Blood-brain barrier transport kinetics of NOTA-modified proteins: the somatropin case

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Abstract

BACKGROUND: Chemical modifications such as PEG, polyamine and radiolabeling on proteins can alter their pharmacokinetic behaviour and their blood-brain barrier (BBB) transport characteristics. NOTA, *i.e.* 1,4,7-triazacyclononane-1,4,7-triacetic acid, is a bifunctional chelating agent that has attracted the interest of the scientific community for its high complexation constant with metals like gallium. Until now, the comparative BBB transport characteristics of NOTA-modified proteins versus unmodified proteins are not yet described.

METHODS: Somatropin (*i.e.* recombinant human growth hormone), NOTA-conjugated somatropin and gallium-labelled NOTA-conjugated somatropin were investigated for their brain penetration characteristics (multiple time regression and capillary depletion) in an *in vivo* mice model to determine the blood-brain transfer properties.

RESULTS: The three compounds showed comparable initial brain influx, with K_{in} = 0.38 ± 0.14 µL/(g×min), 0.36 ± 0.16 µL/(g×min) and 0.28 ± 0.18 µL/(g×min), respectively. Capillary depletion indicated that more than 80% of the influxed compounds reached the brain parenchyma. All three compounds were *in vivo* stable in serum and brain during the time frame of the experiments.

CONCLUSIONS: Our results show that modification of NOTA as well as gallium chelation onto proteins, in casu somatropin, does not lead to a significantly changed pharmacokinetic profile at the blood-brain barrier.

Key words: NOTA-modified somatropins; blood-brain barrier permeability; Gallium chelation; *in vivo* mice model, growth hormone

Introduction

Protein biopharmaceuticals have marched their way into the medicine landscape since the approval of the first recombinant protein insulin, Humilin®, in 1982. Since then, further research on protein modifications, either engineered (*e.g.* polyethylene glycol (PEG)-ylation, drug-conjugation, radiolabeling), originating from the expression host (*e.g.* post-translational modifications (PTM)) or resulting from the manufacturing process, have gained the interest by the biopharmaceutical community. Chemical modifications change the physicochemical properties, hence, alter the pharmacologic activity and pharmacokinetic profile of the protein under investigation. For example, changed elimination via a combination of changed proteolysis, renal ultrafiltration, liver clearance and/or starvation by the immune system, as well as specific interaction and/or accumulation within tissues is observed for PEGylations.[1, 2] Also modifications of proteins with bifunctional chelating agents (BFCA) such as 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) allow the incorporation/chelation of radiometals (*e.g.* ^{68}Ga and ^{67}Cu) and conjugation onto a targeting ligand via a linker, thereby opening the way for theranostic applications.[3-6]

Conjugating moieties typically target functional groups on proteins such as amine, carboxyl and sulfhydryl, which are generally present in multiple copies on the protein and which can lead to complex product outcomes.[3, 7] The conjugate-targeting ligand ratio or substitution degree can influence the target-binding functionalities, the *in vivo* stability, clearance and biodistribution.[8-10]

In general, the ability to freely cross the blood-brain barrier (BBB) depends on the physicochemical properties (*e.g.* molecular size, charge and lipophilicity) and is often a closed route for proteins. However, chemical modifications on proteins such as polyamine modifications have demonstrated a higher permeability at the BBB compared to their unmodified counterparts.[11-13]

In this study, the model protein of interest is somatropin (*i.e.* a recombinant human growth hormone (rhGH), INN) modified with 2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) moieties.[7] Currently, evidence points to the involvement of growth hormone (GH) and its receptor (GHR) on tumour progression.[14-22] The GHR internalizes after signal transduction and nuclear GHR-localization is associated with different cancers, which makes GHR an interesting target to further explore for theranostic purposes.[23-26]

Previously, speculations were made on the passage of somatropin over the BBB, because expression of the GHR has been demonstrated in the central nervous system (CNS) (amongst others choroid plexus, hypothalamus, pituitary, hippocampus and putamen [27-29]) and administration of somatropin has established positive effects on the CNS (reviewed in [30, 31]). Pan *et al.* demonstrated that rat and mouse GH are characterized with a significant blood-to-brain influx transfer constant in rats.[32] However, as chemical modifications of proteins, such as NOTA-conjugation, are often required in radiopharmaceutical medicines, an unresolved question was if those modifications influence the BBB transport and if they do show a different behaviour once complexed with a metal? To our knowledge, the comparative BBB transport characteristics of NOTA-modified proteins versus unmodified proteins are not yet reported. We investigate here the blood-brain barrier behaviour of NOTA-modified somatropin and gallium-chelated NOTA-modified somatropin as model compounds.

Materials and methods

Chemicals and reagents

Zomacton® 4 mg (Ferring, somatropin Ph.Eur.) was obtained from Ghent University Hospital (Ghent, Belgium) and p-SCN-Bn-NOTA was purchased from Macrocyclics Inc. (Dallas, TX, USA). Bovine serum albumin (BSA) was obtained from Merck KGaA (Darmstadt, Germany). Dermorphin was synthesised at Hanhong (Shanghai, China). Ultrapure water was purified with a quality of 18.2 MΩ.cm using an Arium 611 purification system (Sartorius, Göttingen, Germany). For the purification of gallium-chelated NOTA-somatropins, the PD-10 sephadex G-25M column was obtained from GE healthcare (Diegem, Belgium). The Pierce® Pre-Coated iodination tubes, iodine-125 carrier free radionuclide and argent filters used during radiolabeling of the compounds were purchased at Thermo Scientific (Erembodegem, Belgium), Perkin Elmer (Zaventem, Belgium) and Sterlitech (Kent, USA), respectively. The other chemicals and reagents were purchased at Sigma Aldrich (Diegem, Belgium): gallium trichloride, potassium chloride, calcium dichloride dihydrate, sodium lactate, magnesium sulphate, hydrated sodium dihydrogen phosphate, urethane, D-glucose, sodium hydroxide, trichloroacetic acid (TCA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and protease inhibitor cocktail; VWR (Leuven, Belgium): disodium hydrogen phosphate dihydrate and sodium chloride; and AppliChem GmbH (Darmstadt, Germany): dextran. Gradient grade acetonitrile and trifluoroacetic acid (TFA) came from Fisher Scientific (Erembodegem, Belgium).

Animals

The animal experiments were performed according to the Ethical Committee principles of laboratory animal welfare and approved by our institute (Ghent University, Faculty of Veterinary Medicine, Prof. Dr. K. Hermans, approval number EC2014/128, approved on 17th of November 2014). All efforts were made to minimize suffering. Female, Institute for Cancer Research, Caesarean Derived-1 (ICR-CD-1) mice of age 7-10 weeks and weighing 29-32 g, were obtained from Envigo RMS B.V. (Venray, Netherlands).

