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INHIBITION OF p38 MAPK IN THE BRAIN THROUGH NASAL ADMINISTRATION OF p38 INHIBITOR LOADED IN CHITOSAN NANOCAPSULES

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Abstract

Aims: to determine whether a p38MAPK inhibitor incorporated into nanoemulsion-based chitosan nanocapsules is capable of reducing the activity of this kinase in the brain through their nasal administration in mice. **Materials and Methods:** we selected the p38MAPK inhibitor PH797804 (PH), an ATP-competitive inhibitor of p38 α encapsulated in nanoemulsion-based chitosan nanocapsules. Biological effect was evaluated in microglial and neuronal cells *in vitro* and in *ex-vivo* and *in vivo* systems, in a mouse model of Alzheimer's disease. **Results:** encapsulated inhibitor retains enzymatic inhibitory activity and tissue penetration capacity *in vitro*, *ex vivo* and *in vivo*. **Conclusion:** Nasal administration of chitosan nanocapsules can be an effective approach for brain-restricted reduction of p38MAPK activity, thus reducing the side effects of systemic administration.

Introduction

Neuronal cell death is the end point of neurodegenerative diseases, responsible for the loss of particular subsets of neurons. The loss of the neurons occurs during a period of many years, and the pathways leading to neuronal death can vary from disease to disease and even in the same disease. Among the common features preceding neuronal dysfunction and death is the presence of abnormal phosphorylation in functional proteins[1]. One signalling pathway commonly dysregulated in neurodegenerative diseases is that of the p38 mitogen activated protein kinases (MAPK)[2]. p38MAPK comprises four different isoforms: p38 α , p38 β , p38 γ and p38 δ . Physiological p38MAPK activity plays a role in a variety of cellular activities such as differentiation, survival and proliferation[3]. p38MAPK is also activated by numerous cellular stressors and cytokines, such as bacterial lipopolysaccharide (LPS), tumour necrosis factor (TNF) and interleukin-1 (IL-1), giving to p38MAPK a key role in the response of the organism to inflammatory and environmental stress [3]. The aberrant phosphorylation of p38 α and p38 β , expressed in the brain, is thought to contribute to the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Parkinson's disease (PD) [2, 4, 5]. This association has encouraged the field to test the effect of p38MAPK inhibitors in cellular and animal models of these disease [6]. However, inhibitors of p38MAPK, no matter how specific they are, have the potential disadvantage of producing undesired effects in those organs where activation of this pathway at basal levels plays physiological roles. This is especially worrying for chronic treatments such as in the case of neurodegenerative diseases. To overcome this limitation, researchers are exploring the nasal route of drug administration for the treatment of chronic neurological diseases. This appears as a most promising strategy to achieve the concentration of active molecule in the central nervous system with few systemic effects, since it allows the exposure of brain cells to the molecule avoiding to a large extent the blood-brain barrier (BBB) [7]. Furthermore, it is possible to increase the brain penetration capacity of compounds administered in the nostrils by the association of the compound of interest with carriers that increase their stability and bioavailability as well as their penetration in the brain parenchyma. In particular, numerous recent works have shown that nanocapsules based on the natural, biocompatible and biodegradable, cationic polysaccharide chitosan can be an appropriate system for non-invasive drug administration [8-11]. Chitosan is thought to improve drug transport across the epithelium by a dual mechanism: i) by binding to negative sialic residues in the

mucus lining the nasal epithelial cells thereby slowing clearance of the formulation and ii) by direct but transient opening of the tight junctions between epithelial cells, thus allowing paracellular transport of the drug. Experiments performed on epithelial monolayers demonstrated that the exposure time and molecular weight are important parameters for chitosan-induced effects on cellular tight junctions, and that re-establishment of tight junctions is most efficient for formulations containing a high molecular weight chitosan [12]. These studies also showed that solution formulations based on chitosan salts having a molecular weight of greater than 100 kD and with a defined degree of deacetylation improve the bioavailability of nasally administered polypeptides but also non-peptide polar compounds such as morphine. Importantly, exciting results were obtained using simple solution formulations prepared from chitosan in animal models and in humans [13, 14].

In this work, we investigated whether nanocapsules of chitosan based on nanoemulsion core and encapsulating a p38MAPK inhibitor are capable of reducing the activity of this kinase in the brain through their nasal administration in mice. We showed that p38MAPK inhibitor encapsulated in chitosan nanocapsules reduces p38MAPK activity in brain cells in the cerebral cortex and, to a lower extent, also in the hippocampus. This work paves the way for future pre-clinical studies in animal models of p38MAPK-mediated neurodegeneration.

