An MSN-PEG-IP drug delivery system and IL13Rα2 as targeted therapy for glioma

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A combination of gene therapy and chemotherapy has recently received interest as a targeted therapy for glioma. A mesoporous silica nanoparticle (MSN)-based vehicle coated with IL13Rα2-targeted peptide (IP) using polyethylene glycol (PEG), MSN-PEG-IP (MPI), was constructed and confirmed as a potential glioma-targeted drug delivery system in vitro. In this work, tissue microarray (TMA) results revealed that IL13Rα2 was over-expressed in human glioma tissues and that high expression of IL13Rα2 in patients was associated with poor survival. Doxorubicin (DOX)-loaded MPI (MPI/D) crossed the blood–brain barrier, specifically targeting glioma cells and significantly enhancing the cellular uptake of DOX in glioma cells compared with MSN/DOX (M/D) and MSN-PEG/DOX (MP/D), whereas the normal brain was not affected. Magnetic Resonance Imaging (MRI) examinations showed that the tumour size of glioma-bearing rats in the MPI/D-treated group was much smaller than those in the M/D and MP/D treated groups. Immunofluorescence results demonstrated that MPI/D treatment induced more apoptosis and much less proliferation than the other two treatments. However, the therapeutic effect was weak when IL13Rα2 was knocked down. Furthermore, U87 cells treated with IL-13 and MPI together could increase both STAT6 and P63 expression, which attenuated glioma cell proliferation, invasion and migration compared with cells treated with IL-13 alone. The results of the subcutaneous tumour model also revealed that IL13Rα2 knockdown could hinder cell proliferation and induce more apoptosis. The promising results suggested that MPI can not only deliver DOX to glioma in a targeted manner but also occupy IL13Rα2, which can promote IL-13 binding to IL13Rα2 and activation of the JAK-STAT pathway to induce an anti-glioma effect.

1. Introduction

Malignant glioma is the most common devastating type of central nervous system neoplasm and a major cause of death in children and the elderly.1 Although surgery, radiotherapy and chemotherapy have been developed, the prognosis of malignant glioma is still extremely poor due to its highly infiltrating property and the blood–brain barrier (BBB).2 The 5-year survival rate of glioblastoma is one of the lowest in all cancers, with a median survival rate of approximately 14 months.3 Targeted therapies for glioma are progressing to obviate the limitation of the BBB and drug accumulation in normal brain tissue with extra-tumoural damage.4,5 Nanotherapeutics including nanoparticles and specific molecules that target glioma not only enhance therapeutic efficiency but also overcome problems with conventional chemotherapies such as the lack of selectivity, permeability and retention effects.5,6 Recently, many nanoparticle drug delivery systems for tumour-targeting therapy have been reported including liposomes and micelles.7–10 Mesoporous silica nanoparticles (MSNs) have several attractive features such as large pore volume and surface area.11 Researchers have confirmed that MSN-based drug delivery systems have the potential to bypass multidrug resistance mechanisms.12–14 Thus, MSNs have gained attention as an efficient vehicle for use in glioma-targeted therapy.

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IL-13 is a cytokine derived from Th2 cells that can bind to IL13Rα1 and trigger the JAK-STAT pathway, which regulates cell proliferation and apoptosis.15,16 IL13Rα2 is a plasma membrane receptor with high affinity to IL-13 compared with IL13Rα1, and IL13Rα2 over-expression has been confirmed in various cancers including glioma, although it is absent in normal brain tissue.17–20 Thus, IL13Rα2 can be exploited as a potential target for glioma chemotherapy.21–23 The IL-13 peptide (IP) was designed from IL-13, which can bind to IL13Rα2, and has many advantages such as small molecular weight and easy manipulation.24 IP has been confirmed as a practical ligand for glioma targeted therapy with different nanoparticles.25,26 Therefore, in this study, we studied and applied IP as a glioma-specific targeting ligand for IL13Rα2 targeted therapy.

