

PACLITAXEL-LOADED MULTIFUNCTIONAL NANOPARTICLES FOR THE TARGETED TREATMENT OF GLIOBLASTOMA

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Abstract

Introduction: We hypothesised that the active targeting of $\alpha\beta3$ integrin overexpressed in neoangiogenic blood vessels and glioblastoma (GBM) cells combined with magnetic targeting of paclitaxel- and SPIOloaded PLGA-based nanoparticles could improve accumulation of nanoparticles in the tumour and therefore improve the treatment of GBM.

Methods: PTX/SPIO PLGA nanoparticles with or without RGD-grafting were characterised. Their *in vitro* cellular uptake and cytotoxicity was evaluated by fluorospectroscopy and MTT assay. *In vivo* safety and anti-tumour efficacy of different targeting strategies were evaluated in orthotopic U87MG tumour model over multiple intravenous injections.

Results: The nanoparticles of 250 nm were negatively charged. RGD targeted nanoparticles showed a specific and higher cellular uptake than untargeted nanoparticles by activated U87MG and HUVEC cells. *In vitro* IC50 of PTX after 48 h was ~ 1 ng/mL for all the PTX-loaded nanoparticles. The median survival time of the mice treated with magnetic targeted nanoparticles was higher than the control (saline) mice or mice treated with other evaluated strategies. The 6 doses of PTX did not induce any detectable toxic effects on liver, kidney and heart when compared to Taxol.

Conclusion: The magnetic targeting strategy resulted in a better therapeutic effect than the other targeting strategies (passive, active).

Keywords: PLGA nanoparticles; glioblastoma; targeting; nanomedicine; nanotheranostics; paclitaxel

Introduction

Glioblastoma (GBM) is one of the most prevalent and deadliest malignant tumours that occur in central nervous system [1,2]. GBM is featured with rapid proliferation, high invasiveness, extensive infiltration, hyper neoangiogenesis and is very challenging to treat with the current therapies [2]. Advances in drug discovery and therapeutic payload delivery did not significantly improve the survival rates of GBM patients which have remained at an average of less than 18 months [3]. The current standard care for GBM includes maximal safe resection, followed by radiotherapy and concomitant treatment with alkylating agents. However, the standard treatments face limitations such as practically impossible complete resection of tumour, high recurrence rate and overall resistance to therapy [3]. Due to the current survival expectancy and the existing limitations of the available treatments, there is a great need for better and promising therapies for devastating GBM.

The versatility and ability of nanoscale systems as efficient drug targeting and delivery systems for GBM have been previously demonstrated. Nanomedicine has the ability to address some of critical limitations of the conventional drugs such as inability to cross the biological barriers, poor accumulation at the targeted brain tumour site and high systemic side effects [4–6].

Poly(lactic-co-glycolic acid) (PLGA) is one of the most widely used biocompatible and biodegradable polymers for the preparation of nanoparticles (NPs). It is approved by regulatory agencies for parenteral products [7]. PLGA allows encapsulation of potential chemotherapeutic drugs, which face solubility difficulties in conventional forms. Polyethylene glycol (PEG) hydrophilic stabilising sheath around the hydrophobic core of PLGA protect the NPs from the RES clearance [7]. Encapsulation of anticancer drugs in PLGA has been exploited to ameliorate drug delivery in cancer, including GBM [8,9].

Paclitaxel (PTX) is a microtubule stabilising, potent anti-neoplastic agent used as first-line therapy against many tumours such as small cell lung carcinoma, breast cancer [10]. Even though PTX is a highly potent agent, its use in clinical treatment for GBM is limited because of its failure to reach the brain. It has been reported that PTX encapsulated in nanocarriers can cross a disrupted or leaky blood-brain barrier (BBB), exerts a therapeutic effect in preclinical GBM models and lower side effects [11–14].

