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# Transport Characteristics of a Beta Sheet Breaker Peptide Across Excised Bovine Nasal Mucosa

**Alexander Greimel and Andreas Bernkop-Schnürch**
Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innsbruck, Austria

**Maria Dorly Del Curto**
Eurand Int. S.p.A, Pessano con Bornago, (MI), Italy

**Mauro D'Antonio**
Industria Farmaceutica Serono, Ardea, Rome, Italy

**ABSTRACT** The purpose of the present study was to investigate the permeation characteristics of the beta sheet breaker peptide AS 602704 (BSB) on excised bovine nasal mucosa using an Ussing chamber model. The influence of various absorption enhancers such as sodium cholate, sodium dodecyl sulfate (SDS), cetrimidum, sodium caprate, $Na_2EDTA$, polycarbophil (PCP), the thiomer conjugate polycarbophil-cysteine (PCP-Cys), and poly-L-arginine (poly-L-arg; 100 kDa) was evaluated. Additionally, the influence of temperature and pH on the transport rate as well as the stability of the peptide drug against enzymatic degradation were investigated in vitro.

The effective permeability coefficient ($P_{eff}$) of BSB in Krebs-Ringer-buffer (KRB) pH 7.4 was $(1.89 \pm 0.44)^* 10^{-5}$, while in the presence of sodium caprate (0.5%) a $P_{eff}$ of $(9.58 \pm 1.82)^*10^{-5}$ was achieved. Rank order of enhancement ratio was sodium caprate > SDS > sodium cholate > $Na_2EDTA$ > poly-L-arg = PCP-Cys. In case of cetrimidum and PCP even a decrease in the absorption of BSB was determined. $Na_2EDTA$ reduced the enzymatic degradation of BSB when exposed to a nasal tissue homogenate by more than the half. An increased lipophilicity of BSB because of a more acidic milieu (pH 5.5) did not lead to an increased transcellular transport. Permeation studies carried out at 4°C compared to 37°C demonstrated a temperature dependent permeation behaviour suggesting an additional active carrier mediated transport.

The results obtained within these studies should facilitate the development of a nasal delivery system for AS 602704 for the treatment of Alzheimer's disease.

**KEYWORDS** Permeation enhancement, Ussing chamber, Nasal drug delivery, Beta sheet breaker peptide, Alzheimer's disease

Address correspondence to Andreas Bernkop-Schnürch, Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Josef-Möller-Haus, Innrain 52, 6020 Innsbruck, Austria; Tel: +43-512-507-5383; Fax: +43-512-507-2933; E-mail: andreas.bernkop@uibk.ac.at

## INTRODUCTION

Some major advantages offered by the nasal route of administration are rapid absorption, fast onset of therapeutic action, avoidance of gastrointestinal and first pass metabolism, and improved patient compliance (Romeo et al., 1998a). Lipophilic drugs can be expected to demonstrate rapid and efficient

absorption when given nasally, but more polar compounds are poorly absorbed. Various peptide drugs such as desmopressin, calcitonin, and gonadorelin analogues are currently available as nasal presentations, although associated with low bioavailability compared to subcutaneous application (Hussain, 1998; Illum, 2003). Therefore, the use of permeation enhancers in order to modify the mucosal transport is a main strategy to increase the bioavailability of peptides and hydrophilic nonpeptide drugs, respectively, following intranasal administration. A variety of excipients ranging from surfactants, bile salts, phospholipids, and cyclodextrins to chelating agents, polymeric systems, lipids, and miscellaneous systems (microspheres, liposomes, etc.) was introduced into this field of pharmaceutical research (Davis & Illum, 2003; Romeo et al., 1998b)