Compounds under investigation

The BBB behaviour of somatropin, 10:1 NOTA:somatropin and gallium-chelated 10:1 NOTA:somatropin (or Ga-10:1 NOTA:somatropin) was investigated. The synthesis and analytical characterization of 10:1 NOTA:somatropin is described in Bracke *et al.*[7] The NOTA-moieties of 10:1 NOTA:somatropin were chelated with gallium by incubating 1 mg of 10:1 NOTA:somatropin, dissolved in 500 µL ammonium acetate buffer (0.1 M pH 5.5, supplemented with 0.2 mM acetylaceton) together with 1500 µL of a 571 µM gallium solution (285 µL of a 4 mM $GaCl_3$ bulk solution in 0.1 M HCl added to 570 µL 0.05 M NaOH and 1140 µL ammonium acetate buffer) and incubated for 1 h at 37°C in the dark, while shaking at 750 rpm. The sample was loaded onto a PD-10 column (previously rinsed using 25 mL of 25 mM phosphate buffer, pH 7.4) and eluted using 2 mL of a 25 mM phosphate buffer, pH 7.4. BSA and dermorphin were included as negative and positive controls in the MTR study, both dissolved in 25 mM phosphate buffer pH 7. Both control compounds are specific for their BBB-influx purpose, while in other experiments like CD or stability, these 2 controls are of no relevance, and assurance of the experimental results are obtained by good laboratory practices.

Radiolabeling of the compounds

The radiolabeling of the compounds was performed using the Iodogen method.[33-35] In brief, the Pierce® Pre-Coated iodination tubes were pre-rinsed with 1 mL 25 mM phosphate buffer pH 7.4. Then, 50 µL of a 1.1 mM NaI in 500 mM phosphate buffer pH 7.4 and 15 µL $Na^{125}I$ (eq. 1 mCi) were added in the Iodogen tubes, mixed and incubated for six minutes at room temperature. The iodonium solution was transferred to a tube containing 50 µg protein or peptide and the reaction was proceeded for six minutes at room temperature. Purification (*i.e.*

separation of the unbound iodonium vs. radiolabeled protein) was performed by adding 500 µL 25 mM phosphate buffer pH 7.4 to the reaction mixture, followed by filtration through an argent filter. The appropriate protein and peptide concentrations (*i.e.* 30 000 cpm/µL for multiple-time regression analysis (MTR) and 10 000 cpm/µL for capillary depletion (CD)) were prepared using Lactated Ringer's solution containing 1% of BSA (LR/BSA).

Multiple-time regression analysis

After anesthetization of the ICR-CD-1 mice by intraperitoneal injection with a 40% (m/V) urethane solution (3 g/kg), the jugular internalis vein and carotid artery were isolated and 200 µL of the compound solution was injected into the jugular vein. Blood was collected from the carotid artery at regular time points after injection (1, 1, 3, 5, 10, 12.5, 15 and 15 min; one time point per mouse and a total of eight mice per compound, which is in accordance to previous MTR designs reported by our group), followed by immediate decapitation.[35-39] This was done independently for each of the compounds (somatropin, 10:1 NOTA:somatropin, Ga-10:1 NOTA:somatropin and both controls). Next, the brains were collected and weighed, and serum was prepared by centrifugation of the blood at 10 000 *g* for 15 min at 21°C. Radioactivity of the blood, serum and brain was measured using a Wallac Wizard automatic γ-counter (Perkin Elmer, Shelton, CT, USA).

In order to determine the BBB permeability of the compounds, the ratio of the brain and serum concentration (µL/g) was plotted versus the exposure time (Θ).[40] The exposure time is calculated as the integral of the concentration of compounds in the serum (C_p) from start (t=0 min) to the experimental time T (*i.e.* the area under the curve), divided by the concentration of compound in serum at time T: $\Theta = \int_0^T \frac{C_p(t) \cdot dt}{C_p(T)}$ (1). Working with the exposure time instead of the experimental time, allows to correct for the serum radioactivity decay over time and represents the theoretical steady state serum concentration level. A linear model of the blood-brain transfer was used to derive the influx parameters as elaborated by Gjedde and Patlak:

$$\frac{A_m(t)}{C_p(t)} = K_{in}\,\Theta + V_i \qquad (2)$$

where $A_m(t)$ is the amount of radioactivity in brain (cpm/g) at time T, $C_p(t)$ is the amount of radioactivity in serum (cpm/µL) at time T, K_{in} is the influx rate constant and V_i is the initial volume of distribution.[41-43]

The elimination rate constant (k_e) and serum half-life ($T_{1/2}$) were calculated from the serum-concentration time curves, based on a one compartment model: $c = c_0 \times e^{-k_e t}$ (3) and $T_{1/2} = \ln(2)/k_e$ (4).[44]

Capillary depletion

The CD method was performed to distinguish the distribution of the compounds (somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin) between brain parenchyma and cerebral capillaries.[35-39, 45, 46] Each compound was injected in two anesthetized mice as described in the MTR section. Before decapitation at 10 minutes post injection, blood was collected from the abdominal aorta followed by cardiac perfusion with 20 mL LR buffer, after clamping the aorta and severing the jugular veins. Brains were collected and radioactivity was measured, followed by homogenisation of the brain in 0.7 mL ice cold capillary buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 10 mM D-glucose, pH 7.4) and 1.7 mL of ice cold 26% m/V dextran solution in capillary buffer. The resulting homogenate was weighed and centrifuged at 4500 g for 30 min at 4°C. The pellet (*i.e.* capillaries) and supernatant (*i.e.* parenchyma and fat tissue) were weighed and measured in the γ-counter. Serum was prepared from the collected blood by centrifugation at 10 000 g for 15 minutes at 21°C and measured in the γ-counter as well. The compartmental distribution was calculated as follows:

$$Fraction_{capillaries\ or\ parenchyma}(\%) = \frac{A_{capillaries\ or\ parenchyma}}{A_{capillaries} + A_{parenchyma}} \times 100 \quad (5)$$

Tissue distribution after IV injection

From the 15 minutes time points from the MTR study, tissues (spleen, kidney, liver, lungs, heart and serum) were collected after decapitation, weighed (W_{tissue}) and radioactivity was measured in the γ-counter (A_{tissue}). The percentage of injected dose (i.d.) for each tissue was calculated as follows, with $A_{IV\ injected}$ the measured radioactivity of the MTR stock solution and W_{mouse} the weight of the injected mouse:

$$\%\ i.d. = \frac{A_{tissue}/W_{tissue}}{A_{IV\ injected}/W_{mouse}} \times 100 \quad (6)$$