In addition, a suitable targeting strategy can add more value to the whole formulation and increase therapeutic efficacy. Given the selectivity problem of most of the anti-cancer drugs, various efforts are being put forth for specific targeting of tumour cells [15]. Different targeting strategies have been explored. The benefit of nanomedicine can partly be attributed to so-called passive targeting, i.e. the accumulation of NPs via the controversial enhanced permeability and retention (EPR) effect, which is a result of leaky vasculature and/or poor lymphatic drainage system of tumour [16,17]. Active targeting takes advantage of specific moieties expressed on the surface of cancer cells or tumour microenvironments. Specific targeting ligands functionalised on the surface of NPs that have an affinity to interact or bind to those moieties can enhance drug amount and/or intracellular uptake of NPs (active targeting) [17]. GBM cells

are infiltrative in nature as a consequence of expression of integrin that facilitates migration and infiltration into surrounding healthy brain tissue [18]. The expression of $\alpha\beta3$ integrin (a class of cell adhesion receptors) is prominent in GBM biopsy samples at both tumour microvessels and tumour cells [19]. Overexpression of $\alpha\beta3$ integrin is more dominant in highly proliferating and infiltrating areas of GBM [19]. This overexpression can make RGD (tripeptide)-based targeting an effective strategy for drug delivery to GBM tumours as the short peptide sequence is specific and well recognised by $\alpha\beta3$ integrins [12,13,20,21].

Application of external stimuli could also increase the accumulation of NPs into the desired tissues. In particular, application of an external magnetic field can attract magnetic NPs by magnetic targeting [22]. Superparamagnetic iron oxide nanoparticles (SPIOs) were used for imaging as a T_2 contrast agent for magnetic resonance imaging (MRI) and magnetic targeting purposes [23–26]. SPIONs when used in a hybrid targeting strategy (active and/or magnetic) further topped-up the passive accumulation of NPs leading to improved therapeutic efficacy in a colorectal tumour [27].

We have previously shown that magnetic targeting alone induced a significant increase in accumulation of PTX and SPIO loaded PLGA-based NPs at tumour site and prolonged the survival period in orthotopic glioma tumour model [14]. In our current study, we hypothesised that active targeting of $\alpha\beta3$ integrin which is overexpressed in neoangiogenic blood vessels and GBM cells combined with magnetic targeting of PTX-loaded PLGA NPs would improve the treatment of orthotopic GBM model by further enhancing accumulation in the tumour and/or by increasing the endocytosis of the NPs. For this, we have evaluated in an orthotopic U87MG tumour model different targeting strategies: passive targeting by EPR effect as control for other targeting strategies, active targeting via RGD targeting of $\alpha\beta3$ integrin, magnetic targeting of SPIO-loaded formulation and active combined with magnetic targeting.

Material and methods

MATERIALS

Ring-opening polymerisation method was used to synthesise PCL-*b*-PEG (13100–5000); PLGA-*b*-PEG (10040–4600), and fluorescein isothiocyanate (FITC)-PLGA (23,600) as previously described [28–30]. PLGA (50:50 lactide/glycolide; 7000–17,000), sodium hydroxide, iron(III) chloride, iron(II) chloride, oleic acid, hydrochloric acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) PVA (polyvinyl alcohol; 30–70 kDa), GRGDS peptide (linear), anti-human IgG, Human umbilical vein endothelial cells (HUVEC) (200P-05N), Endothelial Cell Growth Medium and xylazine hydrochloride were bought from Sigma-Aldrich (USA). TPD clip 4[3-(trifluoromethyl)diazirin-3-yl]benzoic acid *n*-hydroxysuccinimide ester was obtained from Toronto research chemicals (Canada) and monoclonal anti-human integrin- $\alpha\beta3$ (CD51/61)-phycoerythrin antibody from R&D systems (USA). Poly-D-Lysine hydrobromide (70,000–150,000) was bought from MP Biomedicals (Germany). Trypsin EDTA, penicillin-streptomycin, Foetal

bovine serum (FBS) and Eagle's Minimum Essential Medium were obtained from Gibco® BRL (USA). Ketamine (100 mg/mL; Nimatek) from Eurovet Animal Health B.V. (The Netherlands). U87MG cells (HTB-14™), were obtained from ATCC. Formaldehyde (4% solution) was bought from Merck (Germany). Analytical grade solvents and chemicals were used for all the experiments.

