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A Potential Non-Invasive Glioblastoma Treatment: Nose-to-Brain Delivery of Farnesylthiosalicylic Acid Incorporated Hybrid Nanoparticles

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Abstract

New drug delivery systems are highly needed in research and clinical area to effectively treat gliomas by reaching a high antineoplastic drug concentration at the target site without damaging healthy tissues. Intranasal (IN) administration, an alternative route for non-invasive drug delivery to the brain, bypasses the blood-brain-barrier (BBB) and eliminates systemic side effects. This study evaluated the antitumor efficacy of farnesylthiosalicylic acid (FTA) loaded (lipid-cationic) lipid-PEG-PLGA hybrid nanoparticles (HNPs) after IN application in rats. FTA loaded HNPs were prepared, characterized and evaluated for cytotoxicity. Rat glioma 2 (RG2) cells were implanted unilaterally into the right striatum of female Wistar rats. 10 days later, glioma bearing rats received either no treatment, or 5 repeated doses of 500 µM freshly prepared FTA loaded HNPs via IN or intravenous (IV) application. Pre-treatment and post-treatment tumor sizes were determined with MRI. After a treatment period of 5 days, IN applied FTA loaded HNPs achieved a significant decrease of 55.7% in tumor area, equal to IV applied FTA loaded HNPs. Herewith, we showed the potential utility of IN application of FTA loaded HNPs as a non-invasive approach in glioblastoma treatment.

Keywords: Hybrid nanoparticles, glioblastoma, drug delivery, nose-to-brain, farnesylthiosalicylic acid

Importance of the study: Cancer treatment is nowadays based on lengthening the patients’ survival as well as improving the functional outcomes without impairing the patient’s normal daily living. In order to obtain such good prognostic outcomes, any treatment modality including but not limited to surgery, radiotherapy and chemotherapy need patient compliance. Intranasal delivery is a promising and better application route than intravenous drug administration. Therefore, efforts are made to treat or at least improve the survival for “incurable diseases”. This study shows a proof of concept that glioblastoma can be treated with a non-invasive approach by delivering FTA loaded hybrid nanoparticles into the brain via the nose-to-brain route which can improve patient compliance. Until now, no other study has evaluated the delivery of FTA via nanoparticle formulations and via intranasal delivery against glioblastoma. To this
end, the combination of these approaches in this study, can achieve high clinical impact in either single or combination therapies.
Introduction

Malignant brain tumors account for about 2% of deaths caused by cancer with an incidence of 7.19/100000 a year (1). They are aggressive due to their rapid proliferation and infiltration properties and are highly lethal (2). Nowadays, three main methods are used either as single or in combination in cancer treatment including surgery, radiation therapy, and chemotherapy (3). Even if the majority of the tumor tissue is removed with surgery, some tumor cells may remain depending on the infiltrative nature of gliomas which leads to recurrence of the tumor. Besides, radiotherapy and chemotherapy support the surgical procedure, but they are more effective in early stages of glioma treatment. Hence, it is a challenge to bypass the blood-brain barrier (BBB) to provide high doses of chemotherapeutics to brain tissue without causing systemic side effects that can damage healthy cells and lead to death of patient. To this end, nanoparticle-based drug delivery systems have been intensively investigated for diagnosis, research, imaging and treatment of brain tumors (4-6). Several studies have reported that using nanoparticles in the nose-to-brain route increased drug migration to the brain (7-10). Intranasal (IN) administration is explored as an alternative method for non-invasive drug delivery to the brain. Following the administration to the nasal cavity, it is reported that pharmacological agents are transmitted to the brain via the olfactory and trigeminal nerves (11-14). And although the exact mechanism of nose-to-brain transport is not clarified yet, the dose of the drug absorbed by the nose-to-brain route is dependent on the contact time with the mucus, the rate of the mucus clearance, and with the solubility and metabolic stability of the drug in the mucus (15).

One of the new generation of antineoplastic drugs is S-trans, trans-farnesylthiosalicylic acid (Salirasib, FTA). FTA is a highly specific inhibitor of Ras proteins which are found in most malignant tumors. FTA is a targeted therapeutic agent for MAPK signal transduction pathway, recently, in vitro cell culture studies have shown that FTA is effective against glioma (16). In addition, the in vitro effect of FTA on glioblastoma (17, 18), in vivo effect of FTA via convection-enhanced delivery method on brain tumors (19) and in vivo study of oral delivery of FTA for neurofibromatosis type 1 (20) as well as several clinical
trials with oral pharmacokinetic and pharmacodynamic studies of FTA on other types of tumors (21-24) have been investigated. Altogether, it is concluded that FTA might be considered as a potential anti-cancer drug to block invasiveness, survival, and angiogenesis in glioblastoma. FTA is further known with its poor bioavailability and its limited ability to cross the BBB in an effective concentration (25). To increase bioavailability, nanomicellar formulations of PEG-FTA conjugates have been investigated for other cancer types (26-28).

Despite many studies investigating FTA as a potential anti-cancer drug, there is not any study that investigated FTA loaded nanoparticle formulations in the treatment of glioblastoma. In this study, we modified a previous hybrid nanoparticle formulation (29), consisting of poly lactic-glycolide acid (PLGA) as core polymer, lecithin, amphiphilic 1,2-distearoyl-sn-Glycéro-3-phosphoethanolamine-N-[carboxy poly(ethylene glycol)] (DSPE-PEG) and charged (mono-cationic) lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), in order to obtain hybrid nanoparticles (HNPs) specially designed (increased PEG content) for IN delivery to efficiently penetrate through the nasal mucus and olfactory bulb into brain tissue and provide sustained and/or controlled drug release at the tumor site. In vitro characterization studies, cytotoxicity studies and anti-cancer evaluation of the formulations in RG2-tumor bearing rats were performed and the prepared formulations were either applied IN or intravenous (IV) to rats for which the subsequent changes in tumor volume were analyzed by magnetic resonance imaging (MRI) and histopathological evaluation (Fig. 1). Finally, a biodistribution study of the formulations was investigated in healthy rats. We hypothesized that the non-invasive nose-to-brain delivery of our formulation would bypass the BBB and be as effective as IV delivery of the same formulation against glioblastoma. Our results showed that IN applied, modified FTA loaded [lipid-DOTAP-PEG-PLGA], HNPs (Fig. 2A), were able to accumulate in the olfactory bulb and the brain and showed a significant decrease of tumor size in glioblastoma.
Fig. 1. Graphical representation of investigating the effect of FTA loaded HNP on glioblastoma after IV and IN application in rats.

