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TARGETING PHARMACONS TO THE BRAIN VIA THE NASAL PATHWAY

Summary of Ph.D. thesis

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1. INTRODUCTION

The blood-brain barrier (BBB) is a dynamic interface separating the brain from systemic circulation. Therapeutical compounds to reach the central nervous system need to pass through the brain capillaries, however they prevent the brain uptake of 98% of all potential neurotherapeutics. Due to specialised BBB functions only small lipophilic molecules are able to penetrate freely into the brain tissue by passive transport. In order to enhance the blood to brain transport and deliver drugs in effective concentrations to the brain, several approaches have been attempted. These methods include the modification of the drug molecules, or the manipulation of the BBB. The selection of an application site circumventing the BBB is a third possibility. Pharmacons can be dircetly targeted to the brain tissue, to the cerebrospinal fluid compartments, or to tumor tissue by injections, medical sponges or minipumps. In addition the nasal and ocular routes represent important new alternative gateways to the brain. Intranasal delivery of drugs offers several advantageous properties. This method is non-invasive, essentially painless, can be easily and readily administered by the patient or a physician. Furthermore it ensures rapid absorption, the avoidance of first-pass metabolism in gut and liver and does not require sterile preparations.

The unique anatomical and physiological properties of the olfactory region provide both extracellular and intracellular pathways into the central nervous system that bypass the BBB. The pathways employed for delivery of a particular drug from the nose to the brain is highly dependent on various factors, such as the existence of specific receptors on the olfactory neurons, the lipophilicity and the molecular weight of the drug.

During the formulation of a dosage form intended for intranasal application several aspects should be taken into consideration. Nasally administered drugs are cleared rapidly from the nasal cavity into the gastrointestinal tract by the mucociliary clearance system. The low membrane permeability and the junctional complexes of nasal epithelial cells hinder the trans- and paracellular transport of large molecular weight or polar drugs, and limit their nasal absorption. Moreover, the nasal mucosa

has a metabolic capacity as well, which can contribute to the low transport of peptides and proteins across the nasal membrane. Because of these reasons application of mucoadhesive agents to increase residence time of formulations on the nasal mucosa and absorption enhancers to elevate drug transport across the nasal mucosa is crucial in nasal vehicles.

Although a range of novel nasal products for systemic delivery of therapeutics has reached the market already, there is still no drug exploiting the nasal route to treat CNS diseases. Development of nasal delivery systems that enable rapid and efficient concentration of drugs in the brain is a new and important field of investigation.

2. AIMS

Considering the above mentioned aspects of nasal drug targeting to the brain, the importance of the permeability properties of the olfactory region, and the role of nasal vehicles in drug delivery, the main aims of our experiments were the following:

(1) to reveal the protein composition of the junctional complexes of the olfactory region by immunohistochemistry,
(2) to characterize the permeability of blood vessels in different parts of the olfactory system,
(3) to formulate and characterize carrier systems for nasal delivery containing bioadhesive and/or absorption enhancer components,
(4) to measure plasma and brain pharmacokinetics and bioavailability using different nasal vehicles and as a test molecule fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4.4 kDa (FD-4) in rats,
(5) to test the acute toxicity of nasal vehicles on the olfactory system.

3. MATERIALS AND METHODS

3.1. Antibodies and immunohistochemistry

To detect specific tight junction proteins in fixed olfactory and brain tissue, polyclonal rabbit sera against claudin-1, claudin-3, claudin-5, claudin-19, occludin, ZO-1, ZO-2, connexin-43, and monoclonal mouse antibody against ZO-1 were used as primary antibodies. As secondary antibodies goat anti-mouse and anti-rabbit IgG labelled with cyanin-derivative dye Cy3 or Cy2 were chosen. Nuclei were stained with Sytox or Topro dyes.

3.2. Permeability studies

Vascular permeability of the olfactory system was demonstrated by extravasation of the markers fluorescein (MW 376 Da) and Evan's blue that binds serum albumin (MW 67 kDa) injected intravenously to rats. When the permeability of the blood vessels was tested by electron microscopy, 1% lanthanum nitrate was added to the fixative used for transcardial perfusion.