IL13Rα2 is also considered a decoy receptor because of its high affinity toward IL-13. IL13Rα2 can inhibit the activation of STAT6 of the JAK-STAT pathway by competing with IL13Rα1 binding to IL-13.27–29 IL-13 sequestration may lead to the blockage of downstream activation of the apoptosis gene and help tumour cells escape apoptosis.30 Papageorgis et al. reported that IL13Rα2 silencing could enhance the IL-13 activation of the JAK-STAT pathway and thus increase STAT6 and P63 expression to inhibit tumour metastasis and migration in breast tumours.28 Hsi LC et al. reported that silencing IL13Rα2 promotes glioblastoma cell death.31 Therefore, targeting IL13Rα2 for tumour therapy is attractive, and many biological agents have recently been designed for IL13Rα2 such as IL-13-labelled liposomes and IL-13 ligand-expressing viruses.32

In a previous study, a combination of MSN that was coated with IP using PEG, namely MSN-PEG-IP (MPI), was established as a drug delivery system. We found that MPI successfully transmitted DOX to glioma cells in vitro compared with MSN-PEG (MP) and MSN (M).33 Based on these results, we decided to test the MPI/D targeting anti-glioma effect in vivo by building a glioma-bearing rat model. Moreover, based on the knowledge of IL13Rα2 and the JAK-STAT pathway, we also detected the expression of STAT6 and P63 after glioma cells were treated with MPI and IL13 and assessed the association between the JAK-STAT pathway and tumour proliferation, apoptosis, migration and invasion. We hypothesized that the combination MPI may not only deliver DOX to glioma cells but can also affect glioma cells by occupying the IL-13Rα2 and facilitate IL-13 activation of the JAK-STAT pathway. Therefore, in this study, our aim was to verify if MPI/D is an efficient vector for targeted therapy for glioma using two different methods.

2. Results

2.1 IL-13Rα2 is over-expressed in glioma patients and is associated with poor prognosis

The expression of IL13Rα2 was detected in glioma specimens from 83 patients and in normal brain tissues from 8 epilepsy and brain trauma patients using immunohistochemistry (IHC). As shown in Fig. 1, the TMA results revealed that the IL13Rα2 antibody mainly stained the nuclei and was also in the cytoplasm of glioma cells; thus, cells with IL13Rα2 staining in the nuclei and cytoplasm were considered IL13Rα2-positive cells. The overall expression of IL13Rα2 was upregulated with the degree of malignancy according to the results of IHC (P < 0.05). Kaplan–Meier survival analysis for glioma patients with high or low IL13Rα2 expression.
IL13Rα2 expression in WHO III–IV (high grade) glioma was significantly higher than that of WHO I–II (low grade) glioma ($P < 0.05$) (Fig. 1B).

The X-tile software program-determined cut-off point of 120 was used for statistical analysis. Tissues with a protein staining score at the cut-off point or higher were considered to have high expression and were otherwise considered to have low or no expression. The Kaplan–Meier survival curves showed a significant difference in glioma patients between the high IL13Rα2 expression and low expression groups. Glioma patients with a high IL13Rα2 expression were associated with a shorter survival time than patients with a lower IL13Rα2 expression (Fig. 1C), suggesting that IL13Rα2 expression may be a prognostic factor.

2.2 Cellular uptake and targeting ability study

Cells were incubated with M/D, MP/D and MPI/D for 1 h at a concentration of 50 µg ml$^{-1}$ to study the cellular uptake and targeting ability. As shown in Fig. 2, the loaded doxorubicin almost always accumulated in the nuclei in all cell lines. In GBM cells and C6-IL13Rα2 cells which express IL13Rα2, the fluorescence intensities of DOX in the MPI/D group were significantly higher than those treated with M/D and MP/D but significantly decreased by pre-incubation with excess free IP. In addition, this phenomenon was also observed in C6 cells, which indicated that the peptide IP could also target and bind to rat IL13Rα2. However, in C6-shIL13Rα2 cells and U87-shIL13Rα2 cells, in which IL13Rα2 was knockout, there was no significant difference in the fluorescence intensity between the MPI/D and MP/D groups due to the lack of IL13Rα2. These results showed that the conjugation of IP facilitates MP targeting of glioma cells and transports DOX efficiently.

