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Investigation of imaging the somatostatin receptor by opening the blood-brain barrier with melittin – A feasibility study using positron emission tomography and [^{64}Cu]Cu-DOTATATE

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ABSTRACT

DOTATATE is a somatostatin peptide analog used in the clinic to detect somatostatin receptors which are highly expressed on neuroendocrine tumors. Somatostatin receptors are found naturally in the intestines, pancreas, lungs, and brain (mainly cortex). *In vivo* measurement of the somatostatin receptors in the cortex has been challenging because available tracers cannot cross the blood-brain barrier (BBB) due to their intrinsic polarity. A peptide called melittin, a main component of honeybee venom, has been shown to disrupt plasma membranes and increase the permeability of biological membranes. In this study, we assessed the feasibility of using melittin to facilitate the passage of [^{64}Cu]Cu-DOTATATE through the BBB and its binding to somatostatin receptors in the cortex. Evaluation included *in vitro* autoradiography on Long Evans rat brains to estimate the binding affinity of [^{64}Cu]Cu-DOTATATE to the somatostatin receptors in the cortex and an *in vivo* evaluation of [^{64}Cu]Cu-DOTATATE binding in NMRI mice after injection of melittin. This study found an *in vitro* $B_{max} = 89 \pm 4$ nM and $K_D = 4.5 \pm 0.6$ nM in the cortex, resulting in a theoretical binding potential (BP) calculated as $B_{max}/K_D \approx 20$, which is believed suitable for *in vivo* brain PET imaging. However, the *in vivo* results showed no significant difference between the control and melittin injected mice, indicating that the honeybee venom failed to open the BBB. Additional experiments, potentially involving faster injection rates are required to verify that melittin can increase brain uptake of non-BBB permeable PET tracers. Furthermore, an evaluation of whether a venom with a narrow therapeutic range can be used for clinical purposes needs to be considered.

1. Introduction

In the beginning of the 1970's, the physiological action of the two somatotropin-release inhibitory factors (SRIF) or somatostatins, cleaved from the precursor pre-pro-somatostatin to produce the two forms (SRIF-14 and SRIF-18) were discovered [1–3]. Somatostatin acts as an inhibitor of hormone release or reducer of smooth muscle contractions when binding to one of the five G protein-coupled somatostatin receptors (SSTR1–5) [3]. All the SSTRs are highly expressed in the liver, adrenal gland, thyroid, intestines, pancreas, lungs, and brain (mainly cortex) [4], with SSTR2 as the most expressed receptor [5]. Reduced SSTR levels are seen in neurodegenerative diseases such as Alzheimer's disease, patients with major depressive disorder and schizophrenia, where an increased level have been observed on neuroendocrine tumors [6].

A commonly used tracer for diagnosing and treating neuroendocrine tumors is the radiolabeled form of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid – octreotate (DOTATATE) [7]. DOTATATE is peptide analog of somatostatin, a peptide hormone that binds to SSTRs expressed on the surface of neuroendocrine cells [3]. Comparison

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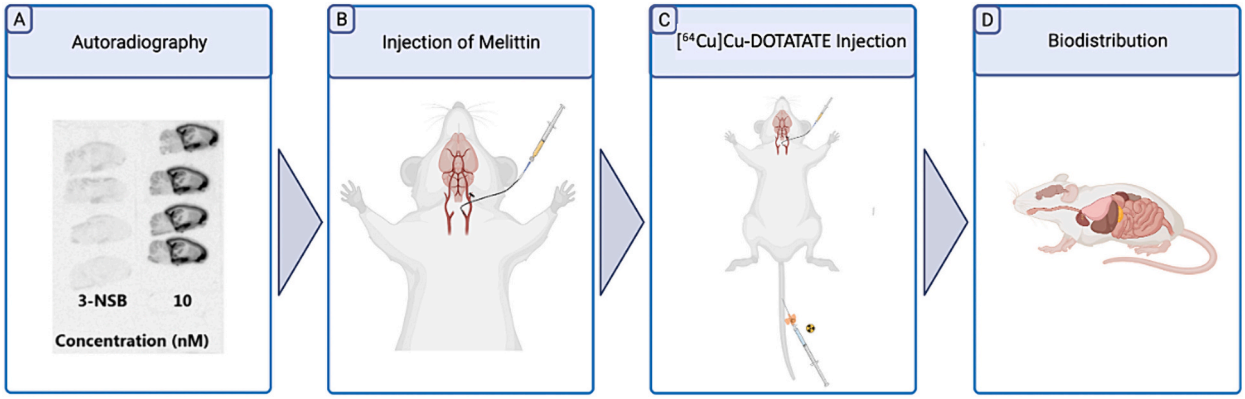

