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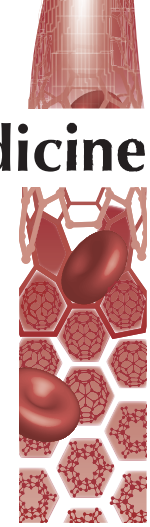

Intranasal administration of molecular-gated mesoporous nanoparticles to increase ponatinib delivery to the brain

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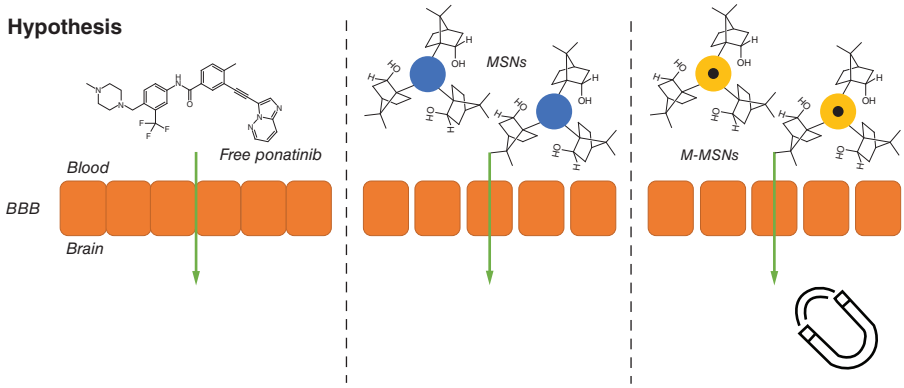
Background: Glioblastoma is the most common and lethal brain cancer. New treatments are needed. However, the presence of the blood–brain barrier is limiting the development of new treatments directed toward the brain, as it restricts the access and distribution of drugs to the CNS. **Materials & methods:** In this work, two different nanoparticles (i.e., mesoporous silica nanoparticles and magnetic mesoporous silica nanoparticles) loaded with ponatinib were prepared. **Results & conclusion:** Both particles were characterized and tested *in vitro* and *in vivo*, proving that they are not toxic for blood–brain barrier cells and they increase the amount of drug reaching the brain when administered intranasally in comparison with the results obtained for the free drug.

Plain language summary: This article presents a couple of promising nanoparticles for the treatment of brain cancer. This research is interesting because the brain and spinal cord are protected by a membrane that prevents toxic substances from reaching them but also hinders the access of drugs. One type of particle has a magnet in its core, so it can be driven with another external magnet until it reaches target; the other type does not have a magnet but has a small size, which would allow it to cross the membrane mentioned above. These particles have been proven to be able to kill cancer cells and to reach the brain after been administered through the nose in a better way than the free drug.

Graphical abstract:



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Keywords: blood–brain barrier • glioblastoma • mesoporous nanoparticles • ponatinib • targeted drug delivery

Future Medicine

Unfortunately, from 2000 to 2019, the prevalence of brain and CNS cancers increased by 46% [1]. Among these cancers, glioblastoma is the most common and lethal one, representing more than 50% of all the primary intracranial tumors [2]. The altered astrocytes and oligodendrocytes that form this type of tumor are able to invade the surrounding tissue very quickly and life expectancy is very low, around 15 months after diagnosis [3]. In fact, only 2.7% of the patients survive 5 years [4–7].

The current treatment of glioblastoma combines surgery, radiation and adjuvant chemotherapy with temozolomide and, in some cases, in combination with bevacizumab [8,9]. Nonetheless, it has been observed that glioblastoma tumors overexpress PDGFR-α and RET, two tyrosine kinases, which may be responsible for the glioblastoma tumors' resistance to treatment with temozolomide [10,11]. Therefore, other drugs that inhibit tyrosine kinases, such as ponatinib, which is currently approved by the US FDA for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia, have been proposed as an alternative for the treatment of several malignancies, including glioblastoma [12]. Despite the potential of ponatinib for treating glioblastoma, the presence of the blood–brain barrier (BBB) is a limiting factor in the development of new treatments directed toward the brain, as it restricts the access and distribution of drugs in the CNS. In fact, ponatinib is a drug with low BBB permeability because it is substrate of efflux transporters and it has a low unbound fraction of drug in plasma, and only the fraction of drug that is not bound to proteins is able to cross the BBB [10,13]. In this scenario, the use of nanotechnology to encapsulate the drug and protect it from efflux transporters and plasma proteins may constitute a good option to overcome the limitations mentioned above.

Among the wide range of nanoparticles, mesoporous silica nanoparticles (MSNs) are highly appealing for drug-delivery applications due to their high surface area, high loading capacity, biocompatibility and easy functionalization [14]. In relation to the use of MSNs and BBB, a relatively recent study in 2016 in the MDCK and RBE4 cells lines as models of the BBB (MDCK and RBE4 cells) showed that bare MSNs had low permeability and external functionalization was necessary to improve BBB penetration [15]. Moreover, functionalization can be used to promote any of the accessing routes that the BBB already has: paracellular diffusion, transcellular diffusion, carrier-mediated transport, receptor-mediated transport, adsorptive-mediated transport and cell-mediated transport [16–20]. For instance, the use of amino acids promotes carrier-mediated transport [20,21]; other macromolecules, such as insulin or transferrin, promote receptor-mediated transport [20,22]; and polycationic substances, such as albumin and other peptides, promote adsorptive-mediated transport [20]. The administration of substances that temporally open the BBB is another strategy to increase the access of substances to the CNS by means of promoting paracellular diffusion. For example, mannitol creates a hyperosmolar environment, which results in a reduction in the size of the endothelial cells, and borneol and other aromatic substances provoke the translocation of the tight junctions proteins back to the cytoplasm, resulting in an opening of the tight junctions and promoting paracellular diffusion [23]. Furthermore, when trying to treat cancer, nanoparticles' functionalization can also be used to foster the active uptake of the nanocarriers by tumor cells, thus reducing side effects [24]. With this objective, different molecules that are ligands of receptors that are overexpressed in tumor cells can be used, such as monoclonal antibodies, antibody Fabs, small peptides, natural proteins, aptamers, carbohydrates or small molecules [24]. Folic acid is one of those molecules that has been widely used to promote the active uptake of nanoparticles by cancer cells, as, due to nutritional requirements, cancer cells overexpress the folate receptor [24–26]. When referring to MSNs, functionalization is also important to ensure that the drug is kept inside the nanoparticle until it reaches its target, where a specific stimulus triggers the drug release. Such capping ensembles are usually known as molecular gates, gatekeepers or nanovalves [27,28]. In addition, because of the extremely restrictive properties of the BBB, which only allows the passage of nanoparticles with a diameter below 100 nm [29], some researchers have started to combine functionalization with the use of an external force to increase the accumulation of drug in the CNS. In this regard, the use of magnetic mesoporous silica nanoparticles (M-MSNs) in combination with an external magnetic field can promote BBB permeation for treating pathologies affecting the CNS [30–32].