Alzheimer's disease (AD) is a devastating neurodegenerative disorder clinically characterized by a progressive cognitive impairment. Recently, much research in the field of AD has been focused on the deposition of amyloid plaques in the brain of patients (Citron, 2002; Clark & Karlawish, 2003; Scarpini et al., 2003). Among the several antiamyloid strategies that have been pursued in the past few years (Barrow, 2002) is the concept of beta sheet breaker (BSB) peptides (Adessi & Soto, 2002; Permanne et al., 2002a; Soto, 1999) as an attempt to develop a treatment with a disease modifying effect. The synthetic pentapeptide N-Ac-Leu-Pro-Phe-Phe-Asp-$NH_2$ (BSB/AS 602704; Fig. 1), which has already shown its capability in preventing amyloid formation and dissolving amyloid plaques in a mouse model of amyloidosis in vivo (Permanne et al., 2002b) belongs to this new class of drugs.

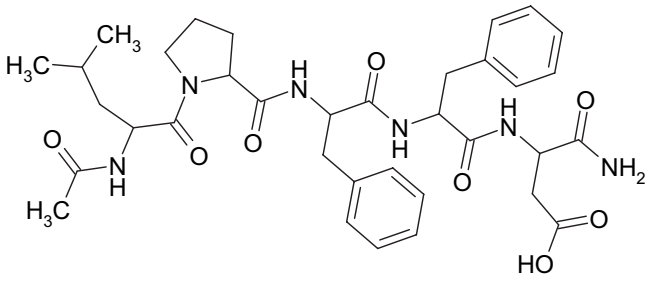

**FIGURE 1** **Chemical Structure of the BSB Peptide (AS 602704; Amino Acid Sequence: Acetyl-Leu-Pro-Phe-Phe-Asp-$NH_2$; MW: 678.76 Da).**

The development of a nasal delivery system for this peptide drug could be an alternative opportunity for the treatment of Alzheimer's disease. Therefore, it was the aim of this study to investigate the permeation characteristics of this promising drug on excised bovine nasal mucosa in vitro, which marks the first approach in the course of the development of a nasal delivery system. Within this study the influence of various permeation enhancers as well as the temperature and the pH was evaluated. Additionally, the stability of the drug was investigated in vitro.

## MATERIALS AND METHODS

### Materials

The BSB peptide AS 602704 was provided by Serono, Italy. Polycarbophil (PCP; Noveon™ AA1) was kindly donated by Noveon Pharma GmbH & Co. KG, Raubling, Germany. The polycarbophil-cysteine conjugate was synthesized by a method described previously (Bernkop-Schnürch et al., 1999). Acetonitril and trifluoracetic acid used for HPLC analysis were both purchased from Acros Organics, Belgium. All other chemicals were of reagent grade and obtained from Sigma, Vienna, Austria.

### In Vitro Nasal Permeation Studies

Tissue with nasal mucosa (conchae nasales) was excised from the noses of freshly slaughtered cattle and stored in Krebs-Ringer-buffer (KRB; ion composition (mM): $MgCl_2$*$6H_20$ 0.492; KCl 4.56; NaCl 119.8; $Na_2HPO_4$ 0.70; $NaH_2PO_4$ 1.5; $NaHCO_3$ 15; D-glucose 10) on ice during transport to the laboratory. Mucosa was separated from the underlying cartilage by blunt stripping using a pair of tweezers and mounted in Ussing-type diffusion chambers displaying a permeation area of 0.64 $cm^2$. The apical side of the tissue was thereby facing the donor compartment. Preheated (37°C) KRB pH 7.4 was added to the donor and acceptor chamber (1 mL each). To ensure oxygenation and agitation a mixture of 95% $O_2$ and 5% $CO_2$ was bubbled through each compartment. The temperature within the chambers was maintained at 37°C (Schmidt et al., 2000). After a preincubation time of 15 min the buffer medium in the donor chamber was substituted by the respective test system or fresh buffer medium as control. The final concentration (m/v) of

