

Aus der Klinik für Allgemein-, Visceral-, und Transplantationsmedizin  
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

## DISSERTATION

# **The direct and systemic influence of immunosuppressive drugs on intestinal glucose absorption, barrier function and chloride secretion in rat models**

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# 1 Introduction

Organ transplantation is nowadays the optimal treatment for patients suffering from end-stage organ failure. Acute cellular rejection is still a big problem in the transplantation field. Apart from antibodies which are currently used as induction or anti acute rejection therapy, immunosuppressive drugs (ISD) are the first line medicaments which allow for successful organ transplantations (1). In the mid-1980s and early 1990s, only corticosteroids in combination with calcineurin inhibitor Cyclosporine A (CyA) and antimetabolite azathioprine were offered on the market. During the past few years some new ISD were approved by the Federal Drug Administration. Microemulsion form of CyA (Neoral®), tacrolimus (TAC, Prograf®), mycophenolate mofetil (MMF, CellCept®), enteric-coated mycophenolate-Na<sup>+</sup> (EC-MPA, Myfortic®), rapamycin (Sir, Sirolimus®) and everolimus (Eve, Certican®) are now widely used in organ transplantation (1, 2). The ISD market is presently still developing new agents, of which the fingolimod (FTY720) is already in phase 2 of the clinical trials (3).

ISD therapy has a wide spectrum of side effects. Among the most common are gastrointestinal side effects, like for example diarrhea. The impact of diarrhea on the transplant recipient can be significant, resulting in dehydration and patient discomfort (4). In general, pathomechanisms of diarrhea can be divided into five groups (Table 1, Page: 8) (5-7).

<b>Diarrhea type</b>	<b>Mechanism</b>
motility disorder-dependent diarrhea	- hypermotility: i.e. hyperthyroidism - hypomotility: i.e. hypothyroidism
malabsorptive diarrhea	malabsorption of nourishment: i.e. glucose
osmotic diarrhea	lack in absorption mechanisms for i.e. lactulose or mannitol
secretory diarrhea	increased Cl <sup>-</sup> secretion by i.e.: E. coli enterotoxin
leak-flux diarrhea	increased intestinal barrier permeability by i.e.: Vibrio cholera

Table 1: Possible diarrhea types and their mechanisms

- (i) Motility disorder diarrhea can take place in a hyper- or hypomotility situation. Hypermotility can be caused for example by hyperthyroidism and leads to a reduced contact time between nourishment and the bowel absorptive area. Hypomotility (i.e. post operative hypomotility, hypothyroidism) on the other hand leads to the prolonged presence of the nutriment in the bowel lumen, causing bacterial overgrowth followed by diarrhea.
- (ii) Malabsorptive diarrhea is caused by solutes, which have not been absorbed in the bowel. Malabsorption of glucose, galactose or tropical sprue are examples of this disorder.
- (iii) Some of the authors see osmotic DIA, which takes place due to a priori absent transport mechanisms for definite substances (lactulose or mannitol), as a separate mechanism (5).
- (iv) Secretory diarrhea is caused by increased net Cl<sup>-</sup> secretion into the bowel lumen. This is due to cAMP, cGMP, PKC or Ca<sup>2+</sup>-dependent activation of a Cl<sup>-</sup> channels and/or inhibition of the Na<sup>+</sup> and Cl<sup>-</sup> resorption in the apical membrane of enterocytes (Figure 1, Page 19). This mechanism is activated for example by different enterotoxins produced by E. coli.
- (v) Leak-flux diarrhea occurs due to a defect of the intestinal barrier function and increased permeability of the intestinal mucosa to small or big molecular solutes (8). *Shigella flexneri*, *Clostridium* spp. or *Vibrio cholerae* induce through their toxins alterations of the tight junction complex and lead to a massive loss of water and solute (9).



Management of diarrhea after transplantation depends on its etiology. There are few factors which have the potential to alter the intestinal physiology in transplanted patients. One of them is an increased risk of infection (e.g. with *C. difficile* or CMV), or graft versus host diseases, post transplantation lymphoproliferative diseases, inflammatory bowel disease, colon cancer and ISD therapy (10). Immunosuppressive drugs and infections are thought to be one of the most common reasons of DIA in transplanted patients (10), however there are numerous other factors that can affect the reported incidence (no standardized questionnaire or standardized recorded histories obtained for DIA events, impact of multiple concomitant medications, ethnicity, transplanted organ type and many others)(4). The incidence of ISD associated diarrhea has been summarized in the Table 2, Page 11. Treatment of DIA in patients receiving ISD is often maintained by dose reduction or withdrawal. It is however known that a reduced dose in some immunosuppression regimens increases the risk of graft loss (4, 11, 12). This has a significant detrimental effect not only on the outcome, but also on the costs of the treatment (13).

<b>ISD</b>	<b>author</b>	<b>group size</b>	<b>application route</b>	<b>study design (drug dose, transplanted organ or disease, study duration)</b>	<b>diarrhea Incidence (%)</b>
<b>CyA</b>	*	266	orally	250-400 ng/ml, orally, liver, 1 year (14)	47
	Pirsch et al.	207	orally	150-400 ng/ml for the first 3 months and 100-300 ng/ml afterwards, kidney, 1 year (15)	40.6
	Levy et al.	251	orally	C <sub>2</sub> level within the target range of 0.8 to 1.2 µg/mL till month 3, and 0.7 to 0.9 µg/mL afterwards, orally, liver, 6 months (16)	14
<b>TAC</b>	Pirsch et al.	205	orally	10-25 ng/ml for the first 3 months and 5-15 ng/ml thereafter, kidney, 1 year (15)	43.9
	*	236	orally	0.2-5 ng p/ml, orally, liver, 1 year (14)	72
	Levy et al.	248	orally	C <sub>0</sub> in the range of 5 to 15 ng/mL till month 3, 5 to 12 ng/mL afterwards, orally, liver, 6 months (16)	29
<b>MMF</b>	Cantarovich et al.	19	orally	1g twice daily , orally, liver, 12 months (one year after transplantation) (17)	18
	Pfitzmann et al.	191	orally	1-2g twice daily, orally, liver, 4 months (18)	24
	Rangel et al.	105	orally	1g twice daily, orally, kidney, (?) (19)	79.2
	Darji et al.	118	orally	500-3000 mg, orally, kidney, (?) (20)	31.4
	Kamar et al.	93	orally	500 mg twice daily, orally, kidney, one year (21)	19.3
<b>EC-MPA</b>	Sumethkul et al.	12	orally	720 mg once daily, orally, kidney, 3-8 months (22)	15
	Darji et al.	118	orally	360-2160 mg, orally, kidney, 3-6 weeks after conversion from CellCept ®, therapy (20)	20.1
	Kamar et al.	37	orally	720 mg once daily, orally, kidney, one year (21)	13.5
	Rangel et al.	60	orally	720 mg twice daily, orally, kidney, (?) (19)	62.3
	<b>SIR</b>	Fairbanks et al.	21	orally	9-12 ng/dl, orally, liver, 64 weeks (23)
Bissler et al.		18	orally	1-5 ng/ml, orally, patients suffering from tuberous sclerosis complex or sporadic lymphangiomyomatosis (not transplanted), 24 months (24)	38
Moro et al.		14	orally	(?), orally, heart, 595 days median follow-up (25)	14.3
<b>EVE</b>	Moro et al.	42	orally	(?), orally, heart, 351 days median follow-up (25)	2,4
	Yao et al.	67	orally	5 or 10 mg/day, patients suffering from low- to intermediate-grade neuroendocrine tumors (not transplanted) (26)	11
	Yee et al.	27	orally	5 or 10 mg/day, patients with different type of leukemia (not transplanted) (27)	33
<b>FTY 720</b>	Kappos et al.	184	orally	1,25 or 5 mg/day, patients suffering from multiple sclerosis (not transplanted), 12 months (28)	10-12

**Table 2: Incidence of immunosuppressive drugs (ISD) associated diarrhea in humans: review of the literature**

(\* The U.S. Multicenter FK506 Liver Study Group).

## **1.1 Immunosuppressive drugs and their side effects**

### **1.1.1 Calcineurin inhibitors**

CyA and TAC are currently the only licensed and widely used drugs of this group. Both drugs possess a wide spectrum of side effects (for example: nephrotoxicity, neurotoxicity and metabolic disorders) (2). Gastrointestinal side effects do not occur as often with the CyA therapy as with the TAC therapy, where 72% of the cases have diarrhea (liver transplant recipients (14)). Cyclosporine was already investigated for its influence on the intestinal transport (29, 30), and Tacrolimus is known for altering the small intestine barrier function (31, 32). Because both of the drugs are often used in the clinical practice, they were included in the study to explain their mechanism of influence on the small bowel.

### **1.1.2 Mycophenolic acid**

Currently two forms of mycophenolic acid (MPA) are available on the market. We analyzed both (MMF and EC-MPA), although they do not differ much in their intestinal side effects (21). MPA is often the cause of diarrhea, nausea, vomiting and abdominal pain, marrow suppression, and so on (2). Its gastrointestinal influence is often a reason for the discontinuation of the MPA therapy. Still the pathophysiology of gastrointestinal disorders after MPA therapy has not yet been clearly explained. Morphological alterations in the small bowel architecture are suspected to be responsible for the post MPA-caused diarrhea (33), but the primary mechanism is still not known. MPA therapy discontinuation, or dose reduction are nowadays the only known treatment options.

### **1.1.3 Mammalian target of rapamycin inhibitors**

Rapamycin and its chemical modification Everolimus belong to the group of mammalian target of rapamycin (mTOR) inhibitors. They both have similar metabolic, hematologic and dermatologic side effects (2). They also both influence the gastrointestinal tract function (34, 35). Sirolimus is suspected of influencing glucose absorption, small intestine barrier function and small bowel morphology. Still nothing is known about the pathophysiology of gastrointestinal disorders caused by everolimus.

#### **1.1.4 FTY720**

FTY720 is a new immunosuppressive agent. It is an analogue of myriocin, a product of ascomycete *Isaria sinclari* (2). It has a wide spectrum of adverse effects and its influence on the gastrointestinal tract is probably comparable to that of MPA (36). FTY720 being a very promising and strong immunosuppressant, was included in the study since no adequate information of its obviously potent influence on the intestinal physiology exists to date (28).

## **1.2 Aim of the study**

The aim was to study the pathomechanism of ISD associated diarrhea.

For the reasons mentioned in the "Introduction", it is of great importance to distinguish between ISD therapy associated DIA and DIA caused by bacterial overgrowth. The differentiation of those two pathomechanisms has a fundamental meaning for the accurate treatment.

The study should answer the following questions in detail:

- Do ISD have a direct influence on the small bowel barrier and transport function?
- Does the 14 days of oral treatment with ISD have an influence on the small bowel and colon barrier and transport function?
- Are those effects dose-dependent?

Using the following experimental setup, a wide spectrum of possible ISD-associated intestine alterations should be characterized. Results of this study should improve the understanding of pathomechanisms of ISD associated diarrhea.

## 2 Materials and Methods

### 2.1 Experimental animals

Male Wistar rats were used as experimental animals, (delivered by the Bundesinstitut für Risikobewertung, Berlin, Deutschland). Rats were included in the experiment 10 days after being delivered. Two to five animals weighing between 280 and 350g were placed in standard cages. Standard rat fodder (V1536-000 sniff R/M-H, Extrudiert, sniff Spezialdiäten GmbH) and water were allowed ad libitum. Principles of laboratory animal care and the current version of the German law on the protection of animals were applied in all experiments (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, Record Number: T 0133/01 from 28.06.2001 and G 0264/03 from 12.02.2004)

### 2.2 Buffers, substances and drugs

#### 2.2.1 Medium used for Ussing chamber experiments

In all Ussing chamber experiments, buffer developed by Schulzke et al. (37) (standard medium, SM) was used.

The water solution of the following substances (in mmol/l) was used as a standard medium:

Na <sup>+</sup>	140,0
Cl <sup>-</sup>	123,8
K <sup>+</sup>	5,4
Ca <sup>2+</sup>	1,2
Mg <sup>2+</sup>	1,2
HPO <sub>4</sub> <sup>2-</sup>	2,4
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	0,6
HCO <sub>3</sub> <sup>-</sup>	21,0

For part of the experiments SM was enriched by (standard medium enriched, SME):

D(+)- Glucose	10,0
B-OH-Butyrate	10,0
Glutamine	0,5
D(+)-Mannose	2,5
Lactulose	200,0

Additionally, 50 mg/l Tobramycin (Brulamycin®) was added to prevent physiological intestinal flora overgrowth (SME only). Both SM and SME were gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, their temperature was kept at 37°C and pH at 7,3.

## **2.2.2 Substances**

Standard laboratory substances were used for all analyses. If not otherwise mentioned, all solutions were made with SM as dissolvent.

### ***2.2.2.1 Immunosuppressive drugs***

Forms of ISD available on the market were used for the experiments:

#### ***2.2.2.1.1 Cyclosporine A***

Optoral (Sandimmun®, 10 mg Kapseln, Novartis Pharma)

#### ***2.2.2.1.2 Tacrolimus***

Prograf® (5 mg Kapseln, Fujisawa Deutschland GmbH)

#### ***2.2.2.1.3 MMF***



CellCept® (250 mg Kapseln., Roche Registration Ltd.)

#### **2.2.2.1.4 EC-MPA**

Myfortic® (powder delivered by producer, Novartis Pharma)

#### **2.2.2.1.5 Sirolimus**

Rapamune® (1 mg, Wyeth Pharma Deutschland)

#### **2.2.2.1.6 Everolimus**

Certican® (0,25 mg Tabletten, Novartis Pharma)

#### **2.2.2.1.7 FTY 720**

FTY720·HCl powder (PKF117-812) from Novartis Pharma was used.

### **2.2.2.2 Bumetanide**

Bumetanide (Sigma, St. Louis, MO, USA) is a member of the so-called loop diuretics. It inhibits an  $\text{Na}^+\text{K}^+2\text{Cl}^-$ -cotransporter (NKCC) in a basolateral membrane of the enterocyte (Figure 1, Page 19). Bumetanide decreases intracellular chloride accumulation and as a result also the electrogenic chloride transport. This effect can be measured by a decrease in short circuit current ( $I_{sc}$ ) value and can be shown as a delta value ( $\Delta\text{NKCC}$ ). Bumetanide was added in a concentration of  $10^{-5}$  mol/l into the serosal side of the Ussing chamber.