Based on the above, the authors report herein the preparation of two new mesoporous nanostructures: MSNs and M-MSNs. The nanoparticles are loaded with ponatinib, which is a tyrosine kinase inhibitor indicated for the treatment of glioblastoma, and are functionalized with borneol and folic acid to increase the access of ponatinib to the CNS and, thus, improve the treatment of glioblastoma.

Once prepared, loaded and functionalized, the particles were studied in different ways. First, they were physically characterized using several techniques: dynamic light scattering (DLS), transmission electron microscopy

(TEM), powder x-ray diffraction (PXRD) analysis, porosimetry (N_2 adsorption–desorption isotherms) and thermogravimetry analysis. Then, *in vitro* tests were carried out: *in vitro* drug release, cytotoxicity and efficacy assay in cell cultures and *in vitro* BBB permeability test. And, finally, when the properties of the particles were considered appropriate, they were tested *in vivo* for evaluating the biodistribution of ponatinib after intravenous and intranasal administration.

Materials & methods

Drugs, cells & products

Ponatinib was purchased from Enamine (Riga, Latvia). Trypan blue, borneol, succinic anhydride and folic acid were purchased from Sigma-Aldrich (Barcelona, Spain). Cetyltrimethylammonium bromide (CTAB), diethanolamine, tetraethylorthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysuccinimide, 4-dimethylaminopiridine, $FeCl_3{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, oleic acid and anhydrous acetonitrile were purchased from Sigma-Aldrich (Madrid, Spain). Chloroform, dimethylformamide, and anhydrous methylene chloride (DCM) were purchased from Acros Organics (Madrid, Spain). Ammonia (32% v/v), ethanol, dimethyl sulfoxide (DMSO) and ethyl acetate were purchased from Scharlab (Barcelona, Spain).

Lysosomal extract was given by Dr. Martinez-Mañez (Valencia, Spain). The MDCK-MDR1 cell line was kindly provided by Dr Gottesman MM (National Institutes of Health, MD, USA) and the U87 cell line was purchased from Sigma-Aldrich (Barcelona, Spain).

DMEM with a high content of glucose, fetal bovine serum, MEM non-essential amino acids, penicillin-streptomycin, L-glutamine, (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) (HEPES), phosphate-buffered saline (PBS), trypsin-EDTA, Hank's balanced salt solution, Tween 80 and a cell proliferation kit I (MTT) were purchased from Sigma-Aldrich (Barcelona, Spain). DMEM with a high content of glucose and high content of pyruvate used with the U87-MG cell line was obtained from Gibco (Barcelona, Spain).

Synthesis of mesoporous nanoparticles

Nonmagnetic MSNs

Nonmagnetic MSNs were prepared based on the protocol described in [33]. Briefly, 1 g of CTAB and 20 ml of deionized water were placed in a two-neck, round-bottom flask and stirred for 30 min at 500 r.p.m. with a rugby-shaped magnet. Then, 160 µl of diethanolamine was added and the temperature was increased to 95°C, at which the mixture was stirred with reflux for 1 h. After that time, 1.5 ml of TEOS were added dropwise and everything was again stirred for 1 h, but at 950 r.p.m. Subsequently, the particles were recovered and washed with water at 13,400× g (20 min) until reaching pH 7 and dried at 70°C overnight in a drying oven. Finally, the calcination procedure was performed at 550°C in an oxidant atmosphere for 5 h in a muffle furnace in order to remove CTAB and obtain the final MSNs.

Ultrasmall superparamagnetic iron oxide nanoparticles

Ultrasmall superparamagnetic iron oxide nanoparticles (USPIONs), which were used later as seeds for preparing M-MSNs, were prepared according to the protocol described in [34]. First, 50 ml of deionized water was placed under Argon atmosphere for 30 min. Then, 12 g of $FeCl_3{\cdot}6H_2O$, 4.9 g of $FeCl_2{\cdot}4H_2O$ and 19.53 ml of ammonia 32% (v/v) were added at 80°C. After 30 min, 2.13 ml of oleic acid was added to the flask and the reaction was left stirring for 90 min at 80°C. Finally, the particles were cooled at room temperature and recovered after washing three times with deionized water and three times with ethanol (12,108× g; 10 min). The material was dried overnight under vacuum and the next day it was resuspended in chloroform to be kept in the fridge until its use.

Magnetic mesoporous silica nanoparticles

M-MSNs were prepared following a slightly modified protocol already described in [34]; 100 mg of CTAB and 10 ml of deionized water were placed in a vial and the mixture was stirred until the CTAB was dissolved. Then, 580 µl of a previously prepared suspension of USPIONs in chloroform (Fe concentration = 3.6 mg/ml) was added to the vial and it was sonicated with a probe sonicator (Branson 450 Sonifier) for 3 min. After sonication, chloroform was evaporated at 70°C with manual agitation and 30 ml of deionized water and 0.547 ml of ammonia 32% (v/v) and the particles were placed in a two-neck, round-bottom flask, where the mixture was stirred at 400 r.p.m. with reflux and a rugby-shaped magnet until the temperature reached 75°C. Later, 0.5 ml of TEOS and 3 ml of ethyl

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acetate were added to the flask (TEOS, dropwise) and the reaction was stirred for 2 min at 850 r.p.m. and for 3 h at 350 r.p.m. At the end of that time, the particles were cooled in an ice bath and they were washed three-times with ethanol (13,400× g; 10 min). Finally, the magnetic particles were separated from the nonmagnetic ones with a magnet; they were dried at 70°C overnight in a drying oven and calcined in the presence of air at 550°C in a muffle furnace.

Drug loading & functionalization of the nanoparticles

MSNs and M-MSNs were loaded following the immersion protocol, in which the particles are left stirring overnight in a solution of the drug: ([0.8 mmol of drug + 30 ml of solvent]/1 g nanoparticle). Two different molecules were loaded in the particles – trypan blue and ponatinib; as trypan blue is soluble in water, the solvent used for loading the particles was deionized water and as ponatinib is not soluble in water but soluble in DMSO, this one was the solvent used for dissolving ponatinib. The day after loading, the particles were filtered and dried under vacuum. Ponatinib is used as glioblastoma treatment and trypan blue is a dye with low access to the CNS that was used as an initial marker for studying the performance of the particles.

Once loaded, both MSNs and M-MSNs were functionalized following the same protocol with the aim of obtaining double-gated nanoparticles with borneol and folic acid (Supplementary Figure 1).