**TABLE 1** **Effective Permeability Coefficients of BSB (AS 602704) on Excised Bovine Nasal Mucosa (Means ± SD; $n = 3$) and Comparison of the Enhancement Ratios of Different Enhancing Systems Compared to Krebs-Ringer-Buffer (KRB; 37°C; pH 7.4). Significantly Different From Control: *$p < 0.001$, **$p < 0.01$, ***$p < 0.02$, ****$p < 0.05$**

| Enhancing system | $P_{eff}$ [*$10^{-5}$ (cm/sec)] | Enhancement ratio |
|---|---|---|
| Sodium caprate 0.5% | 9.58 ± 1.82 | 5.07** |
| SDS 0.5% | 8.66 ± 1.17 | 4.58* |
| Sodium cholate 0.5% | 5.47 ± 0.42 | 2.89* |
| $Na_2EDTA$ 1% | 3.26 ± 0.51 | 1.72**** |
| $Na_2EDTA$ 0.5% | 3.04 ± 0.08 | 1.61*** |
| Poly-L-Arg (100 kDa) 0.5% | 2.07 ± 0.24 | 1.10 |
| PCP-Cys 0.5%/GSH 0.5% | 1.96 ± 0.07 | 1.04 |
| KRB/37°C/pH 7.4 | 1.89 ± 0.44 | 1.00 |
| KRB/37°C/pH 5.5 | 1.78 ± 0.17 | 0.94 |
| unmod. PCP 0.5% | 1.65 ± 0.30 | 0.87 |
| Cetrimidum 0.5% | 0.88 ± 0.13 | 0.47*** |

the various permeation enhancers is listed in Table 1. Furthermore each sample contained 0.02% (m/v) BSB. Over a time period of 90 min samples of 150 µL were withdrawn from the acceptor compartment every 15 min. After centrifugation (24000 g for 10 min) 100 µL of the supernatant were transferred into vials and the amount of permeated peptide drug was quantified by HPLC as described below. Cumulative corrections were made for the previously removed samples.

The viability of the mucosal tissue was evaluated by measuring the transepithelial electrical resistance (TEER) before and after the experiment (EVOM Epithelial Voltohmmeter, World Precision Instruments, Berlin, Germany). Furthermore, the nasal mucosa was investigated microscopically after staining with trypan blue dye at the end of the experiment, whereas fresh tissue served as control.

### *HPLC Conditions*

Analysis of peptide samples by reverse phase HPLC was conducted using a Perkin-Elmer (Norwalk, CT) series 200 LC pump, Perkin-Elmer 200 series auto sampler with a 20 µL injection loop and a diode array detector (Perkin-Elmer 235C). Samples were eluted from a Nucleosil 100 – 5 C18 column (250 × 4 mm), with mobile phase A – 0.1% TFA, B – 90% acetonitrile, 0.1% TFA; flow rate of 1.0 mL $min^{-1}$; detection was at 215 nm. A linear gradient was applied from 90% A to 10 % A over a 22 min period.

### *Data Analysis*

Effective permeability coefficients $P_{eff}$ (cm/s) for the BSB peptide were calculated according to the following equation:

$$P_{eff} = (dC/dt)_{ss}{}^{*}(V/A^{*}C_D) \quad (1)$$

where $(dC/dt)_{ss}$ is the pseudo steady-state change of concentration over time (µg/$cm^3$s), $V$ is the volume of the acceptor compartment ($cm^3$), $A$ is the diffusion area of the Ussing-type diffusion chamber ($cm^2$), and $C_D$ is the initial concentration of the BSB peptide in the donor compartment (µg/$cm^3$).

Transport enhancement ratios were calculated from $P_{eff}$ values by:

$$R = P_{eff}(\text{sample}) / P_{eff}(\text{control}) \quad (2)$$

## Influence of the pH on the Transport Rate of BSB

Additionally, in order to determine the influence of the pH on the transport rate across excised bovine nasal mucosa, the permeation of BSB in buffer medium only at pH 7.4 compared to pH 5.5 (adjusted with 1 M HCl) was investigated under the same experimental setup as described above.