Methods

Reagents

Tween[®] 20, and sodium sulfate anhydrous were purchased from Panreac. Span[®] 85 (sorbitane trioleate), oleic acid (90%) and chitosan (medium molecular weight), were obtained from Sigma-Aldrich. PH-797804 (purity 99,01-99,07%) (Selleckchem S2726), primary anti-p38 antibody (Abcam ab170099), anti-phospho-p38 α Tyr182 (Santa Cruz sc-166182), secondary goat anti-rabbit (1:2500) and goat anti-mouse (1:2500) antibodies were from DAKO); LPS (SIGMA L6143). DiO' (3,3'-dioctadecyloxycarbocyanine perchlorate) and DiD' oil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) were purchased from ThermoFisher Scientific. Syringe filters with PVDF membrane (13 mm, 0.22 μ m pore size) were purchased from Merck Millipore.

Water sterile-filtered, BioReagent, suitable for cell culture (endotoxin $\leq 1\text{EU/mL}$) from Sigma was used in all nanocapsules synthesis.

Synthesis and characterization of inhibitor-loaded chitosan nanocapsules

Chitosan nanocapsules loaded with the p38 MAPK inhibitor were prepared using the following encapsulation method that was slightly adapted from the one reported for the encapsulation of other drugs [15].

5 mg of PH-797804 were mixed with 400 μL absolute ethanol. This solution was then incorporated to an organic solution containing 8.6 mg Span[®] 85 and 40 mg oleic acid in 4 mL absolute ethanol. The mixture was added to an aqueous solution containing 13.6 mg Tween[®] 20 in 8 mL water and kept under magnetic stirring during 15 minutes for the formation of the nanoemulsion core. Then, 2.5 mg from a 5 mg/mL chitosan solution in acetic acid 1% (v/v) were added and again the mixture was left under stirring 15 minutes. Finally, the chitosan-coated nanoemulsion was added to 15 mL of 50 mM Na_2SO_4 . Nanocapsules were separated through ultracentrifugation (69673 G, 30 minutes, 10 °C), they were washed with 10 mL of water, centrifuged again and resuspended in water.

Chitosan nanocapsules labelled with fluorescent dyes were prepared following the same procedure but adding an amount of 50 μg of DiD oil or DiO fluorophores to the organic solution.

The concentration of all nanocapsules suspension was obtained by measuring the weight of 300 μL of sample after freeze-drying.

Encapsulation efficiency (EE, ratio between weight of encapsulated inhibitor and the weight of initially added amount) and drug loading (DL, ratio between the amount of encapsulated inhibitor and to the total weight of the carrier) of PH-797804-loaded nanocapsules were calculated as percentages by extracting the drug just after the encapsulation process. 50 μL of nanocapsule water suspension, (approximately 0.5 mg) were mixed with 0.9 mL of methanol and sonicated during 30 minutes to extract the inhibitor. The absorbance at 306 nm was measured using a Varian Cary 50 UV/Vis spectrophotometer and it was compared with a calibration curve previously performed in the same solvent to obtain the molar extinction coefficient.

Dynamic Light Scattering (DLS) analysis was carried out to measure the size of the obtained chitosan nanocapsules using a Brookhaven 90Plus DLS instrument. Nanoparticle hydrodynamic diameter and polydispersity index (PDI) were measured in milliQ water at the concentration of 0.3 mg/mL. The optimal sample concentration for the analysis was previously established by measuring different concentration of nanocapsules.

DLS analysis was performed under the same conditions resuspending the sample in 5% NaCl to evaluate how the medium affected the colloidal stability and so the behavior of the nanocapsules during administration.

ζ -potential of inhibitor-loaded nanocapsules was determined by measuring the potential of a 0.01 mg/mL nanoparticle suspension in 1 mM KCl with a Plus Particle Size Analyzer (Brookhaven Instruments Corporation). ζ -potential was also measured in a range of pH between 3 and 7. 10 μ L of the sample from a 0.04 mg/mL suspension were dissolved in 3 mL 1mM KCl. Then, the pH was adjusted in each sample using HCl and NaOH solutions. The stability of pH values was confirmed just before ζ -potential measurement.