2.3 Doxorubicin distribution study

The in vivo glioma targeting of MPI/D was studied qualitatively using a fluorescence microscope. As shown in Fig. 3A, analyses of slices after incubating with DAPI show that the loaded doxorubicin mostly accumulated in the nuclei with or without PEG and IP. Among the organs, the kidney, spleen and liver uptakes of doxorubicin were greater than the others in the three groups. Moreover, the uptake of doxorubicin in gliomas in the MPI/D group was distinctly greater than that of the other two groups, but the doxorubicin uptake in normal brain tissue did not increase.

Similar results were also observed using the fluorescence spectrophotometer (Fig. 3B). The results revealed that the fluorescence intensities of doxorubicin in the kidney, liver and spleen were more than in other organs, but there were no significant changes in the three groups. However, there was a significantly higher distribution of doxorubicin in the tumour tissue of the MPI/D group than that of the M/D and MP/D groups ($P < 0.01$), and the doxorubicin uptake in normal brain tissue did not increase.

2.4 In vivo anti-glioma effect of MSN-PEG-IP/DOX

MRI examinations were conducted to assess the anti-glioma effect of the three combinations. As shown in Fig. 4A, the results of the MRI examinations showed that the tumour scope in the control group treated with saline moderately increased in C6 cells and the C6-IL13Rα2 cell glioma model. In the M/D group.

Fig. 2  Fluorescence microscopy images of C6-IL13Rα2, C6-shIL13Rα2, C6, GBM and U87-shIL13Rα2 cells incubated with M/D, MP/D, MPI/D and excess free IP for 1 h. The concentration of nanoparticles was 50 µg ml$^{-1}$. Bar = 25 µm.
group, the tumour scope did not obviously increase after treatment for 2 weeks, almost maintaining the same size. The tumour area was obviously smaller after 2 weeks in the MP/D group even after increasing at 1 week; furthermore, a significantly smaller size was observed in the MPI/D group after 2 weeks in C6-IL13Rα2 cells and the C6 cell glioma model. However, the tumour size increased after 2 weeks when MSN-PEG-IP was injected alone without DOX and the tumour scope of the C6-shIL13Rα2 glioma model treated with MPI/D still increased slowly after 2 weeks.

The histopathological changes in the glioma tissues in all treatment groups were evaluated using immunofluorescence. According to the corresponding results (Fig. 4B and C), less cell proliferation and more cell apoptosis were detected in the MP/D and MPI/D groups than in the M/D group (P < 0.001). Moreover, the effect of MPI/D was stronger than that of MP/D, but there were no changes in the MPI group compared with the control group. These results indicated that MP/D and MPI/D, relying on DOX, could induce enough therapeutic effects for glioma in vivo. In addition, the changes of body weights were recorded to evaluate the toxicity, as shown in Fig. 4D, and the results indicated that there is no evident toxicity of MPI/D.

2.5 Transmission electron microscopy examinations
The normal brain, kidney and glioma tissue of glioma-bearing rats after treatment with MPI/D were examined using TEM. As shown in Fig. 5, the TEM showed many MSNs in kidney tissue as a positive control. It was difficult to find MSNs in normal brain tissue except in some vascular endothelial cells, but a certain amount of MSNs was observed in glioma tissue not only in the vascular endothelial cells but also in glioma cells. These results demonstrated that MPI could cross the BBB and target glioma cells efficiently and avoid damage to normal brain tissue.
Fig. 4  (A) Representative T1-weighted MRI and immunofluorescence images of tumour tissues in glioma-bearing rats after treatment with M/D, MP/D and MPI/D. Bar = 25 μm. Immunofluorescence results for Ki67 (B) and caspase-3 (C). (D) Change of body weight. ***: P < 0.001.