Fig. 1. A schematic illustration of the procedures. A) Autoradiography with [^{64}Cu]Cu-DOTATATE on Long Evans rat brain slices. B) *In vivo* experiments on NMRI mice with melittin injection. C) [^{64}Cu]Cu-DOTATATE injection to perform a PET scan and D) biodistribution of the mice.

studies have shown that DOTATATE had highest affinity for the SSTR2 compared to DOTATOC and DOTANOC [8]. DOTATATE has been successfully radiolabeled with ^{64}Cu, ^{68}Ga, ^{111}In, ^{177}Lu and ^{90}Y, where the two latter radionuclides are mainly used for radiotherapy [9–11]. In the clinic, positron emission tomography (PET) imaging is performed using both ^{64}Cu and ^{68}Ga [12–14]..

Although [^{64}Cu]Cu-DOTATATE allows for detection of peripheral tissue expression of SSTR, which is found to be overexpressed in neuroendocrine tumors, no available PET radiotracers targeting SSTR can enter the brain due to their hydrophilic nature [15]. Several methods for enhancing blood-brain barrier (BBB) delivery of large and hydrophilic compounds have been investigated. These include focused ultrasound (FUS), modulators of the BBB (small molecule, peptides, proteins) and membrane active peptides (MAPs) [16].

Various small molecules, peptides and proteins have been investigated for their potential to open the BBB. Mannitol, for example, is known as a hyperosmolar agent whose function is to dehydrate the cerebrovascular endothelial cells leading to a contraction, which opens the tight junctions in the BBB [15,16]. Recently, the toxic component of bee venom called melittin has been described as a MAP [15,16]. Melittin is a 26 amino acid cationic amphipathic peptide, which has shown to disrupt plasma membranes by toroidal pore mechanism, resulting in an increased calcium influx into the endothelium, leading to disruption of the tight junctions, creating an increased BBB permeability [17–19]. Linville et al. showed that an intraarterial injection of melittin, increased permeability of biological membranes, as the BBB [16]. "Previous studies using mannitol in our group did not result in reproducible data". Hence, we decided to use melittin which has a 3-fold higher focal leak density [16]. Melittin was used to open the BBB so the SSTR2 ligand [^{64}Cu]Cu-DOTATATE could potentially enter the brain (Fig. 1). We decided to use [^{64}Cu]Cu-DOTATATE as copper-64 results in relatively high-resolution images [8].

2. Materials and methods

2.1. General procedure

Reagents and solvents were obtained from suppliers and used without further purification. DOTA-octreotate was kindly provided by Andreas Jensen from the Danish Technical University. Metal free water (Honeywell) was used to make all the standard solutions, and metal free water was used for all experiments unless otherwise stated. Copper-64 (II) chloride was purchased from Risø (Denmark). Analytical HPLC was performed on a Dionex system connected to a P680A pump, a UVD 170 U detector and a Gabi Star radio detector. The system was controlled by Chromeleon 7.2 software.