Materials and Methods

Materials

FTA was purchased from Concordia Pharmaceuticals Inc. (Ft. Lauderdale, FL) and 50:50 PLGA (Resomer RG503) from Boehringer Ingelheim (Ingelheim am Rhein, Germany). DSPE-PEG (2000), lecithin and DOTAP were provided by Avanti Polar Lipids Inc. (Alabaster, AL). Acetic acid was purchased from Merck (Germany). Acetonitrile, methanol and trypan blue were purchased from Sigma Aldrich (USA). PBS, Trypsin-EDTA, L-Glutamin, DMEM/Ham’s F12 (1:1), fetal bovine serum (FBS) and penicillin G sodium-streptomycin sulfate solution, were provided by Biochrom (Berlin, Germany). Rat glioma 2 (RG2) cell lines were purchased from the American Type Culture Collection (Manassas, VA) and L929 cell line (mouse fibroblasts) was purchased from (ATCC, Manassas, VA). Sodium 5-[2,4-disulfophenyl]-2-[4-iodophenyl]-3-[4-nitrophenyl]-2H-tetrazolium inner salt (WST-1) was purchased
from Clontech (Clontech Laboratories Inc., Mountain View, CA, USA). All other chemicals were HPLC grade or research grade.

FTA Assay
In order to obtain reference values, FTA concentrations were determined by high-performance liquid chromatography (HPLC), Agilent HPLC system (Agilent 1100, USA) equipped with UV detector (322 nm) and a column (Waters Spherisorb S10 ODS2 C18 200×4.6 mm). The mobile phase consisted of acetonitrile:water:acetic acid solution (90:10:0.1, v/v) and was used at a rate of 1 mL/min at room temperature (RT). With an injection volume of 100 µL through the HPLC column the peak area was used to determine the FTA concentration.

Formulation of HNPs
HNPs were prepared using the emulsion sonication method (29, 49). Briefly, for one batch to obtain the organic phase, 400 µL of PLGA (2.5 mg/mL dissolved in acetonitrile) and FTA (5% w/w of polymer amount), were mixed. Then, 1 mg/mL of Lecithin, DOTAP and DSPE-PEG were separately dissolved in 4% (v/v) ethanol. A specified amount of lecithin, DOTAP and DSPE-PEG (respectively 1.25:5:30 % w/w of PLGA) was mixed with deionized water to obtain the water phase (organic:water phase ratio, 1:10). The organic phase was dropwisely added to the water phase under continues stirring and then sonicated in a capped glass vial for 5 min using a bath sonicator (Branson B 220 Smith Kline, USA, 42 kHz, 100 W). Subsequently, the nanoparticles were centrifuged in filter-centrifuge tubes (Millipore, 10 kDa), then washed three times with PBS (pH 7.4) and kept in 1 mL PBS solution. The obtained HNPs (consisting of 30% PEG w/w of polymer amount) were freshly prepared for each study.

Characterization of HNPs
Malvern Zetasizer Nano Series ZS device (Malvern Instruments Ltd., Worcestershire, United Kingdom) was used to measure zeta potential, polydispersity-index PDI and size of three batches of freshly prepared HNPs. More detailed size distribution of one batch of freshly prepared empty or FTA loaded HNPs (100x
or 1000x diluted in PBS) were also analyzed by Nano-tracking analysis (NTA, Nanosight LM20, Amesbury, UK).

In order to determine the exact entrapped FTA amount (µg), encapsulation efficiency (EE) and loading capacity (LC), three batches of FTA loaded HNPs were frozen at -80°C and then lyophilized (Heto PowerDry PL 3000, Denmark) for 24 hours. Acetonitrile was added into precisely weighed lyophilized nanoparticles and the suspension was subsequently filtered through a 0.2 µm membrane filter before injection into a HPLC column. The calculations for EE and LC are as follows; EE(%)=[(Entrapped Drug per batch)/Drug added per batch] x 100% and LC(%)=[Entrapped drug per batch/Nanoparticle weight per batch] x 100%.

Surface Morphology
Lyophilized empty and FTA loaded HNPs were evaluated by scanning electron microscopy (SEM) (Nova™ NanoSEM 430, FEI, Muntinlupa City, Philippines, USA). Samples were first fixed on metal plates by two-sided adhesive tape and then coated with 100 Å thick gold in the brand coating device (Bio-Rad Laboratories Inc., Hercules, CA, USA).

In vitro Release of FTA
In vitro FTA release was investigated with a dialysis bag method as described in the literature (50). Briefly, nanoparticles (n=3) were dispersed in 0.1% Tween 20 containing PBS (TPBS) and were placed inside a dialysis membrane (pore diameter 2000 Da) which was fitted inside a glass tube containing 3 mL release media (TPBS). The glass tubes were fully immersed in a stirring water bath at 100 rpm (Heidolph, Germany) and kept at 37°C. FTA release was measured over time until no signal was detected on the HPLC.