## Influence of the Temperature on the Transport Rate of BSB

The permeation of BSB at 37°C was compared with the permeation at 4°C across excised bovine nasal mucosa in KRB pH 7.4 under the same experimental setup as described above. In addition to this investigation, the same study was carried out using sodium fluorescein (NaFlu) as hydrophilic model compound. The final concentration of BSB and NaFlu in the donor compartement was 0.02% and 0.001%, respectively. In brief, over a time period of 90 min samples were withdrawn from the acceptor compartment at predetermined time points and the amount of permeated BSB and NaFlu, respectively, was quantified either by HPLC as described above or photometrically via a fluorescence detector (extinction: 485 nm/emission: 520 nm; Fluostar Galaxy, BMG Labtechnologies,

Germany). Cumulative corrections were made for the previously removed samples. Permeation factors were calculated from $P_{eff}$ values by:

$$R = P_{eff}\,(37°C)/P_{eff}\,(4°C). \quad (3)$$

## Orientating Drug Stability Studies

The stability of BSB regarding a possible enzymatic degradation by peptidases of the nasal mucosa was investigated in vitro. Therefore, pieces of bovine nasal mucosa were mechanically homogenised with a tissue grinder in order to set the intracellular located enzymes free. Then aliquots of 20, 50, and 100 mg of this homogenate were transferred into an Eppendorf vessel and the peptide drug was added in a Tris*HCl buffered medium (1.5 mL; 25 mM, pH=6.8) in a final concentration of 500 µg per ml. Over a time period of 3 hr samples of 150 µL were withdrawn every 30 min and 50 µL of HCl (1 M) were added in order to stop any possible further enzymatic degradation. After centrifugation (24,000 g for 10 min) 100 µL of the supernatant were transferred into vials and the amount of intact BSB was quantified by HPLC as described above.

## Inhibition Studies

The influence of $Na_2EDTA$ on the stability of BSB regarding enzymatic degradation was investigated under the same experimental set up as described above. In brief, aliquots of 100 mg of the nasal tissue homogenate were transferred into Eppendorf vessels containing 0.5% (m/v) $Na_2EDTA$ and the active peptide in a final concentration of 0.05% (m/v). Over a time period of 3 hr samples of 150 µL were withdrawn every 30 min and 50 µL of HCl (1 M) were added in order to stop any possible further enzymatic degradation. After centrifugation (24,000 g for 10 min) 100 µL of the supernatant were transferred into vials and the amount of intact peptide drug was quantified by HPLC as described above. Samples containing the peptide drug in the buffer medium with and without the nasal tissue homogenate served as references.

## Statistical Data Analysis

Statistical data analysis was performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance.

# RESULTS

## Nasal Permeation Studies

Within this study it could be revealed that the permeation enhancing effect on excised bovine nasal mucosa of the assayed permeation enhancing systems (see Table 1) varies in a wide range. The transport of BSB was strongly increased by the addition of anionic compounds such as the sodium salt of a bile acid, a sulfonic acid and a middle chain fatty acid (Fig. 2). In particular, the permeation of BSB in the presence of sodium cholate, sodium dodecyl sulfate (SDS) and sodium caprate was increased 2.9-fold, 4.6-fold and 5.1-fold, respectively. $Na_2EDTA$, used in a final concentration of 0.5% (w/v) as well as 1% (w/v), showed a permeation enhancing effect up to 1.7-fold for the higher concentration, whereas the quaternary ammonium excipient cetrimidum even decreased the amount of permeated peptide drug by half (Fig. 3). The use of polymeric enhancers such as poly-L-arginine and a thiomer conjugate in combination with the permeation mediator glutathione showed only a low and statistically insignificant increase of the permeation rate. The effective permeability coefficients of the various test compounds as well as their enhancement ratios, compared to the permeation of BSB in KRB, are summarised in Table 1.