The fluorescence of labelled chitosan nanocapsules was evaluated using a PerkinElmer, LS 55 fluorescence spectrometer, using quartz cells with a path length of 1 cm. The samples containing DiO' and DiD' oil dyes were irradiated by using an excitation source at 484 and 644nm, respectively. The decays were monitored at 501 nm for DiO' and 665 for DiD' oil.

Chitosan nanocapsules were administered diluted in a 5% NaCl sterile solution with an approximate pH of 4.

p38MAPK inhibition

The p38MAPK inhibitor PH-797804 (Selleckchem S2726) was used in all experiments. Dilution and storage was as in manufacturer's indications. Treatment for *in vitro* experiments: 1 μ M during 24 hours; for *ex vivo*: 1 μ M every 2 days (half of total volume of medium was replaced with fresh medium with new inhibitor up to 1 μ M final concentration) during 12 days; for *in vivo*: 100 μ M administered in 8 μ L in each nostril. Daily dose administrated: 763.68 nanograms.

Microglial cell line

Murine BV-2[16] microglial cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; supplemented with 10% fetal bovine serum (Hyclone Logan, UT) and 1% glutamine in 100 mm plates at 37 °C. Cells were grown to 80% confluency and passaged every 2 days. For experimentation, cells were plated at a density of 20×10^4 per plate and were allowed to grow overnight. The next day, the cells were treated as specified in the figures.

Animal experimentation

18-day pregnant Wistar rats were used for primary culture experiments. Homozygous male hAppNLF/NLF knock-in mice were used for *in vivo* and *ex vivo* (hippocampal slices) experiments [17]. All animals were housed in standard mouse or rat cages (2 pregnant rats/cage, 3-5 mice/cage) with wood-shaving bedding. Food and water were available ad libitum in temperature and humidity controlled rooms with a 12-hour light-dark cycle.

Cortical neurons in culture.

Primary cultures of cortical neurons were prepared from the brains of embryonic day 18 (E18) pregnant Wistar rats. The pregnant rats were killed by an over-dose of anaesthesia, pups removed and the brains placed into ice-cold Hank's solution with 7 mM HEPES and 0.45% glucose. The cortex was dissected and then treated with 0.03% trypsin (trypsin 0.05% EDTA; Invitrogen; Life Technologies Co.) and incubated at 37 °C for 16 min and then treated with DNase (72 mg/mL; Sigma-Aldrich) for 1min at 37 °C. Cortex were washed three times with Hank's solution. Cells were dissociated in 5mL of plating medium (Minimum Essential Medium supplemented with 10% horse serum and 20% glucose) and cells were counted in a Neubauer Chamber. Cells were plated into dishes pre-coated with poly-D-lysine (Sigma-Aldrich) (750,000 cells in a 10 cm dish and 270,000 in a 6 cm dish) and placed into a humidified incubator containing 95% air and 5% CO₂. The plating medium was replaced with equilibrated neurobasal media supplemented with B27 and GlutaMAX (Gibco; Life Technologies Co).

Hippocampal slices

5-7 day-old mice were anaesthetised with isoflurane and decapitated. Hippocampi were extracted in dissection solution (10 mM D-glucose, 4 mM KCl, 26 mM NaHCO₃, 233.7 mM sucrose, 5 mM MgCl₂, 1:1,000 phenol red), oxygen saturated with carbogen (95% O₂/5% CO₂), and sliced in an automatic tissue chopper (McIlwain Tissue Chopper, Standard Table, 220 V, Ted Pella) to obtain 400 µm hippocampal slices, transferred them on to 0.4 µm pore-size cell cultures inserts (Millicell) and maintained in culture medium (Minimum Essential Media MEM) supplemented with 20% (v/v) horse serum, 1 mM glutamine, 1 mM CaCl₂, 2 mM MgSO₄, 1 mg/L insulin, 0.0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose and 5.2 mM NaHCO₃ at pH 7.25, and a final osmolarity of 320 mOsm/L. Cultures were kept at 35 °C for 14 days. Treatments started at day 4; the medium was every two days.

Nasal delivery of chitosan nanocapsules

5 month-old hAPP NL/F mice were randomly divided into groups (n = 3 in each group) and supplied with food and water in plastic cages under conventional conditions at 22 °C, 50–60% humidity and a 12 h light/12 h dark cycle. The day of the experiment, mice were anaesthetized with an Isoflurane vaporizer. Induction was produced in a box at concentration of 5%, and maintained using a concentration of 2%. Using long tips, 4 µL of chitosan capsules in complex with the PH797804 inhibitor or labelled with fluorophore (administered diluted in a 5% NaCl sterile solution with an approximate pH of 4), were inoculated daily in each nostril of the mouse for two times (a total of 16 µL), along 9 days. The mice were anaesthetized 24 hours after last treatment and sacrificed by transcardiac perfusion or decapitated for biochemical analysis.