3.3. Preparation of dosing solutions

Absorption enhancer Cremophor RH40 was dissolved in physiological saline solution. Sodium hyaluronate was added in small amounts to the solution and to ensure the complete solvation samples were rehomogenized after 24 h. The FD-4 was dissolved in the prepared vehicles at a concentration of 1 mg/ml for intranasal and 8 mg/ml for intravenous administration.

Table 1. Compositions of the dosing solutions (in weight percentage)

Vehicle	Physiological saline	Sodium hyaluronate	Cremophor RH40
PhS	100.0%	-	-
AE	90.0%	-	10%
HA	98.5%	1.5%	-
MA	88.5%	1.5%	10%

3.4. Rheological measurements

Rheological measurements were carried out with a Rheostress 1 Haake instrument. A cone-plate measuring device was used in which the cone angle was 1º, and the thickness of the sample was 0.048 mm in the middle of the device. The flow and viscosity curves of the samples were determined by changing the shear rate between 0.01 and 100 s^{-1} at 37ºC.

3.5. *In vitro* drug release studies

Dissolution studies were carried out using ointment cells and small volume dissolution vessels in a Hanson SR8-plus dissolution apparatus. Samples, 0.4 g each, containing 1 mg/ml FD-4 were placed as donor phase on the Porafil membrane filter (pore diameter 0.45 μm). PBS (pH = 7.4, 100 ml) was used as dissolution medium at a temperature of 37ºC. Samples (3 ml each) were taken and immediately replaced with fresh dissolution medium at 15, 30, 60, 120, 180, 240, 300, 360, 420 and 480 min, and further analyzed by spectrofluorometry. Six parallel measurements were performed in case of each dosing solution.

3.6. Animal experiments

Male Wistar rats (316 ± 48 g, 3-month-old) were used for all the studies.

3.6.1. Treatments

The rats were anesthetized ip. with tribromoethanol solution and they were placed in supine position. An average of 40 μl solution was then administrated by micropipette positioned 5 mm deep into the right naris to achieve the longest possible residency time of the vehicle in the nasal cavity. In case of intravenous treatment 500 μl solution was injected into the tail vein of anesthetized animals. There were five treatment groups, and five different time points, 30, 60, 120, 240 and 480 min, during the experiments.

Table 2. The composition of the experimental groups.

Vehicle	**Treatment**	**n**	**V_{FD-4}**	**C_{FD-4}**	**m_{FD-4}**
PhS	intravenous	4	500 µl	8 mg/ml	4000 µg
PhS	intranasal	4	40 µl	1 mg/ml	40 µg
AE	intranasal	4	40 µl	1 mg/ml	40 µg
HA	intranasal	4	40 µl	1 mg/ml	40 µg
MA	intranasal	4	40 µl	1 mg/ml	40 µg

n, number of animals/group; V, volume of the dosing solution given for treatment; C, concentration of FD-4 in dosing solutions; m, quantity of FD-4 given in dosing solutions/animal; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40.

3.6.2. Collection of plasma and brain samples for the measurement of FD-4 levels

In deep anesthesia blood was taken by cardiac puncture into heparinized tubes from all four rats in each treatment group and at each time point, then the animals were transcardially perfused. The brains were removed and 10 brain regions including the olfactory bulb, frontal and parietal cortex from the left and the right side, the hippocampus, the midbrain, the pons and the cerebellum were excised. The weight of the brain samples was measured. Blood samples were centrifuged, and plasma and brain samples were kept frozen until further investigations.

3.7. Determination of FD-4 concentration of samples

Brain samples were homogenized with 7.5% w/v trichloroacetic acid solution in Potter–Elvehjem tissue grinder. Homogenized samples were centrifuged, supernatants were neutralized. The FD-4 content of samples was determined by a PTI spectrofluorometer (Photon Technology International Inc.) at excitation wavelength of 492.5 nm and emission wavelength of 514.5 nm. The sensitivity of the measurement was 1 ng/ml FD-4 in the samples. Linearity of the measurement of FD-4 was $r^2 \geq$

0.998, while the precision was found to be RSD ≤ 9.19 % across all the concentration ranges used in the study.