FORMULATION AND PHYSIOCHEMICAL CHARACTERISATION

PHOTOGRAFTING OF RGD TO PCL-B-PEG COPOLYMER

RGD was grafted to PCL-*b*-PEG copolymer as described by Pourcelle et al. [31]. Briefly, PCL-*b*-PEG was dissolved in acetonitrile with TPD clip (molecular clip). The solution was cast on glass plates and then subjected to evaporation followed by vacuum drying until constant weight. Fully dried copolymer was collected and irradiated in a homemade reactor [3 UV lamps (8 watts for each lamp) placed at a distance of 4.5 cm from rotating quartz flask] at 254 nm under an inert atmosphere for 90 min. The clip grafted copolymer (activated copolymer) was then washed with isopropanol:ethyl acetate (19:1) and dried under vacuum before being mixed with GRGDS peptide 1 mM solution. After 24 h of shaking at room temperature, the RGD grafted polymer was washed 3 times with 5 mM HCl, 5 times with deionised water and vacuum dried. As the molecular clip contain F, ¹⁹F NMR was used to demonstrate the activation of molecular clip, which is a crucial step for the successful grafting of RGD onto NHS ester [31] (see Supplementary data S1).

PREPARATION AND PHYSIOCHEMICAL CHARACTERISATION OF RGD FUNCTIONALISED PTX AND SPIO-LOADED NANOPARTICLES (PTX/SPIO-RGD-NPS)

SPIO coated with oleic acid were synthesised as previously described [32] by coprecipitation method using ferrous and ferric salts in an alkaline medium under argon atmosphere and at 80.

PTX/SPIO-NPs were prepared by an emulsion-diffusion-evaporation method with a few modifications [33]. Initially, PTX (2.5 mg), PLGA (70 mg), PCL-*b*-PEG (15 mg) and PLGA-*b*-PEG (15 mg) in dichloromethane were added with SPIO (7.7 mg/mL iron [Fe] concentration) and mixed well. Secondly, 3% PVA aqueous solution was added to the mixture, vortexed and emulsified at 50 W using an ultrasonicator (3 x 30 s) resulting in an emulsion. Thirdly, the resulted emulsion was added dropwise to a 1% PVA aqueous solution under magnetic stirring at 400 rpm and left for organic solvent evaporation. Then the NPs were washed with water. Finally, the NPs were suspended in 1 ml of water. Same procedures were followed to prepare SPIO-NPs, FITC labeled PLGA NPs and PTX-loaded NPs (PTX-NPs). Similarly, PTX/SPIO-RGD-NPs and FITC labeled PLGA RGD NPs were prepared using RGD grafted PCL-PEG.

PLGA-based NPs loaded with PTX and SPIO (PTX/SPIO-NPs), PTX-NPs, SPIO, SPIO-NPs and FITC labeled PLGA NPs were formulated and characterised for size (dynamic light scattering), zeta potential (laser Doppler velocimetry) using NanoSizer Zeta Series instrument (Malvern, UK). Encapsulation efficiency [percent ratio of entrapped drug (mg) to the total drug (mg)] and drug loading [percent ratio of entrapped drug (mg) to 100 mg polymer] of PTX were measured using by high-performance liquid chromatography with ultraviolet

detection at 227 nm (Agilent 1100 series instrument; Agilent Technologies). Iron loading was determined by electron spin resonance (ESR) spectroscopy (Bruker Optik GmbH, Germany) and validated by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500ce instrument (Agilent Technologies, CA) as previously reported [14,33].