Fig. 5  TEM images of MPI in kidney, normal brain, and glioma after caudal vein injection for 30 min. Kidney was used as a positive control. Lumen or vascular endothelial cells (a, b and c), (d) kidney cells, (e) normal brain cells and (f) glioma cells. The red arrows indicate nanoparticles. Blue triangles indicate cavities. Bar = 1 μm.
2.6 In vivo fluorescence imaging of glioma-bearing mice

Fluorescence imaging of glioma-bearing mice was performed to evaluate the targeting efficiency. The mice were injected with M/D, MP/D and MPI/D on the 19th day. As shown in Fig. 6A, compared with the M/D and MP/D groups, the fluorescence intensity in the tumour region in the MPI/DOX group was obviously higher. The ex vivo organ imaging also revealed the accumulation of MPI/D in glioma tissue, and there was no significant difference between the M/D and MP/D groups. These results exhibited the targeting behaviour of MPI/D.

2.7 IL13Rα2 depletion enhances the IL-13-mediated JAK-STAT6 pathway, weakens glioma cell invasion and migration, inhibits cell proliferation and promotes cell apoptosis in vitro

Western blot analysis was conducted to investigate if IL13Rα2 can regulate the IL-13-mediated JAK-STAT6 pathway because IL13Rα2 acts as a negative regulator of the IL-13 pathway. The results showed that the expression of STAT6 and P63 was significantly upregulated in U87 cells after incubating with IL-13 and MPI compared with IL-13 alone (Fig. 7A and B). Moreover, in U87-shIL13Rα2 cells, IL-13 treatment increased STAT6 and P63 expression dramatically, but when IL-13 and MPI were added at the same time, the expression of the proteins did not increase (Fig. 7C and D). These results indicated that IL-13 can induce STAT6 and P63 expression and that MPI could deplete IL13Rα2 temporarily or permanently, via the IL-13-mediated pathway, and promote STAT6 and P63 expression because the IP peptide can efficiently bind to IL13Rα2.

IL13Rα2 invasion was detected using a matrigel invasion assay. The results showed that the percentage of invading U87 cells did not decrease after IL-13 treatment, but the percentage was significantly lower when IL13 and MPI were added at the same time, and this phenomenon was not observed when MPI was added alone. Moreover, in U87-shIL13Rα2 cells, the percentage of invading cells decreased significantly after IL-13 treatment, but when MPI was added at the same time or alone, the cell invasion did not decrease compared with IL-13 treatment (Fig. 7E and F). In addition, the results of the migration assay showed that MPI and IL-13 can restrain cell migration in U87 cells, but the migration did not decrease in U87-shIL13Rα2 cells when both materials were added. This also means that MPI can deplete IL13Rα2, and the normal IL-13-mediated pathway may weaken cell migration (Fig. 7G and H).

We also detected cell proliferation and apoptosis. The results revealed that there was less cell proliferation and more cell apoptosis in the U87 cells due to MPI and IL-13. However, in U87-shIL13Rα2 cells, IL-13 treatment had the best effect on restraining cell proliferation and promoting cell apoptosis (Fig. 7I and J).

2.8 IL13Rα2 knockdown inhibits tumorigenicity in vivo

The in vivo research inoculating glioma cells into nude mice was used to further discover the characteristics of IL-13Rα2. The results showed that the sizes of tumours formed were smaller in the U87-shIL13Rα2 cells than those in the U87 cell group (Fig. 8A). In addition, the tumour tissues were harvested after 6 weeks and weighed individually. The data showed that the tumour weight in the U87-shIL13Rα2 cell group was lower than that of the U87 cell group (Fig. 8B).

Cell proliferation and apoptosis were also evaluated by immunofluorescence. As shown in Fig. 8C and D, less proliferation and much more cell apoptosis were detected in U87-
Fig. 7  Western blot results for STAT6 and P63 affected by MPI and IL-13 in U87 (A, C) and U87-shIL13Rα2 cells (B, D). Images of U87 and U87-shIL13Rα2 cells traversed the matrigel basement membrane after incubation with MPI and IL-13 for 24 h (I), and corresponding quantitative results (F). Images of U87 and U87-shIL13Rα2 cells migrated for 48 h affected by MPI and IL-13 (G) and corresponding results (H). Cell apoptosis and proliferation induced by MPI and IL-13 for 24 h (I and J) (mean ± SEM). *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Fig. 8  (A) Images of tumour-bearing mice and corresponding tumour volume curves 6 weeks after subcutaneous injection. (B) The tumour tissues were harvested and weighed. (C) Immunofluorescence images of tumour tissues. (D) Quantitative immunofluorescence results of Ki67 and caspase-3. *, P < 0.05, ***, P < 0.001.
shIL13Rα2 cells. These results indicated that the depletion of IL13Rα2 can restrain cell proliferation and promote apoptosis.