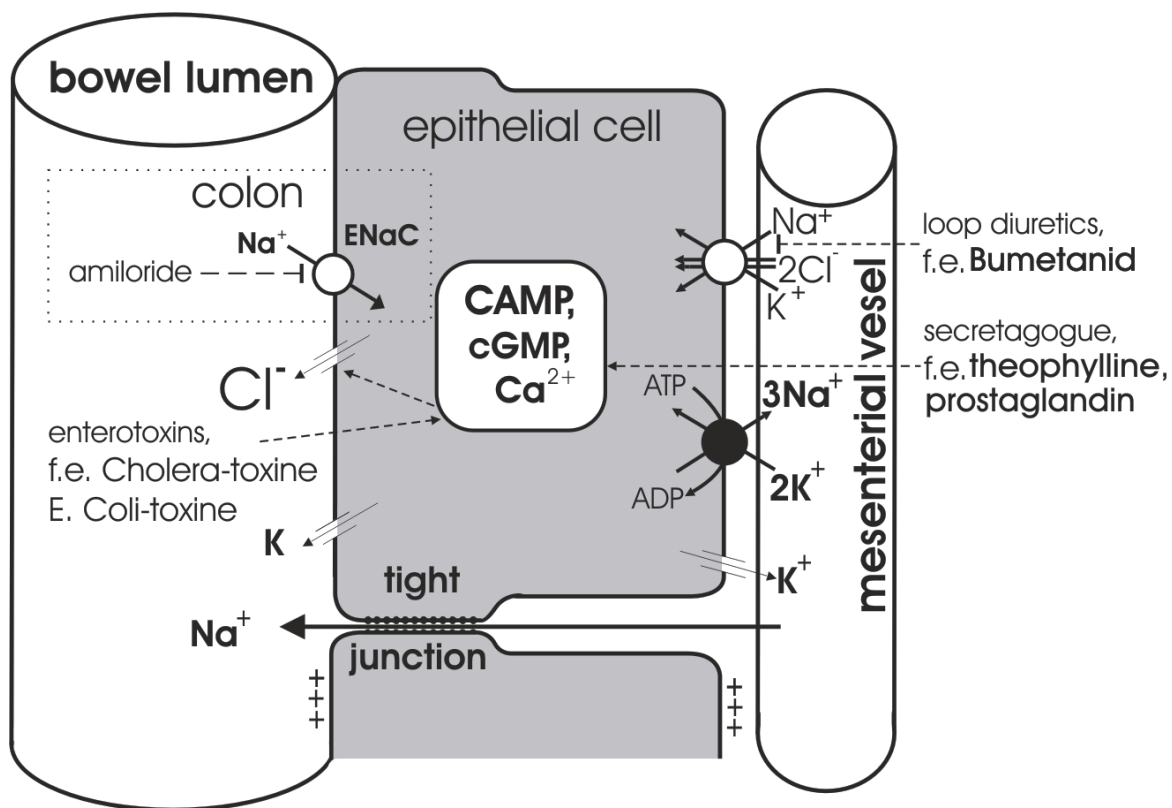
### **2.2.2.3 Theophylline**

Theophylline (Sigma, St. Louis, MO, USA) is a commonly used myorelaxant (for example in bronchial asthma), and works as a dose-dependent inhibitor of intracellular phosphodiesterase. Phosphodiesterase inhibition leads to an increase of the intracellular cyclic adenine mono phosphate (cAMP) concentration, and through a second messenger increases epithelial chloride secretion (Figure 1, page 19). This effect can be measured as increase of the  $I_{sc}$  ( $\Delta\text{cAMP}$ , effect of theophylline given together with prostaglandin  $\text{E}_2$ ).

Theophylline was added in a concentration of  $10^{-2}$  mol/l into both serosal and mucosal sides of the chamber (38).

#### ***2.2.2.4 Prostaglandin E<sub>2</sub>***

Prostaglandin E<sub>2</sub> (PgE<sub>2</sub>) is a metabolite of a cyclooxygenase-dependent arachidonic acid metabolic pathway. PgE<sub>2</sub> works by increasing the intracellular cAMP concentration, which also leads to the up-regulation of the chloride secretion (Figure 1: **Model of electrogenic chloride secretion in the intestinal epithelium and the colonic sodium absorption** page 19) Prostaglandin E<sub>2</sub> (Fluka Chemie GmbH, CH-9471 Buchs) in a concentration of  $10^{-6}$  mol/l (diluted in dimethyl sulfoxide) was added to the serosal side of the chamber (38).



**Figure 1: Model of electrogenic chloride secretion in the intestinal epithelium and the colonic sodium absorption**

Bumetanide inhibits chloride secretion of the epithelial cell by blocking the Na<sup>+</sup>K<sup>+</sup>2Cl<sup>-</sup> basolateral co-transporter. Theophylline and Prostaglandin E<sub>2</sub> activate chloride secretion in a cAMP-dependent manner. Sodium is absorbed in the late distal colon via the ENaC channel, which is specifically inhibited by the amiloride. The figure was based on the work of Hegel et al (39).

### 2.2.2.5 3-O-Methyl-D-glucopyranose

3-O-Methyl-D-glucopyranose (3-OMG) is a non-metabolized glucose analogue. It can be transported by the enterocyte through the Na<sup>+</sup>-glucose co-transporter SGLT1 (SGLT1) and glucose transporter GLUT2 (GLUT2) (placed in the apical and basolateral cell membrane respectively), (Figure 2, Page 21). This transport is electrogenic and can be measured by the increase of an I<sub>sc</sub>. 3-OMG was added to both the serosal and mucosal side of the chamber in different concentrations as explained later.

### 2.2.2.6 Phloridzin

Phloridzin is a specific inhibitor of the SGLT1 (Figure 2, Page 21). For one glucose particle there are always two ions of Na<sup>+</sup> transported, and therefore the phloridzin inhibition effect on the glucose transport can be measured as a change of an electrogenic transport ( $\Delta I_{sc}$ ). Knowing the stoichiometry of the SGLT1 co-transport, one can calculate the exact amount of the absorbed glucose. Together with the Faraday constant (the electric charge amount of one ion,  $F=96450 \text{ C}\cdot\text{mol}^{-1}$ ) it is possible to recalculate the density of the current (I<sub>sc</sub>) to the density of the substance flow:

$$J_{\text{glucose}} = \frac{\Delta I_{sc} \cdot 3600}{2 \cdot F} [\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-1}]$$

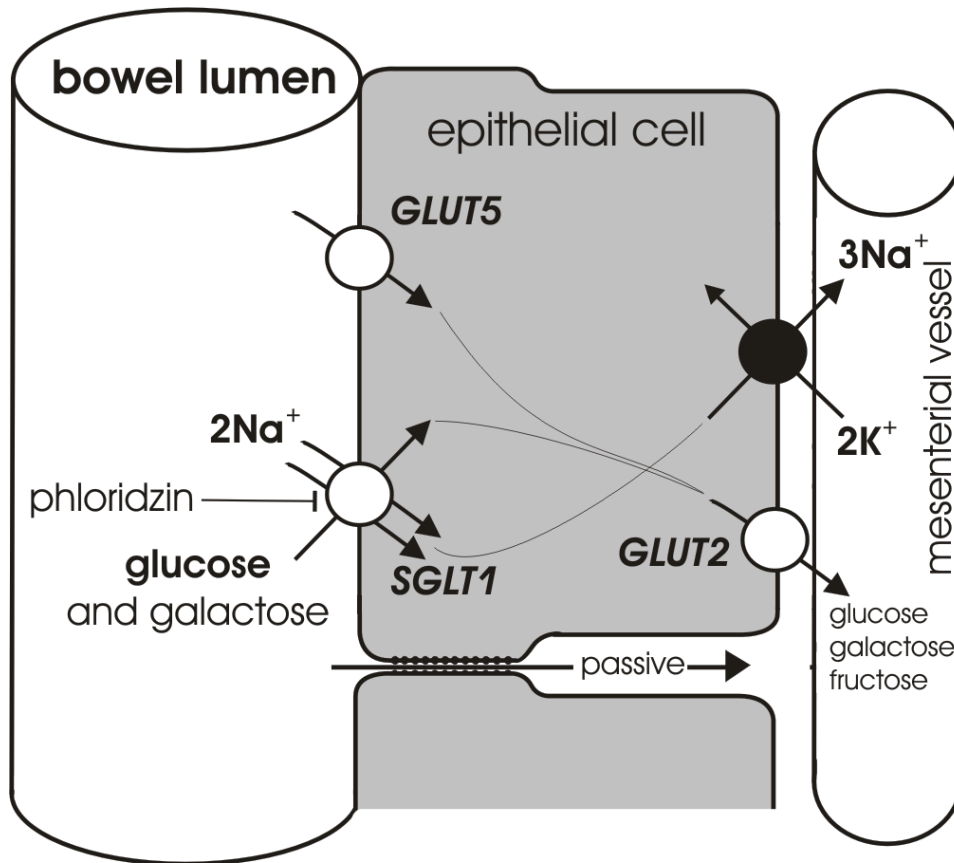
where:

$J_{\text{glucose}}$  is amount of transported glucose

$\Delta I_{sc}$  is difference of the short circuit current

F is Faraday constant

Phloridzin (Sigma, St. Louis, MO, USA) was added in a concentration of  $5 \cdot 10^{-4} \text{ M}$  only to the mucosal chamber side.



**Figure 2: Model of the secondary active glucose absorption by the enterocyte**

Glucose together with two  $\text{Na}^+$  ions (creating necessary gradient) is transported by SGLT1 co-transporter into the enterocyte, and afterwards by GLUT2 into the blood. Phloridzin is a specific inhibitor of SGLT1. Because of the electrogenic specificity of  $\text{Na}^+$ -glucose co-transport, Phloridzin inhibition effect results in a decrease of the recorded  $I_{sc}$  ( $\Delta I_{sc}$ ). The figure is based on the work of Hierzholer and Fromm (40)

### **2.2.2.7 *<sup>3</sup>H-Lactulose***

Lactulose is a disaccharide used as an intestinal permeability marker in vivo (8). It permeates the epithelium specifically through the paracellular way and therefore is a very good marker for big molecule intestinal permeability (8). To assess <sup>3</sup>H-Lactulose flux ( $J_{\text{Lac}}$ ), lactulose marked with isotopes of hydrogen (D-[galactose-6-<sup>3</sup>H], 20 Ci/mmol, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St Louis, MOI, 63146, USA) was used.

### **2.2.2.8 *Amiloride***

Amiloride is a highly specific inhibitor of the sodium/potassium co-transport through the Epithelial sodium (natrium) channel (ENaC) (see Figure 1, Page 19) (41). It was often used to measure ENaC channel function in the Ussing chamber setup (41, 42). The sodium/potassium transport is electrogenic and its decline can be measured by a decrease of the  $I_{\text{sc}}$  value. Amiloride was added to the mucosal and serosal side of the chamber in concentrations of ( $10^{-4}$  mol/l).

## 2.3 Experiments

### 2.3.1 Ussing chamber technique principle

#### 2.3.1.1 Preliminary note

Electrophysiological transport measurements using the Ussing chamber method is well-known and widely used since 1951 (43). To get up an objective transepithelial transport and barrier measuring system, one has to pay attention to the following influencing external forces: hydrostatic pressure, concentration gradient, and transepithelial potential difference. These three forces have to be equalized to allow the measuring of both the passive and active transepithelial transport independently. The Ussing chamber technique fulfills all these conditions. Hydrostatic pressure and concentration gradient are equalized by filling both chambers to the same level (volume) with the same buffer. Through the application of a short circuit current ( $I_{sc}$ ), the spontaneous transepithelial potential difference ( $U_e$ ) is short circuited at 0 mV, therefore the electrical transepithelial gradient does not exist anymore.  $I_{sc}$  is in this situation equal to the net amount of all active electrogenic epithelial ion transports. However, it is not possible to distinguish between the contributions of particular ion types. To do so, the activation, inhibition or flux measurements are needed.

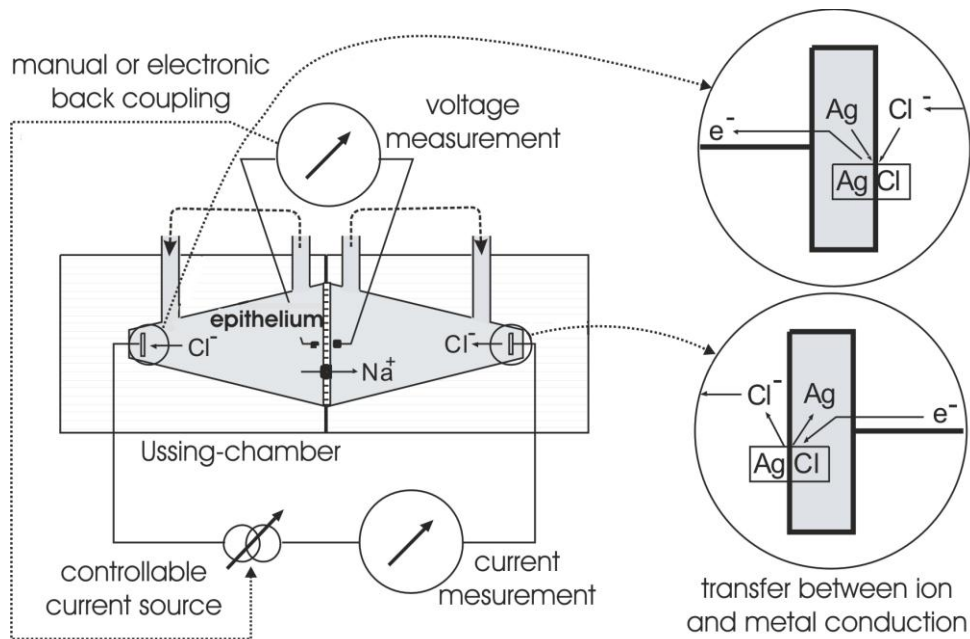
#### 2.3.1.2 Electrical measurement setup

As mentioned above, Ussing et al. described in 1951 a four electrode setup for measuring the transepithelial electrogenic transport (Figure 3: **Principle of a measuring setup in the Ussing chamber**

, Page: 25). The two chambers filled with buffer are separated by the “membrane” made of tensed intestine wall which was glued onto the plastic ring. Apart from the intact epithelium, there is no connection here (ions, water and any other substance or current flow) between the two chambers. The “voltage electrode” ( $U_e$ ) endings (endings of the agar bridges, which connect electrodes with buffer filling the chamber) are placed as close as possible to the epithelium (1-2 mm), which is important because of the influenced  $I_{sc}$  and  $U_e$  fields in the short circuit situation. The fluid layer between electrode spike and epithelium is the reason of an unwanted decline of the potential difference and therefore has to be kept as thin as possible (39).

Two other electrodes inlets are placed as far away from the epithelium as possible. The necessary current now flows through the “current electrodes” and short circuits both epithelial sides at 0 mV. The longest possible distance allows for a nearly equal dispersion of the electrical field in the epithelium. Specific electrode types will be described later on. The overall measurement and short circuit procedure is possible through a specially constructed device (Type CVC8; Fiebig, Berlin), which was already used for similar experiments (44) since it has all the required functions. The control as well as the data acquisition was done on a standard PC with a special measurement program (Fiebig, Berlin).