Cytotoxicity Measurement
In vitro bioactivity evaluation of the prepared nanoparticles was performed on RG2 cell line, which is a good representative model for glioblastoma (54). In order to determine the cytotoxicity of the formulations on healthy cells, L929 cell line was used. Complete medium (CM) was prepared by adding FBS (10%
v/v), penicillin-streptomycin (50 U/mL–50 μg/mL), and L-glutamine (0.002 mM/mL) to DMEM F-12 HAM (for RG2) or High Glucose DMEM (for L929). Cell growth was carried out at 37°C and 5% CO₂. Respectively, 100 μL cell suspension containing 5×10³ RG2 or L929 cells, in the growth phase, were introduced into each well of a transparent flat-bottomed 96 well plate and kept overnight in the incubator. In a previous study (29,53), the LD50 value of FTA incubated for 5 days on RG2 cell line was determined to be 100 μM. For this reason, after overnight incubation, cells were treated with 100 μM free or HNP loaded FTA formulations (dispersed in 0.1% DMSO in CM) for 24 hours. Then, WST-1 was added (1:10) to the wells and the 96-well plate was placed in the incubator (37°C, 5% CO₂) for 2 hours. Finally, the absorbance was measured at 450 nm with a micro-plate reader (ASYS-UVM 304, Austria).

**In Vivo Glioblastoma Induction**

**Animals**

The protocol for in vivo glioblastoma formation was approved by Hacettepe University Animal Experiments Local Ethics Committee (case number:2014/41-3). For the single dose treatment, 54 female Wistar rats (250–300 g) and for the repetitive treatments, 22 female Wistar rats (250–300 g) were housed in a standard animal facility. Controlled temperature and humidity with a regular lighting schedule of 12 hours light were provided. All experiments were carried out in accordance with the institutional guidelines.

**Cyclosporine Treatment**

Once daily treatment of 1 mL (1 mg/mL) cyclosporine in 0.9% NaCL solution, was applied intraperitoneally to all rats for 5 days before tumor cell implantation. This treatment was necessary in order to weaken the immune system to induce tumor development after tumor cell implantation.

**RG2 Cell Implantation**

In order to develop brain tumors in rats, modified methods of Yemisci et al. (51) and Geletneky et al. (52) were used. Briefly, rats were intraperitoneally anesthetized using a mixture of 0.75–1.5 mL/kg ketamin (100 mg/mL) and xylazin (100 mg/mL) with 1:2 ratio, respectively. The body temperature of the animals
was continuously monitored by a rectal probe and maintained at 37.0±1°C with a thermal blanket. The rats were placed in a stereotaxic instrument (Lab Standard Stereotaxic, Stoelting, Chicago, IL, USA). Their scalp was shaved and a burr hole was opened at the level of the bregma in order to inject a 5 µL cell suspension containing 5\times10^5 RG2 cells with a Hamilton glass syringe (Hamilton 32G, Bonaduz, GR, Switzerland) unilaterally into the right striatum with the following coordinates: anteroposterior -0.5 mm, lateral +3.0 mm, and ventral -6 mm. After implantation of RG2 tumor cells, rats were observed for their daily activities and general well-being. Animals who were not able to feed, with no interest to their environment or external stimuli, and or body weight loss of 20% were sacrificed.

**MRI**

MRI was performed with 3 Tesla MR unit (Magnetom Trio, Siemens Healthcare, Erlangen, Germany; slew rate, 200 mT/m/ms; maximum amplitude, 45 mT/m) in the National Magnetic Resonance Research Center (UMRAM). MRI was performed in order to determine the presence and size of the tumor which was inducted in the rat brain. For this purpose, a handmade coil was developed by UMRAM and placed on a semi-cylinder body to support the rat body. 10 days after tumor cell implantation, rats were anesthetized and the pre-treatment tumor size was determined with MRI. 5 days after single dose treatment or next day after 5 days repeated treatments, rats were re-anesthetized and the post-treatment tumor size was determined with MRI. T2-weighted turbo spin-echo imaging was obtained parallel to the long-axis of the rat in prone position (coronal plane) using the following parameters: repetition time /echo time: 4420/94; field of view: 78×78 mm; slice thickness: 2 mm; interslice gap: 0; number of excitations: 5.

**Single Dose Treatment**

After determining pre-treatment tumor size with MRI scans, rats were divided into groups with no treatment (n=8), IV FTA (n=7), IV empty HNPs (n=11), IV FTA loaded HNPs (n=7), IN FTA (n=7), IN empty HNPs (n=7) and IN FTA loaded HNPs (n=7). The average pre-treatment brain tumor size between each group was kept as equal as possible. The used volume of drug dispersion (prepared in TPBS) for both IV and IN application was 20 µL. Formulations were sterilized 30 minutes under UV light before
administration to the rats (52). The drug solution or formulation dispersion was injected into the tail vein or was pipetted into the nasal cavity of the right nostril. The pipette tip was marked for 5 mm to ensure that the drug was delivered into the nasal cavity. For nasal application, 20 µL was applied over a period of 2 minutes while the rat was on supine position. Rats were kept for 30 minutes in supine position after treatment application, and not disturbed in the following 5 days.

Repetitive Dose Treatments
After obtaining results with single dose treatment, IV and IN FTA loaded HNPs have been selected for multiple dosing treatment. For this purpose, brain tumor induced rats were divided into 3 groups; no-treatment (control, n=6), IV FTA loaded HNPs (n=8) and IN FTA loaded HNPs (n=8) and same treatment application procedures were followed as described above. The repeated treatments (5 doses in total) were applied once-daily (as described above) for 5 days. Besides the treatment and control group which were sacrificed (n=22) at day 16 after tumor injection, another group of brain tumor bearing rats (n=14) were observed for survival rate only. Endpoint of survival was based on either death or 20% loss of body weight (sacrificed with overdose anesthesia).