3. Discussion

Traditional chemotherapy drugs for glioma suffer from several limitations such as the lack of selectivity and permeability. Tumour-specific targeting of drug delivery carriers is a promising method to solve these problems. The choice of a promising tumour-specific targeting molecular marker in glioma cells is the first step for glioma chemotherapy.

Consistent with other reports, this study showed that L13Rα2 is a specific molecular marker in glioma that corresponds to tumour grade, whereas normal brain tissue shows a very low or undetectable expression level. The Kaplan–Meier survival analysis indicated that increased IL13Rα2 levels are associated with shorter survival time in glioma patients. Many biological agents have been engineered and are characterized into two groups: IL-13-labelled therapeutic agents and IL13Rα2-targeted immunotherapy. Our study concerns the former, but we applied the peptide IP instead of IL-13 because of its shortcomings such as easy denaturation and large molecular weight.

The blood–brain barrier (BBB) is another problem affecting the efficiency of glioma chemotherapy. Nanoparticles such as micelles, liposomes and inorganic nanoparticles have been reported as promising efficient vehicles that can deliver anti-tumour drugs across the BBB due to their appropriate features. A mesoporous silica nanoparticle (MSN) is a small object, 130 nanometres in size, that can be modified with polyethylene glycol (PEG) and can be easily surface functionalized. In our previous study, MSN-PEG-IP/DOX (MPI/D), a targeted drug-delivery system was designed and confirmed to deliver DOX to glioma cells more efficiently than MSN-PEG/DOX (MP/D) and MSN/DOX (M/D) in vitro (Fig. 9).

In the present study, we utilized MPI/D to further study its targeting ability. The results of the targeting assays for human glioma cell lines from in vitro were in agreement with the previous study. Moreover, MPI could also target GBM primary cells, showing its capacity for clinical use. Interestingly, the same phenomenon was observed in C6 cells, which indicated that IP could also be a potential ligand for rat IL13Rα2. In addition, the fluorescence imaging of glioma-bearing mice further confirmed these results, as the fluorescence signal of MPI/D at the glioma site was much stronger than that of MP/D and M/D. In conclusion, these results demonstrated that MSN-PEG-IP is a practical vehicle for targeted delivery of DOX to glioma in vitro.

To detect its anti-tumour effect in vivo, glioma-bearing rats were applied. Our research demonstrated that the fluorescence intensity of glioma tissue in the MPI/D group was stronger than that in the MP/D and M/D groups, whereas no significant difference in fluorescence intensity in normal brain tissue was detected among the M/D, MP/D and MPI/D groups. Moreover, in the MPI/D group, the fluorescence intensity of the glioma tissue was also stronger than that in normal brain tissue. These results indicated that MPI/D could target the glioma in vivo and reduce the damage to normal brain tissue from DOX. Importantly, according to the MRI examination and immunofluorescence imaging results, although MP/D also showed anti-tumour effects, MPI/D exhibited the highest therapeutic efficacy for glioma and mediated a decrease in proliferation and increased apoptosis of glioma cells. In contrast, when IL-13Rα2 was knocked down, the tumour size of the glioma moderately increased even after treatment with MPI/D for 2 weeks. In addition, if treated with MPI alone, the anti-tumour effect was also detected when glioma cells contain IL13Rα2, which indicated that for MPI/D, in addition to the DOX, the MPI drug delivery system itself can also have an anti-tumour effect. However, the mechanism of the MPI anti-tumour effect is still unclear and should be explored further.

