tissue and for all three tissue types, indicating a slower permeation through pre- than through postmenopausal tissue. Further statistical analysis of our data shows that the permeability differ-

ence between pre- and postmenopausal tissue is more marked in ectocervical tissue. This could be explained by the fact that the ectocervix is a multilayered stratified squamous epithelium, while the endocervix is described as a single layer of mucin-secreting columnar epithelium. Therefore ectocervical tissue may have a stronger barrier function, where differences of marker permeability become more obvious.

Similar marker pair depending results were seen in tissues from patients known to have received hormonal therapy prior to hysterectomy. While hormonal treatment has no impact on the permeability of [^{3}H]Propanolol and [^{14}C]Mannitol, the influence on the transport of FITC-Dextran 4000 and [^{14}C]Dextran 10,000 was close to significance.

One of the main difficulties of drug permeation studies on cervical tissues is that fresh tissues from surgical interventions cannot be obtained on a regular basis. An alternative route would be tissue banking, where patient samples are frozen down and stored for later use. This would greatly improve the conduct of a higher number of parallel experiments under the same conditions. For that purpose the effect of freezing on the permeability of a higher (FITC-Dextran 4000) and a low molecular weight marker ([^{14}C]Mannitol) was studied. The permeation data show very similar permeability coefficients for the transports across fresh and frozen tissue for both markers. For most transport experiments the permeation across frozen tissue was found to be only slightly higher than the permeation across fresh tissue. Statistical evaluation of the P_{app} values before and after freezing reveals that there is no significant difference in the permeability coefficients with respect to fresh or frozen tissue. Therefore we conclude that frozen cervical tissue can be used when fresh tissue is not available.

5. Conclusion

To our knowledge this was the first time that a human *ex vivo* cervical model was established and validated in a wide range for permeability studies. The results showed that [^{14}C]Mannitol and FITC-Dextran 4000 can be used as internal marker molecules, because they exhibit a consistent and distinct difference in their P_{app} values with a low interindividual variability. Furthermore, the fact that the permeability was similar in all three tissue types and the verification that fresh and frozen tissue led to highly comparable permeability rates will facilitate the conduct of larger studies. Future investigations will focus on the intracellular uptake of molecular drugs into the cervical tissue. This approach assumes a viability of the *ex vivo* model and the development of further methods to localize and quantify the compounds inside/outside the cervical cells.

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