In vivo stability

The *in vivo* stability evaluation was performed according to Pan *et al*.[32] Proteins in serum samples from the MTR study were precipitated by addition of 15% TCA. Supernatant and pellet, corresponding to degradation fragments and intact protein, respectively, were separated by a 30 minutes centrifugation step at 4000 g. The brains collected during the MTR study, were homogenized in 1 mL of ice cold LR/BSA solution and 10 µL protease inhibitor cocktail. This mixture was centrifuged for 2 minutes at 4000 g and 500 µL of the supernatant was transferred into a new tube. Intact protein was precipitated by addition of 15% TCA, vortexed and separated from supernatant by a 30 min centrifugation step at 4000 g and 4°C. Radioactivity (A) of the supernatant and the pellet of brains and serum were measured in the γ-counter. The percentage intact protein was calculated as follows $Percentage\ intact\ protein = \frac{A_{pellet}}{A_{pellet} + A_{supernatant}}$.[7] For the kinetic evaluation, the percentage of intact protein at 1 minute post injection was taken as a 100% reference. Assuming first-order kinetics, a linear regression analysis of the ln(percentage intact protein) versus the post-injection time was performed to calculate the half-lives of the somatropins in mouse serum and brain.

Statistics

Regression analysis was performed using the least squares method. Regression lines of the MTR study were statistically compared using the Prism 5 software (Graphpad, La Jolla, USA). Slopes were not statistically significantly different if the P-value was greater than 0.05.

Results

Blood-to-brain transport kinetics

In **Figure 1** and **Table 1**, the MTR results of somatropin, 10:1 NOTA:somatropin, Ga-10:1 NOTA:somatropin and both controls are given. The investigated somatropins all showed a significant influx with a K_{in} between 0.28 and 0.38 µL/(g×min) and similar initial distribution volumes, *i.e.* V_i between 17.12 and 22.11 µL/g, which were not significantly different from each other (**Figure 1A**). The V_i resembles the effective vascular brain distribution volume of the protein including the protein in the vascular space and bound to or accumulated in capillary endothelial cells. Based on the peptide classification according to their BBB influx, somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin have a low influx.[47]

The data of BSA and dermorphin (**Figure 1B**) demonstrated the validity of the results and were comparable to previous influx data published by our group or found in literature:

iodinated BSA shows brain K_{in} values of around 0.13 µL/g×min and brain V_i of around 15 µL/g, which is considered as almost absent, and thus a class 1 compound according to Stalmans *et al.*; dermorphin is considered a positive control which shows a significant but relatively low brain influx (class 2 according to Stalmans *et al.*). [35-39, 47, 48] The combination of both controls serves as a system suitability quality test of the MTR-experiment: a clear difference between the brain influxes of both compounds should be observed to assure this in-vivo experiment.

Overall, the somatropins had a higher K_{in} and V_i value than the negative control BSA.

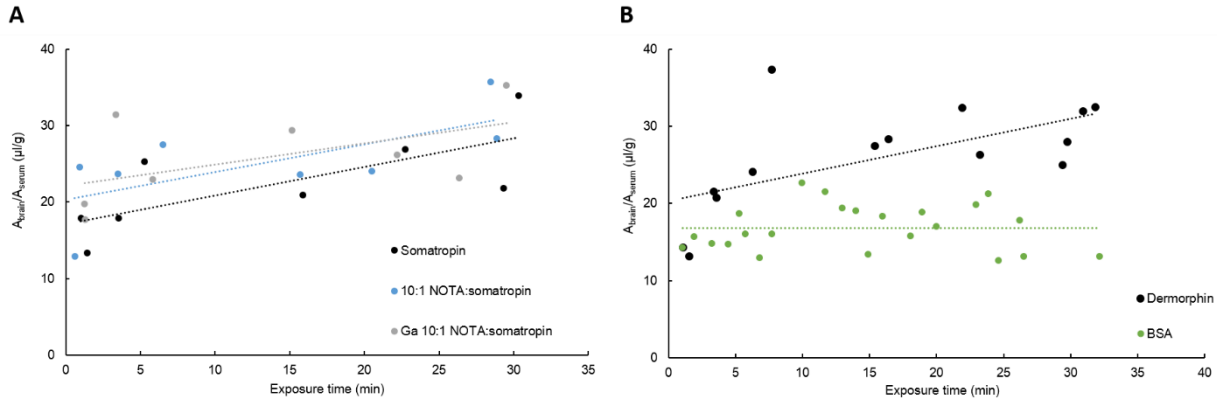

Figure 1: Multiple time regression curves of the exposure time versus the ratio of the brain and serum activity. A: Somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin. B: BSA and dermorphin, negative and positive controls respectively. Data were fitted using the linear Gjedde-Patlak model.

Table 1: Overview of the multiple time regression results, with the 65% confidence interval indicated.

Parameter	Somatropin	10:1 NOTA:somatropin	Ga-10:1 NOTA:somatropin	BSA	Dermorphin
K_{in} (µl/(g × min))	0.38 ± 0.14	0.36 ± 0.16	0.28 ± 0.18	0.13 ± 0.05	0.48 ± 0.09
V_i (µl/g)	17.12 ± 2.60	20.28 ± 2.80	22.11 ± 3.05	15.12 ± 0.69	18.24 ± 1.64

K_{in} = unidirectional influx rate
Vi = initial brain distribution volume

The radioactivity in serum (corrected for injected dose) was plotted versus time (**Figure 2**) and the serum half-lives were estimated. The serum half-lives were in the same order of magnitude, *i.e.* between 7 and 10 min, for somatropin, NOTA-modified somatropin and gallium-labeled NOTA-modified somatropin (**Table 2**).