IN VITRO STUDIES

CELLULAR UPTAKE OF PTX/SPIO-NPS VERSUS PTX/SPIO-RGD-NPS

U87MG cells expression of $\alpha\beta 3$ was evaluated by flow cytometry using monoclonal anti-human integrin- $\alpha\beta 3$ (CD51/61)-phycoerythrin antibody, adapted from [34]. U87MG cells (5×10^4) were incubated with anti-human IgG (1 μg ; 15 min) to block non-specific binding. The cells were incubated with anti-human integrin- $\alpha\beta 3$ (CD51/61)-phycoerythrin monoclonal antibody (0.05 μg ; 4 °C for 60 min), washed with PBS, trypsinized (0.05%) and analysed by flow cytometer (FACScan, Becton Dickinson). The cells incubated without monoclonal antibody was used as a control.

The cellular uptake of NPs was evaluated in U87MG cells (passage 10) and in HUVEC (passage 3) [35]. HUVEC cells were seeded (2×10^4) in 48-well culture plates coated with 0.2% gelatine. U87MG cells were seeded (4×10^4) in 24-well culture plates. After 24 h incubation, the cells were activated with TNF- α (10 ng/mL; to enhance $\alpha\beta 3$ integrin expression) followed by treatment with 0.1% BSA (to inhibit non-specific binding). Then the cells were incubated with FITC-NPs and FITC-RGD-NPs (10 mg/ml polymer concentration) at 37 °C for 4 h. The cells were rinsed with PBS and detached with trypsin (0.05%). A competition assay was performed on activated cells by incubating with an excess of GRGDS peptide for 30 min followed by incubating with NPs for 4 h. The fluorescent NPs uptake was quantified by measuring fluorescence intensity using Packard Fluorocount Microplate fluorometer (Packard Instrument Company, Meriden, CT) at 485/530 nm excitation/emission wavelengths.

CELLULAR CYTOTOXICITY

The cytotoxicity of PTX-NPs, SPIO-NPs and PTX/SPIO-NPs was determined by a MTT cell proliferation assay [36]. U87MG cells were cultured in EMEM culture medium supplemented with 10% FBS, 100 U/mL penicillin G sodium and 100 $\mu\text{g}/\text{mL}$ streptomycin sulphate. For MTT assay, cells were seeded at a density of 5×10^3 cells per well in 96-well plates and after 24 h different formulations (PTX-NPs, SPIO-NPs and PTX/SPIO-NPs, PTX/SPIO-RGD-NPs) at different PTX concentrations [0.01 ng/ml– 10,000 ng/ml PTX (~100 $\mu\text{g}/\text{mL}$ of polymer concentration)] were added to the cells. Post 48 h, cells were washed with PBS and incubated with the medium containing MTT (5 mg/ml) for an additional 4 h. Then MTT solution was removed and DMSO was added. Absorbance of treated and untreated cells was measured (BioRad microplate reader, USA) at 570 nm. The results are presented as relative percentage of living cell survival compared to the untreated cells (negative control). IC₅₀ values were calculated using Graph pad prism software (San Diego, CA).

***IN VIVO* STUDIES**

All experiments were compliant with Belgian national regulation guidelines and approved by the animal care ethical committee of the Université Catholique de Louvain (2014/UCL/MD/004).

ORTHOTOPIC U87MG TUMOUR MODEL

NMRI-nude mice (female, 6 weeks old; Janvier, France) were procured followed by 1-week acclimatisation. Mice were anaesthetised (intraperitoneal injection of xylazine (13 mg/kg) and ketamine (100 mg/kg)) and were positioned into a stereotaxic frame. For tumour inoculation, a 3 μ L intracranial injection of U87MG cells in culture medium (3×10^4 cells) was performed into the right frontal lobe (striatum; coordinates: 2.1 mm lateral from bregma, 0.5 mm anterior and 3 mm deep from the outer border of the cranium) using a 5 μ L Hamilton® syringe fitted with a 26 G needle.