2.2. Radiochemistry

Radiosynthesis of [^{64}Cu]Cu-DOTATATE (1), a solution of [^{64}Cu]$CuCl_2$ in 1 mM HCl (100 μL, 100–120 MBq) was mixed with ammonium acetate buffer (1 M, pH 5, 15 μL), gentisic acid solution (10 μL of 10 mg/mL solution in water) and DOTA-octreotate (5 μL of 2 mg/mL solution in water). The resulting mixture (pH 4) was placed on an agitating mixer and shaken at 600 rpm for 5 min at 60 °C. Then pentetic acid solution (10 μL of 10 mM solution in phosphate buffer) was added to the mixture, and 10 min later the mixture was diluted with phosphate buffer (500–600 μL, 0.1 M pH 7).

The resulting [^{64}Cu]Cu-DOTATATE solution contained <10 % unchelated copper. The radio chemical purity (RCP) of [^{64}Cu]Cu-DOTATATE was 86–88 % (assessed by radio-HPLC, SI Fig. S1) and the molar activity was 15–20 GBq/μmol.

2.3. Animals

Ten NMRI male mice weighing 30 ± 5 g (Janvier Labs, La Rochelle, France), were housed in a cage of 5 mice per cage. The two female Long Evans rats (Charles River, Calco, Italy) were housed in cages of 2–3. The animals were kept in a climate-controlled facility with a 12-h light/dark cycle. The cages contained a biting stick, fed *ad libitum* with commercial breeding diet (1310 FORTI- Avlsfoder, Brogaarden, Altromin International, Denmark) and had full access to water. All procedures were conducted in accordance with the European Commission's Directive 2010/63/EU, FELASA and ARRIVE guidelines for animal research and, with approval from The Danish Council for Animal Ethics (license numbers 2017-15-0201-01283) as well as the Department of Experimental Medicine, University of Copenhagen.

2.4. In vitro autoradiography

The 4–5 months old Long Evans rats were guillotined, and their brains were taken out and frozen at −80 °C. Autoradiography was performed using 20 μm sagittal cryosections containing the cortex of the brains. The frozen brains were sectioned on a cryostat (Leica CM1800, Leica Biosystems, Buffalo Grove, IL, USA) and mounted on Superfrost Plus™ adhesion microscope slides (Thermo Fischer Scientific, MS, USA). Sections were stored at −80 °C for the remaining period of the study. The sections were thawed at 4 °C before the study, and consequently pre-incubated for 30 min with assay buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$ and 0.1 % BSA. The original concentration of [^{64}Cu]Cu-DOTATATE (60 μM) was diluted with the same assay buffer to obtain the following concentration sequence: 30 nM, 10 nM, 3 nM, 1, 0.3 nM and 0.1 nM in duplicates. These concentrations were incubated on the slides for 1 h. In parallel the four highest concentrations were used on negative

control slides to determine non-specific binding, using an excess (10 μM) of cold octreotide acetate (ADooQ Bioscience, USA). After 1 h samples of the solution on the slide were taken and measured on the gamma counter (Packard Cobra II Auto-Gamma Counter Model, Downers Grove, IL, USA) to check for ligand depletion. The solutions were removed from the slides and the slides were washed with assay buffer for 3 min and water for 30 s, respectively. The slides were subjected to a gentle air flow until dry. A calibration curve was prepared consisting of 6 consecutive 3-fold dilutions of [64Cu]Cu-DOTATATE starting from 200-fold dilution of the original stock; 1 μL aliquots were placed on a silica TLC plate at a distance of 5–10 mm from each other. The TLC plate was placed atop an empty glass slide with the same dimensions as the glass slides with mounted brain slices. Slides with washed and dried tissue slices and the TLC plate with the calibration curve aliquots were exposed side by side against phosphor storage screens (Perkin-Elmer, USA) overnight. The screens were then read in the Cyclone Plus Storage Phosphor System (PerkinElmer, Inc. Waltham, USA), and the images were analyzed in Optiquant 3.0 Software (Packard Instruments Company, Washington, USA). Regions of interest (ROIs) were drawn around cortical and cerebellar regions of the brain slices (Fig. S2), while circular regions of interest were drawn around the borders of calibration curve spots. For each ROI, the measured exposure in digital light units per square millimeter (DLU/mm2) was exported from Optiquant to Microsoft Excel (version 16.59, Redmond, Washington, USA). The correspondence between exposure values in DLU/mm2 and radioligand concentration in nmol/L was established by linear regression using calibration curve spots. The obtained regression formula was used to convert DLU/mm2 values for brain ROIs into nmol/L. Obtained nmol/L values were then exported from Microsoft Excel to Graphpad Prism 8.0 (GraphPad Software, Boston, Massachusetts USA), where they were used for non-linear regression using single-site saturation binding model with non-specific binding to calculate affinity and binding site density for [64Cu]Cu-DOTATATE in rat brain slices.