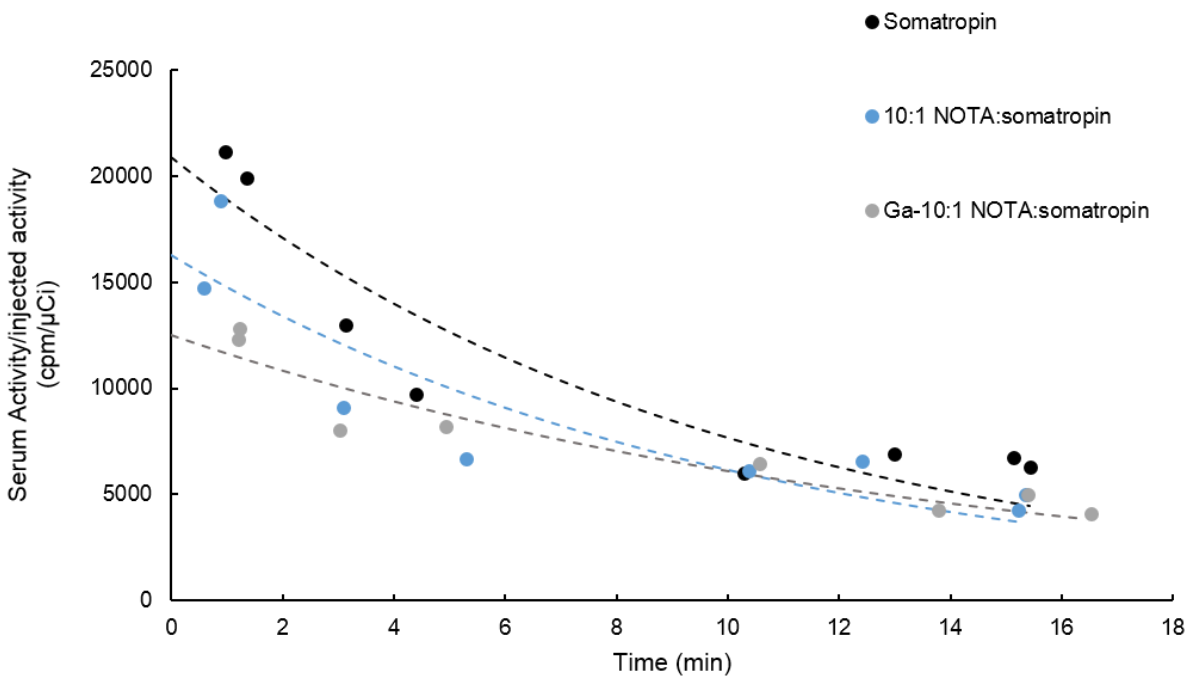

Figure 2: Serum concentrations of somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin in function of time (min), as obtained during the MTR experiments. Data were fitted using a one compartment model (dashed lines).

Table 2: Serum radioactivity time curve parameters.

Parameter	Somatropin	10:1 NOTA:somatropin	Ga-10:1 NOTA:somatropin
k_e (min^{-1})	0.100	0.097	0.072
c_0	20883	16245	12617
$T_{1/2}$ (min)	6.911	7.138	9.673
R^2	0.859	0.795	0.899

Parameters are based on a one compartment model

Capillary depletion

The compartmental distribution of the radiolabelled compounds in the brain was estimated using the capillary depletion method. In the CD method, a cardiac perfusion step washes out the residual radioactivity in the brain vasculature, thereby allowing to distinguish between compounds that have reached the brain parenchyma and compounds that are trapped in the capillaries. The parenchymal fraction amounted 80% for somatropin, which is comparable to previous obtained results and confirms the validity of the method.[32] Parenchymal fractions of 82% and 81% were obtained for 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin, respectively (**Figure 3**). This indicates that only about 20% of the measured radioactivity was trapped in the brain capillaries.

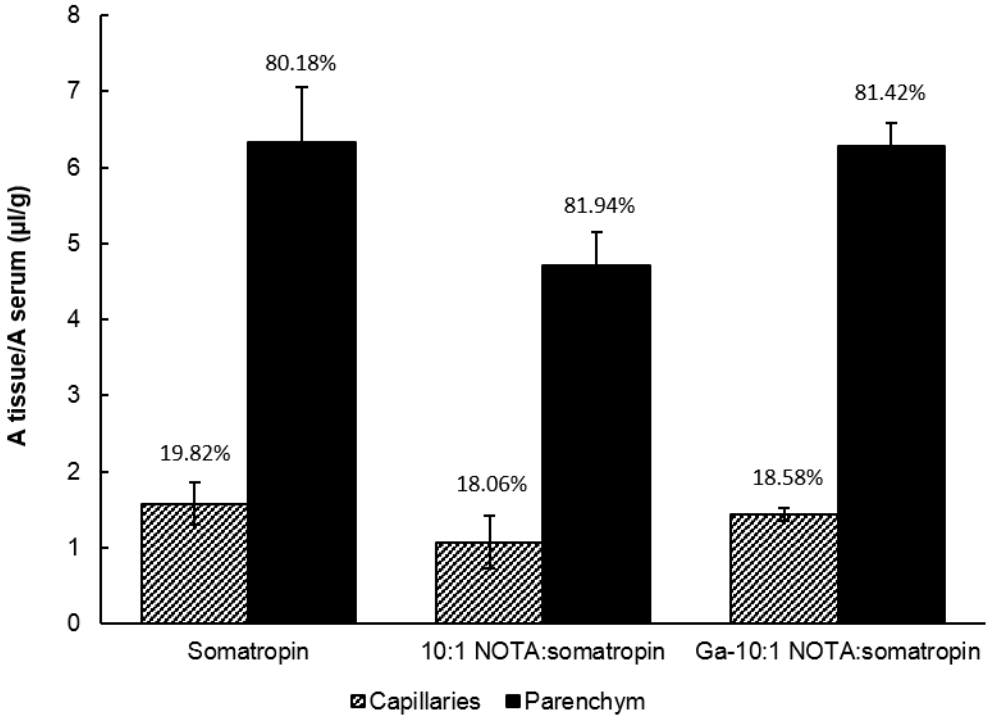

Figure 3: Capillary depletion compartmental distribution results normalized for brain weight (mean absolute amounts in µL/g ± SEM, n=2), showing a very high brain parenchymal penetration for all investigated compounds. A_{tissue} and A_{serum} are the measured activities in the concerned tissue and serum, respectively.