The IL13Rα2 is a monomeric plasma membrane receptor that binds IL-13 but is not homologous to IL-4 and is a high-affinity binding IL-13 receptor compared with IL13Rα1. According to current research, IL13Rα2 is a decoy receptor for IL-13 and inhibits IL-13 binding to IL13Rα1, which can trigger the JAK-STAT6 pathway. Depletion of IL13Rα2 was reported to weaken breast cancer cell migration and suppress cell metastasis because the lack of STAT6 impedes downstream genes such as P63, which is a tumour suppressor. In this study, we found a similar phenomenon in glioma cells, and IL13 treatment of U87 cells with IL13Rα2 knockdown resulted in a significant delay in cell invasion, migration, proliferation, and promotion of cell apoptosis compared with the control. Furthermore, for U87 cells without IL13Rα2 knockdown, incubating with MPI and IL-13 together also led to an inhibition of cell migration and promotion of cell apoptosis, whereas cells incubated with IL13 alone did not show such an effect. In addition, our observations confirm that STAT6 and P63 expression increased when U87 cells were incubated with MPI and IL-13 compared with other groups. The study of the subcutaneous tumour model also suggests that IL13Rα2 promotes tumourigenicity. These results indicated that IL-13Rα2 might...
inhibit the activation of the JAK-STAT pathway by restraining IL-13-targeted IL-13Rα1 and decreasing STAT6 and P63 expression which play a role in tumour inhibition.

4. Conclusion

In summary, for the first time, MPI/D was confirmed as a targeted delivery system for targeted therapy for glioma cells in vitro and in vivo. It not only delivers DOX to glioma cells to induce a chemotherapeutic effect but can also occupy IL-13Rα2 temporarily or permanently and facilitate the IL13 activation of the JAK-STAT pathway to induce a series of anti-tumour effects. The details of the mechanism should be investigated further as these promising results suggested that the MSN-PEG-IP targeted drug delivery system has great potential for clinical applications in targeted therapy for glioma.

5. Materials and methods

5.1 Materials

IL-13 peptide (IP) with the sequence “VDKLL LHLKK LFREG QFNRN FESII ICRDR TC” was synthesized and characterized by Ziyu Biotechnology Company (Shanghai, China). The targeted M/D, MP/D, MPI/D and MPI/D delivery systems were constructed by Fudan University (Shanghai, China). Lentiviral vectors were purchased from Ruilu Biotechnology Company (Shanghai, China), and siRNA was purchased from Shengnuo Kang Biotechnology Company (Jiangsu, China).

5.2 TMA construction and analysis

All of the tissues were obtained from patients with a diagnosis of glioma that was confirmed by pathology at the Affiliated Hospital of Nantong University. Core tissue samples with a 2 mm diameter were taken for TMAs. TMA specimens were cut into 5 µm sections and mounted on glass slides coated with 10% polylysine. All of the sections were blocked in 0.3% hydrogen peroxide for 30 min, and then antigen retrieval was performed in citrate buffer heated to 121 °C in a autoclave for 15 min. When the sections cooled to room temperature, they were blocked with 10% goat serum for 1 h to prohibit non-specific reactions. IL13Rα2 expression was detected using the IL13Rα2 antibody (1: 500, ab55275, Abcam) at 4 °C overnight. Anti-mouse IgG was used as the secondary antibody. The expression of IL13Rα2 was scored based on the intensity of staining and the percentage of cells at that intensity. The staining intensity was scored as 0 (no staining), 1 (weak intensity), 2 (moderate intensity), and 3 (strong intensity). The final staining scores were evaluated by two pathologists and calculated from the sum of the four intensity percentage scores. The outcome was analysed using SPSS 20.0.

5.3 Cell culture and transfection

The C6 glioma cell line and U87 cell line were obtained from the cell library of the Chinese Academy of Sciences (Shanghai, China). The GBM cell line (GBM) was derived from patients with glioma at the Department of Neurosurgery of the Affiliated Hospital of Nantong University. The C6 cell line was cultured in F12 K nutrient mixture (Gibco BRL, Grand Island, NY, USA) with 12.5% horse serum and 2.5% foetal bovine serum (Gibco BRL, Grand Island, NY, USA). The GBM and U87 cells were cultured in DMEM (high glucose) with 20% foetal bovine serum. All of the cell lines were cultured at 37 °C with 5% CO2. The two lentiviruses (shRNA contain IL13Rα2 and shRNA against IL13Rα2) were separately added to the C6 cells, and shRNA against IL13Rα2 was transfected into U87 cells in 6-well plates (Corning, NY, USA) for 24 h and 4 h at 37 °C; the infection efficiency was determined by GFP expression using flow cytometry. C6-IL13Rα2, C6-shIL13Rα2 and U87-shIL13Rα2 cells were obtained for subsequent experiments.