SDS-PAGE and western blotting

Cells and slices were treated with the p38MAPK inhibitor PH797804 at concentration mentioned above. After treatment, cells were washed once with ice-cold PBS 1X and scraped, as slices and brain were directly lysed in RIPA lysis buffer with Protease Inhibitor Cocktail Set III (Merck Millipore) and PhosStop (Phosphatase inhibitor cocktail (Roche). Samples were sonicated and centrifuged (10,000 rpm for 10 min at 4 °C). Post-nuclear supernatant was recovered and stored at -20 °C. Protein quantification was determined with BCA Protein Assay (Thermo scientific). After the extracted lysates were mixed in loading buffer and then boiled for 5 min, equivalent amounts of protein (30 µg) were loaded and run on a single

track of a 10% or 8% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes (LifeScience). The membranes were blocked with 5% BSA-TBST for 1 h at room temperature and then incubated with primary antibodies (in TBST-5% BSA) overnight at 4 °C. Total p38 antibody was from Abcam (ab170099), used at a concentration of 1:1000. Phospho-p38 α Tyr182 antibody was from Santa Cruz (sc-166182) used at a concentration of 1:1000. After incubation, the blots were washed four times for 15 min each in TBS-Tween and then incubated with the secondary antibodies goat anti-rabbit (1:2500) or goat anti-mouse (1:2500) (DAKO) (in TBST-BSA 5%) for 1 h at room temperature. Next, the blots were again washed four times for 15 min each in TBST and then detected with enhanced chemiluminescence. The blots were developed with ECLWestern blot Detection Reagents (Thermofisher).

Histological procedures

Mice were deeply anesthetized, then sequentially perfused with PBS 1:3 and PBS 1:3 PFA 4%. After perfusion, animals were decapitated and brains were collected, fixed, and cryopreserved by complete immersion in PBS 1:3 sucrose 30% with gentle shaking overnight at 4 °C. Brains were included in agarose-sucrose and cut into 40 μ M slices and finally mounted on Superfrost Plus coated slides (Menzel-Gleaser, Madison, WI). Images were taken in a LSM710 Zeiss confocal microscope (10X objective). Images were processed using ImageJ software.

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. of the values from the independent experiments performed, as indicated in the corresponding figures legends. Student's t-test was used for statistical analysis of the data using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Results

Synthesis and characterization of PH797804-loaded chitosan nanocapsules (PH-NC).

We selected for our studies the p38MAPK inhibitor PH797804 (PH), an ATP-competitive inhibitor of p38 α with IC₅₀ of 26 nM on the recombinant protein, about 4-fold lower than for p38 β , which has been

also reported to potentially inhibit p38MAPK-regulated processes in animal models[18, 19]. PH797804 was successfully incorporated in nanocapsules based on a nanoemulsion core and stabilized with a chitosan shell. The nanoemulsion system is formed by a reverse micelle, oily core, and a hydrophilic shell. So, it offers the advantage of retaining both lipophilic and hydrophilic molecules. A schematic representation of the delivery system is reported in Figure 1A.

This delivery system was chosen since it is a spontaneous and soft encapsulation method that does not require any high energy input, so it is recommended to preserve the properties of the active molecule [20]. Moreover, it was successfully applied in a previous work for the encapsulation of bedaquiline, an antimicrobial drug [15].

In this case, the p38MAPK inhibitor PH797804 was added during the preparation of the nanoemulsion dissolved in the organic phase to be entrapped in the nanocapsules upon formation of the hydrogel shell. Nanocapsules in suspension were diluted in methanol to extract the molecule by sonication and to determine the encapsulation efficiency (EE) and drug loading (DL) by spectrophotometric measurement. A calibration curve of PH797804 in the same solvent was previously carried out. The inhibitor was encapsulated with an efficiency of 21.5 ± 2.7 % and the final drug loading percentage was 3.5 ± 0.5 %.