**Figure 3: Principle of a measuring setup in the Ussing chamber**

The epithelium was tensed and placed into the Ussing chamber to separate the two chambers filled with buffer from each other. Voltage ( $U_e$ ) was measured between “voltage electrodes”, and a short circuit current ( $I_{sc}$ ) was applied to “clamp” the electrodes at 0 V (see Paragraph 2.3.1.1, Page 23). Transfer from ion to electrical conductivity (from liquid to metal) was possible by using Ag/AgCl<sup>-</sup> electrodes. The figure is based on the work of Hegel et al (39).

### **2.3.1.3 Warm-up exchanger, gassing and circulation**

A complex glass device (science workshops at UKBF, Berlin) labeled as “warm-up exchanger” (

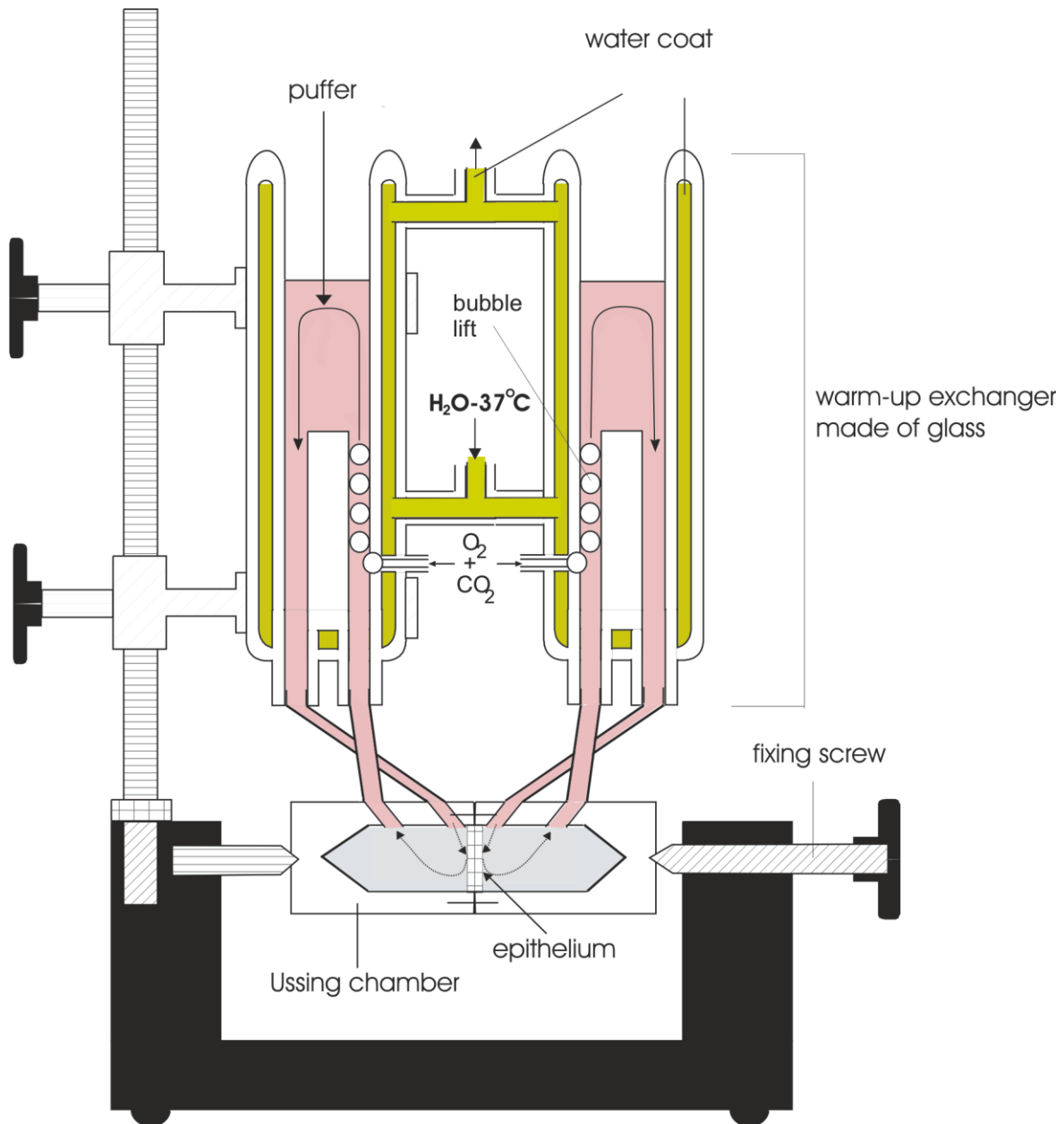
Figure 4: Ussing-chambers with connected warm-up exchanger and gassing system **(bubble lift)**.

was used to secured the heating, gassing and circulation of the buffer which fills up the Ussing chamber. It consists of two double-walls and cylinder shape solution reservoirs, which keeps the temperature constant at  $37 \pm 0.2$  C°. This device is connected to a water bath (P5, Haake, Berlin) with pumps, which ensures a steady flow between the spaces of the double-walls, thus keeping the temperature of the fill-in solution constant. Each solution reservoir is connected by two thin elastic tubes to the respective container side.

Gassing was ensured by a 20G needle connected to the channels (extension of the above mentioned thin elastic tubes) which supply the device with gas (95%O<sub>2</sub> and 5%CO<sub>2</sub>, flow- 8 l/hour). The gas leaving the needle tips in the channels creates bubbles, which carry some of the chamber solution in an upward direction. In the second chamber half, the solution was passively moved in a contrary direction. The fill-in solution stream continuously flushes the epithelium area and mixes the content of the reservoir. This mechanism is called “bubble lift” (

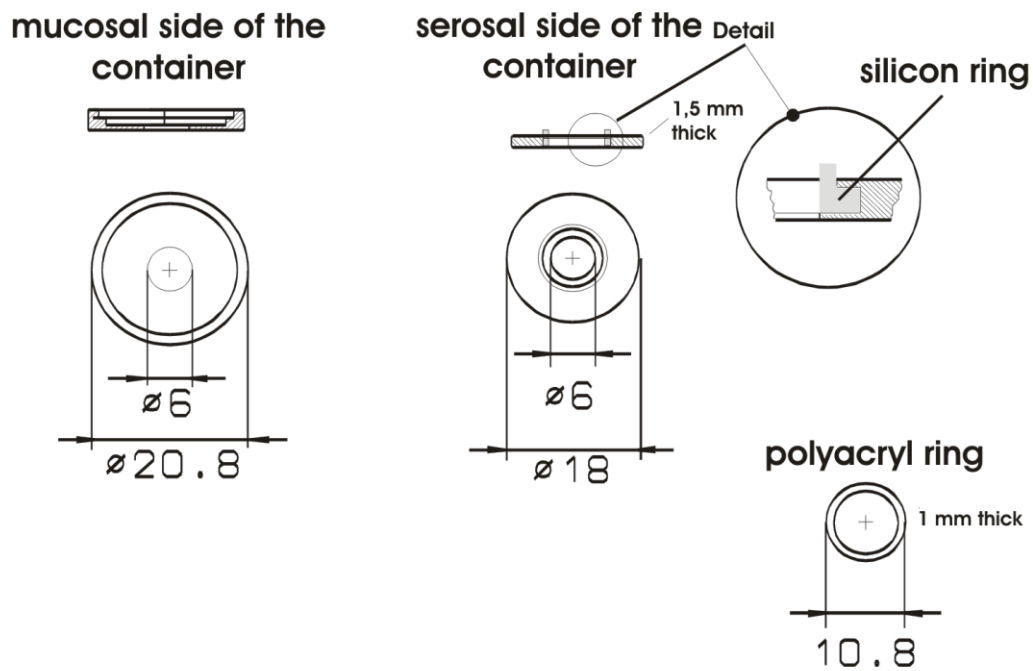
Figure 4: Ussing-chambers with connected warm-up exchanger and gassing system **(bubble lift)**.

and assures not only good gassing and mixing, but also keeps the pH of the solution at a stable level. The stream which is continuously flushing the epithelium removes all obstacles (mucus, small gas bubbles) from its surface, ensuring good tissue conductivity.



**Figure 4: Ussing-chambers with connected warm-up exchanger and gassing system (bubble lift).**

After mounting the epithelium between the two halves of the Ussing chamber, they are held in place with a screw and then connected to the warm-up exchanger by silicon tubes. The circulating buffer was heated up to 37°C by circulating in the warm-up exchanger hot water, and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Bubble lift secured the continuous mixing of the buffer in the chamber. Buffer stream leaving the silicon tube was directed to the epithelium to remove gas bubbles or mucus from the tissue, ensuring a good conductivity.



**Figure 5: Epithelium container**

A polyacryl ring was glued to the serosal side of the tightened intestine wall, and then placed onto the silicon ring (part of the serosal side of the container) and covered with the mucosal side of the container. In the container, the tissue was pressed between the edge of the plastic mucosal side and the silicon ring to minimize the “edge damage” (see paragraph 2.3.1.4). An area of  $0,28 \text{ mm}^2$  was exposed to the buffer, filling both chambers.

#### **2.3.1.4 0.28 cm<sup>2</sup> containers**

For all experiments performed, so called “small” chambers modified by Schultz & Zalusky (45), with 0.28 mm<sup>2</sup> exposed intestine wall area were used. They have two symmetric parts made of acryl glass and a volume of 0,5 ml. Each part has a cone-shaped cavity with one end open at the medial side, and a lateral blind end. The upper part of the cavity is connected to the bubble-lift endings (

Figure 4: Ussing-chambers with connected warm-up exchanger and gassing system **(bubble lift)**.

The medial side is deepened to mount the epithelium container. The epithelium-container is pressed between the two acryl glass parts. The epithelium container consists of two plastic parts, which have a 6 mm diameter hole in the middle (identical to the 0.28 mm<sup>2</sup> area). The specially formed silicon ring (Elastosil RT604, A:B mixed 9:1, Darwin Vertriebs GmbH, Ottobrunn) was attached to one half of the container to ensure that the epithelium is pressed into the ring shape (0.28 mm<sup>2</sup>) by the second chamber half (

Figure 5: Epithelium container The intestinal wall itself was tensed and fixed with Histoacryl glue (B. Braun, Tuttlingen, Germany) on a polyacryl ring, then pressed by the silicon ring onto the second part of the epithelium container. The construction of the chamber was made so, that the pressure was carried by the container itself not by the tissue, thus reducing any “edge damage” (46).

#### **2.3.1.5 Chambers setup**

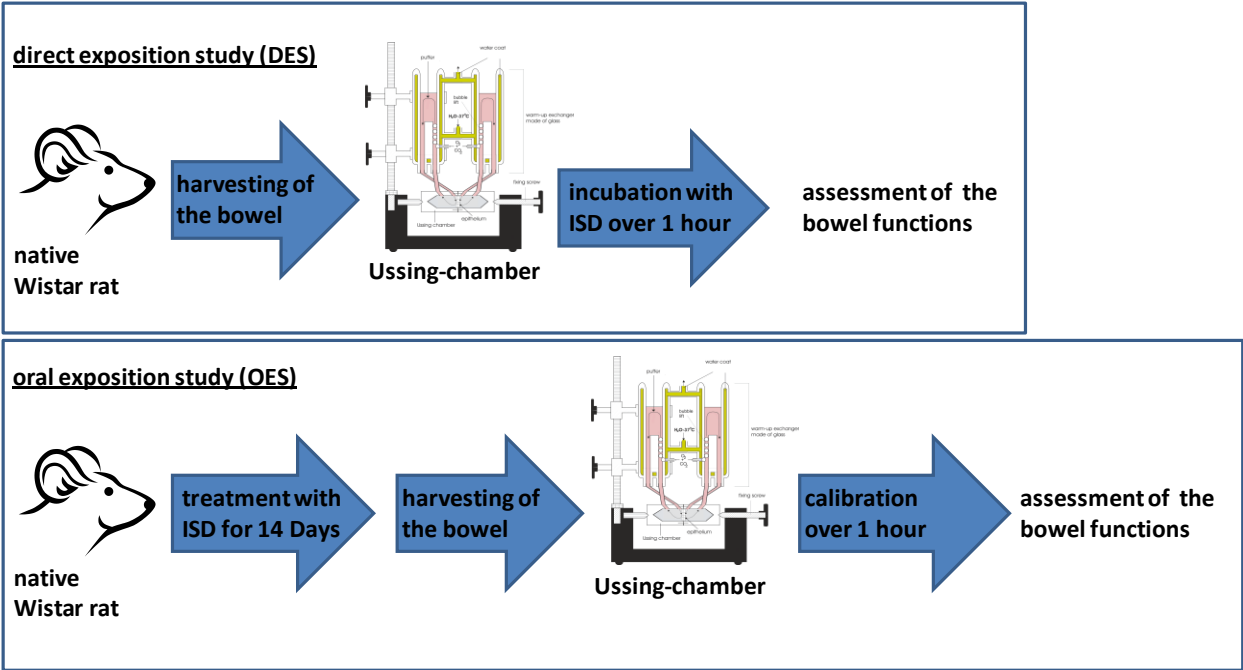
Two “voltage electrodes” (Mettler Toledo, Inlab® 301 Reference) were connected to the chamber by so called “agar bridges”. The electrode was immersed in a container filled with 3 mol/l KCl solution, and the one end of the thin (1 mm diameter) tube was filled with 3 g/dl Agar (Oxoid, Purified) in 0.5 mol KCl (agar bridge). The second agar bridge end was put through a canal into the medial part of the chamber, and placed as close as possible to the epithelium. The tip was cut at a 45° angle, so that the electric field between the two agar bridges was nearly linear. “Current electrodes” were made from the silver bar mounted in a 3 mol/l KCl solution (science workshops at UKBF, Berlin) and connected as voltage electrodes to the chamber by the agar bridges. However, the agar bridge chamber

channels were located near the top of the cone-shaped chamber gap, ensuring that the agar bridge endings were as far away as possible from the epithelium.

## 2.4 Experimental course

### 2.4.1 Preliminary note

The study was divided in two parts. The *direct exposition study* was performed on bowel from animals, which were not treated (in vivo) before. Instead, bowel was prepared, put into the chamber and then incubated with the analyzed drug. In contrary rats being used in *oral exposition study* were treated with the analyzed drug for 14 days prior to experiments. After two weeks bowel was prepared and experiments performed without ex-vivo incubation of the bowel with the drug. Such experimental set up was necessary to fulfill the aim of the study and differentiate between direct and systemic influence of immunosuppressive drugs on the bowel (see Figure 6, page 32).



**Figure 6: Experimental course**



### 2.4.2 Calibration of empty chambers

Thirty minutes before the tissue was placed into the container, the empty chamber was calibrated. The chamber was assembled as described above, but without tissue. Buffer resistance and “empty” potential difference were saved and then used as a correction value for experiments. “Empty” chambers filled with SM were then started-up, and after 30 minutes the “empty” voltage and resistance values were checked for absence of a significant aberration (lack in the electrodes stability). The  $I_{sc}$  values were corrected as described by Tai and Tai (47) to not underestimate the real  $I_{sc}$ .