Tissue distribution

After 15 minutes post injection, both somatropin and 10:1 NOTA:somatropin were mainly found in serum, kidney and liver. Once gallium is complexed in 10:1 NOTA:somatropin, the compound was mainly distributed in serum (**Figure 4**). The tissue distribution of the positive and negative control corresponded well with previously obtained research results.[35, 37-39]

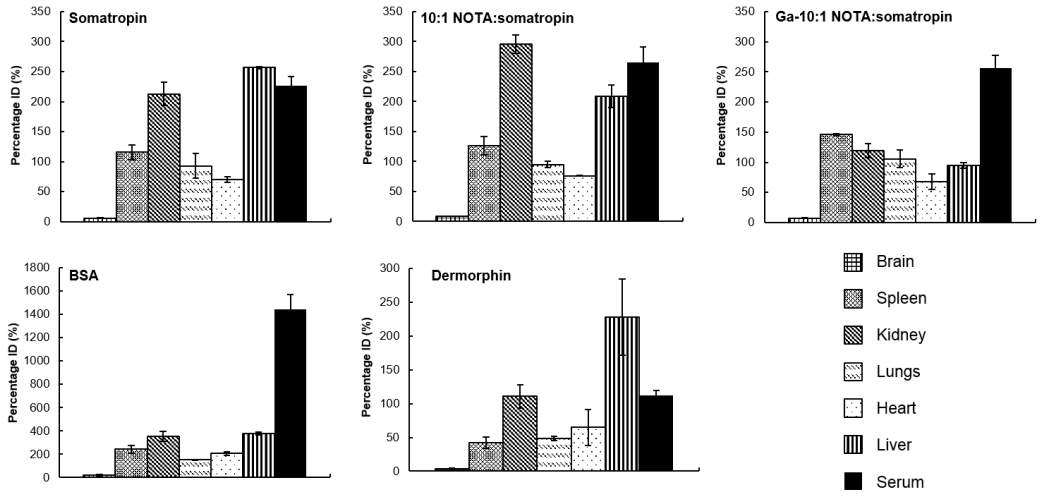

Figure 4: Relative tissue distribution at 15 minutes post injection, expressed as the percentage of the injected dose (ID) (mean ± SEM, n=2). From left to right: brain, spleen, kidney, lungs, heart, liver and serum. A 100% ID indicates that the concentration of the compound within the tissue is the same as the mean concentration over the entire animal (*i.e.* homogeneous distribution).

In vivo stability

The *in vivo* stability was evaluated according to the TCA precipitation method of Pan *et al.*[32] Using this method, a separation is made between intact protein (precipitate) and degradation products (*e.g.* metabolites (peptides) or free iodine as a result of deiodination; supernatant). A first-order kinetic is assumed in the calculation of the rate constant (k) and related half-life (**Figure 5**). The calculated metabolic half-lives were for all three compounds between 17 and 30 min in serum and brain.

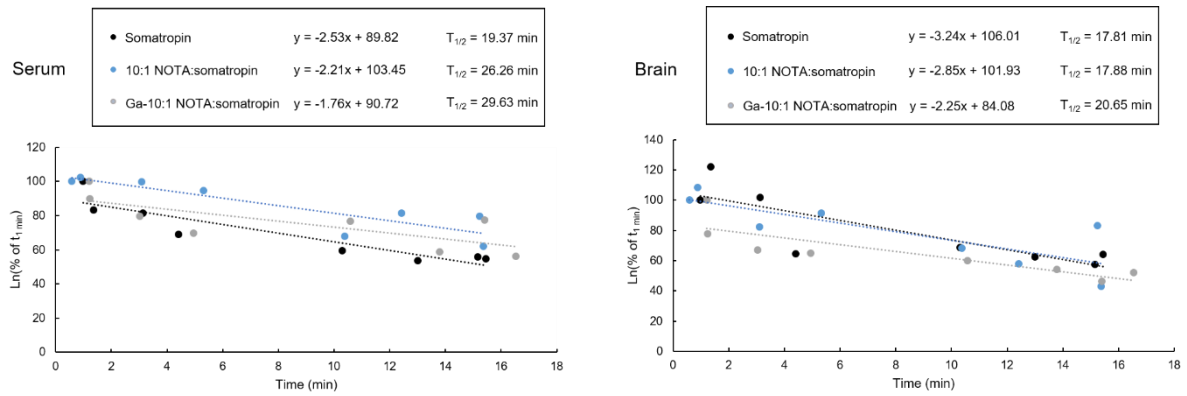

Figure 5: First-order kinetics of the in vivo stability in serum (left) and brains (right). Black: somatropin, gray: 10:1 NOTA:somatropin and blue: Ga-10:1 NOTA:somatropin. Fitting results and calculated half-lives are shown in the insets.

Discussion

The effect of NOTA-conjugation on the BBB transport characteristics of proteins, has not yet been described. The BBB is a unique, selective interface formed by the tight junctions between the endothelial cells that line the cerebral capillaries, as well as the metabolic barrier and the specific transport systems.[49, 50] Molecules that readily pass the BBB under normal conditions are traditionally explicitly small and lipophilic; however, saturable transport systems are described for different proteins, *e.g.* interleukins, tumour necrosis factor α and interferons.[46, 51-57] This means that molecules can diffuse passively through the BBB (mainly small, lipophilic molecules) or make use of a carrier- or receptor-mediated transport system as also for peptides and proteins.[58] Receptor-mediated transport systems are now strategies used for the transport of protein therapeutics across the BBB, *e.g.* bispecific antibodies with binding to the transferrin receptor (TfR).[59] Some proteins however, do not pass this BBB and are here used as vascular markers, characterized by a negligible vascular brain distribution volume (on average 8.0 µL/g).[41, 60] BSA is a known protein vascular marker, but radioiodination of BSA has been demonstrated to show some nonspecific binding to cerebral capillaries in comparison to the tritiated form, explaining the observed brain influx and higher

initial brain distribution volume (V_i = 15 µL/g).[61] In fact, molecular modifications on substances is one of the nine mechanisms influencing brain penetration of compounds, but unfortunately, the pharmacokinetic outcome is often difficult to predict.[62] As exemplified by Banks *et al.*, PEGylations can both enhance and reduce the BBB permeation. Also glucose modifications enhanced the BBB penetration, but these modifications did not target the expected glucose transporter GLUT1.[62-63] Therefore, the inclusion of a BBB permeability study is recommended to investigate the potential of drugs and their modification moieties to target the CNS, with (i) intentional passage *e.g.* for treatment of CNS diseases or (ii) unintentional passage *e.g.* to estimate non-selective tissue distribution in CNS during ligand-targeted radiopharmaceutical development.[64]

Rat GH was previously characterized by a low brain influx (K_{in} of 0.23 and 0.32 µL/(g×min) in mice and rat, respectively) demonstrated that a recombinant, human GH (somatropin) with cross-species reactivity (*i.e.* human ligand binds mouse receptor), has a similar brain influx of 0.38 ± 0.14 µL/(g×min) in the mouse model.[32, 65, 66] The 10:1 NOTA:somatropin sample contains a mixture of products with different NOTA-substitution degrees and positional isomers.[7] No altered functional receptor binding potency was demonstrated for 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin compared to unmodified somatropin.[67] In this study, we could not demonstrate a significantly different BBB kinetic transport behaviour for these three compounds. Moreover, the compartmental distribution in the brain parenchyma and brain capillaries was also similar for the three compounds, with an estimated higher parenchymal localisation (≥ 80%). From our results, we can conclude that for 10:1 NOTA:somatropin with or without gallium chelation, there was no significantly different BBB behaviour compared to unmodified somatropin.