2.5. In vivo animal experiment

NMRI mice were weighed, placed in an induction box, and induced with 4 % sevoflurane in 1–2 L/min of oxygen. When sedated the mice were placed with a nasal/mouth cone in a lateral decubitus position and the paws were taped. The animals were shaved with an electric shaver and 0.5 % lidocaine was administered. Micro scissors were used for opening the animal in a midline longitudinal incision. The occipital artery and the pterygopalatine artery were closed with 9–0 ligatures (Ethicon) and then a microcatheter (100,828 Mouse Arterial Catheter with bead, VeruTech, Sweden) was placed into the external carotid artery and permanently ligated. The pressure from the arteria after the microcatheter had been placed was kept by using a hospital saline bag. Melittin was administered over a span of 7 min (3 μM in 150 μL solution) utilizing an infusion pump (Harvard PHD 2000 dual syringe pump, Holliston Massachusetts, USA). The microcatheter was blocked permanently making it possible to move the animal into the PET scanner. The animals were kept warm by a heating plate and monitored during surgery by thermometer. The intra-arterial cannulation was performed as described by Santillan [20].

2.6. PET scan

The mice were anesthetized with 4 % sevoflurane in 1–2 L/min of oxygen in the scanner. First a computer tomography (CT) scan was performed. Subsequently, a tail vein catheter was used to inject the tracer [^{64}Cu]Cu-DOTATATE (15–20 MBq/100 μL), simultaneously with the start of the dynamic positron emission tomography (PET) (Preclinical PET/CT Inveon, Siemens Medical Solution, Malvern, PA, USA) scan, which lasted 0-1 h. Reconstruction of the sinogram was performed by 3-dimensional posteriori algorithm with CT attenuation and scatter corrections. The PET scans were transformed into 33 dynamic frames of (6 × 10, 6 × 20, 6 × 60, 8 × 120, and 7 × 300 s). CT imaging was conducted with the parameters: 360 projections, 65 kV voltage, 500 μA current and 450 ms exposure. The PET and CT images were analyzed by Inveon Research Workplace 4.2 software (Siemens Molecular Imaging, USA). The mean %ID was extracted manually by creating ROIs on the CT images and overlaid on the raw PET images. Pictures were taken with the software PMOD 3.7 (Technologies, Zurich, Switzerland).

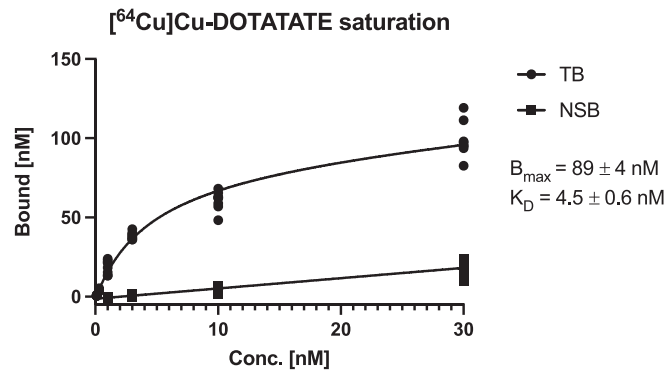

Fig. 2. Binding saturation study of [^{64}Cu]Cu-DOTATATE to somatostatin receptors in the cortex of Long Evans rats. TB: tissue binding, NSB: Nonspecific binding. The B_{max} = 89 ± 4 nM and the K_D = 4.5 ± 0.6 nM.