### 2.4.3 Voltage, resistance and short circuit current measurements

Transepithelial potential difference ( $U_e$ ) was measured directly. According to the Ohm’s law, transmural resistance ( $R_t$ ) was calculated using the potential differences ( $U_1$  and  $U_2$ ) which resulted from application of current  $I_1$  (+10 $\mu$ A) and  $I_2$  (-10 $\mu$ A).

Formula 1:

$$U_1 = U_e + R \cdot I_1 \quad \text{and} \quad U_2 = U_e + R \cdot I_2$$

The resistance is calculated with formula 2:

$$R = \frac{U_1 - U_2}{I_1 - I_2}$$

To obtain the  $R_t$ , the “empty buffer” resistance ( $R_e$ ) was subtracted (Formula 3):

$$R_t = R - R_e$$

The  $I_{sc}$  was then calculated as a ratio of  $U_e$  and  $R_t$  (Formula 4):

$$I_{sc} = \frac{U_e}{R_t}$$

These equations only refer to the open-circuit current situation (not clamped). After the clamp procedure (short-circuit mode), the  $R_t$  and short circuit current ( $I_{sc}$ ) were measured, and  $U_e$  was then calculated with formula 4.

#### **2.4.4 Direct exposition study (DES)**

##### **2.4.4.1 Principle**

The direct exposition study (DES) was performed to characterize a direct influence of the ISD on the small bowel barrier and transport function. In clinical practice, ISD are usually administered orally, and therefore the biggest concentration is in the small bowel and supposedly also influences parts of the gastrointestinal tract. However, an accurate estimation of the exact drug concentration in parts of the bowel is not possible. The blood concentration changes over time from peak to trough levels. The intestinal wall is then exposed to the different drug concentrations from both the lumen (mucosal), and the opposite basolateral (serosal) side.

##### **2.4.4.2 Preparation**

Rats were shaved, and under Isofluran anesthesia the abdomen and thorax were opened and a heart apex was cut. Afterwards the small intestine (between 5<sup>th</sup> and 15<sup>th</sup> cm distally from the hepato-duodenal ligament) was prepared, flushed with ice cold water and gassed with SM to remove any deposits, bile and mucus. The probe was then cut open along the mesentery and divided into two parts with one immersed into SM and a second into SME. Both buffers were placed in ice cold water and gassed. The specimen immersed in SM was then used for glucose absorption experiments and was then immersed in SME for <sup>3</sup>H-Lactulose flux and chloride secretion experiments.

Tissue with the serosal side up was placed tightly on a silicon plate and fixed with pins. Then polyacryl ring was glued to the intestine wall and cut out from the rest of the surrounding tissue. The “membrane” was then placed in the container and then into the chamber. Tissue tension was kept at the same level, and the intestinal mucus and possible artifacts (i.e. air bubbles) were removed by flushing the container’s tissue holes with SM

stream. The time between the rats death and the beginning of the Ussing chamber measurement was kept under 60 min., since the tissue in the buffer was not allowed to dry. Both chambers were then filled carefully with 10 ml of the buffer to avoid tissue damage. The experiment time was only started once all chambers (8 separate chambers were used at the same time) were mounted. Aliquot of the examined drug was added to the specific chamber (see Table 2 for concentration in the chamber).

#### ***2.4.4.3 Experimental groups***

In the DES low (LD<sub>d</sub>) and high dose (HD<sub>d</sub>) groups were used. The LD<sub>d</sub> are related to a target serum level of the drugs used in humans, and HD<sub>s</sub> were 100 x higher (toxic level). In each group, 14 experiments were performed with the tissue samples from 14 separate rats. Each HD<sub>d</sub> and LD<sub>d</sub> group was divided into two. In the first seven experiments, a drug was added to the chamber flushing mucosal, and in the other seven to the serosal side of the intestinal wall.

	<b>CyA</b>	<b>TAC</b>	<b>Myf</b>	<b>MMF</b>	<b>Sir</b>	<b>Eve</b>	<b>FTY</b>
<b>LD<sub>d</sub></b>	0,2	0,01	2,16	3	0,01	0,008	8
<b>HD<sub>d</sub></b>	20	1	216	300	1	0,8	800

**Table 3: Immunosuppressive drugs concentrations in the direct exposition study**

Concentrations of the immunosuppressive drugs in the direct exposition study were chosen as follows: Low doses according to the target therapeutic serum level of the drugs by human. High doses were one hundred times higher than the low dose (both [ $\mu\text{g}/\text{ml}$ ]). The drugs were added to either the serosal or the mucosal side of the chamber.

#### **2.4.4.4 Experimental course**

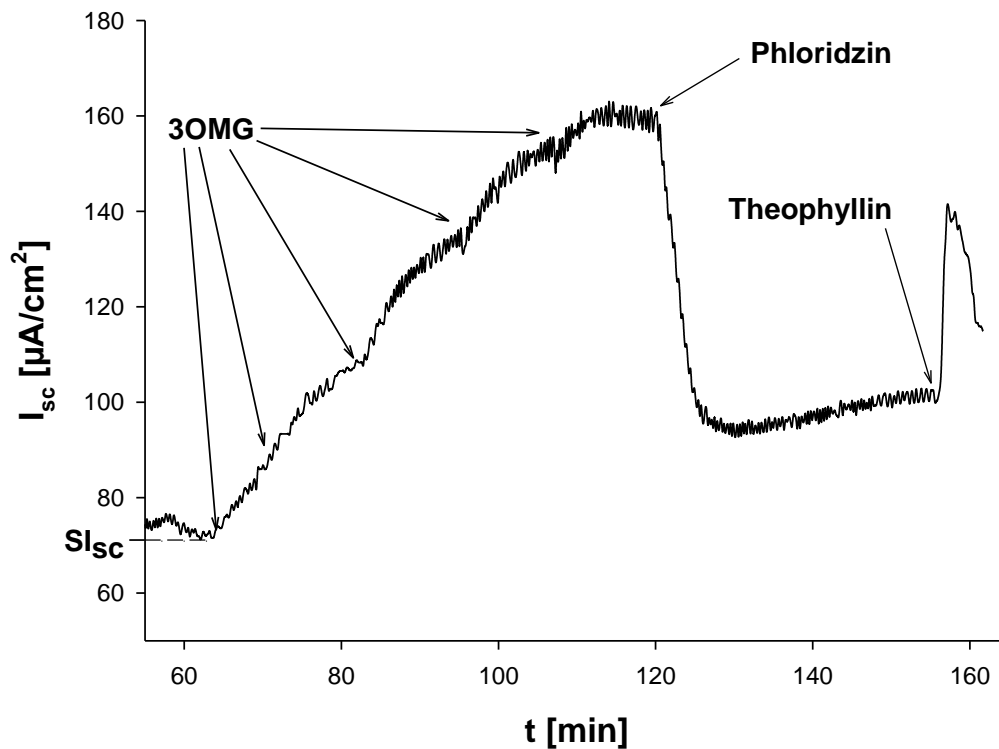
##### **2.4.4.4.1 Small intestine glucose absorption**

For glucose absorption experiments, the chambers were filled with SM. After 60 min incubation with the drug, aliquots of SM supplemented with 3OMG were added. Due to this procedure, 3OMG chamber concentrations increased to 4, 8, 16, 32 and 48 mmol/l every 10 min (see Figure 7: Glucose absorption kinetics measurement

$\Delta I_{sc}^{max}$  ( $V_{max}$ ) and  $K_M$  were then calculated from Lineweaver-Burk and Eady-Hofstee plots (data were corrected to reach a similar  $V_{max}$  from both methods because of their different sensibility for high and low substrate concentration). Ten minutes after last 3OMG aliquot (when  $I_{sc}$  plot reached plateau level by 48 mmol/l 3OMG concentration), phloridzin was added ( $5 \cdot 10^{-4}$  mol/l, mucosal) to inhibit the SGLT1-dependent glucose transport. To estimate the integrity of the epithelium, its secretory response was tested by adding theophylline ( $10^{-2}$  mmol/l) to both chambers for 20 min. after phloridzin was added. Samples which responded to theophylline only very weakly or not at all, or those which had a low resistance of less than 20 Ohms were excluded from further analyses.

##### **2.4.4.4.2 Overall small intestine transport**

Two factors describing overall transport in the small intestine were assessed: intestinal transport with (from experiments where SME was used,  $EI_{sc}$ ) or without (from experiments where SM was used,  $SI_{sc}$ ) carbohydrates in the tissue flushing buffer.  $EI_{sc}$  was calculated as an  $I_{sc}$  mean value from  $J_{Lac}$  (the same time period as for  $R_t$  mean equation, see page 40), and  $SI_{sc}$  is an  $I_{sc}$  value taken just before the 3OMG was added in the glucose absorption experiments (see Figure 88, Page 40).



**Figure 7: Glucose absorption kinetics measurement**

The jejunum was immersed in SM and incubated for 60 min with one of the immunosuppressive drugs (DES) or left for 20 min. to reach the “steady” status (OES). Afterwards  $I_{sc}$  was recorded ( $SI_{sc}$ ), and glucose absorption kinetics were measured (see Paragraph 2.4.4.4.1, Page 37). Then phloridzin and after 20 min. theophylline were added.  $I_{sc}$  “answer” to theophyllin was noted as a control of the tissue reactivity.

### **2.4.4.4.3 Small intestine barrier function**

#### **2.4.4.4.4 Preliminary note**

The assessment of intestinal barrier function in the human focus mainly on the non invasive tests like urinary excretion of orally administered substances i.e. lactulose(48), L-rhamnose(49), or polyethylene glycols(50). These three substances leave the intestinal lumen by the paracellular (lactulose), transcellular “aqueous” (L-rhamnose) and transcellular “lipid” (polyethylene glycols) way, being therefore markers for the pathology connected to different parts of the intestinal wall(8). However urinary excretion tests can only describe the intestinal permeability in “global” and cannot differentiate between small and big bowel pathology. As well the recognition of small molecule permeability is difficult using only non invasive tests. To analyze the big and small molecule permeability by two factors:  $^3\text{H}$ -Lactulose flux ( $J_{\text{Lac}}$ ) and transepithelial resistance ( $R_t$ ), the invasive experimental method described by Schulz and Zaluski(51) was used in this study. Lactulose is the biggest molecule of all commonly used in the urinary tests(8). The  $R_t$  correlates with the number of tight-junction strands and therefore with the amount of ions permeating in the transepithelial direction (52).

##### **2.4.4.4.4.1 $^3\text{H}$ -Lactulose flux.**

Measurement of the  $^3\text{H}$ -Lactulose flux was based on the method described by Shultz and Zaluski(51). For those experiments, buffer (SME) containing 20 mmol/l Lactulose was used as medium. After one hour of incubation with one of the ISD, the tissue was clamped (see Figure 3, Page 25) and a 1 ml “empty” probe was taken from the serosal chamber side (S0). After another 20 min., a series of four 1 ml samples (S1, S2, S3, S4) were taken every 15 min.  $^3\text{H}$ -Lactulose ( $\sim 0,005$  mCi) was added to the mucosal side between S0 and S1. M1 and M2 probes (each 100  $\mu\text{l}$ ) were taken between S1 and S2 to estimate the mucosal chamber medium radioactivity level. After each probe was taken, the chambers were refilled with the same amount of SME. 5 ml Ultima Gold XR (Perkin Elmer, Boston, MA, USA) was added to the samples, whereafter the radioactivity (counts per minute – CPM) was measured with the Tri-Carb 1900TR Liquid Scintillation Analyser (Packard Canberra Company). Fluxes [ $\text{nmol/h} \times \text{cm}^2$ ] were calculated according to the standard method described by Schultz and

Zalusky (51). Mean value of three flux periods (between S1-S2, S2-S3 and S3-S4) is shown as  $J_{\text{Lac}}$ .

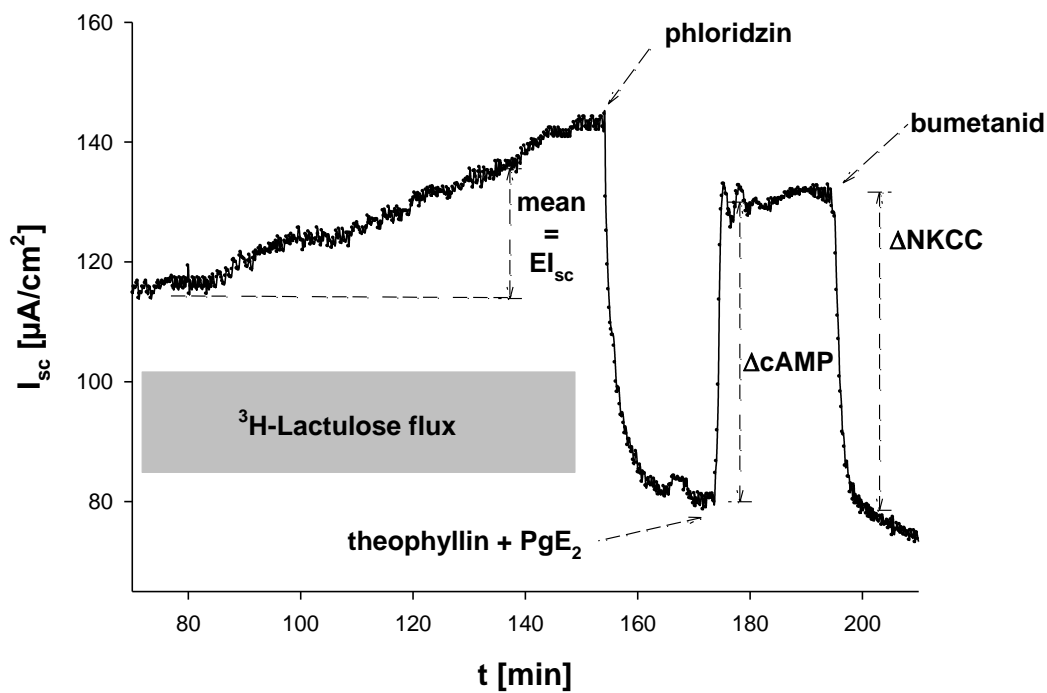
#### **2.4.4.4.2 Transmural resistance**

Transmural resistance ( $R_t$ ) (see Paragraph 2.4.3, Page 33) value was taken from the  $J_{\text{Lac}}$  experiments. A mean  $R_t$  value from a constant time period (between 80 and 135 min after experiment start) was used.

#### **2.4.4.4.5 Small bowel chloride secretion**

After  $J_{\text{Lac}}$  experiments and phloridzin addition, chloride secretion of the small bowel was assessed by a series of stimulation and inhibition experiments. Theophyllin with  $\text{PgE}_2$  ( $10^{-2}$  M both sides, and  $10^{-6}$  mol/l serosal), and 20 min. later bumetanide ( $10^{-5}$  mol/l serosal) were added to the chamber. Delta  $I_{\text{sc}}$  values for both stimulation and inhibition were calculated ( $\Delta\text{cAMP}$  and  $\Delta\text{NKCC}$ , respectively, [ $\mu\text{A}/\text{cm}^2$ ], Figure 8, Page 41).