The obtained nanocapsules, with inhibitor entrapped, were also characterized in terms of their physicochemical properties and in particular their hydrodynamic diameter and Z-potential were determined. The diameter obtained was bigger than empty nanocapsules of the same kind [21]. This difference could be addressed to the interaction of the molecule of the inhibitor with nanocapsule structure. To better characterize the obtained nanomaterial, Z-potential measurements were performed. A negative surface potential of -18.5 ± 2.9 mV was observed.

As the nanocapsules need to be resuspended in 5 % NaCl for nasal administration, their stability in this medium was evaluated by dynamic light scattering comparing the hydrodynamic diameter obtained in 5% NaCl with the one observed in pure water (Figure 1B). Data showed that the presence of salt did not affect the aggregation state of the nanocapsules since no significant difference between the diameter of the PH797804-loaded nanocapsules (PH-NC) in the two media was observed.

Finally, the surface potential of the nanocapsules was measured at different pH values to investigate at which conditions the highest positive potential value of the surface was observed (Figure 1C). This parameter indicates that amino groups of chitosan are protonated and that the best mucoadhesive

properties of the nanocapsules could be obtained. Because of the modification of the nanomaterial structure, due to the interaction with the inhibitor inside, the point of zero charge of the surface was found to be slightly shifted with respect to empty nanocapsules of the same nature and the highest positive charge was observed below pH 5. To take advantage of this property, nanocapsules were administered in a 5% NaCl sterile solution with an approximate pH of 4.

Synthesis and characterization of fluorescently labelled chitosan nanocapsules.

The fluorescently labelled nanocapsules were obtained by adding the fluorescent dye (either DiD' oil or DiO') to the organic phase during the formation of the nanoemulsion core of the carrier. Thanks to the lipophilic nature of these molecules, they were successfully retained in the oily core of the chitosan nanocapsules.

The fluorescence of the obtained nanocapsules was determined by spectrofluorimetric measurements, confirming the almost 100% encapsulation efficiency as well as that both dyes maintained their fluorescent properties after encapsulation. Spectra of the fluorescently labelled nanocapsules are reported in Figure S1 of the Supporting Information.

***In vitro* validation approach I: effect of PH797804-loaded nanocapsules on p38 MAPK phosphorylation in microglia cells.**

Inflammation plays a pivotal role in the pathogenesis of many diseases in the central nervous system. An experimental approach of great utility to study the mechanisms by which inflammation leads to disease of the nervous system is through the use of LPS [22]. LPS induces a dysregulated inflammatory response through the activation of MAPKs and other pathways, which produce bioactive substances including tumour necrosis factor (TNF) [23]. In particular, p38 α MAPK is an important regulator for TNF- α expression [24]. In order to assess whether the p38 α inhibitor PH-797804 could be a tool for the inhibition of p38MAPK signalling when encapsulated in chitosan nanocapsules, we first explored the inhibitory capacity of the complex in a simple *in vitro* model of brain inflammation: immortalized murine microglia-like BV-2 cells [16, 25]. To increase p38MAPK activity levels, and therefore to have better chances of seeing an effect, BV-2 cells were exposed to a single dose of LPS (1 μ g/mL; Figure 2A). In Figure 2B, LPS untreated control cells and LPS-treated cells incubated with the PH-797804 inhibitor in nanocapsules (PH-

NC) or PH-797804 alone are compared. The addition of 1 μ M of encapsulated inhibitor (corresponding to 0.489 mg/mL of PH-NC and 12.4 mg/ml Chitosan) resulted in the significant reduction of p38MAPK activity (Figure 2B and quantification in Figure 2C). Comparatively, the level of kinase inhibition by inhibitor loaded in chitosan nanocapsules (PH-NC+LPS) was similar to that obtained with the free-in-solution inhibitor (compare PH-NC+LPS with PH+LPS in Figure 2B). Chitosan nanocapsules without PH797804 did not reduce p38MAPK activity levels (compare LPS with PH-NC+LPS in Figure 2B), giving us confidence that the effect observed with the nanocapsules with inhibitor is specific. Quantifications are shown in Figure 2C.