3.8. Electron microscopy

Rats were transcardially perfused with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The olfactory tissue was dissected out and postfixed in the identical fixative for additional 4 h, and then stored in cacodylate until further processed. Cerebral cortex, olfactory bulb, nasal mucosa were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer and then dehydrated in an ethanol series. The specimens were embedded, ultrathin sections were cut by ultramicrotome, mounted on pioloform-coated copper grids, contrasted with lead citrate and analyzed and documented with an EM10A (Zeiss) electron microscope.

3.9. Statistical analysis

All data presented are means ± SEM or SD. The values were compared using GraphPad Prism software. The analysis of variance was followed by Newman–Keuls multiple comparison test. Changes were considered statistically significant at $P < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Rheological measurements

Viscosity of the vehicle may influence the diffusion speed of the incorporated drug, in this way play important role in the drug release process. Low shear stress values and a linear correlation between shear stress and shear rate were measured for both the physiological saline solution and the absorption enhancer containing system, typical for Newtonian flow behavior. The addition of the surface active agent in AE slightly increased the slope, reflecting the viscosity of the sample. The viscosity values of HA and MA vehicles containing sodium hyaluronate were higher, than for PhS and AE samples. HA and MA solutions can be characterized by pseudoplastic flow behavior.

4.2. *In vitro* drug release studies

Carriers PhS and AE, not containing biopolymer, showed similar dissolution kinetics and profiles but in the case of vehicles containing the mucoadhesive hyaluronan, HA and MA, different dissolution profile could be detected. The addition of hyaluronan slowed the release of FD-4 between 30-240 min, but the same amount of FD-4 was released from the vehicles at 8 hours.

4.3. The morphological basis of nasal pathway

4.3.1. Immunostainings of junctional proteins in the olfactory system

Epithelial and endothelial barriers separate the organisms from the external environment and the body compartments from each other. The intercellular seals responsible for the effective separation are called tight junctions (TJ). In our studies we revealed by immunohistochemistry the junctional protein compositions of the major barriers of the olfactory system. These are (i) the layers of epithelial cells connected by apical TJ complexes, (ii) endothelial cells of the blood vessels in the lamina propria and (iii) the olfactory ensheathing cells (OECs) and perineural cells protecting the axons of the sensory olfactory neurons which are projected from the periphery to the CNS.

Table 3. Summary of the immunohistochemical findings in the major barriers of the olfactory system.

Junctional protein	Epithelial cells	Endothelial cells	OECs
ZO-1	+++	+++	+++
ZO-2	++	-	-
Occludin	+++	+	+++
Claudin-1	+++	-	-
Claudin-3	+++	-	-
Claudin-5	+++	+	++
Claudin-19	++	-	-

The TJ protein pattern of OECs is similar to that of endothelial cells, and may consist of claudin-5, occludin and ZO1. According to our data the olfactory epithelial layer presents the most complex and possibly the most tight barrier from the point of view of drug targeting.

4.3.2. Permeability properties of the olfactory region

Dye study

We found that the blood vessels of the lamina propria in both the respiratory and olfactory regions are highly permeable to the dyes fluorescein and Evan's blue in contrast to the vessels of the brain. These findings are in agreement with our data on the protein composition of the endothelial TJs of the olfactory mucosa.