**Figure 8:  $^3\text{H}$ -Lactulose flux,  $EI_{sc}$  and chloride secretion assessment in the jejunum**

After 60 minutes of incubation with one of the ISD (DES) or after a 20 minute stabilization time (OES), the  $^3\text{H}$ -Lactulose flux was measured. Afterwards,  $\Delta\text{cAMP}$  (theophyllin with  $\text{PgE}_2$ ) and  $\Delta\text{NKCC}$  (bumetanide) were measured. Additionally,  $EI_{sc}$  was calculated as a mean  $I_{sc}$  of the constant period of time (80 to 135 min).

## **2.4.5 Oral exposition study (OES)**

### ***2.4.5.1 Principle***

In the oral exposition study (OES), the influence of two weeks treatment with ISD on the intestinal functions was analyzed. In clinical practice, immunosuppressive drugs are usually given orally, and the intestinal side effects are most probably the result of both a systemic and ex-lumen influence on the bowel. To imitate this situation we decide to treat the rats orally for two weeks. To ensure the exact dosage, 1 mm of a drug solution was injected directly into the animal stomach with a dull metal needle. The medicament was applied between 8.00 and 10.00 a.m.; rats were allowed to feed and drink ad libitum. Weight gain was calculated between the first and fourteenth day.

### ***2.4.5.2 Experimental groups***

Rats were divided into low and high dose groups (LD<sub>o</sub> and HD<sub>o</sub>, respectively, n=7). Doses of ISD are shown in (Table 4: Immunosuppressive drug doses, oral exposition study 43). Low and high doses conform to the normal therapeutic doses used in rat transplantations. The doses for rats are higher than those for humans, because of different body area/volume ratio and the much faster metabolism in the rat. As a control, 1 mm tap water was injected for the same period of time (n=10). The ISD formulas currently available on the market were used (see Paragraph 2.2.2.1, Page 16).

	<b>TAC</b>	<b>CyA</b>	<b>EC-MPA</b>	<b>MMF</b>	<b>SIR</b>	<b>EVE</b>	<b>FTY</b>
<b>LD<sub>0</sub></b>	0,3	1,5	10	15	0,25	0,5	0,3
<b>HD<sub>0</sub></b>	1	5	30	40	0,5	3	1

**Table 4: Immunosuppressive drug doses, oral exposition study**

In the oral exposition study, rats were fed with the same doses of immunosuppressive drugs as used in rat transplantations. The drugs were dissolved in tap water and injected with a dull needle (1 ml) directly into the stomach. Since rats have a faster metabolism than humans due to a higher body surface/volume index, it is difficult to find a drug dose which will be equivalent to that of humans. In this study low and high therapeutic doses were used [mg/kg b.w. once daily].

### **2.4.5.3 Preparation - jejunum**

After the rats had been treated for two weeks, they were starved overnight and weighed. The jejunum was then prepared as described before (see Paragraph 2.4.4.2, Page 34).

### **2.4.5.4 Preparation – distal colon**

The late distal part of the colon (last 3 cm) was then harvested and used for the experiments. The bowel was cut open and immersed in gassed and ice cold SME. The muscularis propria layer was then removed (53) and placed on a silicon plate, and mounted into the Ussing chamber (see Paragraph 2.4.4.2, Page 34). The enriched medium (SME) was only used for experiments with the colon.

### **2.4.5.5 Experimental course**

#### **2.4.5.5.1 Jejunum measurements**

All small intestine measurements (glucose absorption, barrier function, chloride secretion and overall intestinal transport) were similar to the short time exposition experiments. The only difference is that the tissue was not incubated with an ISD prior to the experiment. Instead, a 20 minute time period was used to reach the steady status of the intestinal transport (measurements were started 20 minutes after the tissue was mounted into the chamber).

#### **2.4.5.5.2 Barrier function of the distal colon**

The barrier function assessment of the distal colon was similar to that of the small bowel in two aspects:  $^3\text{H}$ -Lactulose flux ( $J_{\text{Lac}}$ ) and transmural resistance ( $R_t$ ). Both parameters were assessed with the same protocol as for the jejunum (see Paragraph 2.4.4.4.3, Page 39).

#### **2.4.5.5.3 Chloride secretion in the distal colon**

In the OES, colonic chloride secretion was measured in a manner similar to the chloride secretion assessment in the jejunum (see Paragraph 2.4.4.4.5, Page 40). Following  $J_{\text{Lac}}$  experiments,  $\Delta\text{cAMP}$  after theophyllin with  $\text{PGE}_2$  addition was recorded. In the separate

chamber filled with SME, bumetanide was added 60 minutes after the stabilization period and then  $\Delta\text{NKCC}$  was measured.

#### ***2.4.5.5.4 Sodium transport via Epithelial Sodium Channel in the distal colon***

Before the  $J_{\text{Lac}}$  experiments, amiloride ( $10^{-4}$  mol/l) was added to both chamber sides to inhibit the ENaC sodium channel function. Afterwards  $\Delta I_{\text{sc}}$  ( $\Delta\text{ENaC}$ ) was calculated.

## **2.5 Statistical analysis**

All parameters were compared using an SPSS 13.0 for Windows software. Multivariable testing (Mann-Whitney Rank Sum Test) was used to compare parameters, and according to the Bonferroni-Holm correction p values lower than 0.007 ( $p=0.05$  divided by 7) were defined as statistically significant.

Differences between the groups were compared using the two sided student test for the groups with equal variation (p value under 0.05 was consider as statistically significant).

The statistical analysis was performed in cooperation with Prof. Dr. P. Martus from the Institut für Biometrie und Klinische Epidemiologie, Charité, Berlin.

## 3 Results

### 3.1 Direct exposition study

In the LD<sub>d</sub> and as HD<sub>d</sub> subgroups (serosal and mucosal), measured parameters did not differ from each other, therefore they will not be shown separately. If not mentioned otherwise, all significant tests were performed between the control group and one other group.

#### 3.1.1 Small intestine glucose absorption

##### 3.1.1.1 Low concentration

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
<b>V<sub>max</sub></b>	85.5 ±9	92.3 ±1	92.6 ±6	84.3 ±7	82.1 ±7	95.8 ±1	70.9 ±5	68.8 ±6
<b>K<sub>M</sub></b>	24.2 ±2	21.1 ±2	29.4 ±3	29.5 ±3	29.7 ±3	22.7 ±2	23.1 ±2	25.8 ±3
<b>EI<sub>sc</sub></b>	122 ±9	137 ±15	136 ±11	129 ±15	82 ±13 <sup>†</sup>	131 ±15	129 ±12	118 ±17
<b>SI<sub>sc</sub></b>	74 ±5	70 ±10	68 ±6	72 ±5	63 ±5	77 ±8	57 ±5 <sup>†</sup>	55 ±5 <sup>†</sup>
<b>N</b>	21	9	12	15	17	14	14	14

**Table 5: Glucose absorption in the LDd groups**

None of the glucose absorption parameters in the direct exposition study of the low dose groups reached a significance level. (mean values ±SEM, † -p<0.05)

### 3.1.1.2 High concentration

	control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
$V_{\max}$	85 ±9	95 ±11	80 ±11	78 ±11	72 ±8	67 ±8	65 ±5	70 ±6
$K_M$	24.2 ±2	17.9 ±2 <sup>†</sup>	28.6 ±2	22.9 ±2	26.7 ±2	25.0 ±1	23.8 ±3	26.0 ±3
$EI_{sc}$	122 ±9	161 ±18 <sup>†</sup>	123 ±14	123 ±16	86 ±11 <sup>†</sup>	113 ±13	124 ±11	83 ±11 <sup>†</sup>
$SI_{sc}$	74 ±5	71 ±7	69 ±5	76 ±7	57 ±6 <sup>†</sup>	70 ±5	58 ±5 <sup>†</sup>	63 ±6
<b>N</b>	21	13	13	14	20	14	16	13

**Table 6: Glucose absorption in the HDd groups**

In the direct exposition study, the CyA high concentrations groups showed an increase of the glucose absorption process without achieving statistical significance after Bonferroni correction (decreased  $K_M$ , and increased  $EI_{sc}$ ,  $p < 0.05$ ). Rats treated with EC-MPA had a decreased overall intestinal transport without carbohydrates in the medium ( $p < 0.05$ ). None of the parameters in the study reached any significant level. (mean values  $\pm$ SEM, † -  $p < 0.05$ )



### 3.1.2 Small intestine barrier function

#### 3.1.2.1 Low concentration

	control	CyA	TAC	MMF	MYF	SIR	EVE	FTY
$R_t$	35.8 ±2	33.7 ±2	31.5 ±2	35.4 ±2	38.4 ±3	34.6 ±3	32.6 ±3	37.7 ±5
$J_{Lac}$	218 ±11	215 ±12	257 ±27	214 ±24	233 ±19	209 ±17	201 ±23	206 ±17
N	44	14	16	16	16	15	15	14

**Table 7: Barrier function in the LDd groups**

There were no statistical differences in the small bowel barrier function parameters in the low concentration groups compared to the control group. (mean values ±SEM)

#### 3.1.2.2 High concentration

	control	CyA	TAC	MMF	MYF	SIR	EVE	FTY
$R_t$	35.8 ±2	32.1 ±3	33.3 ±2	38.6 ±3	38.1 ±3	33.2 ±2	29.6 ±2 <sup>†</sup>	37.8 ±3
$J_{Lac}$	218 ±11	232 ±28	246 ±25	166 ±12 <sup>†</sup>	226 ±19	274 ±35 <sup>†</sup>	302 ±30 <sup>‡</sup>	255 ±27
N	44	15	15	20	21	18	17	15

**Table 8: Barrier function in the HDd groups**

In the EVE HD<sub>d</sub> group, the small bowel barrier function was not significantly impaired ( $R_t$  was decreased ( $p < 0.05$ ), and  $J_{Lac}$  significantly increased ( $p < 0.007$ )). The reduced  $J_{Lac}$  in the SIR group cannot be confirmed by other measured parameters ( $p < 0.05$ ). However, the

reduced  $J_{\text{Lac}}$  in the MMF group ( $p < 0.05$ ) is consisted with the results of the OES. (mean values  $\pm$ SEM, †- $p < 0.05$ , ‡- $p < 0.007$ )

### 3.1.3 Chloride secretion

#### 3.1.3.1 Low concentration

	control	CyA	TAC	MMF	MYF	SIR	EVE	FTY
$\Delta$ cAMP	68.9 $\pm 3$	62.3 $\pm 5$	60.6 $\pm 4$	66.1 $\pm 5$	62.8 $\pm 4$	72.6 $\pm 4$	78.0 $\pm 7$	83.8 $\pm 6^\dagger$
$\Delta$ NKCC	65.1 $\pm 3$	55.1 $\pm 5$	58.9 $\pm 5$	57.6 $\pm 5$	58.8 $\pm 3$	76.6 $\pm 6$	72.9 $\pm 5$	75.9 $\pm 6$
<b>n</b>	40	15	15	15	16	12	12	13

**Table 9: Chloride secretion in the LDd groups**

Only FTY LD<sub>a</sub> had a reduced cAMP maximal activation capacity compare to the control group ( $p < 0.05$ ). This effect however cannot be confirmed by any other results of the present study (mean values  $\pm$ SEM, † - $p < 0.05$ )

#### 3.1.3.2 High concentration

	control	CyA	TAC	MMF	MYF	SIR	EVE	FTY
$\Delta$ cAMP	68.9 $\pm 3$	71.6 $\pm 5$	69.6 $\pm 5$	52.1 $\pm 5 \ddagger$	53.2 $\pm 4 \ddagger$	54.8 $\pm 8^\dagger$	56.8 $\pm 5^\dagger$	59.8 $\pm 5$
$\Delta$ NKCC	65.1 $\pm 3$	68.9 $\pm 6$	68.0 $\pm 4$	38.6 $\pm 4 \ddagger$	48.6 $\pm 3 \ddagger$	53.3 $\pm 3^\dagger$	63.5 $\pm 5$	54.9 $\pm 4$
<b>n</b>	40	14	16	17	20	16	17	15

**Table 10: Chloride secretion in the HDd groups**

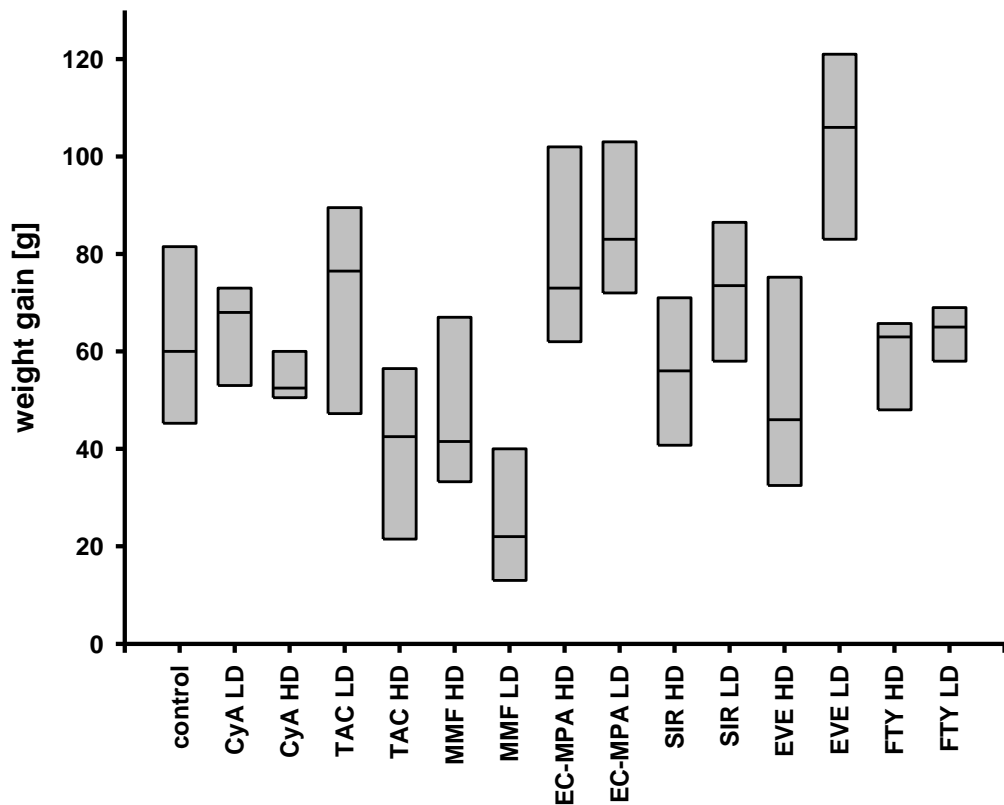
The cAMP-activated and the NKCC-dependent chloride secretion was reduced in the SIR HD<sub>d</sub> group (p<0.05). Everolimus reduced cAMP-dependent chloride secretion (p<0.05); this effect is described in the oral exposition part of the study. MMF and EC-MPA reduced both cAMP- and NKCC-dependent chloride secretion, and are the only groups that reached a significance level. (mean values ±SEM, † -p<0.05, ‡ -p<0.007)

## **3.2 Oral exposition study**

The study groups were compared to the control group if not mentioned otherwise.