H&E Staining
In order to perform histopathological analysis, rats were sacrificed after the second MRI study, and their brains were removed and post-fixed in 10% formalin. Respectively, the brains were cut in 2 mm thick slices in the coronal plane and embedded in paraffin. Serial 6 µm thick sections were obtained and stained with hematoxylin and eosin to visualize with microscopy. The largest transverse and longitudinal diameters (product of the two is the tumor area in mm²) of the tumors were measured on the slices at 5x magnification. Images of the sections were obtained at 5x, 20x, 40x or 100x magnification with an upright light microscope (LEICA, DM 3000 LED, Wetzlar, Germany).

Disposition of FTA
The distribution of FTA throughout the rat body was investigated by using liquid chromatography/tandem mass spectrometry (LC/MS/MS) method. Blood samples were collected from healthy 30 female Wistar
rats (250–300 g) (approved by Koç University Animal Experiments Local Ethics Committee with case number:2016.HADYEK.027), which received either no treatment (n=1), IV FTA (n=6), IV FTA loaded HNPs (n=9), IN FTA (n=5) or IN FTA loaded HNPs (n=9) with a FTA dose of 500 µM within an administration volume of 20 µL per animal. Same IV and IN administration procedures were maintained as described earlier in this study. Blood sampling time points were 1, 4, 24, 72, and 120 hours. Blood plasma was extracted and subsequently precipitated with 1:3 ratio plasma:acetonitrile, vortexed for 30 seconds and further centrifuged for 5 min at 10 000 RPM at 4 °C. Then, the subsequent supernatant was taken and placed in a chromatography vial for LC/MS/MS.

From each treatment group, rats were euthanized with an overdose of anesthesia after 4, 24 and 120 hours of drug administration. Subsequently, non-perfused brain, olfactory bulb, liver and spleen were harvested and a specified (50 mg for olfactory bulb, 500 mg for other organs) amount of each organ was added to PBS and subsequently oscillated for 1 min at 30 Hz with the use of a magnetic bead in a tissue lyser device (Qiagen). After precipitation of residue:acetonitrile with 1:3 ratio, it was vortexed for 30 seconds and centrifuged for 5 min at 10 000 RPM and 4 °C. The supernatant was carefully taken and frozen at -80 °C (freezing point acetonitrile is -45 °C) and subsequently lyophilized for 24 hours. The obtained powder was dissolved in acetonitrile and vortexed for 30 seconds before centrifugation for 5 min at 10 000 RPM at 4 °C. The supernatant was carefully taken and placed in a chromatography vial for LC/MS/MS.

LC/MS/MS analysis was performed on an Agilent 1260 Series HPLC system equipped with an Agilent 6460 Triple Quadrupole LC-MS system (Agilent Technologies Inc., CA, USA). Separation was performed on a reversed phase Poroshell 120-EC-C18 column (3.0 mm x 50 mm x 2.7 mm). Obtained data was processed by Agilent MassHunter software. (Detailed description of the LC/MS/MS setup is provided in Table S3.

Statistical Analysis

In vitro cytotoxicity data was analyzed by unpaired t-test (two tailed) and in vivo glioblastoma treatment data were analyzed by Mann Whitney Test (one tailed) with Prism 5.0 (GraphPad Software). For the
Kaplan Meyer curve, a Log rank test was performed. Values of \( p < 0.05 \) were considered statistically significant. MRI obtained tumor sizes away from \( \text{mean} \pm 2 \text{SD} \) per group were considered outliers and were excluded from the whole animal study.

**Results**

**Characterization of HNPs**

FTA loaded HNPs yielded a size of \( 164.3 \pm 10.3 \) nm, PDI of \( 0.192 \pm 0.06 \) and zeta-potential of \( -12.0 \pm 1.3 \) mV (mean±SD, for \( n=3 \) batches) and were larger in size compared to empty HNPs with a size of \( 111.1 \pm 4.7 \) nm, PDI of \( 0.146 \pm 0.02 \), and zeta potential of \( -11.2 \pm 1.4 \) mV (mean±SD, for \( n=3 \) batches) (Table 1). PDI results showed a homogenous distribution for both formulations. Next to freshly prepared HNPs, nanoparticle formulations were also lyophilized and analyzed by SEM which yielded particle sizes in micrometer range and showed a large and spherical shape for these particles (Fig. 2B).

**Table 1.** Size, PDI and zeta potential values for both empty and FTA loaded HNP formulations as well as the exact amount of FTA, EE and LC per batch are listed. Data is shown as mean±SD for \( n=3 \).

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Charge (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTA loaded HNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>175.5±0.5</td>
<td>0.114±0.011</td>
<td>-12.7±1.7</td>
</tr>
<tr>
<td>2</td>
<td>163.9±4.0</td>
<td>0.222±0.031</td>
<td>-11.2±0.7</td>
</tr>
<tr>
<td>3</td>
<td>153.6±6.9</td>
<td>0.239±0.004</td>
<td>-12.0±1.1</td>
</tr>
<tr>
<td>Empty HNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>106.6±2.4</td>
<td>0.150±0.004</td>
<td>-10.1±0.3</td>
</tr>
<tr>
<td>2</td>
<td>116.4±1.0</td>
<td>0.141±0.016</td>
<td>-10.6±0.8</td>
</tr>
<tr>
<td>3</td>
<td>110.2±3.1</td>
<td>0.148±0.026</td>
<td>-12.8±0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Batch</th>
<th>FTA (µg)</th>
<th>EE (%)</th>
<th>LC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTA loaded HNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>49.3±0.1</td>
<td>98.6±0.3</td>
<td>3.5±0.01</td>
</tr>
<tr>
<td>2</td>
<td>47.8±0.8</td>
<td>95.5±1.5</td>
<td>3.4±0.05</td>
</tr>
<tr>
<td>3</td>
<td>49.5±0.7</td>
<td>99.0±1.5</td>
<td>3.5±0.05</td>
</tr>
</tbody>
</table>
Also, NTA was used to measure the size distribution of our freshly prepared formulations. NTA showed a homogeneous size distribution with peaks at 163 nm and 130 nm for FTA loaded HNPs and empty HNPs respectively (Fig. 2C). Video frames of empty and FTA loaded HNPs are provided in Video SI & S2.