5.4 Cellular uptake and the intracellular tracking process

C6-IL13Rα2, C6-shIL13Rα2, C6, U87-shIL13Rα2 and GBM cells were seeded in 24-well cell culture plates at a density of 1 × 10⁴ cells per well for 24 h, washed with PBS, and incubated with M/D, MP/D or MPI/D at a concentration of 50 μg ml⁻¹ for 60 min. After washing twice with PBS, the nuclei were stained with DAPI for 5 min. Fluorescence microscopy images were obtained using an Olympus IX71.

5.5 In vitro cell invasion and migration assays

24-Well Transwell plates with 8 µm polycarbonate filters were used for the cell invasion assay. U87 and U87-shIL13Rα2 cells were seeded in serum-free DMEM on the membrane, which was coated with a matrigel basement membrane matrix (100 μg cm⁻²), and DMEM with 10% serum was added in the bottom of the plates. Both cell lines were cultured with or without 20 ng ml⁻¹ IL-13 and MPI for 48 h. The invasive cells on the lower surface of the membrane were fixed in 4% paraformaldehyde for 20 min. After staining with crystal violet (0.1%) for 10 min, the cells that migrated to the lower side were determined using an Olympus IX71 microscope. 6-Well plates were used for the migration assay, U87 cells and U87-shIL13Rα2 cells were cultured, and a scratch was made in the monol culture using a 100 μl pipette tip. Three reference marks were made in the wells, and light-phase photographs were taken at 0 h and 48 h post-wounding.

5.6 Cell proliferation and apoptosis analysis

The U87 and U87-shIL13Rα2 cells were seeded in 96-well plates at a density of 2 × 10³ cells per well with 100 μl DMEM medium. After treatment with IL-13 and MPI, 10 μl cell counting kit-8 (CCK-8) was added to each well for proliferation measurement. The plates were incubated at 37 °C, and the absorbance was determined at 450 nm using a microplate reader (Bio-Rad, CA, USA). The U87 and U87-shIL13Rα2 cells were also incubated with Annexin V and 7-AAD for 15 min after treatment with or without 20 ng ml⁻¹ IL-13 and MSN-PEG-IP. Apoptotic cells were analysed using a FACSCalibur flow cytometer (Becton-Dickinson, NJ, USA).
5.7 Glioma model

All Wistar rats and nude mice were purchased from the animal experimental centre of Nantong University (male rats, average 200 g, 4–6 weeks). C6-IL13Ra2 and C6-shIL13Ra2 cells for injection were washed twice with PBS and resuspended in a F12 K nutrient mixture without serum to a final concentration of 10^5 cells per 10 µl and then maintained at 37 °C. After the rats were anesthetized with 10% chloral hydrate at 0.9 ml, a hole was made in the skull 0.5 mm posterior and 2 mm lateral to the bregma after the rat was fixed in the stereotactic frame. Then, 10 µl of cell suspension was slowly injected into the right putamen at a rate of 1 µl min⁻¹. The rats were housed under standard conditions with free access to food and tap water. All of the animal experiments were in accordance with institutional animal care guidelines and approved by the ethics committee of the Affiliated Hospital of Nantong University.

5.8 In vivo distribution study

The glioma-bearing rats were established as described above. Injection with M/D, MP/D and MPI/D was at a dose of 6 mg doxorubicin per g body weight for 2 h. Some of the rats were anesthetized with 10% chloral hydrate followed by heart perfusion with a saline solution and 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde for 24 h at 4 °C before dehydration with 20% and 30% sucrose solutions. The brains were frozen at −80 °C with OCT (Sakura, Torrance, CA, USA), and 10 µm sections were obtained. After staining with DAPI for 5 min at room temperature, images were obtained using an Olympus BX41. The organs including the brain, heart, liver, spleen, lung and kidney were harvested without heart perfusion, only washed carefully with distilled water, weighed and homogenized. The intensity of doxorubicin in each organ was measured using a fluorescence spectrometer.