### **3.2.1 Experimental animals**

There were no differences in the weight gain between the control and the other groups. All animals survived the experimental period (Figure 9, Page 53)



**Figure 9: Weight gain of the experimental animals**

All animals survived the experimental period in a good condition. Despite the gastrointestinal disturbances, no significant weight loss was noticed. In the figure, mean values of the animals' weight and a low and high quartile is shown as a box plot. No significant differences compared to the control group were observed.

## 3.2.2 Jejunum

### 3.2.2.1 Glucose absorption

#### 3.2.2.1.1 Low dose

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$V_{\max}$	123.1	94.8	131.0	132.8	73.9	73.1	77.0	115.0
	± 17	± 13	± 26	± 22	± 11	± 18	± 9	± 21
$K_M$	22.0	23.5	22.7	18.1	19.8	15.0	25.7	22.5
	± 3	± 4	± 2	± 2	± 3	± 2	± 4	± 4
$EI_{sc}$	90.2	106.0	80.8	110.1	93.5	80.0	65.8	82.9
	± 20	± 29	± 15	± 23	± 15	± 21	± 11	± 14
$SI_{sc}$	65.6	53.6	55.2	57.4	54.6	54.7	54.3	39.9
	± 5	± 6	± 10	± 7	± 9	± 11	± 3	± 11
<b>N</b>	9	9	7	7	7	6	8	6

**Table 11: Glucose absorption in the LD<sub>0</sub> groups**

None of the glucose absorption parameters had changed significantly in the LD<sub>0</sub> groups.  
(mean values ± SEM)

### 3.2.2.1.2 High dose

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$V_{\max}$	123.1	103.7	52.9	152.0	68.2	70.0	65.7	118.2
	±17	± 17	± 11‡	± 22	± 9†	± 8	± 18†	± 14
$K_M$	22.0	21.6	18.0	22.3	22.7	24.2	25.4	22.8
	± 3	± 2	±3	± 1	± 2	± 5	± 4	± 3
$EI_{sc}$	90.2	156.7	70.3	99.1	53.2	90.0	86.8	110.7
	±20	±42	±24	±16	±7	±25	±26	±24
$SI_{sc}$	65.6	45.5	46.6	58.9	43.6	50.0	47.2	64.8
	±5	±4	±2†	±10	±7†	±10	±5	±6
<b>n</b>	9	8	7	7	7	6	6	10

**Table 12: Glucose absorption in the HD<sub>o</sub> groups**

In the HD<sub>o</sub> groups, TAC ( $p < 0.007$ ), EVE and EC-MPA (both  $p < 0.05$ ) decreased  $V_{\max}$  compared to the control group. Also, the overall intestinal transport without carbohydrate in the buffer ( $SI_{sc}$ ) was reduced in the EC-MPA and TAC groups ( $p < 0.05$ ). (mean values ±SEM, † - $p < 0.05$ , ‡ - $p < 0.007$ )

### 3.2.2.2 Chloride secretion

#### 3.2.2.2.1 Low dose

	control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
$\Delta$ cAMP	72.1 ±12	67.0 ±12	103.0 ±11	55.7 ±5	43.5 ±5†	51.6 ±8	34.7 ±3†	72.3 ±13
$\Delta$ NKCC	63.0 ±5	65.7 ±9	63.1 ±15	57.3 ±6	45.9 ±7	47.9 ±5	44.1 ±3	66.4 ±9
<b>n</b>	8	10	7	9	8	8	7	9

**Table 13: Chloride secretion in the LDo groups**

In the EC-MPA and EVE HD<sub>0</sub> groups, the cAMP-dependent chloride secretion was decreased achieving statistical significance after Bonferroni correction in the small bowel ( $p < 0.05$ ). (mean values ±SEM, † - $p < 0.05$ )



### 3.2.2.2.2 High dose

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
<b>ΔcAMP</b>	72.1 ±12	100.0 ±9†	63.12 ±10	69.1 ±7	37.9 ±5‡	70.9 ±7	34.3 ±8‡	64.7 ±7
<b>ΔNKCC</b>	63.0 ±5	69.1 ±5	51.9 ±5	68.5 ±7	43.1 ±4	49.4 ±5†	37.1 ±6†	52.6 ±8
<b>n</b>	8	8	8	8	7	10	8	11

**Table 14: Chloride secretion in the HD<sub>o</sub> groups**

In the HD<sub>o</sub> groups, EVE decreased cAMP- ( $p < 0.007$ ) and NKCC- ( $p < 0.05$ ) dependent chloride secretion in the small bowel. Also, EC-MPA (cAMP,  $p < 0.007$ ) and SIR (NKCC,  $p < 0.05$ ) impaired the chloride secretion. In contrast however, CyA did not significantly increased the cAMP-dependent chloride secretion. (mean values  $\pm$ SEM, † - $p < 0.05$ , ‡ - $p < 0.007$ )

### 3.2.2.3 Barrier function

#### 3.2.2.3.1 Low dose

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$R_t$	44.4 ±5	32.1 ±3†	32.8 ±2†	30.6 ±2‡	35.8 ±3	40.8 ±4	42.2 ±3	34.5 ±3†
$J_{Lac}$	207.0 ±26	325.5 ±66	293.6 ±39	322.2 ±30	303.3 ±41	261.8 ±28	284.3 ±21	237.1 ±46
<b>n</b>	9	6	7	8	8	7	8	8

**Table 15: Small bowel barrier function in the LDo groups**

Changes of the  $R_t$  were only noticed in the LDo groups: MMF ( $p < 0.007$ ), CyA, TAC and FTY ( $p < 0.05$ ) impaired the small bowel barrier function compared to the control group. (mean values ±SEM † - $p < 0.05$ , ‡ - $p < 0.007$ )

### 3.2.2.3.2 High dose

	Control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
<b>R<sub>t</sub></b>	44.4 ±5	31.2 ±3†	41.0 ±5	31.4 ±2†	44.6 ±7	45.0 ±7	36.9 ±3	35.1 ±4
<b>J<sub>Lac</sub></b>	207.0 ±26	258.2 ±34	290.8 ±32	344.2 ±55‡	332.5 ±55†	238.1 ±27	351.7 ±19‡	274.4 ±28
<b>n</b>	9	8	9	7	7	8	8	10

**Table 16: Small bowel barrier function in the HD<sub>o</sub> groups**

In the HD<sub>o</sub> groups, CyA and MMF decreased R<sub>t</sub> (p<0.05). J<sub>Lac</sub> values were increased in the MMF, EVE (p<0.007), and EC-MPA groups (p<0.05) (mean values ±SEM, † -p<0.05, ‡ -p<0.007)

### 3.2.3 Colon

#### 3.2.3.1 Chloride secretion

##### 3.2.3.1.1 Low dose

	Control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$\Delta$ cAMP	43,9 $\pm 10$	46,4 $\pm 10$	29,3 $\pm 4$	37,1 $\pm 9$	60,8 $\pm 6$	50,8 $\pm 5$	61,5 $\pm 7$	45,9 $\pm 3$
$\Delta$ NKCC	-8,2 $\pm 5$	29,6 $\pm 11$	12,0 $\pm 11$	8,0 $\pm 9$	-1,8 $\pm 6$	9,2 $\pm 19$	-16,6 $\pm 6$	8,2 $\pm 4$
n	8	7	8	8	8	7	7	8

Table 17: Chloride secretion in the LD<sub>0</sub> groups

None of the LD<sub>0</sub> groups significantly altered chloride secretion in the colon (mean values  $\pm$ SEM).

### 3.2.3.1.2 High dose

	Control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
$\Delta$ cAMP	43,9 ±10	52,7 ±12	54,0 ±7	67,4 ±6	74,8 ±11†	57,5 ±9	64,9 ±7	52,9 ±6
$\Delta$ NKCC	-8,2 ±5	11,0 ±2	-17,7 ±5	-12,7 ±5	-20,2 ±5	-4,9 ±9	-14,1 ±5	-15,2 ±2
n	8	8	7	8	8	8	7	8

**Table 18: Chloride secretion in the HDo groups**

In the EC-MPA group the cAMP-activated chloride secretion was increased in the distal colon ( $p < 0.05$ ); none of the other groups had any significant changes compared to the control group (mean values  $\pm$ SEM, †  $-p < 0.05$ ).

### 3.2.3.2 Colon barrier function

#### 3.2.3.2.1 Low dose

	Control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$R_t$	174,3 ±10	146,5 ±11	123,7 ±11	151,1 ±10	164,7 ±14	162,8 ±20	154,1 ±18	170,0 ±17
$J_{Lac}$	34,1 ±8	58,8 ±12	45,7 ±14	56,6 ±15	73,17 ±7	47,7 ±10	60,0 ±11	60,3 ±13
N	8	8	8	7	8	8	7	8

**Table 19: Colon barrier function in the LDo groups**

No significant differences of the colon barrier function parameters were noticed in the LDo groups when compared to the control group (mean values ±SEM).

#### 3.2.3.2.2 High dose

	control	CyA	TAC	MMF	EC- MPA	SIR	EVE	FTY
$R_t$	174,3 ±10	128,8 ±7	146,5 ±7	182,7 ±23	157,7 ±23	141,8 ±14	158,7 ±19	150,3 ±6
$J_{Lac}$	34,1 ±8	53,4 ±8	51,4 ±6	51,0 ±8	70,1 ±20	68,5 ±16	53,6 ±12	52,9 ±13
N	8	7	8	7	7	8	8	8

**Table 20: Colon barrier function in the HDo groups**

In the HDo groups there were also no significant differences of the colon barrier function parameters compared to the control group (mean values ±SEM).

### 3.2.3.3 ENaC function

#### 3.2.3.3.1 Low dose

	control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
$\Delta\text{ENaC}$	6,2 $\pm$ 3	9,4 $\pm$ 4	5,8 $\pm$ 3	19,0 $\pm$ 7	12,0 $\pm$ 5	13,6 $\pm$ 6	19,4 $\pm$ 12 <sup>†</sup>	6,0 $\pm$ 3
<b>n</b>	8	8	7	8	8	7	7	8

**Table 21: Colon sodium absorption in the LDo groups**

Amiloride sensitive sodium absorption was increased in the EVE group ( $p < 0.05$ ), however all other groups remained unchanged when compared to the control group (mean values  $\pm$ SEM, <sup>†</sup> - $p < 0.05$ ).

#### 3.2.3.3.2 High dose

	control	CyA	TAC	MMF	EC-MPA	SIR	EVE	FTY
$\Delta\text{ENaC}$	6,2 $\pm$ 3	5,0 $\pm$ 1	9,4 $\pm$ 2	13,0 $\pm$ 5	18,0 $\pm$ 7 <sup>†</sup>	7,2 $\pm$ 3	26,3 $\pm$ 4 <sup>‡</sup>	4,0 $\pm$ 1
<b>n</b>	8	7	8	8	8	7	8	8

**Table 22: Colon sodium absorption in the HDo groups**

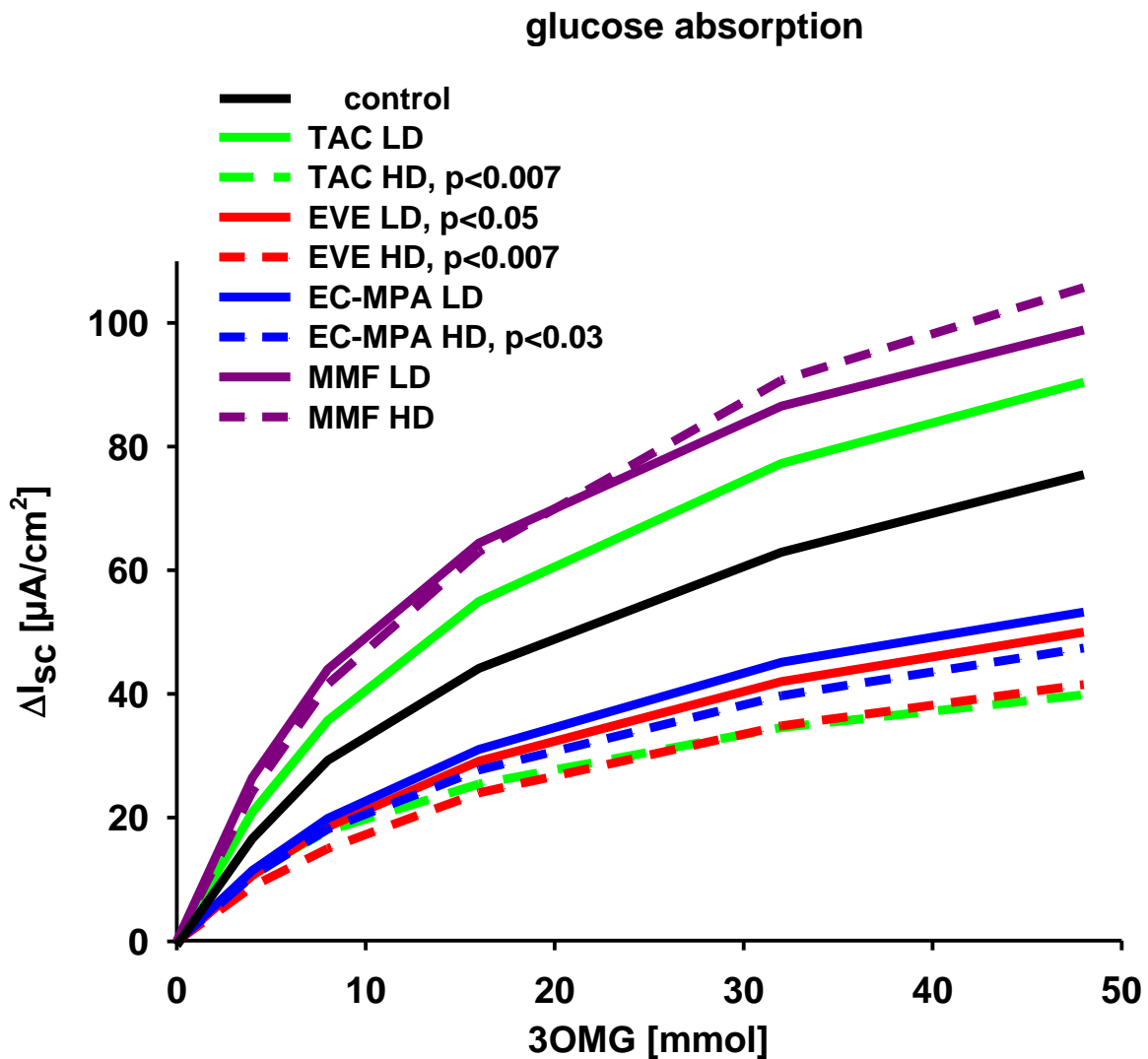
In the EVE and EC-MPA groups, amiloride-sensitive sodium absorption was increased ( $p < 0.007$  and  $p < 0.05$  respectively). All other groups remained unchanged compared to the control group (mean values  $\pm$ SEM, <sup>†</sup> - $p < 0.05$ , <sup>‡</sup> - $p < 0.007$ ).