First, to allow borneol to attach to the nanoparticles, it was modified to obtain BO-SA active ester, as described in [26]. Then, loaded nanoparticles were functionalized with APTES in anhydrous acetonitrile ([6 mmol APTES + 30 ml solvent]/1 g nanoparticles) for 5.5 h at room temperature. The resulting nanoparticles were isolated by filtration and dried under vacuum. For synthesis of the final nanoparticles, BO-SA active ester (0.3 mmol/100 mg nanoparticles), folic acid (0.3 mmol/100 mg nanoparticles), EDC (3 mmol/100 mg nanoparticles), N-hydroxysuccinimide (3 mmol/100 mg nanoparticles), dimethylformamide (3 ml/100 mg nanoparticles) and DMSO (1 ml/100 mg nanoparticles) were mixed together in a vial overnight at room temperature. Finally, the particles were washed four times with dimethylformamide and four times with deionized water and dried at 37°C.

Drug stability

The stability of ponatinib was evaluated in PBS, plasma and brain homogenate. Solutions with an initial concentration of free drug of 100 μM were prepared and the amount of drug present in the solutions was measured at times 0, 24 and 48 h. Solutions were kept at 4°C and 37°C until the sample times arrived. Samples were diluted with cold methanol, centrifuged (5 min; 10,000 r.p.m. Eppendorf Centrifuge 5424, Rotor FA-45-24-11) and kept in the freezer at -20°C until their analysis.

Characterization of the nanoparticles

Dynamic light scattering

DLS experiments were conducted with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). This technique was used for measuring the hydrodynamic size, the polydispersity index and the zeta potential of the particles. Suspensions of 1 mg/ml of nanoparticles were prepared in water and the characteristics mentioned above were measured thrice.

Transmission electron microscopy

The proper size and shape of the MSNs and M-MSNs were checked in a 100 kV JEOL JEM-1010 transmission electronic microscope operated with AMT image capture engine software.

PXRD analysis

The x-ray diffractograms of the USPIONS, the MSNs and the M-MSNs (as-made, calcined, calcined and functionalized with borneol and folic acid and loaded with trypan blue and functionalized with borneol and folic acid) were obtained with a Bruker D8 Advance diffractometer (Bruker, Coventry, UK).

Porosimetry

A Micromeritics TriStar II Plus automated analyzer (Micromeritics Instrument Corporation, GA, USA) was used for recording the N_2 adsorption–desorption isotherms of the MSNs and M-MSNs. The samples were degassed at 120°C in vacuum overnight. A Brunauer–Emmett–Teller model was used for calculating the specific surface areas

from the adsorption data in the low-pressure range. On the other hand, the Barrett–Joyner–Halenda method was used to determine the size and volume of the pores present in the particles.

Thermogravimetry

A TGA/SDTA 851e thermobalance from Mettler Toledo (Mettler Toledo Inc., Schwarzenbach, Switzerland) was used to obtain thermograms for different solid samples and evaluate the organic content of loaded and functionalized nanoparticles. Thus, the percentage of drug loaded in the MSNs and M-MSNs could be obtained. Briefly, samples were heated in a dynamic step at 10°C/min, from 25°C to 100°C. Then, the temperature was maintained at 100°C for 60 min; the temperature was increased again until 1000°C at 10°C/min. The samples were kept at 1000°C for 30 min. Total organic content was evaluated in the range between 100° and 800°C.

In vitro drug release

Release studies were carried out at 37°C for both types of nanoparticles, MSNs and M-MSNs, loaded with trypan blue and ponatinib. First, a suspension of each type of particle, with a particle concentration of 10 μg/μl, was prepared in PBS and divided into two eppendorfs. Then, PBS or lysosomal extract was added until reaching a final concentration of particles of 1 μg/μl (final volume: 1 ml). Samples (100 μl) were taken at different times (2 min, 30 min, 1 h, 2.5 h, 4 h and 5.5 h) and the volume was replaced with new PBS. All samples were diluted with cold methanol, centrifuged (5 min; 10,000 r.p.m. Eppendorf Centrifuge 5424, Rotor FA-45-24-11) and kept in the freezer at -20°C until their analysis.

Cytotoxicity & efficacy assay *in vitro*

The cytotoxicity and efficacy of the MSNs and M-MSNs functionalized with borneol and folic acid and loaded with ponatinib and functionalized with borneol and folic acid were evaluated in two different cell types (U87-MG glioblastoma cells and MDCK-MDR1 BBB cells) using an MTT kit.

MDCK-MDR1 cells were maintained in an incubator at 37°C with an atmosphere of 5% CO_2 and 90% humidity inside flasks of 75 cm^2 with DMEM with a high content of glucose completed with fetal bovine serum (10% [v/v]), MEM non-essential amino acids (1% [v/v]), penicillin-streptomycin (1% [v/v]), L-glutamine (1% [v/v]) and HEPES (1% [v/v]). U87-MG cells were kept using the same protocol as for MDCK-MDR1 but with a cell culture medium with a higher concentration of pyruvate. When the cells reached 80% confluence, they were split and subcultured in new flasks. For detaching them and allowing the subculturing procedure, a trypsin-EDTA:PBS (2:8) solution was used. The day after subculturing, the medium of the flasks was replaced with new, fresh medium to remove all the dead cells.

To carry out the cytotoxicity assay, 100 μl of cells was seeded in each well of a 96-well plate (2.5×10^4 cells/well) and, after 24 h at 37°C, the medium was removed and replaced with 100 μl of a ponatinib solution or a particle suspension with ponatinib concentration ranging from 0.002 to 200 μM. After 72 h, the solutions/suspensions were aspirated and replaced with 100 μl of fresh culture medium. Then, 10 μl of the MTT labeling reagent was added to the wells and the cells were kept in the incubator for 4 h; 100 μl of solubilization solution was added to the plates and incubated overnight. Finally, the absorbance of the plate was measured at 570 and 630 nm using a microplate reader (Microplate Reader MB-850, Heales®).

BBB permeability

The penetrability of the formulations (MSN and M-MSN) was evaluated in MDCK-MDR1 monolayers. Cells were seeded in six-transwell plates with a pore size of 0.4 micron, an effective area of 4.2 cm^2 and a pore density of $(100 \pm 10) \times 10^6$ pores/cm^2 and they were maintained, changing the culture medium every 2 days, during 8 days until confluence. Once the cells were confluent (trans-endothelial electrical resistance [TEER]: 120–140 kΩ/cm^2), the permeability study was carried out using an orbital shaker at 37°C and 100 r.p.m.

In the experiments with the M-MSNs, circular neodymium magnets were placed under each well. Samples were taken from basolateral at 15, 30, 60, 90, 120, 180 and 240 min and the mass balance was checked by means of taking two samples from apical at times 0 and 240 min, a sample of the particles adhered to the cells and a sample after disrupting the cells with methanol. Samples were kept at -20°C until their analysis.