Furthermore, with a retention time of approximately 9.5 minutes in HPLC runs, FTA had a linear curve for FTA concentrations between 0.097-25 µg/mL (Fig. 3A). This data was used to determine both the encapsulation efficiency of FTA in HNPs and FTA release from HNPs in vitro. The encapsulation efficiency of FTA in HNPs was 97.7%±2.0 (mean±SD, for n=3 batches) and the loading capacity of FTA in the nanoparticle was 3.5%±0.1 (mean±SD, for n=3 batches) (Table 1). Next, these formulations were analyzed for FTA release by using the dialysis bag method which showed that freshly prepared FTA loaded HNPs caused a release of 45.8%±8.3 (mean±SD, for n=3) of drug within 12 hours and a total FTA release for even longer than 120 hours (Fig. 3B).
Fig. 2. HNP characterizations. A) Schematic drawing of the composition of the FTA loaded HNP (size ~160 nm). B) SEM images of lyophilized empty and FTA loaded HNPs. C) Characterization of empty and FTA loaded HNP with NTA. Left panel represents a NTA video snapshot, middle panel represents the size distribution and the right panel represents a 3D scatterplot of nanoparticle size (horizontal) vs. light scattering intensity (height) vs. concentration (vertical).
Fig. 3. FTA assay and the release and toxicity characteristics of the HNPs. A) FTA concentration assay analyzed by HPLC. B) Cumulative release study of FTA loaded HNPs over time with the use of the dialysis bag method (n=3). C) Cytotoxicity of control (complete medium+0.1% DMSO), FTA, empty HNP and FTA loaded HNP on RG2 cells. D) Cytotoxicity of control (complete medium+0.1% DMSO), FTA, and FTA loaded HNP on L929 cells. For both C & D, cell viability is shown as a measurement of surviving percentage after treatment of 24 hours with formulations. Results are shown as mean±SD for n=6 (except for Control (n=5) and FTA (n=11) in Figure F). Significance between groups was analyzed with unpaired t-test (two tailed). *: P<0.05, **: P<0.01, ***: P<0.001.

Cytotoxicity of HNPs

The IC50 value of FTA in a previous study was based on 5 days incubation of free FTA on RG2 cells. In our study we incubated FTA and FTA loaded HNPs on RG2 cells for 24 hours. With our formulation it
was not possible to incubate free FTA and FTA loaded HNPs for 5 days to compare for toxicity due to rapid toxicity of our FTA loaded HNP formulation on RG2 cells. Therefore, free FTA is not at its IC50 point yet after 24 hours (Fig. 3C). Compared to the control group (complete medium+0.1% DMSO), FTA and FTA loaded HNPs showed a significant cytotoxic effect on cell viability of RG2 cells after 24 hours (Fig. 3C). FTA loaded HNPs significantly decreased tumor cell viability with approximately 60% (p<0.001), where the same concentration of FTA only caused a decrease of approximately 13% (p<0.05). Empty HNPs on the other hand, did not show a significant cytotoxicity on RG2 cells and were considered as non-toxic.

To investigate whether free FTA and FTA loaded HNPs are toxic for healthy cells, L929 cells were incubated with the formulations for 24 hours and subsequently the cytotoxicity was analyzed. Compared to control group (CM+0.1% DMSO), FTA showed a significant toxic effect (p<0.01) whereas FTA loaded HNPs did not show a significant toxicity (Fig. 3D).

**Antitumor Efficacy of HNPs after Single Dose**

During the single dose experiment, rats with additional conditions (hematoma, hydrocephalus and abscess) observed by MRI were directly sacrificed and excluded from the whole experiment. Also, due to the malignant symptoms or large size of the brain tumor, a number of rats died before data was collected. These animals were also excluded from the whole experiment. The drop-outs are listed in Supplementary Table 4. These drop-outs in the experimental groups caused a variation in the pre-treatment tumor volumes which were kept homogenous before the treatment started.

On MRI scans it was noted that when brain tumor induced rats received IV FTA loaded HNPs, the tumor area was decreased after 5 days (Fig. 4). The same pattern was seen in the case of IN FTA loaded HNPs. From pre-treatment and post-treatment MRI scanning images of rats, the tumor area size (mm²) was determined by multiplying the transverse and longitudinal diameters of the tumor (Fig. 4 & 5A).
**Fig. 4.** Representative MRI images of rat brains initial/before and follow up/after treatment with different formulations. Corresponding coronal brain sections, taken from rats which were decapitalized after post-treatment MRI, are stained with H&E. Dark blue areas in these coronal sections show the tumor in the brain.
The tumor area in the non-treated group was around 25 mm$^2$ after 15 days (10 days tumor development and 5 days ‘treatment’ period) (Fig. 5A). When anti-cancer treatment with FTA loaded HNPs was applied to tumor bearing rats, the tumor area decreased. Data showed that post-treatment tumor area was decreased compared to pre-treatment tumor area by 57.3% and 31.0% for a single dose of IV FTA loaded HNPs and IN FTA loaded HNPs, respectively.

In order to make a statement about the significance of the fold changes in tumor growth or tumor reduction, the Log power of 2 of the ratio post/pre tumor areas for each rat were taken and a box-and-whisker plot was generated for each group. When the application of IV and IN FTA loaded HNP groups were compared to non-treated rats, the fold change in the decrease of the tumor area was significant ($p<0.05$) for both groups (Fig. 5B). On the other hand, IV and IN application of free FTA and empty HNPs had no significant anti-cancer effect in vivo compared to non-treated group (Fig. 5B). Within the IN applied series, FTA loaded HNPs had a significant anti-cancer effect compared to FTA and empty HNPs which was in line with our in vitro cytotoxicity results on RG2 cells.