Histological studies demonstrated the viability of the tissue as no dead cells were found after staining

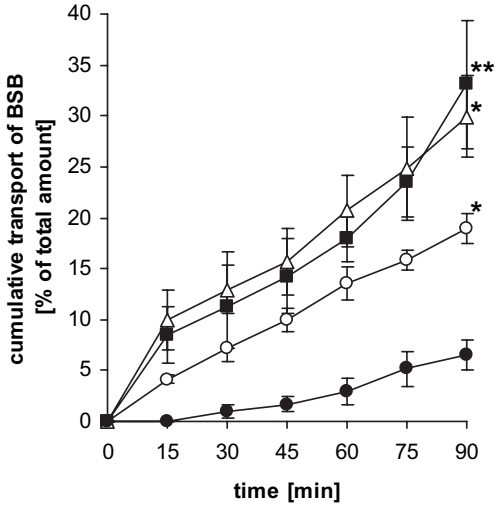


**FIGURE 2** **Transport of BSB (AS 602704) Across Excised Bovine Nasal Mucosa in the Presence of 0.5% (m/v) Sodium Caprate (■), Sodium Dodecyl Sulfate (SDS; Δ) and Sodium Cholate (○) Compared With Krebs-Ringer-Buffer (KRB; pH 7.4; ●). Differs From Control: *$p < 0.001$, **$p < 0.01$. Indicated Values are Means (± SD, $n = 3$).**

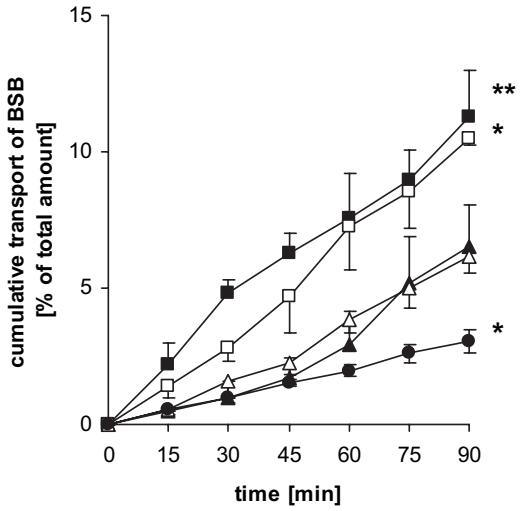


**FIGURE 3** Transport of BSB (AS 602704) Across Excised Bovine Nasal Mucosa in the Presence of 1% (m/v) $Na_2EDTA$ (■), 0.5% (m/v) $Na_2EDTA$ (□) and 0.5% (m/v) Cetrimidum (●), Compared to KRB pH 7.4 (▲). Transport of BSB (AS 602704) Across Excised Bovine Nasal Mucosa at pH 5.5 (Δ) Compared to KRB pH 7.4. Differs From Control: *$p < 0.02$, **$p < 0.05$. Indicated Values are Means (±SD, $n = 3$).

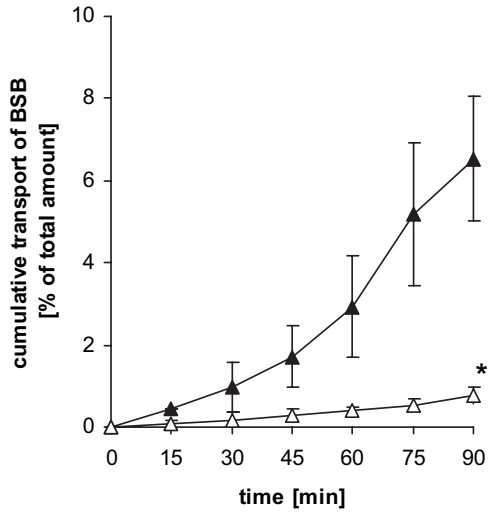


**FIGURE 4** Transport of BSB (AS 602704) Across Excised Bovine Nasal Mucosa in Krebs-Ringer-Buffer (KRB; pH 7.4) at 37°C (▲) Compared to 4°C (Δ). *Differs From 37°C, $p < 0.01$. Indicated Values are Means (±SD, $n = 3$).

with trypan blue under microscopic investigation. Furthermore, no significant change in the TEER could be observed after the experiment. The TEER of the tissue was determined to be in the range of 80 $\Omega.cm^2$.