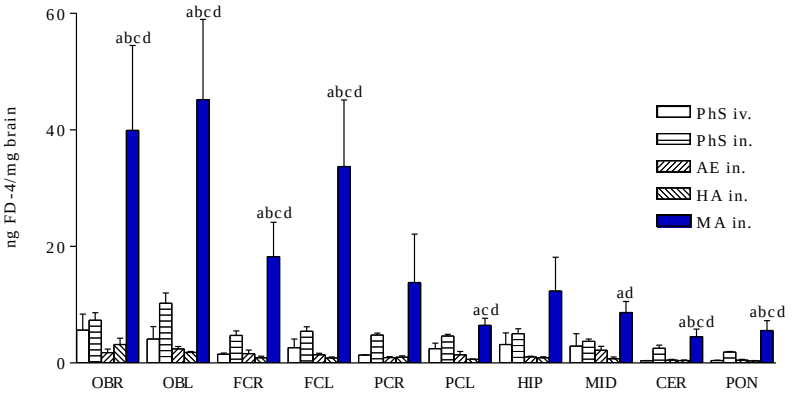
Lanthanum study

We demonstrated for the first time that the blood vessels of the lamina propria of the olfactory mucosa are leaky for perfused lanthanum nitrate as an accepted electron microscopical tracer. The OEC TJs form a barrier for lanthanum nitrate, which is incomplete allowing a low amount of leakage from the interstitial to the periaxonal space. This finding explains the morphological basis of the transport pathway between the nasal cavity and the CNS via the olfactory nerve. Our data also indicate, that if a molecule is transported across the olfactory epithelium with the help of an appropriate nasal vehicle, the nasal pathway demonstrated for lanthanum can be used to reach the CNS.

4.4. Nasal targeting of FD-4 test molecule by different vehicles in rat

The main finding of our studies is that the combination of the surfactant Cremophor, as an absorption enhancer, and hyaluronic acid, as a viscosifying and bioadhesive polymer, could significantly increase the nose to brain transport of the test molecule FD-4, especially in the olfactory bulb and frontal cortex regions, 4 hours after administration.

Fig. 1. FD-4 concentrations in different brain regions measured 240 min after different treatments



OBR: olfactory bulb from right side; OBL: olfactory bulb from the left side; FCR: frontal cortex from the right side; FCL: frontal cortex from the left side; PCR: parietal cortex from the right side; PCL: parietal cortex from the left side; HIP: hippocampus; MID: midbrain; CER: cerebellum; PON: pons. Statistically significant differences ($P < 0.05$) were detected in the MA group compared to the values measured in [a]PhS iv. group; [b]PhS in. group; [c]AE group; [d]HA group. Data are presented as mean ± SEM, n = 4.

We hypothesize that peptides or peptide fragments in the size range of 1-4 kDa acting on neuropeptide or hormon receptors in the nervous system, eg. peptides regulating appetite and body weight, could be targeted with the MA formulation for therapeutical purposes.

4.4.1. Surfactants as absorption enhancers

The surfactant Cremophor RH40 was used as an absorption enhancer. Although surfactants, which are non-specific permeation enhancers, can improve the absorption of drugs by increasing their paracellular and transcellular transport via the modification of the cell membrane, no increase in the brain FD-4 levels was observed after application of AE vehicle compared to that of the PhS. A possible explanation can be the fast removal of both vehicle from the nasal cavity by the mucociliar

activity. The higher test molecule levels in brain regions using MA formulation as compared to HA clearly indicates that Cremophor played an important role in enhancing the permeability for FD-4 of the main barrier of the olfactory system, namely the epithelium.

4.4.2. Role of mucoadhesion in nasal vehicles

Although viscosity and mucoadhesion are key factors in nasal drug delivery, and hyaluronates have excellent mucoadhesive properties, natural biocompatibility, and lack of immunogenicity, only one study tested viscous sodium hyaluronate solutions in nasal absorption so far, but pharmacokinetics in the blood or in the CNS were not determined.

According to our data in contrast to the combination of hyaluronan with Cremophor RH40 (MA vehicle) sodium hyaluronate alone (HA vehicle) did not increase the amount of FD-4 transported to the brain in our experiments.

Table 4. Pharmacokinetic parameters in brain.

Vehicle	**c_{max} [ng FD-4/mg brain] mean ± SD**	**t_{max} [min]**	**AUC mean ± SD**	**Relative BA mean ± SD**
PhS iv.	1.72 ± 0.90	240	598 ± 178	1.0 ± 0.3
PhS in.	4.49 ± 0.14	120	1523 ± 97	254.7 ± 16.3
AE in.	2.07 ± 0.21	30	554 ± 83	92.7 ± 13.9
HA in.	1.39 ± 0.09	60	431 ± 52	72.2 ± 22.7
MA in.	15.21 ± 9.11	240	3358 ± 1713	561.5 ± 286.5

AUC, area under the concentration-versus-time curve; BA, bioavailability compared to the PhS iv. group; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40; n = 4.