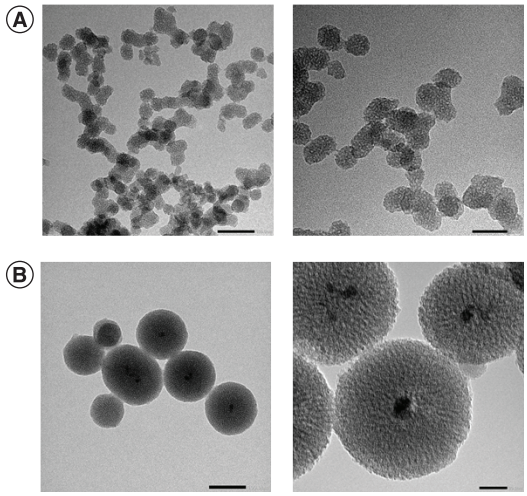

Figure 1. Transmission electron microscopy images of (A) calcined mesoporous silica nanoparticles and (B) calcined magnetic mesoporous silica nanoparticles.

Biodistribution *in vivo*

The biodistribution of the particles was evaluated in rats. The *in vivo* experiments were approved by the ethical committee of Miguel Hernández University (2021/VSC/PEA/0133 type 2). The number of animals used was the minimum possible to obtain significant results, a total of 290 rats divided into eight groups (A: n = 48, free ponatinib administered intravenously; B: n = 48, MSNs loaded with ponatinib administered intravenously; C: n = 48, M-MSNs loaded with ponatinib administered intravenously; D: n = 1, vehicle administered intravenously; E: n = 48, free ponatinib administered intranasally; F: n = 48, MSNs loaded with ponatinib administered intranasally; G: n = 48, M-MSNs loaded with ponatinib administered intranasally and H: n = 1, vehicle administered intranasally).

After intraperitoneal anesthesia, healthy Wistar rats (~300 g) were administered intravenously or intranasally with a solution of ponatinib or a suspension of MSNs or M-MSNs loaded with ponatinib with a concentration of drug of 3 mg/kg. The rats were previously cannulated at the jugular vein to allow intravenous administration and blood sampling. In addition, the skulls of the rats that received the M-MSNs were shaved and a neodymium magnet was attached between the ears and the eyes. In the intravenous pathway, 5 ml/kg of solvent (DMSO:Tween 80:PBS; 2:1:7) was used to administer the drug as done in [10]. In the intranasal administration, 20 µl/nostril of the same solvent mixture was used [35]. At different times, blood samples were taken by the cannula. In addition, when the animals were euthanized, their blood was removed with physiological serum and their brains and other organs were extracted to evaluate the amount of drug present on them. Proteins were precipitated using cold methanol and, in half of the samples, particles were forced to be opened with sodium hydroxide with DMSO (1%, v/v); the other half did not receive sodium hydroxide with DMSO (1%, v/v). All the samples were kept at -20°C until their analysis.

Analysis of the samples

Trypan blue was analyzed by HPLC using a Waters 2695 separation module, a Waters 2487 UV detector and a XBridge C18 column (3.5 µM, 4.6 × 100 mm; Barcelona, Spain). The wavelength (λ) used for measuring trypan blue was 300 nm, with a mobile phase of 20% H_2O and 80% acetonitrile at 30°C and a flow rate of 1 ml/min. The peak appeared after 0.750 min and a calibration curve with a coefficient of determination (r^2) of 0.998 was obtained.

Ponatinib samples were sent to Valencia University to be analyzed in a QTRAP 6500+ LC–MS/MS system.

Results

MSNs and M-MSNs were prepared following previously reported procedures [33,34]. Four different solids were used for the experiments: Calc: calcined; BO_FA: calcined and functionalized with borneol and folic acid; TB_BO_FA: calcined, loaded with trypan blue and functionalized with borneol and folic acid; and P_BO_FA: calcined, loaded with ponatinib and functionalized with borneol and folic acid.

Figure 1 shows TEM images of both types of particles. It can be observed that MSNs (Figure 1A) have a grape form and a particle size (42.8 ± 9.2 nm) that corresponds with the one obtained in the article from which the

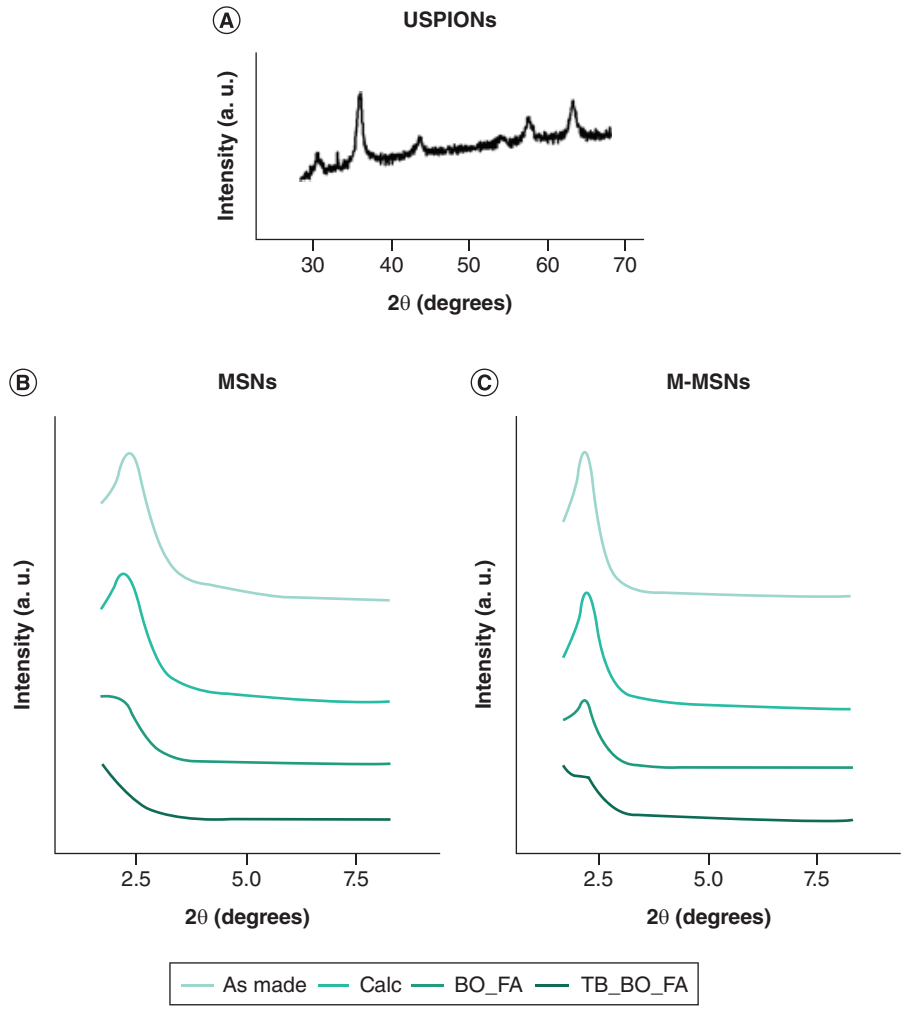

Figure 2. x-ray diffractograms for (A) ultrasmall superparamagnetic iron oxide nanoparticles, (B) mesoporous silica nanoparticles and (C) magnetic mesoporous silica nanoparticles.
BO_FA: Calcined and functionalized with borneol and folic acid; Calc: Calcined; TB_BO_FA: Calcined loaded with trypan blue and functionalized with borneol and folic acid.

synthesis method was extracted [33]. In Figure 1B, a core-shell structure can be observed in which the magnetic core is surrounded by mesoporous material, which creates wormhole-like channels.