***In vitro* validation approach II: effect of PH797804-loaded nanocapsules on p38 MAPK phosphorylation in rat cortical neurons.**

In addition to the activation of glial cells, the development of neuroinflammatory pathology affects neurons [22]. Therefore, in the next series of experiments we tested the effect of PH797804-loaded nanocapsules in cortical neurons *in vitro* subjected to LPS stimulation. 15 days *in vitro* (15 DIV) cortical neurons were exposed to 1 μ M PH797804, either free-in-solution or in chitosan nanocapsules, and 24 hours later they were exposed to LPS (1 μ g/mL) for 5 h, then cells were solubilised and processed for SDS-PAGE/western blotting for the detection of phosphorylated p38MAPK levels. Treatment with the PH797804-loaded nanocapsules effectively reduced the levels of phosphorylated p38MAPK, as much as the free-in-solution inhibitor (Figure 2D-E). Chitosan nanocapsules alone did not reduce p38MAPK activity induced by LPS (LPS compared with PH-NC+LPS in Figure 2D-E), suggesting that also in neurons the inhibitor remains active even when conjugated with chitosal in the nanoparticles.

***Ex vivo* validation approach: effect of PH797804-loaded nanocapsules on p38 MAPK phosphorylation in hippocampal slices from hAPP NL/F mice**

Although the previous *in vitro* experiments served to demonstrate that PH797804 retains pharmacological activity even when it is encapsulated in chitosan nanocapsules, we needed to show, before moving to the *in vivo* animal experimentation, that this complex also has the capacity to penetrate into nervous tissue. To determine if this is the case, we used an *ex vivo* system, consisting of 400 μ m thick hippocampal slices kept in culture for 2 weeks. We used hippocampal slices from early postnatal (5 days old) hAPP NL-F mice [17], since p38MAPK activity levels are significantly higher in the slices from these mice than in slices from wild type mice after 2 weeks *in vitro* (Figure 3A). To assess the effectiveness of

PH797804-loaded nanocapsules in this system, slices were incubated during 12 days (starting on day 3) with 1 μ M of inhibitor encapsulated in chitosan nanocapsules or with 1 μ M of free-in-solution inhibitor. Medium with PH-NC or free inhibitor was freshly added every 48 hours. Both, free-in-solution PH797804 or in chitosan nanocapsules reduced p38MAPK activity, but not empty chitosan nanocapsules (Figure 3A, B), altogether indicative that PH797804 retains, to a great extent, p38MAPK inhibitory function and capacity to penetrate through tissue even when entrapped in the carrier. These results encouraged to take the next step: *in vivo* administration of PH-NC.

***In vivo* validation (I): effect of the nasal administration of PH797804-loaded nanocapsules on p38 MAPK phosphorylation in hAPP NL/F mice.**

To determine if the nasal administration of the PH797804-loaded nanocapsules was able to reduce the activity of this pathway *in vivo*, chitosan nanocapsules with and without PH797804 were administered in the nostrils of hAPP NLF mice. These mice present a significant increase in brain A β 42/40 ratio with amyloid plaques appearing on month 9-12, and cognitive deficits at month 18, preceded by increased neuroinflammation and glial reactivity [17]. However, contrary to what has been indicated in previous studies with other animal models of this disease, p38MAPK activity in the hAPP NL/F mice is high at 2 months of age, decreasing at later time points. Thus, we took advantage of the early upregulation of cerebral p38MAPK to test the effect of nasal administration of PH797804-loaded nanocapsules. As shown in Figure 4, the nasal administration of (16 μ L of 100 μ M) PH797804-loaded nanocapsules during 9 consecutive days resulted in the significant reduction of p38MAPK activity in the olfactory bulb and in the cortex, but not in the hippocampus. Importantly, the administration of the same inhibitor in solution, without complexing, in the nostrils of hAPP NL/F mice did not reduce p38MAPK activity, neither in the olfactory bulb nor in the cortex, the two structures (Figure S2), that do show an effect when the inhibitor is administered in the nanocarriers (see above).

***In vivo* validation (II): nasally administered fluorescently-labelled chitosan nanocapsules reach the mouse cortex and hippocampus**

The preferential effect of the PH-NC in cortex but not in hippocampus suggest the existence of preferential transport route of the inhibitor from the nasal mucosa to the cortex, at least in the time-course and dose paradigm used here. In agreement with this possibility, nasal administration of nanocapsules of chitosan labelled with the lipophilic dye DiD' oil during 3, 6, or 9 days resulted in the presence of fluorescent signal in olfactory bulb and cortex, but not in the hippocampus (Figure 5). Importantly, the signal in the olfactory bulb and cortex was only evident at the late time-points (6 and 9 days), indicative, together with the absence of signal in the hippocampus, that signal in the cortex and olfactory bulb is not the consequence of auto-fluorescence. Despite these results, we would like to indicate that the nasal administration of chitosan nanocapsules labelled with a different fluorescent dye (DiO') resulted in the presence of fluorescence in the hippocampus, even after only 3 days of nasal administration (Figure S2), (see Discussion for possible explanation).