### 3.3 Differences between the groups and dose dependency

In Figure 10 (Page 65), glucose absorption kinetic plots from significant ISD groups (oral exposition) are shown. A clear dose dependency of the tacrolimus effect can only be seen in a high dose group, namely the glucose absorption course was decreased (tacrolimus low dose vs. high dose  $p < 0.03$ ). In the EVE and EC-MPA groups glucose absorption was reduced similarly by low and high doses. In contrast to EC-MPA, MMF does not decrease the glucose absorption kinetics. The difference between MMF and EC-MPA is significant in the case of  $V_{max}$  ( $p < 0.01$ ). Other parameters were not significantly different between the MMF and EC-MPA groups.

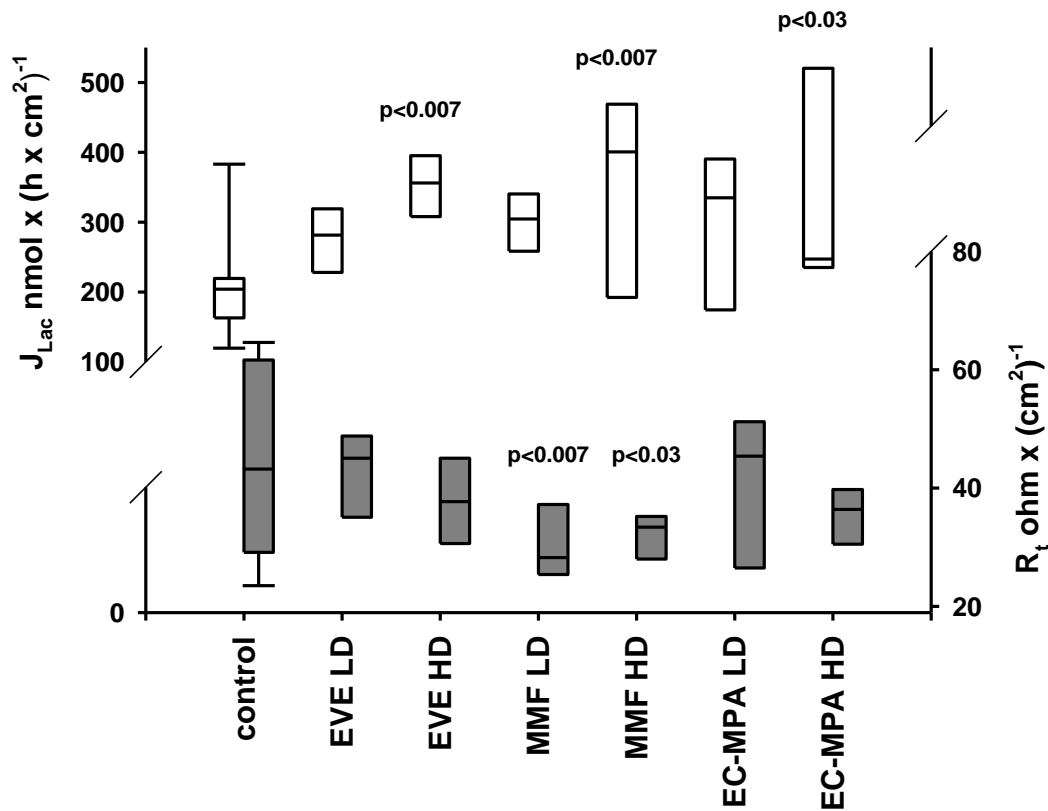
EVE, MMF and EC-MPA altered the small bowel barrier function in a dose-dependent manner.  $J_{Lac}$  was increased in all three high-dose groups, while all low-dose groups were not significantly different compared to the control group. However, only the EVE high- and low-dose differed significantly ( $p < 0.05$ ).  $R_t$  was diminished with both a high and low doses of MMF (Figure 11, Page 66).





**Figure 10: 3OMG absorption kinetics plots of selected groups, oral exposition.**

Glucose absorption kinetic plots (3OMG absorption) of selected groups from the oral exposition study are presented. The current flow rises ( $\Delta I_{sc}$ ) due to the increase of glucose/sodium electrogenic transport through SGLT1 channel (see the method part). A clear dose dependency was observed with tacrolimus.



**Figure 11: Small bowel barrier function of selected groups, oral exposition.**

Small bowel barrier function in the groups, which significantly influenced lactulose flux ( $J_{Lac}$ ) or transepithelial resistance ( $R_t$ ) in the oral exposition study.  $J_{Lac}$  as a marker for large molecules small bowel barrier function is increased (bigger leak) in all high dose groups (EVE, MMF, EC-MPA) compared to the control group.  $R_t$  – parameter describing small molecule small bowel barrier function is altered in the MMF low and high dose group (decreased value – bigger leak). Significant values vs. control group.

## **4 Discussion**

### **4.1 Discussion of the method**

#### **4.1.1 Introduction**

Aim of the study was to elucidate the pathomechanism of the immunosuppressive drugs (ISD) associated with diarrhea. Three of those mechanisms (see Table 1, page 8) were analyzed in this study. Small bowel glucose absorption, barrier function and chloride secretion as well as distal colon barrier function, chloride secretion and sodium absorption were studied in a rat model.

#### **4.1.2 Ussing chambers measurements and the study design**

The Ussing chamber method was chosen to analyze the bowel functions mentioned in the forgoing. This method is well known and has already been used to analyze different biological membrane functions i.e.: epithelial sodium channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) (54) and pathologies i.e. in: HIV Infection (44), Crohn Disease (55) or collagenous colitis (56). Two protocols were used to measure the small bowel function: the direct exposition study (DES, exposition ex-vivo to measure the direct influence of ISD on the jejunum), and the oral exposition study (OES, exposition in-vivo to simulate clinical therapeutic regimen). Drugs were used in two doses in order to see if a dose dependency exists. In DES the drug concentrations respond to the therapeutic serum and toxic concentrations. As explained in paragraph 2.4.4.1, page 34, by using such doses it is possible to simulate variations of the drug concentrations in the bowel lumen and in the blood. Either the mucosal or serosal side of the intestine was exposed to the ISD to additionally clarify if the drug formulations (oral or venous) differ regarding their influence on the small intestine function. Such a difference could not be confirmed, thus there is no difference of the analyzed ISD influence on the small bowel barrier and transport function, regardless of whether it influenced the mucosal or serosal bowel side or not. In the OES low and high therapeutic doses were applied as in experimental rat transplantation studies. The differences between these dosages are discussed later.

## **4.2 Discussion of the results**

### **4.2.1 Introduction**

In Table 2, page 10, the incidence of diarrhea in patients treated with ISD is shown. Unfortunately, there are significant differences in the incidence numbers observed by separate authors. The reasons for this discrepancy are that different diarrhea definitions have been used in the studies. Additionally, the differentiation between ISD-associated and others (e.g. infectious) is difficult and might confuse reported numbers. Thus the real incidence of diarrhea accompanying individual ISD therapies is unknown.

The possible pathomechanisms of ISD-associated diarrhea found in this study are summarized in Table 23, page 69.

<b>ISD</b>	<b>possible pathomechanisms of ISD-associated diarrhea: result of this study</b>
CyA	no significant influence on small bowel or colon transport or on barrier function
TAC	reduced glucose absorption and secondary increased Na <sup>+</sup> colon re-absorption (n.s.) –malabsorptive diarrhea
MMF	altered small bowel barrier function –leak flux diarrhea
EC-MPA	<ul style="list-style-type: none"> <li>➤ reduced glucose absorption and secondary increased Na<sup>+</sup> colon absorption (n.s.) – malabsorptive diarrhea</li> <li>➤ altered small bowel barrier function –leak flux diarrhea</li> </ul>
SIR	no significant influence on small bowel or colon transport or on barrier function
EVE	<ul style="list-style-type: none"> <li>➤ reduced glucose absorption and secondary increased Na<sup>+</sup> colon re-absorption</li> <li>➤ impaired small bowel barrier function-</li> <li>➤ reduced chloride secretion</li> </ul> <p>overall diminished mucosal small bowel function -malabsorptive and leak flux diarrhea</p>
FTY 720	no significant influence on small bowel and colon transport and barrier function

**Table 23: Immunosuppressive drug associated diarrhea – proposed pathomechanisms**

Pathomechanisms of ISD influence on the bowel function found in this study are summarized in table 23. Malabsorptive diarrhea can occur in the case of TAC, EC-MPA or EVE therapy. Leak flux due to the impaired small bowel barrier function was observed in the MMF, EC-MPA and EVE treated rats. In the case of TAC and EVE, the pathomechanisms were partially dose-dependent. No significant alterations of the small or big bowel barrier or transport function were noticed in the other immunosuppression groups.

#### **4.2.1.1 Calcineurin inhibitors (CNI)**

Cyclosporine A and tacrolimus are commonly used for the treatment of transplanted patients. The mechanism of action of CNI is the inhibition of T cell activation. After entering the cytoplasm, CNIs form complexes with immunophilins (Cyclosporin A with cyclophilin, and tacrolimus with FKBP-12). The CNI-immunophilin complexes inhibit calcineurin activity and hence prevent nuclear translocation of NF-AT and cytokine gene transcription. Finally, CNIs block the production of cytokines such as IL-2 and inhibit T cell activation and proliferation (2).

CNIs have a wide spectrum of side effects, with diarrhea being the most common one. 14%-47% of the patients receiving the Sandimmune® or Neoral® (new microemulsion formulation of Cyclosporin A) therapy have diarrhea. In patients treated with Tacrolimus, the diarrhea incidence is between 37% and 72% (see Table 2, Page 11).

##### **4.2.1.1.1 Cyclosporin A**

It is not clear in which mechanism Cyclosporin A (CyA) induces diarrhea. Some authors proposed that CyA causes malabsorption due to the inhibition of glucose absorption in the intestine, as in the kidney tubular cell line (57). Only a few experimental studies show CyA's deleterious effect on intestinal glucose absorption (29, 58, 59) (either longer exposition time or higher dose of CyA than in the present study), in other studies however the glucose absorption is up-regulated (30). Interestingly, a decrease in glucose transport appears only in the jejunum, and is then up-regulated in the ileum. Thus, the direct inhibition of glucose transporters seems not to take place here. However, it is possible that a high dose of CyA influencing the proximal jejunum (before the drug is absorbed) has some toxic effect on the glucose absorption. It is known that CyA nephrotoxicity is caused by increased vasoconstriction and that withdrawal of the drug could lead to kidney fibrosis (60). If the same mechanism takes place in the intestine, this persistent ischemia could lead to down-regulation of the glucose transporter due to insufficient energy needed for this ATP-dependent transport. At the same time glucose absorption can still take place in the ileum due to an increased concentration of the substrate, which has not been absorbed in jejunum. This also explains the lack of effect in tissue taken ex vivo which is supplied with oxygen due to diffusion rather than in the physiological manner. In our experiments only toxic levels of CyA influenced glucose absorption.

The deleterious effect of the CyA on the bile salt and bile fluid output, as well as diminished absorption of stearic and linolenic acid was found by Sigalet et. al. (58). An increased intestinal villous surface area was found by two other authors after CyA therapy (29, 30), which may be a sign of accommodational changes of the mucosa. On the other hand we did not noticed any increased glucose transport which would take place in the case of increased villous surface.

Longer exposition to CyA therapy should be analyzed in order to explain the relative high incidence of diarrhea in patients treated with CyA.

#### **4.2.1.1.2 Tacrolimus**

Tacrolimus (TAC) is suspected of having a specific effect on the mitochondria of the enterocyte, reducing ATP production. As a result, secondary changes appear in the intestinal mucosa function such as reduced small intestine barrier function and decreased glucose absorption capability (31, 32, 61). In our experiments, TAC showed no influence on the intestinal barrier function even when used in a toxic concentration. We however showed a decreased intestinal glucose transport capacity in rats treated with a high dose of TAC. This effect was dose-dependent. Possibly too short treatment time with tacrolimus is responsible for a normal small intestine barrier function, as seen in our experiments. Glucose transport mechanisms seem to be more sensitive towards a lack in cellular energy resources than other bowel functions. Interestingly, no changes in the measured parameters appear after direct exposition to a toxic TAC concentration (1µg/ml). Thus, we suppose that the direct influence of Tacrolimus is not toxic to the enterocyte cell (even when exposed from a basolateral side). It may be that some metabolite of tacrolimus influences the intestinal mucosa or its influence is indirect and is mediated by the endo-paracryne, neuronal (vegetative) system, or by changes similar to CyA induced vasoconstriction in the kidney vessels. The increased systolic blood pressure in rats after treatment with 1 mg/kg for 30 days was recently described (62). However it has failed to show the increased perfusion pressure due to noradrenaline/sodium nitroprusside stimulation in the isolated mesentery of the rat. Thus, if there are circulatory (blood pressure, vasoconstriction) changes during TAC therapy, they seem to be more of central than peripheral origin. No changes in the colon transport or barrier function were found in

the study. Even sodium absorption in the colon was not increased, which could have been expected by glucose malabsorption observed in the jejunum.

To summarize, tacrolimus induces the glucose malabsorption in the small bowel in a dose-dependent manner.

#### **4.2.1.2 Mycophenolic acid**

MMF and EC-MPA both deliver mycophenolic acid (MPA) as an active substance. EC-MPA was developed to decrease the MMF-associated gastrointestinal side effects by protecting the upper gastrointestinal tract (enteric-coating). The target of MPA is inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in the de novo synthesis of guanosine nucleotides, which are the essential factors for DNA synthesis. Lymphocytes do not possess the salvage pathway like most of the other cells. This results in the selective blockade of the lymphocytes proliferation. MPA inhibits the preferentially activated lymphocyte population (2).

Recently, a few authors noted that diarrhea occurrence does not significantly differ between therapies with MMF and EC-MPA (21, 63). Gastrointestinal side effects are the reasons for therapy reduction or discontinuation in 10% of the adult patients (10, 64), and also very often pediatric patients treated with MPA (65). Diarrhea tends to disappear after MMF withdrawal (21, 33, 66), and its occurrence seems dose-dependent (67). Thus, the gastrointestinal side effects of MPA are often treated by reducing the dosage. The impact of MMF dosage reduction on clinical outcome has recently been studied intensively, and there is no doubt that MMF dose reduction caused by gastrointestinal side effects is correlated to a poor clinical renal transplant outcome. This is due to the strong correlation between AUC of MPA, and the probability of acute graft rejection (11-13, 64). Gastrointestinal side effects after MMF therapy correlate more with  $C_{max}$  (peak level) than with the area under the curve (AUC). To avoid high peak MPA concentrations, the daily dose should be split into three per day or a low-release formula should be used (68). In the presented study no significant dose dependency was observed in the case of MPA influence on the intestine function.