The hydrodynamic diameter, the surface charge and the polydispersity of the particles were analyzed in a Zetasizer (Supplementary Figure 2). Both types of particles have a negative charge on their surface once they are prepared and calcined, but once they are functionalized with borneol and folic acid, the charge becomes positive with independence of the loading. In terms of size, the MSNs are smaller than the M-MSNs, but once functionalized, they have a greater polydispersity index (0.360 ± 0.030 vs 0.166 ± 0.009), so the batch is less homogeneous.

Figure 2 shows the x-ray diffractograms (XRDs) for the USPIONS, MSNs and M-MSNs (as-made, calcined, calcined and functionalized with borneol and folic acid and loaded with trypan blue and functionalized with borneol and folic acid). XRDs obtained for the USPIONS showed a highly crystalline structure with the presence of six sharp diffraction peaks at 2θ of 30.17, 35.67, 43.21, 53.53, 57.08, 62.81. The 2 position and relative intensity of the peaks are in concordance with the Bragg reflections of magnetite, which were indexed as (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) [34]. On the other hand, the noncalcined MSNs and M-MSNs XRD patterns showed one intense single diffraction peak at 2θ 2.17 in both cases, which indicates that there is a periodic pattern that repeats within the mesoporous structure [34]. As can be seen in Figure 2A & B, the removal of the surfactant and the condensation of silanol groups during the calcination step are reflected in a slight shift of these peaks.

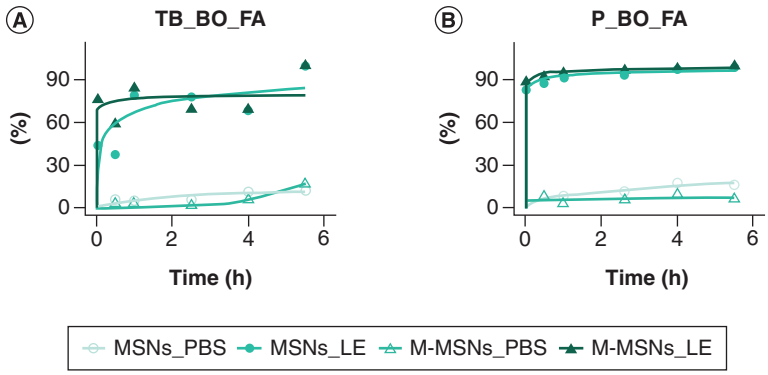

Figure 3. Release profiles for the different nanoparticles with and without stimulus to open the borneol and folic acid gate. (A) MSNs and M-MSNs loaded with trypan blue and **(B)** MSNs and M-MSNs loaded with ponatinib.
LE: Lysosomal extract; MSN: Mesoporous silica nanoparticle; PBS: Phosphate-buffered saline.

The existence of these reflection peaks in the PXRD pattern of calcined and functionalized MSNs and M-MSNs shows that the functionalization processes did not alter the 3D mesoporous structure of the scaffold and did not, to a large, extent modify the mesoporous scaffolding.

The porosimetry analysis showed that, after being prepared and calcined, both types of particles (MSNs and M-MSNs) had a similar surface area, pore volume and pore size. In addition, as seen in Supplementary Figure 3, the adsorption and desorption isotherms were also very similar, were type IV and corresponded to mesoporous material [36]. In contrast, the N2 isotherm for the solids once functionalized showed a significant reduction in the absorption process and indicated pore blocking with borneol and folic acid gating system. Application of the Barrett–Joyner–Halenda resulted in the determination of the pore diameter and volume values (2.919 nm and 0.858 cm^3 g^{-1} for MSNs and 2.871 nm and 0.802 cm^3 g^{-1} for M-MSNs). Application of the Brunauer–Emmett–Teller model allowed calculation of the total specific surface area (989.317 m^2 g^{-1} for MSNs and 896.343 m^2 g^{-1} for M-MSNs) of the prepared nanomaterials.

Finally, from thermogravimetry studies, the organic content of the particles was calculated (Supplementary Table 1). The percentages of trypan blue in the MSNs and M-MSNs once calcined and functionalized with borneol and folic acid were 11.4% and 13.9%, respectively. In the case of ponatinib, the percentages of drug loaded in the MSNs once calcined and functionalized with borneol and folic acid were 19.0% and 14.8% in the M-MSNs once calcined and functionalized with borneol and folic acid. (The percentages of trypan blue in loaded BO_FA_MSNs and BO_FA_ M-MSNs were 11.4% and 13.9%, respectively. In the case of ponatinib, the percentages of drug loaded in BO_FA_MSNs were 19.0% and 14.8% in the BO_FA_M-MSNs.)

Once the particles were prepared and characterized, the ability of the gate to keep the drug inside them and release it in the presence of the stimulus (the presence of lysozymes) was evaluated with an *in vitro* release test. Figure 3 shows the release profiles for both MSNs and M-MSNs loaded with trypan blue (Figure 3A) or with ponatinib (Figure 3B). It can be seen that when the particles were resuspended in PBS, no release of the drug was observed, but in the presence of lysozymes, from lysosomal extract, the content of the particles was rapidly released. Profiles are described with a Weibull kinetics model.

In terms of cytotoxicity and efficacy, two types of studies were carried out. First, as can be seen in Figure 4, the lack of toxicity of the empty nanoparticles functionalized with borneol and folic acid was evaluated in an *in vitro* BBB model (MDCK-MDR1). It can be observed that at the highest concentrations tested, 20 and 200 µM, the free ponatinib was able to kill both types of cells, as previously observed by Zhang *et al.* in 2014 [37]. Nonetheless, the empty nanoparticles did not reduce the viability of the cells at any concentration after 72 h. This fact confirms that both, the case and the gate of the particles, are not toxic.