MRI IMAGING

MRI was performed using an 11.7 T Biospec small-animal MR scanner (Bruker BioSpin GmbH, Rheinstetten, Germany) to obtain tumour location and volume. Mice were anaesthetised using isoflurane (2.5 for induction and 1% for maintenance). Animal respiration was monitored during all the experiment and core temperature was maintained using hot water circulation in a blanket. To monitor the tumour growth anatomical images were obtained using T₂-weighted rapid acquisition with refocused echoes sequence [RARE]. Parameters were as following: number of averages = 8, matrix size = 200 x 200, field of view [FOV] – 2 x 2 cm, repetition time [TR] - 2500 ms, effective echo time [TE_{eff}] - 30 ms, number of continuous slices = 25, slice thickness = 0.3 mm, in-plane resolution = 100 μ m². Tumour volume was determined from manually drawn region of interest (ROI) using Paravision 6.1 software (Bruker BioSpin), on day 7 and day 28.

IN VIVO ANTI-TUMOUR EFFICACY

The anti-tumour efficacy of the formulated NPs was evaluated in the orthotopic U87MG tumour model. Mice bearing tumours were assigned uniformly into groups ($n \geq 5$). Dosing regimen was adapted based on previously established protocols [37,38]. All the groups except the untreated/control (saline) group received six intravenous injections (5 mg/kg PTX and 14 mg/kg Fe; 150 μ L) over three weeks (Supplementary data S2). The experimental endpoint was determined as 20% weight loss or 10% weight loss with tumour related sickness (moribund mouse or mouse lacking complete mobility).

Group 1: Injection of PBS.

Group 2: Injection of PTX/SPIO-NPs (passive targeting).

Group 3: PTX/SPIO-NPs injection with post application of 1.4 T neodymium magnet for 4 h (magnetic targeting).

Group 4: Injection of PTX/SPIO-RGD-NPs (active targeting).

Group 5: Injection of PTX/SPIO-RGD-NPs with application of 1.4 T neodymium magnet for 4 h (magnetic + active targeting).

IN VIVO SAFETY EVALUATION

Mice (Swiss mice; Janvier) were randomly assigned into three groups (n - 6) and each group received six intravenous injections of PTX/SPIO-NPs (5 mg/kg PTX), Taxol® (5 mg/kg PTX) or control (saline) over 3 weeks. Daily monitoring of body weight and collection of blood and tissue samples (at 24 h and 7 days) were performed. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatine kinase (isoenzymes CKM and CKB; CKMB) levels were assayed using a Fujifilm DRI-CHEM NX500i Analyser (Fujifilm, Tokyo, Japan). Haematoxylin and Eosin staining were performed for histological analysis on liver tissue [14].

STATISTICAL ANALYSES

All the results were presented as the mean \pm standard deviation (SD). One-way ANOVA with Tukey post-tests, Two way ANOVA with Bonferroni post-test and Kaplan–Meier survival rate curves were performed using the software Graph Pad Prism 5 for Windows (GraphPad Software, San Diego, CA) to demonstrate significant differences among treatment groups (*p < .05, **p < .01, ***p < .001).

Table 1. Physicochemical characterisation of various NPs FITC-NPs, FITC RGD-NPs, SPIO-NPs, PTX-NPs, PTX/SPIO-NPs and PTX/SPIO-RGD-NPs (n \geq 3).

	FITC - NPs	FITC RGD - NPs	SPIO-NPs	PTX-NPs	PTX/SPIO-NPs	PTX/SPIO-RGD-NPs
Size (nm)	220 \pm 8	229 \pm 10	230 \pm 5	224 \pm 12	247 \pm 10	255 \pm 15
PDI	0.14	0.18	0.15	0.11	0.17	0.15
Zeta potential (mV)	-16 \pm 2	-18 \pm 4	-14 \pm 3	-22 \pm 5	-17 \pm 7	-19 \pm 7
Drug encapsulation efficiency (%)	NA	NA	NA	29 \pm 3	31 \pm 5	27 \pm 5
Drug loading (%)	NA	NA	NA	0.88 \pm 0.4	0.83 \pm 0.5	0.80 \pm 0.3
Iron loading (%)	NA	NA	3 \pm 1	NA	3.5 \pm 0.5	3 \pm 1