**Antitumor Efficacy of HNPs after Repetitive Treatments**

After evaluating the MRI images (Fig. 6A & Supplementary Figures S1, S2 & S3) of non-treated, IV FTA loaded HNP and IN FTA loaded HNP rats which were treated for multiple doses, it was shown that in both treatment groups of FTA loaded HNPs the tumor area decreased equally with 55% (Fig. 6B), whereas the non-treated group showed an increase of 43% in tumor area. The fold changes in tumor area for both FTA loaded HNP treated groups were significant ($p<0.01$) compared to the no-treatment group (Fig. 6C). Furthermore, there was no significant difference in the fold changes of tumor area between IV FTA loaded HNP and IN FTA loaded HNP groups. In both IV and IN FTA loaded HNPs treatment groups and no-treatment group, 2 rats in each group died due to tumor progression (see Supplementary Table 4). Since the follow up MRI was missing for these animals, these were excluded from all data except for the Kaplan Meyer curve. Until the end of the study, no other drop-outs have occurred. Moreover, from brain tumor bearing rats which were not treated but analyzed for survival, 5 out of 14 died from which 2 were
sacrificed due to 20% loss of body weight. The no-treatment group (n=6 included in data & n=2 drop-outs) and survival observation group (n=14) were combined for the Kaplan Meyer analysis for the duration of the experiment and is shown in a Kaplan Meyer curve in Fig. 6D.

**Fig. 5. A&B** Effect of HNP formulations on tumor size. A) In vivo pre-treatment and post-treatment tumor sizes for each group. Data is shown as mean±SEM for no treatment (n=8), IV FTA (n=7), IV empty HNP (n=11), IV FTA loaded HNP (n=7), IN FTA (n=7), IN empty HNP (n=7) and IN FTA loaded HNP (n=7). B) Boxplot (Whiskers: min. to max.) of fold changes in tumor size with [Log 2(post-treatment tumor area/pre-treatment tumor area)]. Significance between groups was analyzed by Mann Whitney Test (one tailed). *: p<0.05. **C,D&E** Pathological brain analysis after treatment. C) Two post-ex rat brains with upper image showing tumor present on the right hemisphere and lower image showing injection site. D) Correlation of post-pathological tumor size versus post-MRI tumor size is presented in a scatterplot. The moderate correlation between the two groups is shown by the R value. E) Coronal plane slices of rat
brains stained with H&E and visualized with light microscopy. Upper panel shows tumor area in the right striatum whereas the lower panel shows cellular organization of tumor cells after for either no treatment or treatment with IV FTA loaded HNPs or IN FTA loaded HNPs from left to right, respectively. Left panels show abundant tumor cells with tight organizations, whereas middle and right panel shows disturbed tumor cells structure and organization with the presence of many inflammation cells.
**Fig. 6.** A) Initial/pre-treatment and follow up/post-treatment MRI images of rat brains from non-treated or after repetitive treatments with IV or IN FTA loaded HNP formulations and their corresponding coronal brain sections stained with H&E are shown. In the coronal brain sections, the upper panels show a dense tumor area in the right striatum of non-treated rats whereas the middle and lower panels show cellular re-organization of tumor cells after treatment with IV FTA loaded HNPs or IN FTA loaded HNPs, respectively. Presence of inflammatory response is shown by the abundant presence of histiocytes and lymphocytes. B&C Effect of repeated treatments with FTA loaded HNP formulations on tumor size. B) In vivo pre-treatment and post-treatment tumor sizes for each group. Data is shown as mean±SEM for no treatment (n=6), IV FTA loaded HNP (n=8), and IN FTA loaded HNP (n=8). C) Boxplot (Whiskers: min. to max.) of fold changes in tumor size with \( \log_2(\text{post-treatment tumor area/pre-treatment tumor area}) \). Significance between groups was analyzed by Mann Whitney Test (one tailed). \(*⁺: p<0.01. \) D) Kaplan Meyer curve for the survival rates of the animals used in the repetitive treatment study. At day 16, both treatment groups (n=8 for each group) and tumor control group (n=6) were sacrificed for histopathological evaluation. These numbers are censored. Animals, still alive at day 35 are also censored. Group sizes for the Kaplan Meyer curve are: No-treatment (n=22.), IV FTA loaded HNP (n=10) and IN FTA loaded HNP (n=10). Log rank test: \( p<1 \), no significance.

**Histopathological Analysis**

Rats were sacrificed after post-treatment MRI and subsequently fixed in formalin. Several isolated brains were clearly showing tumors on the surface of the brain while other brains showed only the injection site (Fig. 5C&E & 6A). From histopathological results, it was observed that tumor eradicated brains were remarkable with a small lesion at the initial tumor site. Brain slices on the coronal plane were visualized and post-pathological tumor areas were obtained. These post-pathological tumor areas were compared to tumor area values obtained after post-treatment MRI (Fig. 5D). A moderate correlation was found between both groups (R value of 0.39).
When pathological analysis was performed on the stained brain slices, different cellular organizations were found for both IV and IN FTA loaded HNP applied groups compared to the non-treated group (Fig. 5E & 6A). Namely, either a lesion, increased necrosis, inflammation, increased (reactive) histiocytes, non-neoplasia cells, reactive glial cells, presence of (perivascular) lymphocytes or combination of them were found in these treated groups.

**Distribution of FTA**

FTA distribution was investigated in plasma and non-perfused brain, olfactory bulb, liver and spleen tissues of healthy rats. According to the data shown in Fig. 7A & B (For kinetics data see Table S1 & S2), FTA plasma concentration was the highest for IV FTA loaded HNPs with $40.4\pm20.7\ %\text{ID}$ (mean±SEM, n=9) and lowest for IN FTA with $0.13\pm0.02\ %\text{ID}$ (mean±SEM, n=6) after 1 hour of administration. Both Fig. 7A & B, showed a rapid distribution of IV and IN administered free FTA within the first 4 hours whereas IV and IN FTA loaded HNPs remained present in plasma for at least 24 hours, and even for 120 hours with concentrations very close to the detection limit of the analyzing method. Moreover, the half-life for IV and IN FTA loaded HNPs was increased with 5- and 6-folds respectively compared to IV administered free FTA.