The difference between the efficacy of nasal vehicles HA and MA may indicate, that optimal viscosity, supposed mucoadhesivity, longer residence time and absorption enhancement induced by the nonionic surfactant all contributed to the increased brain penetration of the test molecule.

The effect of MA is not related to elevation in plasma levels of FD-4, since FD-4, injected intravenously, resulted in a high concentration in plasma, and a low concentration in brain, due to the highly selective and strictly regulated transport processes of the BBB. The nasal formulations led to negligible plasma concentration, while the brain concentration of FD-4 in MA group exceeded that of the intravenously administered FD-4. This clearly indicates the circumvention of the BBB and a direct access to the CNS.

Table 3. Pharmacokinetic parameters in plasma.

Vehicle	**c_{max} [ng FD-4/ml plasma] mean ± SD**	**t_{max} [min]**	**AUC mean ± SD**	**Relative BA mean ± SD**
PhS iv.	8253 ± 848	30	592411 ± 82638	1.00 ± 0.14
PhS in.	6.52 ± 3.16	30	1887 ± 730	0.32 ± 0.12
AE in.	3.04 ± 2.35	30	1129 ± 567	0.19 ± 0.10
HA in.	4.36 ± 0.53	30	1719 ± 79	0.29 ± 0.01
MA in.	9.73 ± 6.27	120	1847 ± 1060	0.31 ± 0.18

AUC, area under the concentration-versus-time curve; BA, bioavailability compared to the PhS iv. group; PhS, physiological saline solution; AE, vehicle containing absorption enhancer Cremophor RH40; HA, vehicle containing hyaluronan alone; MA, vehicle containing mucoadhesive hyaluronan and absorption enhancer Cremophor RH40; n = 4.

Toxicity is a major issue in the development of formulations for the nasal route. It is very important to note, that in our study the vehicle containing sodium hyaluronate and Cremophor RH 40 did not induce tissue damage, epithelial or subepithelial toxicity, or ciliotoxicity demonstrated by electron microscopy.

5. SUMMARY

The delivery of hydrophilic drugs or large biopharmaceuticals to the nervous system is still a big challenge. The blood-brain barrier prevents the brain penetration of the great majority of possible therapeutical molecules. Targeting drugs to brain tissue requires transport through the blood-brain barrier, or utilization of alternative routes. Due to the specific anatomical and physiological properties of the olfactory region, the nasal pathway can be exploited as a direct route to the nervous system.

The three major barriers of the olfactory system are the epithelial cells, the endothelial cells of the blood vessels in the lamina propria and the olfactory ensheating cells which protect the axons of the olfactory neurons. We revealed by immunohistochemistry, that the tight junction protein pattern of rat endothelial and olfactory ensheating cells are similar and consist of claudin-5, occludin and ZO-1. The olfactory epithelial cells express also ZO-2, claudin-1, -3 and -19 in addition to the proteins found in endothelial cells, indicating that they represent the most complex and the tightest barrier from the point of view of drug targeting. In accordance with these data we found that blood vessels of the lamina propria of the olfactory mucosa are leaky for lanthanum, an electron microscopical tracer. The olfactory ensheating cells also form an incomplete barrier for lanthanum allowing leakage from the interstitial to the periaxonal space. This finding explains for the first time the morphological basis of the transport pathway between the nasal cavity and the nervous system via the olfactory nerve.

We designed and tested nasal vehicles to overcome these barriers and target the test molecule fluorescein-labelled 4 kDa dextran by the nasal pathway to the brain in rats. From the four tested vehicle, the combination of the surfactant Cremophor, as an absorption enhancer, and hyaluronic acid, as a viscosifying and bioadhesive polymer, could significantly increase the nose to brain transport of the test molecule, especially in the olfactory bulb and frontal cortex regions. Morphological examination of the olfactory system revealed no toxicity of the vehicles.

In conclusion, the nasal pathway has a potential for targeting therapeutical drugs to the central nervous system by circumventing the blood-brain barrier.