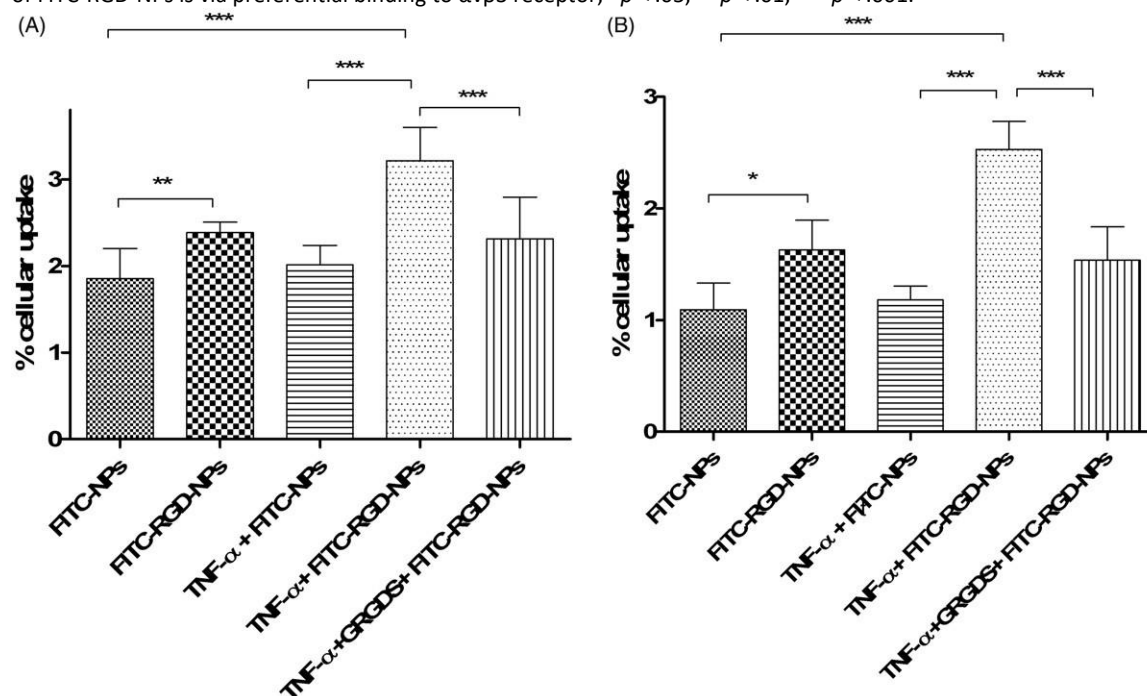
Results

PHYSICOCHEMICAL PROPERTIES OF PTX AND SPIO-LOADED NANOPARTICLES

The formulated NPs were characterised for size, zeta potential, encapsulation efficiency, drug and iron loading as applicable and are summarised in Table 1. The PTX-NPs, SPIO-NPs, PTX/SPIO-NPs and PTX/SPIO-RGD-NPs were in size range of ~ 230–255 nm and PDI was \leq 0.2. All the NPs were negatively charged (less than - 15 mV). The PTX encapsulation efficiency was ~ 30% and drug loading was ~ 0.8%. The iron loading was found to be ~ 3%. The TEM images and superparamagnetic properties of the prepared NPs have

already been published by our group [33]. Consistent with our previous reports [27], the inclusion of ligand (RGD) grafted copolymer did not alter the size or other physicochemical characters.

Figure 1. (A) *In vitro* cellular uptake of FITC-NPs versus FITC-RGD-NPs by U87MG cells ($n = 6$). (B) *In vitro* cellular uptake of FITC-NPs versus FITC-RGD-NPs by HUVEC cells ($n = 6$). In both the cell lines, there was nearly two-fold increase in cellular internalisation of FITC-RGD-NPs with TNF- α activated cells. A competition assay with excess of GRGDS peptide showed that the enhanced uptake of FITC-RGD-NPs is via preferential binding to $\alpha v \beta 3$ receptor; * $p < .05$, ** $p < .01$, *** $p < .001$.