Discussion

The results obtained in this work suggest that the nasal administration of chitosan-nanocapsules encapsulating p38MAPK inhibitors may be a good strategy for future pre-clinical trials with other, perhaps more specific/potent, p38MAPK inhibitors. Moreover, the nanocarrier used here has been developed as a multi-pocket reservoir since it is able to encapsulate both hydrophilic and hydrophobic molecules, therefore representing a very versatile system for the delivery of drugs of both nature at the same time. The potential usefulness of this type of nanocarrier is evidenced in this work. In fact, the encapsulated PH797804 not only retains inhibitory function in an LPS-based system of p38 activation *in vitro*, in two different cell types, neurons and microglia, but also when added to thick (400 μ m) brain slices and when administered in the nose of living mice. Of course, this is not to say that the systemic administration of p38MAPK inhibitors cannot be beneficial for the treatment of nervous system conditions with increased activity of p38MAPK. What our results do imply is that the formulation used here can achieve beneficial effects in the brain while having less pronounced peripheral consequences.

While the inhibitory effect in the cortex was significant, we did not observe inhibition in the hippocampus. This is certainly a limitation of our studies as any attempt to offer a therapeutic alternative for Alzheimer's disease should demonstrate effectiveness in this structure. But if we take the fact that our experimental

approach was effective at the level of the cortex, we can speculate that increasing the concentration of nanocarriers/compound or, fundamentally, use a greater number of injections in the nostrils during a longer period of this may also affect the hippocampus. However, a change in experimental paradigm was beyond the purpose of this work, which remains to demonstrate that the use of nanocarriers administered in the nose can be a suitable approach to produce functional effects in the brain without some of the risks of systemic administration. This work does not pretend to be a pre-clinical study for Alzheimer's disease. As a matter of fact, the Alzheimer's mouse model used in this study was just an *in vivo* "read-out" to test the effect of p38MAPK inhibition via nanocarriers. On the other hand, we believe to have some logical explanations for the lack of effect in the hippocampus. One possibility is that the number of cells that have taken up the inhibitor in the cortex is higher than in the hippocampus. In this regard it is worth noting that stimuli in the nasal mucosa are conveyed to the olfactory bulbs and from there conducted by the lateral olfactory tract to the primary olfactory cortex and to the orbito-frontal cortex [26]. In this scenario, one can envision that the inhibitor may become dissociated from the nanocapsules upon contact with the membrane of the olfactory neurons, enter and exert an effect on these neurons (in fact, p38MAPK inhibition is strong in the olfactory cells) and also travel to the cortex by anterograde transport. On the other hand, the chitosan nanocapsules that did not enter the olfactory cells may have entered the cerebrospinal fluid, reaching all structures, including the hippocampus. This possibility could be tested in future studies using chitosan nanocapsules containing fluorescently labelled inhibitor, though labelling may interfere with the inhibitor's action. In any event, to have shown that a particular type of p38MAPK inhibitor in chitosan nanocapsules is capable of inhibiting activity *in vivo* paves the way for more clinically oriented studies.

For the *in vivo* demonstration that PH797804 can exert its inhibitory effect even when in complex with chitosan, we used the human APP NL/F knock-in mice. These mice present a significant increase in A β 42/40 ratio with amyloid plaques appearing on month 9-12, and cognitive deficits at month 18, preceded by increased neuroinflammation and glial reactivity [17]. However, contrary to what has been indicated in previous studies with other animal models of this disease, p38MAPK activity in the hAPP NL/F mice is high at 2 months of age, decreasing at later time points. This lack of temporal correlation between AD signs (A β -plaques-neuroinflammation) and high p38MAPK activity precluded us from analysing whether the chitosan nanoparticle-PH797804 inhibition of p38MAPK could reduce AD signs. Future work in a

different mouse model of p38MAPK-disease, for example the mouse APP / PS1 / tau in which one of the symptoms of disease can be rescued by inhibiting the p38MAPK pathway (ref), is needed to test if the nasal-chitosan/inhibitor complex can prevent/rescue functional deficits produced by the aberrant activity of this kinase. The results that we present in this work seem to indicate that this is a certain possibility.