Then, with the aim of proving that the particles loaded with ponatinib were able to kill the cancer cells, an extra assay was carried out in the U87-MG cell line after resuspending the particles in lysosomal extract (Figure 5). In this second case, MSNs and M-MSNs with lysozymes were able to kill the glioblastoma cells at the highest concentration tested; although the levels of toxicity were not as high as the ones obtained with the free drug, this can be explained by the difficulties of the drug in getting out of the nanoparticle.

When evaluating the *in vitro* BBB permeability of the nanoparticles, it was observed that just in the case of the ones loaded with ponatinib there was a slight increment of the influx clearance (Figure 6); this phenomenon was not observed when the particles loaded with trypan blue were tested.

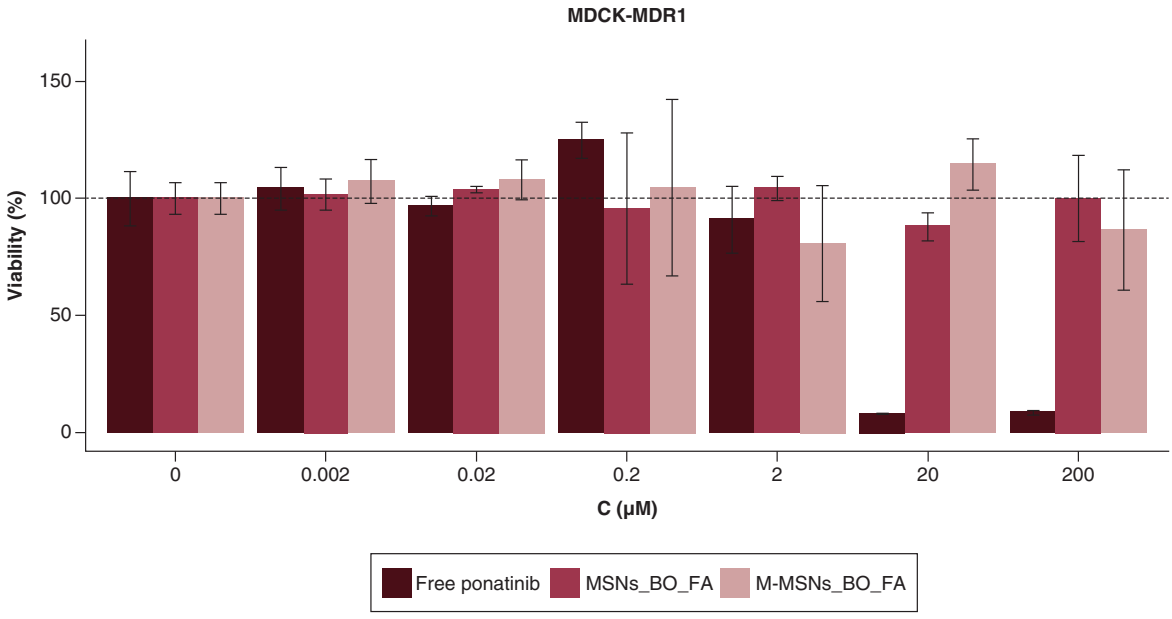

Figure 4. Cytotoxicity results obtained with an MTT assay in an *in vitro* blood–brain barrier model after administering different concentrations of free drug and empty nanoparticles.
M-MSNs_BO_FA: Magnetic mesoporous silica nanoparticles functionalized with borneol and folic acid; MSN_BO_FA: Mesoporous silica nanoparticles functionalized with borneol and folic acid; MSN: Mesoporous silica nanoparticle.

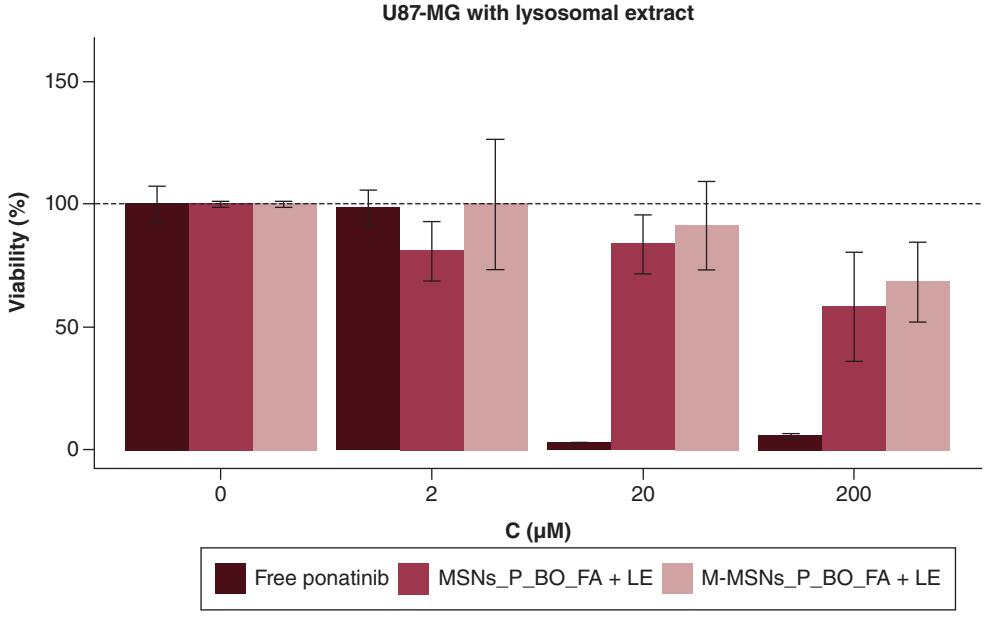

Figure 5. Efficacy results obtained with an MTT assay in an *in vitro* glioblastoma model when adding lysosomal extract with the loaded nanoparticles.
M-MSNs_BO_FA: Magnetic mesoporous silica nanoparticles mesoporous silica nanoparticles loaded with ponatinib, functionalized with borneol and folic acid and in presence of lysosomal extract; MSNs_BO_FA: Mesoporous silica nanoparticles loaded with ponatinib, functionalized with borneol and folic acid and in presence of lysosomal extract.

The MSNs and M-MSNs loaded with ponatinib were selected to evaluate the *in vivo* biodistribution in rats. These studies revealed that neither the particles nor the drug was able to cross the BBB after being administered intravenously. Nonetheless, when administration was done intranasally a clear increase in the accumulation of drug in the brain was observed with both the MSNs and M-MSNs, in comparison with the free ponatinib (Figure 7).