From organ distribution, it was clearly remarkable that all formulations were present in the brain after 4 hours, however, the highest accumulation was observed for IN and IV FTA loaded HNPs and these even remained present till 120 hours after application. Moreover, IN FTA loaded HNPs were able to accumulate in a higher degree in the olfactory bulb, which shows that FTA was delivered to the brain via the olfactory bulb. Within all formulations, only IV FTA loaded HNP caused a high accumulation in the liver and spleen.

**Discussion**

Despite recent advances in the multiple treatment approaches, mean survival time of malignant glioma patients varies between 3 to 16 months. Therefore, new brain targeted strategies have become a need in
the clinical and scientific field of neuro-oncology. The main challenge here is to cross or bypass the BBB which prevents many drugs from reaching the brain. If nanoparticles are designed with targeting agents or injected into the desired regions, they can provide continuous and high amount of drugs in the targeted tissues. Whereas, they must be designed in a special way to increase both their uptake and transport of drugs across the BBB. This situation led to intensive research on these systems in the last few years especially for the treatment of gliomas. One of the approaches used in recent studies is to design nanoparticles with functionalized lipid-PEG cationic lipid systems in other words, preparation of hybrid formulations.

**Fig. 7.** Biodistribution study of the formulations in healthy rats. A) Plasma FTA concentration versus time profile is represented for the treatment formulations. Data is shown a mean±SEM, or as single point for n<2. B) The distribution of FTA in the brain, olfactory bulb, liver and spleen of healthy rats after 4, 24 and 120 hour of formulation administration. Data is shown as mean±SEM. Data with n<2 are shown without error bars.
While PLGA was preferred in our nanoparticle formulation due to its long acting controlled release (30), DOTAP was used in this study because of its ability to increase cellular uptake in the brain (31). PEG on the other hand, is a useful hydrophilic polymer to coat nanoparticles or to co-polymerize (PEGylation) the used polymer in order to achieve a longer retention time of nanoparticles in the bloodstream (32-35). PEGylation of nanoparticles causes not only extension in the retention time, it also provides increase in cellular uptake (36, 37). In addition, PEG provides cationic charge to the nanoparticle surface thereby increases the diffusion of the nanoparticle through the mucus and subsequently increases its transition into the brain (7, 38-42). With regard to the ability of PEG that can enhance drug transport from nose to brain, the preparation of our nanoparticle formulation with 30% PEG/polymer mass ratio was intended to increase mucosal absorption.

We prepared and characterized our HNP formulations which showed particle size<200 nm and a homogenous distribution. However, SEM revealed a large size for the HNPs after lyophilization. Presumably, the high lipid content in these formulations may have caused clotting during lyophilization and therefore may have increased the size. NTA measurements on the other hand, showed that our freshly prepared formulations were homogenously distributed with particle size <200 nm. Lyophilized formulations were not used due to their extreme large size and therefore freshly prepared HNPs were used in further experiments. The release study showed first a burst release but then stabilized to release FTA till at least 5 days. This latter is very important in avoiding recurrent tumor formation during treatments.

After evaluating the toxicity of the HNPs on RG2 and L929 cells, it was shown that FTA loaded HNPs were able to significantly kill tumor cells, whereas no toxicity was observed on healthy cells. This result is in line with literature (56), demonstrating that although Ras/MAPK pathway is active in normal cells, MAPK pathway is highly upregulated in glioblastoma cells. FTA is a Ras antagonist, and Ras is abundantly present on tumor cells for example RG2 cells. That is why a special pharmacological inhibitor of this pathway at the higher point of the cascade blocks the overactivation while it has limited side effects in normal systems. Therefore, healthy cells are less affected by the FTA. Even more, we showed that free
FTA was slightly toxic on healthy cells compared to FTA loaded HNPs, which indicates that the HNP formulation decreases toxicity of the anti-cancer drug on healthy cells, while it mediates killing tumor cells. The latter might be due to more uptake of FTA loaded HNPs by tumor cells than FTA only. HNPs can therefore be considered as a potential cell entry vehicle for FTA. From both data (toxicity on RG2 cells and L929 cells), we can assume that our HNPs are potential shuttles for delivery of FTA into brain tumor cells by simultaneously decreasing toxicity of the anti-cancer drug on healthy cells.

After characterization and evaluation of our formulations in vitro, tumor cells were seeded in rat brains in order to evaluate the antitumor efficacy of a single dose treatment with FTA, empty HNP and FTA loaded HNPs after IV or IN administration. IV application was performed in order to compare the effectiveness of IN application. From the IN applied series, it is clear that administering anti-cancer drug FTA is not sufficient to treat glioblastoma by itself alone. On the other hand, a system where FTA is incorporated in HNPs has the potential in reducing tumor area in the brain. Furthermore, when IV route was compared to IN route it was obvious that both groups had a significant anti-cancer effect in vivo by decreasing the tumor area and possibly inhibiting tumor cell growth. Although, IV application decreased tumor area in a higher degree than IN application, it is expected that IN route might have potential as IV route due to its low plasma levels and for that reason might be a better option in anti-cancer treatment where non-invasive methods with less systemic side-effects are desired. One reason for the less effect of IN FTA loaded HNP application compared to IV application is the possibility of systemic drug loss during application. For this, a controlled infusion pump might be of great value. In addition, the single dose treatment may not be sufficient for the IN pathway and may need repeated doses to show a higher effectiveness of the route. Therefore, a repetitive treatment study was performed to show the efficacy of the nasal route. The obtained results showed that the administration of multiple doses with the nasal route is as effective as IV administration and caused a significant decrease in the tumor area. Moreover, the nose-to-brain route is not yet clear and/or fully understood but there seemed to be a potential response to our anti-cancer therapy. Tumor cells were clearly disturbed in both IN and IV FTA loaded HNPs treated groups, whereas
in non-treated rat, only central necrosis was present in the tumor area. The latter is mainly caused by no vascular feeding of the core of the tumor and subsequent necrosis of the cells in this region. Moreover, the present cellular organizations at the initial tumor site after both IV and IN applied FTA loaded HNPs are quite similar and represent a recovery process.