5.9 In vivo imaging of MSN-PEG-IP/DOX in glioma-bearing mice

The glioma-bearing mice were established as the glioma-bearing rats were, using U87 cells. M/D, MP/D and MPI/D were injected into the mice at a dose of 100 µg at 18 days after implantation, and in vivo imaging was performed at various points (2 h, 4 h and 8 h) post-injection using an In Vivo IVIS spectrum imaging system (PerkinElmer, USA). The mice were sacrificed at 8 h post-injection. The major organs (brain, heart, liver, spleen, lung, and kidney) were harvested, and ex vivo imaging of the organs was conducted.

5.10 MRI examinations

MRI was performed using a 1.5 T horizontal Bruker magnet. The glioma-bearing rats were anesthetized with 10% chloral hydrate before MRI examination, and the rats were placed into a head holder within the magnet. The first MRI examination was performed at two weeks after C6-IL13Ra2 cell implantation, and then the rats were injected with saline, M/D, MP/D and MPI/D by the caudal vein at a dose of 6 mg doxorubicin per g body weight. The second and third examinations were taken at 1 week and 2 weeks after injection. Ten contiguous slices (1.5 mm) were obtained for diffusion-weighted imaging.

5.11 Immunofluorescence

After MRI examination, the glioma-bearing rats were anesthetized and intracardially perfused with a saline solution (250 ml) and then perfused with paraformaldehyde (4%, 250 ml). After the brains were removed, they were dehydrated with a 20% sucrose solution followed by a 30% sucrose solution; then, 5 serial sections were placed on glass slides coated with 10% polylysine using a freezing microtome (Thermo Scientific, Germany). All sections were blocked in 0.3% hydrogen peroxide for 30 min, and then the antigen was retrieved with citrate buffer heated to 121 °C in an autoclave for 15 min. When the sections cooled to room temperature, they were blocked with 10% goat serum for 1 h to prohibit nonspecific reactions and incubated with Ki67 antibody (ab16667) and caspase-3 antibody (ab47131) at 4 °C overnight. After washing with PBS, the sections were incubated with relevant secondary antibodies and DAPI. Fluorescence microscopy images were obtained using an Olympus BX41.

5.12 Western blotting

All of the protein preparations were run on sodium dodecyl sulphate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% skimmed milk and then incubated with primary antibodies against STAT6 and P63 (1 : 1000, Abcam, USA) overnight at 4 °C. After rinsing in Tris-buffered saline-Tween (TBST), the blots were incubated with anti-mouse secondary antibody (1 : 2000, Beyotime Institute of Biotechnology, China). The protein band results were visualized using an enhanced chemiluminescence system, and the membranes were stripped to incubate with GAPDH (1 : 3000, Abcam, USA), which was detected as a gel loading control. All western bands were quantified using densitometry and are presented in the form of bar graphs.

5.13 Transmission electron microscopy (TEM)

The brains and kidneys of glioma-bearing rats administered MSN-PEG-IP/DOX after heart infusion with normal saline were fixed in 2.5% glutaraldehyde at 4 °C overnight. To observe the detailed mechanism of MSN-PEG-IP/DOX crossing the blood-tumour barrier and permeating the glioma, transmission electron microscopy was performed using a HITACHI HT7700 with an acceleration voltage of 200 kV.

5.14 Statistical analysis

All results are presented as the mean ± standard deviation. Comparison groups were analysed using one-way analysis of variance followed by Tukey’s post hoc multiple comparison tests. Survival curves were calculated using the Kaplan–Meier method, and a log-rank test was used for survival analysis. All data were analysed using SPSS 20.0 and GraphPad prism sof-
ware (v.6). P-Values less than 0.05 were regarded as statistically significant.

Conflict of interest

The authors declare no competing financial interest.

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