2.7. Biodistribution

The mice were euthanized with a guillotine after the scan and the organs (brain, blood, heart, liver, spleen, kidneys, muscle, intestine, bladder, and lungs) were collected and measured using a gamma counter (Wizard2, Perkin Elmer, Turku, Finland). The data were corrected for decay, tissue weight and injected amount of radioactivity. Data analyzed with the use of Microsoft excel version 16.59, Redmond, Washington, USA.

3. Results and discussion

3.1. In vitro binding assay

To investigate the binding of [^{64}Cu]Cu-DOTATATE in the brain, in vitro autoradiographic saturation studies were performed using rat brain slices (SI, Fig. S2). Autoradiography images showed highest binding in the cortex and lowest binding in the cerebellum, which was used as a reference region. The [^{64}Cu]Cu-DOTATATE is known to bind mainly to the SSTR2 and SSTR5 [19]. The autoradiography experiment resulted in a B_{max} = 89 ± 4 nM and K_D = 4.5 ± 0.6 nM using the cortex as the region of interest (Fig. 2).

Our results were compared with previous saturation studies done with different tracers and isotopes (SI, Table S1) to demonstrate that our DOTATATE had sufficient binding potential in the cortex. In this regard, the theoretical binding potential (BP) was calculated from $B_{max}/K_D \approx 20$, which is considered sufficient for detectable binding in *in vivo* experiments [21].

3.2. In vivo experiment with melittin

The purpose of the *in vivo* study was to increase the permeability of the BBB using melittin injection and subsequently investigate whether [^{64}Cu]Cu-DOTATATE PET can quantify somatostatin receptor density in the brain. A microcatheter was placed in the external carotid artery, in order to inject melittin directly into the internal carotid artery (ICA), without interrupting the blood flow from the common carotid artery [20]. Four NMRI mice were injected with melittin, and four mice were injected with only [^{64}Cu]Cu-DOTATATE. The PET scans were conducted over 1 h, starting from the time of injection of [^{64}Cu]Cu-DOTATATE.

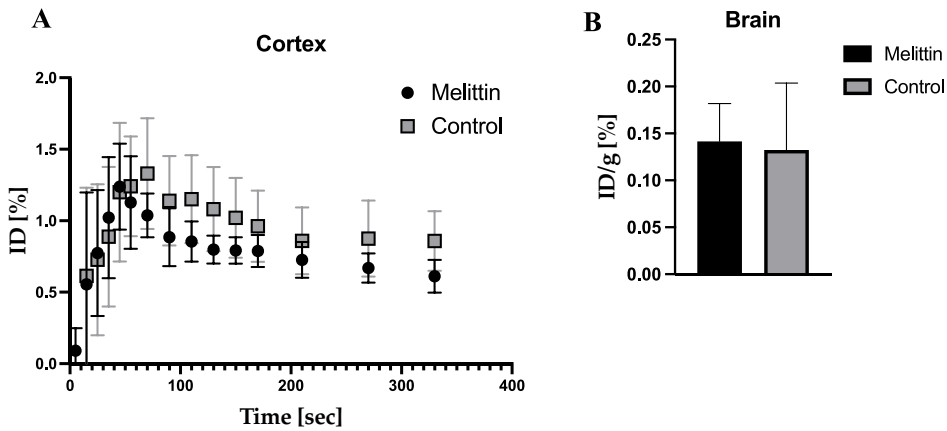

Fig. 3. *In vivo* administration of melittin to open the BBB. Black: Melittin injected mice and afterwards injected with [^{64}Cu]Cu-DOTATATE to see the binding in the brain. Gray: [^{64}Cu]Cu-DOTATATE injection only, the values in experiment A is given as injected dose ID [%] and B as ID/g [%] with $n = 8$, (full scan values (ID [%] and SUV see SI, Fig. S4 and Table S2). A) Values collected from the PET scan and analyzed with PMOD. B) Values from the biodistribution study analyzed with gamma counter showed no significant difference between melittin injected: mean = 0.142, SD = 0.04 and control: mean = 0.132, SD = 0.07, *t*-test(3) = 0.2, p = 0.857) (SI, Fig. S5). Results are given in mean values and error bars as standard deviation.