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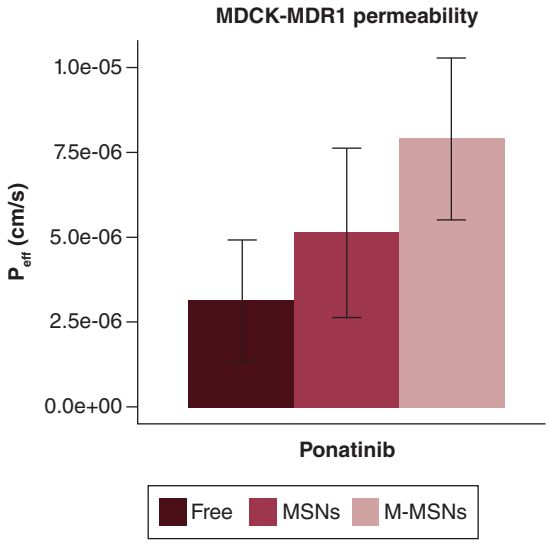

Figure 6. *In vitro* blood–brain barrier permeability of ponatinib tested in MDCK-MDR1 monolayers as free drug or loaded in mesoporous silica nanoparticles and magnetic mesoporous silica nanoparticles functionalized with borneol and folic acid.
M-MSN: Magnetic mesoporous silica nanoparticle; MSN: Mesoporous silica nanoparticle.

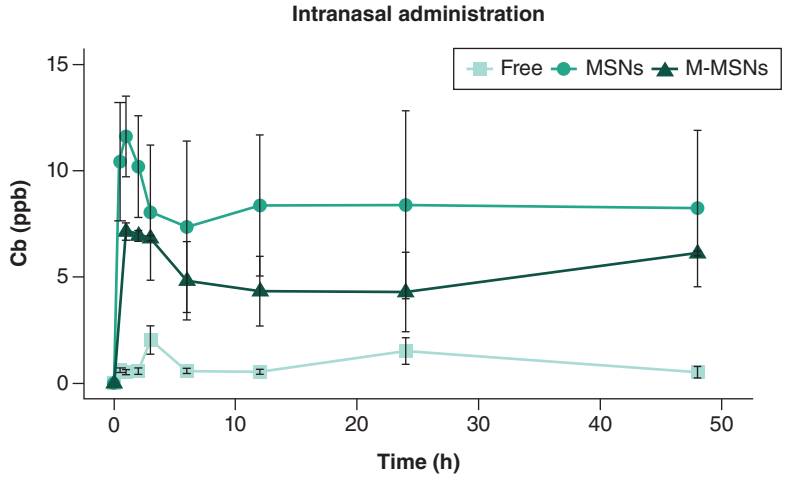

Figure 7. Brain profiles obtained after the intranasal administration of a free ponatinib solution or mesoporous silica nanoparticles and magnetic mesoporous silica nanoparticles loaded with ponatinib and functionalized with borneol and folic acid suspensions (dose: 3 mg/kg).
M-MSN: Magnetic mesoporous silica nanoparticle; MSN: Mesoporous silica nanoparticle.

In addition, the accumulation of drug in three other organs was measured: in the liver and the kidneys, because they are the main excretory organs, and in the lungs, because they are at the end of the respiratory route, after the nostrils. Analysis of these organs gave values under the limit of quantification, so no drug was accumulated in these organs.

Results from the stability study carried out with free ponatinib (Figure 8) showed that the encapsulation of this drug is an excellent idea because, when it is freely dissolved in PBS, plasma or brain homogenate, it disappears in 24 h (37°C).

Discussion

It has been proved that nanoparticles whose size is below 100 nm are able to cross the BBB [29]; this is the case for the particles prepared in this investigation (Figure 1). In both the MSNs and M-MSNs, the real particle size, measured by TEM, was below 100 nm, which might allow the particles to cross the BBB. However, when comparing the sizes obtained by TEM with the data obtained by DLS (Supplementary Figure 2), it was observed that the DLS size was bigger. The differences between TEM and DLS values can be explained because the hydrodynamic particle diameter measured by DLS is bigger than particle diameter measured by TEM because, when a liquid medium

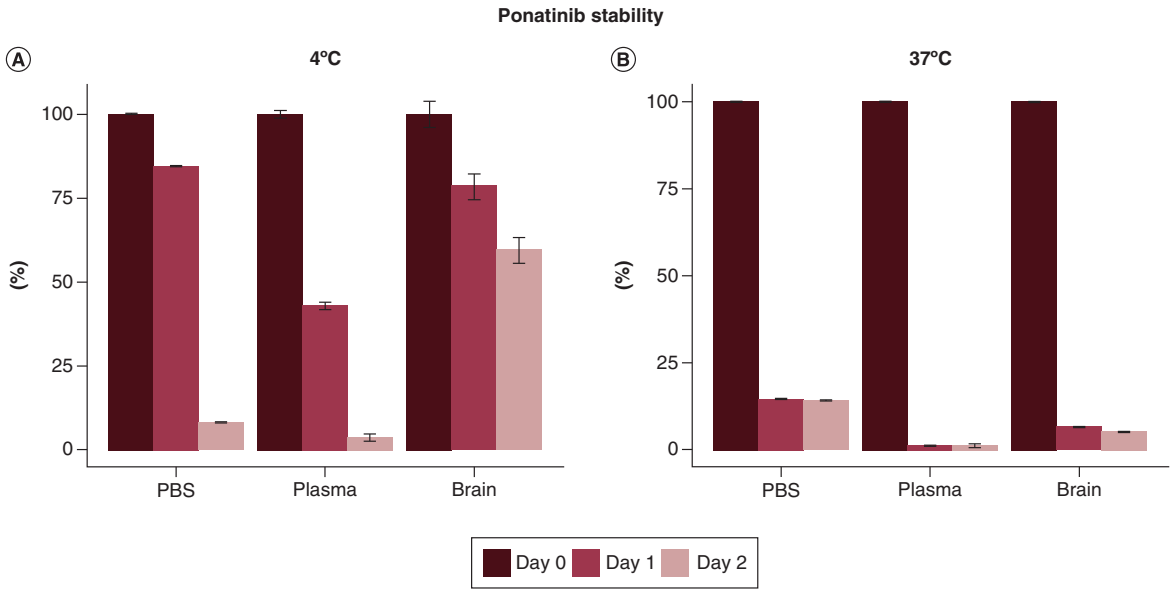

Figure 8. 48 h ponatinib stability in phosphate-buffered saline, plasma and brain homogenate at (A) 4°C and (B) 37°C.

moves a dispersed particle, a dipole electrical layer is attached to the particle's surface that influences the movement of the particle and causes the diameter measured by DLS to be larger.

Not only the size of the particles is important for obtaining a good nanocarrier to deliver drugs to the CNS. That is why other characterization tests were carried out. The XRDs (Figure 2) and the porosimetry analysis (Supplementary Figure 3) proved, on the one hand, the proper structure of the magnetic seeds and the mesoporous materials [34,36] and, on the other hand, how the pores were covered by the borneol and folic acid gating system.