## Influence of the pH on the Transport Rate of BSB

Permeation studies carried out at pH 5.5 demonstrated that a more acidic milieu has no significant influence on the permeation behaviour of BSB. As shown in Table 1 and Fig. 3, the shift from pH 7.4 to the lower value did not alter the amount permeated across excised bovine nasal mucosa.

## Influence of the Temperature on the Transport Rate of BSB

Within this study the influence of the temperature on the permeation properties of BSB across excised bovine nasal mucosa was evaluated. Additionally, the permeation behaviour of NaFlu was investigated under the same experimental set up. Results as shown in Fig. 4 demonstrates that the amount of permeated BSB across the nasal mucosa was 8.6-fold lower at 4°C compared to 37°C. On the contrary, this testing revealed no significant difference between the transport rate of NaFlu at 37°C and 4°C, respectively. The effective permeability coefficients of both compounds concerning the various test conditions as well as the permeation factors are summarised in Table 2.

**TABLE 2** Effective Permeability Coefficients of BSB (AS 602704) and Sodium Fluorescein (NaFlu) on Excised Bovine Nasal Mucosa in Krebs-Ringer-Buffer pH 7.4 at 37°C Compared to 4°C (Means ± SD; $n = 3$). Permeation Factors Were Calculated According to the Equation $R = P_{eff}$ (37°C)/$P_{eff}$ (4°C); *$p < 0.01$

| Compounds | Temperature | $P_{eff}$ [$*10^{-5}$ (cm/sec)] | Permeation factor |
|---|---|---|---|
| BSB | 4°C | 0.22 ± 0.06 | 8.59* |
| | 37°C | 1.89 ± 0.44 | |
| NaFlu | 4°C | 1.67 ± 0.26 | 1.56 |
| | 37°C | 2.61 ± 0.81 | |

## Drug Stability and Inhibition Studies

First orientating studies showed a decrease of intact BSB in the presence of 100 mg and 50 mg of the nasal mucosal homogenate. When BSB was exposed to only 20 mg of it, no degradation could be observed (data not shown). This obvious concentration dependent trend of enzymatic degradation of intact BSB in the presence of 100 mg of a bovine nasal tissue homogenate could be proven within further studies concerning the influence of $Na_2EDTA$ on the degradation

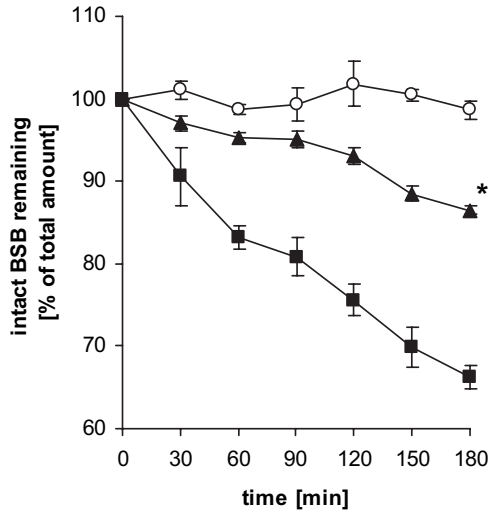


**FIGURE 5** **Comparison of the Decrease of Intact BSB (AS 602704) in the Presence of Nasal Tissue Homogenate: BSB (■); BSB Plus 0.5% (m/v) $Na_2EDTA$ (▲). BSB Incubated in Buffer Medium Without Tissue Homogenate Served as Control (o). *Differs From BSB, $p < 0.001$. Indicated Values are Means (±SD, $n = 3$).**

level. After 3 hr one third of the initial amount of the peptide drug was degraded as a result of the exposure to the tissue homogenate compared to the control, BSB incubated in Tris*HCl buffer only. The addition of 0.5% (w/v) $Na_2EDTA$ could reduce the enzymatic degradation by more than the half (Fig. 5).