In Fig. 3, the *in vivo* experiments are presented. No statistical differences from a t-test were seen between cortical [^{64}Cu]Cu-DOTATATE uptake in the melittin and the control groups (Fig. 3A and B). Several parameters were changed in order to make it less invasive and to keep the mice alive, which could explain why the outcome achieved for opening of the BBB was contradictive to previously shown by Linville et al. [16] Linville and co-workers inserted the microcatheter into the common carotid artery (CCA), which was permanently ligated [16]. In this investigation, the catheter was inserted into the external carotid artery (ECA), and sutures were used to block the pterygopalatine artery (PPA) and the occipital artery (OCA) (SI, Fig. S3) [20]. This allowed melittin to be administered into the bloodstream and kept the blood supply to the brain's left side open. A decrease in the radioactive response was seen in the injected site of the brain compared to the non-operated site even though the blood flow was maintained (Fig. 3 and SI, Fig. S6). Furthermore, an injection with the same rate of 3 μM/min with a speed of 150 μL/min was impossible, as it would have increased the interatrial pressure at site of injection, leading to a backward flow ending up in the lungs. To circumvent this, the injection was administered over a span of 7 min, which likely resulted in a blood concentration of melittin too low to increase BBB permeability. Two animals perished despite lengthier exposure times, while others experienced erratic breathing. An additional contributing factor that could further reduce melittin's efficiency might be the heavier mice in our study. The higher mice weight leads to an increase in blood volume, which further lowers melittin concentration. It took maximum 30 min from the time melittin was injected until the mice were cannulated and placed in the scanner, at which point the tracer was injected. This time delay most likely resulted in an even lower concentration of melittin and reduced BBB permeability at the time of [^{64}Cu]Cu-DOTATATE administration. From our observations, a higher dose would most likely have killed the mice, even though it was estimated by Linville et al. 2021 to be ~10 times lower than the LD_{50} with an injection of 3 μM/min [16]. In the paper from Linville et al., they demonstrated no response of 1 μM/min, but with 5 μM/min the animals died of neurological toxicity [16]. The PET scans showed further reduced radioactivity in the brain from the injected site, which could have resulted from toxicity from melittin injection (SI, Fig. S6). In our study, the utilization of a different mouse strain and anesthesia compared to those used Linville et al., could contribute to why melittin had reduced efficacy [16]. However, this seems unlikely since previous research involving adult NMRI and C57BL/6 J mice given isoflurane or sevoflurane has only shown subtle genotype-specific changes in metabolic and stress-related blood markers [22].

4. Conclusion

In this study, [^{64}Cu]Cu-DOTATATE binding affinity towards SSTR2 was investigated on the autoradiography brain slides, which resulted in a $B_{max} = 89 \pm 4$ μM and $K_D = 4.5 \pm 0.6$ nM, with a theoretically binding potential of 20. These results indicated that if the BBB could be opened, a binding *in vivo* would have been seen. Unfortunately, we were not able to replicate the BBB opening and conduct a significant better binding in the brain with the use of melittin infusion. From our perspective more research using melittin is required in order to find optimal dose for imaging of SSTR2 using [^{64}Cu]Cu-DOTATATE. Alternative approaches could be more pertinent, such as FUS or other modulators for openings of the BBB.

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CRediT authorship contribution statement

Ida Vang Andersen: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Natasha Shalina Rajani Bidesi:** Methodology, Formal analysis. **Vladimir Shalgunov:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Jesper Tranekjær Jørgensen:** Methodology, Investigation. **Tobias Gustavsson:** Writing – review & editing. **Kristian Strømgaard:** Resources, Funding acquisition. **Andreas T. Ingemann Jensen:** Resources. **Andreas Kjær:** Resources, Funding acquisition. **Matthias M. Herth:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nucmedbio.2024.108905.

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