The particles, MSNs and M-MSNs, were able to retain drugs inside them, both trypan blue and ponatinib, and release them only in the presence of lysozymes, the stimuli responsible for opening the molecular gate. For both molecules and particles, the release profile was described with a Weibull kinetics model (Figure 3), which reflects a complex release mechanism [38]. Probably, the diffusion of drug from the particle is happening simultaneously with the destruction of the gate by the lysosomal enzymes.

The cytotoxicity (Figure 4) and efficacy (Figure 5) tests proved that the case and the gate of particles were not toxic for the cells; however, once loaded with ponatinib, they were able to kill cancer cells (U87-MG). Nonetheless, for this toxic effect, it is necessary that the particles reach the CNS and this fact was confirmed with the *in vitro* permeability test.

Figure 6 shows how the influx clearance was greater when ponatinib was administered in particles than when it was administered on its own. The increment in permeability was not observed in the particles loaded with trypan blue. Theoretically, both molecules, trypan blue and ponatinib, have low access to the CNS. In fact, trypan blue was used in the 20th Century when the BBB was discovered, as it was observed that after an intravenous injection of this dye, the brain and spinal cord were not stained [39]. Nonetheless, the toxicity of trypan blue in cell cultures has also been described [40–42], so it is considered that the high permeability rates obtained with both the free trypan blue and the trypan blue loaded in the nanoparticles are due to that toxicity, which may alter the tight junctions in the monolayers. Ponatinib is a substrate of efflux transporters and it has a low $f_{u,\ plasma}$ [10]. In this *in vitro* test, the particles increased the permeability rate of this drug, because they prevented the drug from binding to the efflux transporters and they opened the tight junctions due to the presence of borneol [23]. The TEER measurements, taken during the experiment, proved the activity of borneol, as after an hour, the monolayers in contact with the nanoparticles showed a reduction in their TEER value of 81 ± 22%, while the monolayers that were in contact with the free drug kept their resistance constant.

In Figure 7, it can be observed how the use of the nanoparticles increased both the penetration and the retention of ponatinib in the brain of the rats after a single intranasal administration. Probably, it is because the particles were better adhered to the nasal mucosa than the free drug, which may have been expelled out of the nostrils or moved

down to the respiratory system. Specifically, it can be observed that, after 48 h, the concentration of ponatinib after the administration of MSNs was 8.9-times the concentration of free ponatinib and after the administration of M-MSNs it was 4.1-times the concentration of free ponatinib. One could expect a greater accumulation when the M-MSNs are used, due to the additional force of the magnet, but it is also true that this type of particle was bigger than the MSNs (Figure 1). In fact, in 2020, a study carried out with a 'nose–brain' *in vitro* cell model showed that the cut-off point for silver nanoparticles to have good access to the brain through the nasal epithelium was 60 nm [43] and, according to the TEM images, the MSNs prepared in this thesis were below 60 nm, but the M-MSNs were above this limit. In addition, the presence of the USPIONs in the core of the M-MSNs can promote its aggregation, due to magnetic forces, increasing even more the final size of the particles present in the administered suspension and hindering passage to the CNS.

As shown in Figure 8, the encapsulation of ponatinib is a good idea not only because the particles increase the drug's access to the CNS but also because they protect ponatinib from degradation, which disappears extremely fast at 37°C.

The use of borneol in intranasal administration has also been proved to effectively promote the delivery of ponatinib to the CNS, as it has with other drugs [44–48]. In fact, in 2019 Tang *et al.* prepared nanoparticles with borneol for treating Parkinson's disease and they observed that the use of borneol prevented the particles from being excreted by the mucociliary system, as it increased permeability through the epithelial membrane [47]. In addition, when human nasal epithelial cell monolayers are exposed to borneol, they reduce the tight junctions, therefore their TEER value and the fluidity of their cellular membrane is increased [46].

The previous information paves the way for enhanced delivery of ponatinib to the CNS and thus for the treatment of glioblastoma. It is important to highlight the novelty of this investigation, as, at the moment these lines are written, only 17 articles can be obtained when 'mesoporous silica nanoparticles' and 'glioblastoma' keywords are used. In addition, one of the novelties of this work is the combination in mesoporous silica nanoparticles of borneol, for opening the tight junctions, folic acid for targeting cancer cells and intranasal administration for targeting the brain when crossing the BBB is not possible [26,43]. On the other hand, the most relevant advantage of these systems and the characteristic that was most difficult to obtain was their small size. This fact differentiates these nanocarriers from the other silica nanoparticles whose size is greater than 100 nm, making them incapable of crossing the BBB.

Conclusion

Considering all the results presented previously, it can be concluded that two new mesoporous nanostructures, a magnetic and a nonmagnetic one, which increase the access of ponatinib to the CNS intranasal administration have been developed, characterized and adequately evaluated *in vitro* and *in vivo*. Encapsulation of ponatinib overcomes the stability problem of ponatinib. Moreover, these smart nanoparticles have been functionalized with borneol to be efficient in crossing the BBB and both types of designed nanosystems are able to increase the penetration and the retention of ponatinib in the brains of rats after a single intranasal administration. This kind of nanoparticle is nontoxic and constitutes a promising delivery system in the treatment of glioblastoma, the most lethal brain tumor worldwide and constitutes an interesting platform to administer other drugs to access CNS.

Future studies will be necessary to carried out to confirm cellular uptake and competition binding assays in cell cultures or experiments in animal models with glioblastoma to evaluate the efficacy and specificity of the formulations.

In the last 10 years, the number of articles about nanoparticles has hugely increased. In addition, the prevalence of CNS cancers has also increased. These tendencies make us think that the interest in this theme will continue growing during the next decade. Nonetheless, we think that a proper translation to society will be difficult, as most of the investigations are carried out by public institutions with a lower budget than private enterprises.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2023-0131

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The *in vivo* experiments were approved by the ethical committee of Miguel Hernández University (2021/VSC/PEA/0133 type 2). The authors state that they have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

Summary points

- Glioblastoma is the most common and lethal brain cancer and new treatments to combat it are needed.
- In this work, mesoporous nanoparticles (magnetic and nonmagnetic) loaded with ponatinib for the treatment of glioblastoma were prepared.
- Particles were functionalized with borneol, to open the tight junctions of the blood–brain barrier, and folic acid, to direct the particles toward cancer cells.
- The particles had a proper size, below 100 nm, and porosity and they released the drug only in contact with lysosomal extract, the stimulus to open the gate.
- Both type of particles, when tested *in vitro* in cell cultures, were not toxic for blood–brain barrier cells.
- The particles increased the permeability rate of ponatinib *in vitro*, because they prevented the drug from binding to the efflux transporters and they opened the tight junctions (trans-endothelial electrical resistance values decreased).
- When administered intranasally to healthy rats, both particles increased the amount of drug that reached the brain.
- More studies in animal models with glioblastoma are necessary to evaluate the efficacy and specificity of the formulations.

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