## DISCUSSION

Within this study various well-established absorption enhancers from different classes such as bile salts, surfactants, fatty acids, chelating agents and polymers were evaluated concerning their permeation enhancing effects on BSB across excised bovine nasal mucosa. Because of its reproducible quality, this model membrane seems to be well-suited for studies on the nasal permeation characteristics of peptides (Merkle et al., 1998; Lang et al., 1996). Additionally, a good in vitro in vivo correlation could be achieved in recent studies with this model (Leitner et al, 2004).

The present study demonstrated that all of the three transcellular permeation enhancers (sodium cholate as well as sodium laurylsulfonate and sodium caprate) used display a strong permeation enhancing effect. Among these anionic low molecular mass permeation enhancers sodium caprate displayed the highest enhancement ratio. The permeation of BSB was increased up to 5.1-fold compared to the buffer medium. On the contrary, the addition of cetrimidum even decreased the amount of permeated drug by half. It can be assumed that ionic interactions between the free carboxylic group of the asparaginic acid moiety of BSB and the cationic structure of the quaternary ammonium compound are responsible for this effect. Because BSB displays per se good permeation properties, the formation of ion-pairs leading to a reduced solubility could in such a case negatively influence the transport rate to some extent. The effective permeability coefficient of BSB on excised bovine nasal mucosa is in good correlation with those of buserelin and salmon as well as human calcitonin – peptide drugs nasally administered in daily clinical practice (Lang et al., 1998).

Poly-L-arginine and the thiomer conjugate (polycarbophil-cysteine) in combination with the permeation mediator glutathione (Clausen et al., 2002) revealed to have a weak, but statistically insignificant effect on the permeation rate. Nevertheless, these two polymeric enhancers, which facilitate the paracellular uptake of hydrophilic drugs, have already shown their enhancement potential on the nasal mucosa of rats in vivo (Leitner et al., 2004; Natsume et al., 1999; Ohtake et al., 2002). In contrast to these findings $Na_2EDTA$ increased the permeation of BSB in a statistically significant way up to 1.7-fold. In addition to its permeation enhancing properties $Na_2EDTA$ also inhibited the degradation of the peptide drug when exposed to a homogenate of bovine nasal tissue. This enzyme inhibiting properties of $Na_2EDTA$ are in good correlation with results obtained by Sayani et al. (1993).

Permeation studies carried out at pH 5.5 failed to support the theory that the peptide molecule should pass the mucosa to a higher ratio in a more acidic milieu as a result of the higher extent of protonation of its carboxylic acid group. Shifting the hydrophilic-lipophilic-balance of the peptide to the lipophilic side did not increase the transport of BSB via the transcellular route.

Furthermore, it could be shown that at a temperature as low as 4°C the amount of BSB permeated across the nasal mucosa was 8.6-fold lower than at 37°C. In comparison to the outcome of the studies carried out with sodium fluorescein – no significant difference could be observed there –, this seems to indicate an additional active carrier mediated mechanism of uptake on the nasal mucosa. In this context Adessi et al. (2003) suggested a saturable transport mechanism, most likely a receptor mediated transport for the crossing of the blood brain barrier of a derivative

of BSB. Even unknown at present, the transport systems at both membranes are eventually underlying the same mechanism of action.

## CONCLUSION

Nasal administration of the BSB peptide AS 602704 could be an alternative opportunity for the treatment of Alzheimer's disease. Therefore, the mucosal barrier in the nasal cavity has to be overcome. Within this study various low molecular weight permeation enhancers strongly increased the transport rate of the peptide drug across excised bovine nasal mucosa in vitro. A more acidic milieu leading to an increased lipophility of BSB did not influence the transport rate. Furthermore, a temperature dependent permeation behaviour could be revealed assuming an additional carrier mediated transport across nasal mucosa. These results might represent substantial information for the development of nasal delivery systems for BSB.

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