The serum half-lives for somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin were calculated to be 6.9, 7.1 and 9.7 minutes, respectively. As seen from the tissue distribution after 15 minutes post injection, somatropin and 10:1 NOTA:somatropin are highly distributed in serum, liver and kidney. The kidney is the main clearance site for GH, the liver is the main target site for GH action with GHR expression; and the extracellular part of the GHR, known as the growth hormone binding protein (GHBp), circulates separately in the blood at nanomolar concentrations, where it will bind with a substantial part of plasma GH (40-50%).[68-73] Binding to GHBp can positively influence the serum half-life. The Ga-chelated 10:1 NOTA:somatropin showed a different tissue distribution profile: significantly high serum levels were found and no preferential tissue uptake was observed.

Next to the tissue distribution, also the *in vivo* metabolic stability was evaluated which can contribute to the serum half-lives. All three compounds (somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin) were relatively stable during the 15 minutes time period of the MTR experiment with metabolic half-lives between 17-30 minutes in serum and brain. Because the compounds were labeled with ^{125}I and the TCA precipitation method was used (precipitate vs. soluble radioactivity), no direct conclusions could be made for the *in vivo* stability of the lysine-isothiocyanate(linker)-NOTA conjugation and the Ga-NOTA chelation. However, isothiocyanates are amine-reactive groups, form thiourea bonds onto lysine residues and are more stable than other amine-reactive linkers such as *N*-hydroxysuccinimide (NHS) esters.[4] The Ga-NOTA chelate is characterized with a high complexation constant ($Log(K_ML)$ = 31.0) and remains intact in nitric acid over a period of 6 months, indicative for a high stability.[74-75] Indeed, gallium-p-SCN-Bn-NOTA-peptide conjugates have been reported to be stable in serum for >90% over 4 h, in the presence of apotransferrin.[76]

GH has been related to effects in the CNS such as sleep, mood, cognitive function, memory but also on neuroprotection, apetite and feeding behaviour.[30, 31, 77] The expression of the GHR in the different brain regions support these functional observations. At this moment, data on GH mechanisms on brain function remains limited and has to be further elucidated.[78] The NOTA-modified somatropins offer opportunities not only for targeting of GHR-overexpressing tumours as diagnostic and/or therapeutic agent, but also for imaging toward more fundamental, mechanistic research in the brain.

Conclusions

The BBB transport properties of somatropin after NOTA-conjugation and gallium chelation were investigated. Somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin all showed a low brain influx rate in the *in vivo* mice model, which were not significantly different from each other. In comparison to somatropin and 10:1 NOTA:somatropin, both having a pronounced tissue distribution to liver, kidney and serum, Ga-10:1 NOTA:somatropin showed a somewhat different tissue distribution profile with a high serum level and no preferential tissue distribution. The compounds had a relatively high *in vivo* metabolic stability during the timeframe of the study ($T_{1/2}$ between 17-30 minutes). Our results thus indicate that NOTA-modification, including gallium chelation, onto a protein characterized with a low brain influx, does not lead to significantly altered pharmacokinetic profile at the blood-brain barrier.

References

1. Mehvar R. Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation. J Pharm Pharm Sci 2000;3:125-36.
2. Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. Adv Drug Deliv Rev 2003;55:1261-77.
3. Wynendaele E, Bracke N, Stalmans S, De Spiegeleer B. Development of Peptide and Protein Based Radiopharmaceuticals. Curr Pharm Des 2013.
4. Liu S. Bifunctional coupling agents for radiolabeling of biomolecules and target-specific delivery of metallic radionuclides. Adv Drug Deliv Rev 2008;60:1347-70.
5. D'Huyvetter M, Xavier C, Caveliers V, Lahoutte T, Muyldermans S, Devoogdt N. Radiolabeled nanobodies as theranostic tools in targeted radionuclide therapy of cancer. Expert Opinion on Drug Delivery 2014;11:1939-54.
6. Lee DY, Li KC. Molecular theranostics: a primer for the imaging professional. AJR Am J Roentgenol 2011;197:318-24.
7. Bracke N, Wynendaele E, D'Hondt M, Haselberg R, Somsen GW, Pauwels E, et al. Analytical characterization of NOTA-modified somatropins. J Pharm Biomed Anal 2014;96:1-9.
8. Shen BQ, Xu K, Liu L, Raab H, Bhakta S, Kenrick M, et al. Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. Nat Biotechnol 2012;30:184-9.
9. Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. Site-specific antibody drug conjugates for cancer therapy. MAbs 2014;6:34-45.
10. Hamblett KJ, Senter PD, Chace DF, Sun MM, Lenox J, Cerveny CG, et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clin Cancer Res 2004;10:7063-70.
11. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 2001;46:3-26.
12. Gabathuler R. Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. Neurobiol Dis 2010;37:48-57.
13. Poduslo JF, Curran GL. Polyamine modification increases the permeability of proteins at the blood-nerve and blood-brain barriers. J Neurochem 1996;66:1599-609.

14. Clayton PE, Banerjee I, Murray PG, Renehan AG. Growth hormone, the insulin-like growth factor axis, insulin and cancer risk. Nat Rev Endocrinol 2011;7:11-24.
15. Perry JK, Emerald BS, Mertani HC, Lobie PE. The oncogenic potential of growth hormone. Growth Horm IGF Res 2006;16:277-89.
16. Perry JK, Liu DX, Wu ZS, Zhu T, Lobie PE. Growth hormone and cancer: an update on progress. Curr Opin Endocrinol Diabetes Obes 2013;20:307-13.
17. Holly JM, Gunnell DJ, Davey Smith G. Growth hormone, IGF-I and cancer. Less intervention to avoid cancer? More intervention to prevent cancer? J Endocrinol 1999;162:321-30.
18. Jenkins PJ, Mukherjee A, Shalet SM. Does growth hormone cause cancer? Clin Endocrinol (Oxf) 2006;64:115-21.
19. Waters MJ, Barclay JL. Does growth hormone drive breast and other cancers? Endocrinology 2007;148:4533-5.
20. Harvey S, Martinez-Moreno CG, Luna M, Aramburo C. Autocrine/paracrine roles of extrapituitary growth hormone and prolactin in health and disease: An overview. Gen Comp Endocrinol 2015;220:103-11.
21. Heaney AP, Melmed S. Molecular targets in pituitary tumours. Nature Reviews Cancer 2004;4:285-95.
22. Brooks AJ, Waters MJ. The growth hormone receptor: mechanism of activation and clinical implications. Nat Rev Endocrinol 2010;6:515-25.
23. van Kerkhof P, Govers R, Alves dos Santos CM, Strous GJ. Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. J Biol Chem 2000;275:1575-80.
24. Lincoln DT, Sinowatz F, Kolle S, Takahashi H, Parsons P, Waters M. Up-regulation of growth hormone receptor immunoreactivity in human melanoma. Anticancer Res 1999;19:1919-31.
25. Garcia-Caballero T, Mertani HM, Lambert A, Gallego R, Fraga M, Pintos E, et al. Increased expression of growth hormone and prolactin receptors in hepatocellular carcinomas. Endocrine 2000;12:265-71.
26. Conway-Campbell BL, Wooh JW, Brooks AJ, Gordon D, Brown RJ, Lichanska AM, et al. Nuclear targeting of the growth hormone receptor results in dysregulation of cell proliferation and tumorigenesis. Proc Natl Acad Sci U S A. 2007;104:13331-6.