Due to its mechanism of action and antitumor activity in preclinical studies, FTA is currently in clinical development as oral treatment (21-24). Many preclinical studies evaluated FTA via oral (43), intrathecal (44), intraperitoneal (45), local (46) intratumoral and IV administration (29). However, the pharmacokinetics of the IN delivery route of FTA has not been investigated yet. Therefore our data is the first study describing the IN delivery of FTA and FTA loaded HNPs as well as the IN related pharmacokinetics of FTA and FTA loaded HNPs in the treatment of glioblastoma.

After oral administration in patients with solid tumors, Tsimberidou et al. observed a slow absorption and a rapid elimination of Salirasib after a single dose (23). Our IV FTA loaded HNP formulation caused increase in the retention time of FTA in the blood circulation with 5 folds compared to IV FTA only. Whereas no interpolation could be made for IN free FTA data, it is remarkable that compared to free IV FTA, a 6 fold increase in the retention time of FTA loaded HNP was observed after administration via the nasal cavity. The latter may be influenced by the slow and/or absent process of absorption and transporting of FTA loaded HNPs from nasal mucosa into the blood circulation, instead it is distributed to the olfactory bulb in a higher degree. Also, as expected, IN free FTA was not able to reach the circulation due to its limited ability of crossing the mucus barrier in the nasal cavity. Additionally, a certain amount may have remained in the nasal tract and therefore extended its pathway to the blood circulation as can be noticed from the evidence that a small amount of free FTA is present in the olfactory bulb and brain after 4 hours. IN FTA loaded HNPs, were able to cross the mucus and were present in the brain and olfactory bulb in a higher concentration compared to IV FTA loaded HNP after 4 hours. The lower plasma concentration of IN FTA loaded HNP were as expected since the blood circulation is avoided in the nose-to-brain pathway. In addition, the presence of FTA in both brain and olfactory bulb is significantly higher.
for IN FTA loaded HNPs compared to IV FTA loaded HNPs. This result indicates that IN FTA loaded HNPs are being transported via the olfactory bulb to the brain bypassing the blood circulation and the BBB in a very efficient and quick manner.

FTA might be quickly eliminated from the circulation due to nonspecific uptake. Nanoparticle formulations (especially included PEGylation) affects the biodistribution and blood circulation half-life of circulating nanoparticles by reducing the level of nonspecific uptake, delaying opsonization, and increasing the extent of tissue specific accumulation (55). Therefore, the overall levels of FTA is much higher for the HNP formulations compared to free FTA. This effect could be more instrumental and also more beneficial to treat glioblastoma with FTA loaded nanoparticles compared to free FTA. Besides, the delivery of the HNPs to the brain is mediated by passive transport of HNPs through the BBB via lipophilicity and small size (<200 nm). With tumor presence, the damaged BBB is even more permeable for IV administered HNPs. On the other hand, IN administered HNPs are more likely transported via the axonal route through the olfactory bulb, thereby bypassing the BBB issue.

In the presence of brain tumors, inadequate constitution of the BBB properties results in edema formation as well as increased BBB permeability (47). Since our biodistribution data is related to healthy rats, the accumulation of our formulation in glioblastoma bearing brain might be higher. Also, although non-perfused brain tissue is not effecting the obtained accumulation dose of the drug due to negligible vascularized percentage (48), the obtained doses for FTA in highly vascularized liver and spleen might be even lower in perfused animals. Besides, FTA was used in this study in an extremely low concentration (14 µg/kg) whereas in other studies a dose of 5 mg/kg FTA in rats (45) and up to 800 mg in humans (21-24) have been used.

Altogether, we obtained promising results for nasal administration. Especially, the presence of low accumulation of IN FTA loaded HNPs (approximately 10 folds lower than IV FTA loaded HNPs) in the liver and spleen, indicates for a safer profile with regard to side-effects of the treatment. Upon these findings, further studies are needed to demonstrate clinical efficacy.
Conclusion

Due to recurrence of most brain tumors and serious suffering of these patients, IN FTA loaded HNP delivery, which is a non-invasive method and has also low systemic accumulation and side effects, is a promising novel approach and can be chosen over IV application in order to improve patient compliance. In this study we were able to show the proof of concept for the effectiveness of intranasal application of FTA loaded HNPs on brain tumors in rats. Further studies could include an extended treatment program with analyzing the survival rate of non-treated rats versus IV and IN FTA loaded HNP treated rats. Nonetheless, it is quite likely that this non-invasive treatment method will develop multidisciplinary collaborations as well as become a more economically effective treatment method or even be part of a combination therapy.
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Supplementary Materials

**Figure S1:** MRI images and corresponding coronal brain sections of rat brains before and after repetitive treatments with IV FTA loaded HNPs.

**Figure S2:** MRI images and corresponding coronal brain sections of rat brains before and after repetitive treatments with IN FTA loaded HNPs.

**Figure S3:** Initial and follow up MRI images and corresponding coronal brain sections of non-treated rat brains.

**Table S1:** Plasma concentration-time profile of FTA

**Table S2:** Pharmacokinetic parameters of FTA distribution

**Table S3:** Details of the LC/MS/MS setup

**Video S1:** Tracking analysis of empty HNPs

**Video S2:** Tracking analysis of FTA loaded HNPs
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