Conclusion

Several publications support the view that nasal delivery of drugs/compounds is a suitable strategy to modify some of the abnormal biochemical reactions that occur in the brain in the course of disease. On the one hand, it appears that drugs administered through the nose can overcome blood brain barrier (BBB) and thus reach high concentrations that would not be obtained after systemic administration. In addition, it is considered that drugs administered in the nose would not reach significant levels in peripheral organs which would help to reduce undesired side effects. This last feature appears especially important in the case of p38MAPK. In fact, although the abnormal activation of p38MAPK is one of the components of the inflammatory process thought to play a role in the appearance of signs and symptoms of different chronic pathologies of the nervous system, including Alzheimer's disease, the inhibition of this enzyme with systemic treatments can lead to defects associated with the lack of function of this pathway in organs where basal levels of expression play an important physiological role. For this reason, the nasal administration of p38 inhibitors appeared to us as a way to both by-pass the blood-brain-barrier and to reach effective concentration in the central nervous system with a minimum effect in peripheral organs. In agreement with our expectations, in this work we demonstrate that the encapsulation of a p38MAPK inhibitor administered in the nostrils in a delivery system based on nanoemulsion-based chitosan nanocapsules led to the significant inhibition of p38MAPK enzymatic activity in the brain of a mouse model of Alzheimer's disease. We are convinced that the use of last generation p38MAPK inhibitors, which show greater specificity and more inhibitory power, will add pre-clinical validity to the results presented here. In any case, the results here presented constitute a strong indication that nasal administration of p38MAPK inhibitors in chitosan nanocapsules can be an effective strategy to reduce the exaggerated activity of this enzyme as it occurs in pathologies like Alzheimer's disease.

Future Perspectives

One of the greatest challenges of modern society is to target the brain and find effective treatments for neurodegenerative diseases such as Alzheimer's. Unfortunately, treatments approved by government agencies (FDA, EMA) are modestly effective, at best. While the reasons behind the lack of efficacy are multiple, it is likely that the increase of the levels of active principle could improve the treatments efficacy, mainly for those drugs designed to interfere with physiological biochemical pathways that are abnormally elevated in the course of pathology (e.g. γ -secretase). In this sense, the use of the intranasal route appears as most promising. Consistently, a number of studies have reported an improvement in some features of learning after the intranasal administration of insulin in individuals with amnesic memory impairment (aMCI) or mild Alzheimer's disease. Therefore, it is expected that the use of formulations able to improve the delivery of compounds in the brain, such as nano-capsules of chitosan, will have a great impact on medicine, especially for the treatment of brain diseases. Naturally, the packaging formulation of the compounds must be optimized for each compound, for which the use of pre-clinical experimentation models is essential. On the other hand, we ought to be realistic and should expect that increasing the availability of drugs in the brain will always have beneficial effects. Only further experimentation will be able to tell which "localised" formulations are effective and which are not. However, when it comes to the inhibitor used in this work, we can confirm that the presence of inhibitor in the brain after its nasal administration in nanocapsules did not produce counter-productive effects, at least at the histological and biochemical level analysed here. Only future work will be able to determine whether or not the strategy used here is beneficial in the pre-clinical and clinical context.

Summary

1. We used for our studies the p38MAPK inhibitor PH797804 (PH), an ATP-competitive inhibitor of p38 α
2. PH797804 was incorporated in nanocapsules based on a nanoemulsion core and stabilized with a chitosan shell.

3. Addition of chitosan-PH797804 nanocapsules to the culture medium of LPS-activated microglial cells efficiently inhibited p38 α , to a level similar of free PH-797804 inhibition.
4. Addition of chitosan-PH797804 nanocapsules to the culture medium of LPS-activated mouse cortical neurons efficiently inhibited p38 α , to levels similar of free PH-797804 inhibition.
5. Two week long treatment of hippocampal slices from hAPP NL-F mice with chitosan-PH797804 nanocapsules inhibited p38 α to levels similar of free PH-797804 inhibition.
6. Daily application (9 days) of chitosan-PH797804 nanocapsules in the nose of hAPP NL-F mice inhibited p38 α activity in the olfactory bulb and cerebral cortex, something that could not be reached by free PH797804.
7. Fluorescence microscopy revealed that the lipophilic dye DiD' incorporated into chitosan nanocapsules reaches the cerebral cortex after the endonasal administration of the nanocapsules.
8. Fluorescence microscopy revealed the presence of the fluorescent dye (DiO') in the cerebral cortex and hippocampus after the endonasal administration of dye-filled chitosan nanocapsules.

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