27. Lai ZN, Roos P, Zhai QZ, Olsson Y, Fholenhag K, Larsson C, et al. Age-Related Reduction of Human Growth Hormone-Binding Sites in the Human Brain. Brain Res 1993;621:260-6.
28. Zhai Q, Lai Z, Roos P, Nyberg F. Characterization of growth hormone binding sites in rat brain. Acta Paediatr Suppl 1994;406:92-5.
29. Lobie PE, Garcia-Aragon J, Lincoln DT, Barnard R, Wilcox JN, Waters MJ. Localization and ontogeny of growth hormone receptor gene expression in the central nervous system. Brain Res Dev Brain Res 1993;74:225-33.
30. Aberg ND, Brywe KG, Isgaard J. Aspects of growth hormone and insulin-like growth factor-I related to neuroprotection, regeneration, and functional plasticity in the adult brain. ScientificWorldJournal 2006;6:53-80.
31. Hallberg M, Nyberg F. Growth hormone receptors in the brain and their potential as therapeutic targets in central nervous system disorders. The Open Endocrinology Journal 2012;6:27-33.
32. Pan W, Yu Y, Cain CM, Nyberg F, Couraud PO, Kastin AJ. Permeation of growth hormone across the blood-brain barrier. Endocrinology 2005;146:4898-904.
33. Vergote V, Bode S, Peremans K, Vanbree H, Baert B, Slegers G, et al. Analysis of iodinated peptides by LC-DAD/ESI ion trap mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2007;850:213-20.
34. Vergote V, Baert B, Vandermeulen E, Peremans K, van Bree H, Slegers G, et al. LC-UV/MS characterization and DOE optimization of the iodinated peptide obestatin. J Pharm Biomed Anal 2008;46:127-36.
35. Stalmans S, Bracke N, Wynendaele E, Gevaert B, Peremans K, Burvenich C, et al. Cell-Penetrating Peptides Selectively Cross the Blood-Brain Barrier In Vivo. PLoS One 2015;10.
36. Stalmans S, Wynendaele E, Bracke N, Knappe D, Hoffmann R, Peremans K, et al. Blood-Brain Barrier Transport of Short Proline-Rich Antimicrobial Peptides. Protein and Peptide Letters 2014;21:399-406.
37. Verbeken M, Wynendaele E, Mauchauffee E, Bracke N, Stalmans S, Bojnik E, et al. Blood-brain transfer and antinociception of linear and cyclic N-methyl-guanidine and thiourea-enkephalins. Peptides 2015;63:10-21.
38. Wynendaele E, Verbeke F, Stalmans S, Gevaert B, Janssens Y, Van De Wiele C, et al. Quorum Sensing Peptides Selectively Penetrate the Blood-Brain Barrier. PLoS One 2015;10:e0142071.

39. Gevaert B, Wynendaele E, Stalmans S, Bracke N, D'Hondt M, Smolders I, et al. Blood-brain barrier transport kinetics of the neuromedin peptides NMU, NMN, NMB and NT. Neuropharmacology 2016;107:460-70.
40. Kastin AJ, Akerstrom V, Pan W. Validity of multiple-time regression analysis in measurement of tritiated and iodinated leptin crossing the blood-brain barrier: meaningful controls. Peptides 2001;22:2127-36.
41. Gjedde A. High-Affinity and Low-Affinity Transport of D-Glucose from Blood to Brain. J Neurochem 1981;36:1463-71.
42. Patlak CS, Blasberg RG, Fenstermacher JD. Graphical Evaluation of Blood-to-Brain Transfer Constants from Multiple-Time Uptake Data. J Cereb Blood Flow Metab 1983;3:1-7.
43. Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. Neuropharmacology 1998;37:1553-61.
44. Fan J, de Lannoy IA. Pharmacokinetics. Biochem Pharmacol 2014;87:93-120.
45. Triguero D, Buciak J, Pardridge WM. Capillary Depletion Method for Quantification of Blood-Brain-Barrier Transport of Circulating Peptides and Plasma-Proteins. J Neurochem 1990;54:1882-8.
46. Gutierrez EG, Banks WA, Kastin AJ. Murine Tumor-Necrosis-Factor-Alpha Is Transported from Blood to Brain in the Mouse. J Neuroimmunol 1993;47:169-76.
47. Stalmans S, Gevaert B, Wynendaele E, Nielandt J, De Tre G, Peremans K, et al. Classification of peptides according to their blood-brain barrier influx. Protein and peptide letters 2015;22:768-75.
48. Kastin AJ, Akerstrom V. Entry of exendin-4 into brain is rapid but may be limited at high doses. Int J Obesity 2003;27:313-8.
49. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis 2010;37:13-25.
50. Abbott NJ. Blood-brain barrier structure and function and the challenges for CNS drug delivery. J Inherit Metab Dis 2013;36:437-49.
51. Ohtsuki S, Terasaki T. Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain; Importance for CNS drug discovery and development. Pharm Res 2007;24:1745-58.
52. Cecchelli R, Berezowski V, Lundquist S, Culot M, Renftel M, Dehouck MP, et al. Modelling of the blood-brain barrier in drug discovery and development. Nat Rev Drug Discov 2007;6:650-61.

53. Banks WA, Kastin AJ, Durham DA. Bidirectional transport of interleukin-1 alpha across the blood-brain barrier. Brain Res Bull 1989;23:433-7.

54. Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human Interleukin (Il) 1 Alpha, Murine Il-1 Alpha and Murine Il-1 Beta Are Transported from Blood to Brain in the Mouse by a Shared Saturable Mechanism. J Pharmacol Exp Ther 1991;259:988-96.