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7. PUBLICATIONS, POSTERS AND CONFERENCE LECTURES

Publications related to the subject of the thesis

I. Wolburg H., Wolburg-Buchholz K.,·Sam K., **Horvát S.**, Deli M.A., Mack A.F.
Epithelial and endothelial barriers in the olfactory region of the nasal cavity of the rat.
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IF: 2.320 (2008)

II. **Horvát S.**, Fehér A., Wolburg H., Sipos P., Veszelka S., Tóth A., Kis L., Kurunczi A., Balogh G., Kürti L., Erős I., Szabó-Révész P., Deli M.A.
Sodium hyaluronate as a mucoadhesive component in nasal formulation enhances delivery of molecules to brain tissue.
European Journal of Pharmaceutics and Biopharmaceutics; 72: 252-259, 2009.
IF: 3.344 (2008)

III. **Horvát S.**, Kis L., Dr. Szabóné Dr. Révész P., Dr. Erős I., Dr. Deli M.
Hatóanyagok agyba juttatása a nazális útvonalon keresztül**.**
Gyógyszerészet; 53: 259-266, 2009.
IF: -

Posters and conference lectures related to the subject of the thesis

PP = Poster presentation, OP = Oral presentation

1. Deli M.A., Fehér A., Kurunczi A., Sipos-Bodó E., Veszelka S., **Horvát S.**, Penke B., Szabó-Révész P.,Targeting molecules to brain by intranasal delivery.
9th Symposium on Signal Transduction in the Blood-Brain Barriers, Salzburg, Austria, September 7-10, 2006 OP

2. Kurunczi A., Fehér A., Veszelka S., Sipos E., **Horvát S.**, Szabó-Révész P., Erős I., Párducz Á., Penke B., Deli M., β-amiloid peptid bejuttatás agyba intranazális úton - egy új lehetséges Alzheimer-kór modell?
X. Alzheimer-kór konferencia, Szeged, Hungary, September 20-22, 2006 PP

3. Fehér A., Kurunczi A., Veszelka S., Sipos E., **Horvát S.**, Párducz Á., Penke B., Deli M., Erős I., Révész P., B-amiloid peptid központi idegrendszerbe irányuló transzportjának vizsgálata intranazális alkalmazás esetén.
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4. **Horvát S.**, Fehér A., Kurunczi A., Veszelka S., Penke B., Erős I., Szabó-Révész P., Deli M., Molekulák agyba juttatása intranazális beadással.
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5. Deli M. A., **Horvát S.**, Fehér A., Veszelka S., Kurunczi A., Kürti L., Penke B., Erős I., Szabó-Révész P., Targeting molecules to brain by intranasal delivery.
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6.. Fehér A, **Horvát S.**, Veszelka S., Kurunczi A., Kürti L., Erős I., Deli M.A., Révész P., Study of drug transport to the central nervous system following intranasal administration.
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7. **Horvát S.**, Fehér A., Veszelka S., Kurunczi A., Kürti L., Penke B., Erős I., Szabó-Révész P., Deli M.A., Bioadhesive formulations increase the brain targeting of molecules by the nasal pathway.

Signal Transduction in the Blood-Brain Barriers, Potsdam, Germany, September 13-16, 2007 PP

8. **Horvát S.**, Fehér A., Veszelka S., Kurunczi A., Kürti L., Penke B., Erős I., Szabó-Révész P., Deli M.A., Bioadhesive formulations increase the brain targeting of molecules by the nasal pathway.
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9. **Horvát S.**, Fehér A., Veszelka S., Tóth A., Kis L., Sipos P., Kürti L., Erős I., Szabó-Révész P., Deli M.A., Sodium hyaluronate as a mucoadhesive component in nasal formulation enhances delivery of molecules to brain tissue.
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10. **Horvát S.**, Fehér A., Sipos P., Veszelka S., Tóth A., Kis L., Kurunczi A., Kürti L., Erős I., Szabó-Révész P., Deli M.A., A hialuronsav, mint mukoadhezív komponens, növeli a molekulák agyba juttatását a nazális útvonalon keresztül.
    A Magyar Gyógyszerésztudományi Társaság Ipari Szervezete, Gyógyszertechnológiai és Gyógyszerkutatási Szakosztályának Konferenciája, Sopron, Hungary, September 25-27, 2008 PP

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    Sófi József Alapítvány Konferencia, Szeged, Hungary, March 25, 2009 OP