There are only a few studies and theories describing the MPA influence on the gastrointestinal tract. Some authors describe the intestinal villous atrophy followed by the malabsorption syndrome (33, 66, 69-71) and some the inflammatory changes (63, 72). MPA inhibits selectively the de-novo purine biosynthesis pathway, whereas enterocytes are dependent in only approx. 50%. They fill the guanosine nucleotides pool by salvage



pathway as well. However, because of the great proliferative activity of the intestinal mucosa purine de-novo biosynthesis pathway depletion induced by MMF treatment might be of great importance (68). Also important is the fact that MPA is present in the epithelial cells of the gastrointestinal tract in a high concentration (68). Infection is probably not often a reason for post MPA diarrhea (21, 33, 70, 71), however MMF therapy can be a reason for rare gastrointestinal infections, like i.e. microsporidiosis (68). MPA itself is probably not as toxic for the intestinal mucosa as its carboxyl-linked glucuronide metabolite AcMPAG. It binds to such proteins as ATPase/ATP synthetase, protein disulfide isomerase (controls redox state of the cell) and selenium binding protein, decreasing energetic cell status and influencing its red-ox state (73). AcMPAG undergoes biliary-intestinal circulation and could be a reason for post MPA gastrointestinal disorders, especially when renal function is seriously impaired (74).

In the presented study, the direct exposition of MMF or EC-MPA did not cause any significant pathophysiological changes in the intestinal mucosa. In a toxic dose both MMF and EC-MPA decreased the chloride secretion, however a 14-day treatment with MPA did not influence chloride secretion.

In an oral exposition study, MMF influenced the intestinal barrier function, which was not seen in the EC-MPA groups (only an insignificant increase of  $J_{Lac}$  in HD,  $p=0,054$ ). EC-MPA in the high dose oral exposition group reduced glucose absorption ( $p<0.05$ ). These results support the theory about the toxic effect of MPA metabolite (AcMPAG) and not MPA itself (no direct influence on barrier and transport function). It is not clear why after 14 days of therapy with MMF and EC-MPA there are two different pathophysiological pictures of intestinal function alteration. It should be noted that MMF, at least during the early treatment period, influences the intestine barrier function more than EC-MPA. It diminishes the tight-junction function, though it could lead to leak-flux diarrhea. EC-MPA's inhibition of glucose transport (without achieving statistical significance after Bonferroni correction) could lead to malabsorptive diarrhea, however this effect is weaker than the similar effect of the EVE or TAC. That MPA reduces the effect on energetic and red-ox cell status, as mentioned above, is a possible reason for the observed changes in both MMF and EC-MPA groups.

### **4.2.1.3 mTOR inhibitors**

In the study the two ISD sirolimus (SIR) and everolimus (EVE), which belong to the mammalian target of the rapamycin (mTOR) inhibitor family, were analyzed. They both have similar molecules, and similar side effects (2).

SIR and EVE bind to the intracellular protein FKBP12, and unlike tacrolimus do not inhibit calcineurin but mTOR kinase. Inhibition of mTOR has a profound effect on the cell signaling pathway required for cell-cycle progression and cellular proliferation. The net effect is the blockade of T cell activation by preventing progression of the cell cycle from the G1 to the S phase. In addition to their immunosuppressive effects, mTOR-inhibitors inhibit fibroblast growth factors required for tissue repair, which can result in wound healing problems.

#### **4.2.1.3.1 Sirolimus**

Occurrence of Sirolimus (SIR) associated with diarrhea is not very clear. Some authors report it as low, some even as often as 33% (see Table 2, Page 11). This side effect is also dose-dependent (75, 76). Pathophysiology of sirolimus-associated diarrhea is not known. After two weeks of treatment with either a low or high therapeutic dose of rapamycin, we found nearly no significant alteration in any of the analyzed small bowel functions. One hour incubation with a potentially toxic dose (10 $\mu$ g/dl vs. therapeutic serum level of 10-15 ng/dl) of sirolimus altered the intestinal barrier function and chloride secretion (77). Dias et. al. (30) analyzed the intestinal function in rabbits treated for 10 days with 0,25 or 1 mg/kg of SIR. In that study, a significant weight gain and a food intake decrease were observed in both groups, and interestingly, increased glucose absorption in the 1 mg/kg group was found. Also the intestinal barrier function was altered in this group. Increased glucose transport was achieved by a higher dose and longer treatment periods than in that study (OES). However, such effects did not occur in the HD<sub>a</sub> (toxic concentration, direct exposition) group. The same authors also reported on the decreased mucosa weight and intestinal absorptive area (small bowel villous density, height and width), which is in contrast to the increased glucose absorption. SIR is also shown to inhibit GLUT2 trafficking (78) and therefore passive glucose absorption (GLUT2 trafficking and increase of passive glucose absorption takes place in much higher (48 mmol/l) concentrations than observed in that study (79)). In another study Dias et. al (59) also showed a decreased food intake, intestinal villous area and weight gain in rabbits treated with 1m/kg sirolimus for 10 days.

No influence on D-glucose transport was shown in this study. After 6 weeks of treatment with 2mg/kg every second day the rats were still losing weight and hence the food intake was decreased (80). They also had a decreased ileal (but not jejunal) villous area. Recently it has been shown that mTOR plays a key role in cell growth, proliferation and metabolism. It regulates a wide array of cellular functions (translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization and autophagy function) (81, 82). mTOR inhibition in the small intestine leads to mucosal atrophy, and probably also alters the tight-junction function (82). In our study, SIR treatment for two weeks caused no significant alterations of the small bowel chloride secretion, glucose absorption or barrier function. However we cannot exclude such effects after a longer treatment period.

#### **4.2.1.3.2 Everolimus**

Patients treated with everolimus (EVE) often suffer from diarrhea, and there are no data available on the pathomechanism of this disorder.

Among all analyzed ISD, EVE influences the small bowel function most in this study. ISD is the only group of drugs that diminishes the small bowel barrier function also after direct exposition, and decreases glucose absorption and chloride secretion. In the colon we measured an increased sodium absorption (Epithelial Sodium Channel –EnaC- function), which could be secondary to the glucose malabsorption in the jejunum. EVE's influence on the jejunum transport and barrier function is much more potent than that of SIR.

We agree with the above mentioned findings that mTOR inhibitors influence many cell functions (EVE >> SIR). A disturbed barrier function was seen in both the direct and oral exposition study, however glucose absorption and chloride secretion reduction are measured only in the animals treated for two weeks. This observation may lead to a point that the most important pathomechanism of EVE-associated small bowel toxicity is the barrier function alteration. However mTOR inhibitors have an influence on many cell cycles and proteins such as hypoxia inducible factor1 $\alpha$ , VEGF, PDGF, GLUT1 and others (27). Till now, no data exist about EVE's influence on a transport and tight-junction proteins in the bowel.

EVE like TAC is known for its relative narrow therapeutic spectrum, and this serum-level controlled therapy is often used in clinical practice. This study confirms the dose-dependent influence of EVE on the small bowel barrier function.

In summary, we have observed a global decrease of enterocyte (in the jejunum) functions after EVE therapy.

#### **4.2.1.4 FTY 720**

FTY 720 is a synthetic analogue of myriocin, a product of the ascomycete *Isaria sinclairii*. In vivo FTY720 is phosphorylated to the active metabolite FTY720-P, and this molecule targets the cell receptors for the natural lipid sphingosine 1-phosphate. Due to their increased sensitivity, homing cytokines lead to the sequestration of lymphocytes in the lymphoid organs and reduction of circulating lymphocyte population (2).

There are very few data about FTY influence on the gastrointestinal system. In a multicenter, randomized, phase III study of 696 de novo renal transplant patients, Tedesco-Silva et al. compared FTY720 in a 2.5 and 5 mg dosage with Cyclosporine A, and showed that FTY gastrointestinal side effects are comparable to MMF (83). In his other study he estimated the diarrhea incidence to be 14-20% for doses of 0,25 to 2,5 mg FTY in the renal transplant recipients, which was comparable with MMF (both protocols included Cyclosporine and corticosteroids) (36). One can assume that FTY therapy might be toxic to the intestine, but in our study neither 1,0 mg given for 14 days nor toxic dose directly influenced the intestinal transport or barrier function.

#### 4.2.2 Conclusions

In this study three common pathophysiological mechanisms of diarrhea were analyzed: malabsorption of glucose, secretion of chloride ions and impaired bowel barrier function (leak-flux). Dysmotility of the bowel as well as malabsorption of lactulose and fructose have to be analyzed in the future, since they might play a role in the development of post transplantation gastrointestinal disorders. In the complex clinical situation where a patient treated with ISD suffers from diarrhea, the role of applied drugs cannot be underestimated. In fact, when no bacteriological or viral pathogen can be found, it should be a reasonable approach to change the ISD scheme. Due to this change, the patient's chance of survival can be diminished and the transplant function can suffer when under-/over-immunosuppression occurs. For this reason it is of great importance to apply an ISD combination therapy with very limited potential side effects. According to results of the study, the therapy with TAC, MMF, EC-MPA or EVE might alone induce a small bowel or colon dysfunction and thus lead to diarrhea by the rat. In the case of TAC and EVE the changes observed were dose-dependent. The combination therapy with TAC, MMF, EC-MPA or EVE might lead to accumulation of adverse effects on the bowel by rat.

## 5 Summary

Malinowski M. Direct and systemic influence of immunosuppressive drugs on glucose absorption, barrier function and chloride secretion in the small and large bowel of the rat. Dissertation, Charité – Universitätsmedizin Berlin, Department: Human Medicine, Berlin 2010

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Transplantation is nowadays an optimal treatment for multiple end stage organ diseases. However, a lifelong immunosuppressive therapy is still necessary to prevent cellular and humoral rejection of the transplanted organ. This therapy is accompanied by serious side effects such as diarrhea. The impact of diarrhea can be significant due to patient dehydration and low quality of life. However most important is the poor outcome due to reduction or withdrawal of the immunosuppressive drugs (ISD) when diarrhea occurs.

There are five pathomechanisms leading to diarrhea: motility disorder, defect of the absorption mechanisms of i.e. lactulose (osmotic), malabsorption of nourishments (malabsorptive), increased chloride ion secretion (secretory), and impaired bowel barrier function (leak flux diarrhea). In the transplanted patient, bacterial overgrowth and influence of the ISD can induce diarrhea. In this study, malabsorptive, secretory and leak flux diarrhea were analyzed in the rat model. ISD nowadays used in the clinical practice were analyzed: Cyclosporine A (CyA), tacrolimus (TAC), mycophenolate mofetil (MMF), enteric coated mycophenolic acid (EC-MPA), sirolimus (SIR), everolimus (EVE) and FTY 720.

To assess the bowel transport and barrier function, the Ussing chamber method was used. The study was divided into two parts: direct (DES) and oral exposition study (OES). Male Wistar rats were used for all experiments. In the DES, the proximal jejunum of the rat was prepared and incubated for one hour with a low or high (toxic) concentration of the ISD. In the OES the rats were treated with a low or high therapeutic dose of the ISD. Afterwards glucose absorption, chloride secretion and barrier function of the jejunum, and chloride secretion, barrier function and sodium absorption of the late distal colon were measured.

In the CyA groups, no significant changes of the measured parameters were noticed. Rats treated with tacrolimus developed alterations of the glucose absorption, which was dose-dependent (OES). MMF caused impairment of the small bowel barrier function (OES) and a

decrease of the chloride secretion in the small bowel (DES). In the case of EC-MPA, impaired small bowel barrier function and showed a tendency to reduce the glucose absorption capacity (OES). SIR did not change significantly any of the measured parameters. Rats treated with EVE developed global dysfunction of the small bowel. Reduced glucose absorption, dose-dependent impaired small bowel barrier function and diminished chloride secretion (OES) were observed. FTY 720 had no significant influence on the small bowel transport or barrier function.

In conclusion, the direct exposition to the toxic dose of MMF, EC-MPA (chloride secretion) and EVE (small bowel barrier function) altered the small bowel transport or barrier function. TAC, MMF, EC-MPA as well as EVE significantly impaired the small bowel barrier or transport function after 14 days of treatment. Those effects were dose-dependent in the case of TAC and EVE. The combination therapy with TAC, MMF, EC-MPA or EVE might lead to accumulation of adverse effects on the bowel by rat.

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## Abbreviations

ISD -	immunosuppressive drugs
CNI-	calcineurin inhibitors
CyA-	cyclosporine A
TAC-	tacrolimus®
MPA-	Mycophenolic acid
MMF-	mycophenolate mofetil®
EC-MPA-	myfortic®
mTOR-	mammalian target of rapamycin
SIR-	sirolimus®
EVE-	everolimus®
FTY720	fingolimod
SM -	standard medium
SME -	standard medium enriched
$U_e$ -	transepithelial potential difference
$I_{sc}$ -	short circuit current
$R_t$ -	transepithelial resistance
$V_{max}$ -	epithelial 3OMG transport capacity by the 48 [mol] substrat concentration
$K_M$ -	3OMG concentration by $0.5 \cdot V_{max}$
$J_{Lac}$ -	$^3H$ -Lactulose flux
NKCC-	$Na^+2Cl^-K^+$ -cotransporter
$\Delta NKCC$ -	$\Delta I_{sc}$ after inhibition of the chloride transport with bumetanide
cAMP-	cyclic AMP
$PgE_2$ -	Prostaglandin $E_2$
$\Delta cAMP$ -	$\Delta I_{sc}$ after stimulation of the chloride transport with Theophylline and $PgE_2$
3OMG-	3-O-Methyl-D-gluckopyranose
SGLT1-	enterocyte apical $Na^+$ -glucose cotransporter
GLUT2-	enterocyte basolateral glucose transporter
$EI_{sc}$ -	intestinal basal overall transport with carbohydrates in the buffer
$SI_{sc}$ -	intestinal basal overall transport without carbohydrates in the buffer
$\Delta ENaC$ -	ENaC function measured as $\Delta I_{sc}$ after inhibition with amiloride
DES-	direct exposition study

LD<sub>d</sub>- low doses of the Immunosuppression drug in an DES  
HD<sub>d</sub>- high doses of the Immunosuppression drug in an DES  
OES- oral exposition study  
LD<sub>o</sub>- low doses of the Immunosuppression drug in an OES  
HD<sub>o</sub>- high doses of the Immunosuppression drug in OES



Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Addendum

## Selbstständigkeitserklärung

„Ich, Maciej Malinowski, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „ The direct and systemic influence of immunosuppressive drugs on intestinal glucose absorption, barrier function and chloride secretion in rat models“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum

Unterschrift

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