55. Banks WA, Kastin AJ, Gutierrez EG. Penetration of Interleukin-6 across the Murine Blood-Brain-Barrier. Neurosci Lett 1994;179:53-6.

56. Banks WA, Kastin AJ, Ehrensing CA. Blood-Borne Interleukin-1-Alpha Is Transported across the Endothelial Blood-Spinal Cord Barrier of Mice. Journal of Physiology-London 1994;479:257-64.

57. Pan W, Banks WA, Kastin AJ. Permeability of the blood-brain and blood-spinal cord barriers to interferons. J Neuroimmunol 1997;76:105-11.

58. Pardridge WM. Drug transport across the blood-brain barrier. J Cereb Blood Flow Metab 2012;32:1959-72.

59. Yu YJ, Atwal JK, Zhang Y, Tong RK, Wildsmith KR, Tan C, et al. Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. Sci Transl Med 2014;6.

60. Gjedde A, Rasmussen M. Pentobarbital anesthesia reduces blood-brain glucose transfer in the rat. J Neurochem 1980;35:1382-7.

61. Pardridge WM, Eisenberg J, Cefalu WT. Absence of albumin receptor on brain capillaries in vivo or in vitro. Am J Physiol 1985;249:E264-7.

62. Banks WA. From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery. Nat Rev Drug Discov 2016;15:275-92.

63. Polt R, Dhanasekaran M, Keyari CM. Glycosylated neuropeptides: a new vista for neuropsychopharmacology? Med Res Rev 2005;25:557-85.

64. Pardridge WM. The blood-brain barrier: bottleneck in brain drug development. NeuroRx 2005;2:3-14.

65. Rowlinson SW, Waters MJ, Lewis UJ, Barnard R. Human growth hormone fragments 1-43 and 44-191: In vitro somatogenic activity and receptor binding characteristics in human and nonprimate systems. Endocrinology 1996;137:90-5.

66. Souza SC, Frick GP, Wang XZ, Kopchick JJ, Lobo RB, Goodman HM. A Single Arginine Residue Determines Species-Specificity of the Human Growth-Hormone Receptor. Proc Natl Acad Sci U S A 1995;92:959-63.

67. Bracke N, Yao H, Wynendaele E, Verbeke F, Xu X, Gevaert B, et al. In vitro functional quality characterization of NOTA-modified somatropins. Anal Chem 2017;89:9.
68. Johnson V, Maack T. Renal Extraction, Filtration, Absorption, and Catabolism of Growth-Hormone. Am J Physiol 1977;233:F185-F96.
69. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. Endocr Rev 2001;22:53-74.
70. Kopchick JJ, Andry JM. Growth hormone (GH), GH receptor, and signal transduction. Mol Genet Metab 2000;71:293-314.
71. Hadden DR, Prout TE. A Growth Hormone Binding Protein in Normal Human Serum. Nature 1964;202:1342-3.
72. Baumann G, Stolar MW, Amburn K, Barsano CP, Devries BC. A Specific Growth Hormone-Binding Protein in Human-Plasma - Initial Characterization. J Clin Endocrinol Metab 1986;62:134-41.
73. Baumann G, Amburn K, Shaw MA. The circulating growth hormone (GH)-binding protein complex: a major constituent of plasma GH in man. Endocrinology 1988;122:976-84.
74. Clarke ET, Martell AE. Stabilities of the Fe(Iii), Ga(Iii) and in(Iii) Chelates of N,N',N''-Triazacyclononanetriacetic Acid. Inorganica Chimica Acta 1991;181:273-80.
75. Broan CJ, Cox JPL, Craig AS, Kataky R, Parker D, Harrison A, et al. Structure and Solution Stability of Indium and Gallium Complexes of 1,4,7-Triazacyclononanetriacetate and of Yttrium Complexes of 1,4,7,10-Tetraazacyclododecanetetraacetate and Related Ligands - Kinetically Stable Complexes for Use in Imaging and Radioimmunotherapy - X-Ray Molecular-Structure of the Indium and Gallium Complexes of 1,4,7-Triazacyclononane-1,4,7-Triacetic Acid. Journal of the Chemical Society-Perkin Transactions 2. 1991:87-99.
76. Ferreira CL, Yapp DTT, Mandel D, Gill RK, Boros E, Wong MQ, et al. Ga-68 Small Peptide Imaging: Comparison of NOTA and PCTA. Bioconjug Chem. 2012;23(11):2239-46.
77. Schneider HJ, Pagotto U, Stalla GK. Central effects of the somatotropic system. Eur J Endocrinol 2003;149:377-92.
78. Aramburo C, Alba-Betancourt C, Luna M, Harvey S. Expression and function of growth hormone in the nervous system: a brief review. Gen Comp Endocrinol 2014;203:35-42.

Authors' contributions

All experiments were performed by N.B. and Y.J.; N.B., together with B.D.S. designed the experiments and wrote the manuscript. E.W., A.M., C.V.D.W. and M.S. commented the results and reviewed the manuscript. B.D.S. directed the research.

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Conflicts of interest

The authors declare that they have no conflict of interest and that no competing financial interests exist.

TITLES OF TABLES

Table 1: Overview of the multiple time regression results, with the 65% confidence interval indicated.

Table 2: Serum radioactivity time curve parameters

TITLES OF FIGURES

Figure 1: Multiple time regression curves of the exposure time versus the ratio of the brain and serum activity. A: Somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin. B: BSA and dermorphin, negative and positive controls respectively. Data were fitted using the linear Gjedde-Patlak model.

Figure 2: Serum concentrations of somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin in function of time (min), as obtained during the MTR experiments. Data were fitted using a one compartment model (dashed lines).

Figure 3: Capillary depletion compartmental distribution results normalized for brain weight (mean absolute amounts in µl/g ±SEM, n=2), showing a very high brain parenchymal penetration for all investigated compounds. A_{tissue} and A_{serum} are the measured activities in the concerned tissue and serum, respectively.

Figure 4: Relative tissue distribution at 15 minutes post injection, expressed as the percentage of the injected dose (ID) (mean ± SEM, n=2). From left to right: brain, spleen, kidney, lungs, heart, liver and serum. A 100% ID indicates that the concentration of the compound within the tissue is the same as the mean concentration over the entire animal (*i.e.* homogeneous distribution).

Figure 5: First-order kinetics of the *in vivo* stability in serum (left) and brains (right). Black: somatropin, gray: 10:1 NOTA:somatropin and blue: Ga-10:1 NOTA:somatropin. Fitting results and calculated half-lives are shown in the insets.