IN VITRO CELLULAR UPTAKE STUDIES

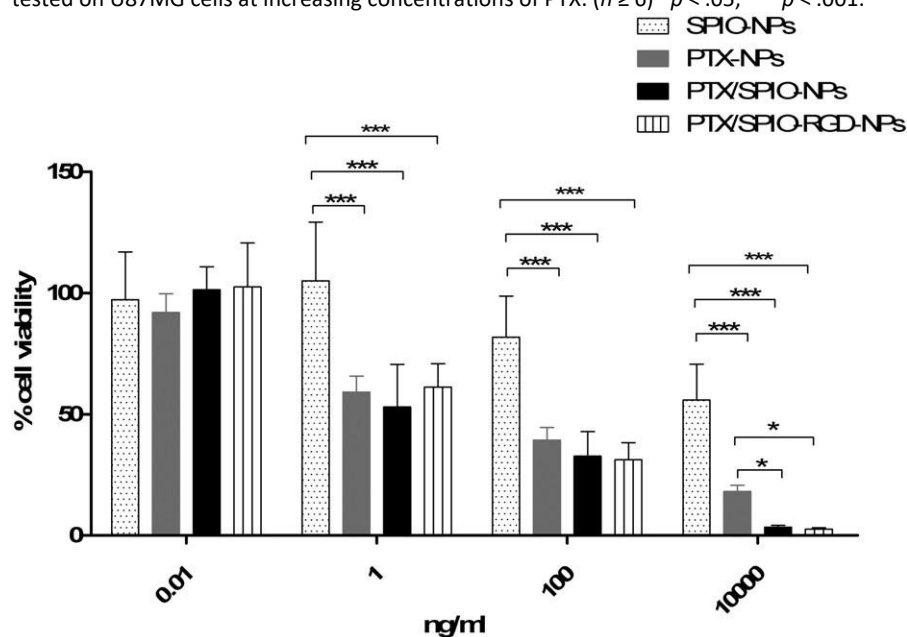
The purpose of *in vitro* cellular uptake studies was to evaluate the effect of RGD on cellular internalisation of NPs in U87MG and HUVEC cell lines (Figure 1). Expression of $\alpha v \beta 3$ integrin by U87MG cells was verified before quantitative analysis of the cellular uptake of PLGA-FITC-based fluorescent NP.

The flow-cytometry analysis confirmed that the used U87MG cells expressed $\alpha v \beta 3$ (Supplementary data S3) in compliance with the literature [34]. HUVEC cells activated by TNF- α [39] served as positive control for $\alpha v \beta 3$ integrin expression. Both cell lines showed increased uptake of FITC-RGD-NPs ($p < .001$) after activation by TNF- α whereas there was no significant change in the case of FITC-NPs uptake. HUVEC cells displayed two-fold higher uptake of FITC-RGD-NPs ($p < .001$). Competition assay with excess concentration of GRGDS peptide or anti- $\alpha v \beta 3$ integrin inhibited the enhanced uptake of RGD-NPs by both HUVEC and U87MG cells. This confirms that RGD enhanced the cellular uptake through its favoured binding to $\alpha v \beta 3$ receptor [35].

IN VITRO CYTOTOXICITY STUDIES

In vitro cytotoxicity of NPs was performed on U87MG cell line (Figure 2). At the concentration of 0.01 ng/mL, no significant cytotoxic effect was observed. With increasing concentrations of NPs, cell viability significantly decreased except in case of SPIO-NPs, confirming that the cytotoxic effect of the formulation was mainly due to PTX. After 48 h, the IC₅₀ values of PTX was 1 ng/mL whatever the NPs (0.9 ng/mL for PTX-NPs, 0.8 ng/mL for PTX/SPIO-NPs and 0.9 ng/mL for PTX/SPIO-RGD-NPs), indicating that, inclusion of RGD-grafted PCL-PEG into the formulation did not enhance cytotoxicity effects of PTX/SPIO-NPs.

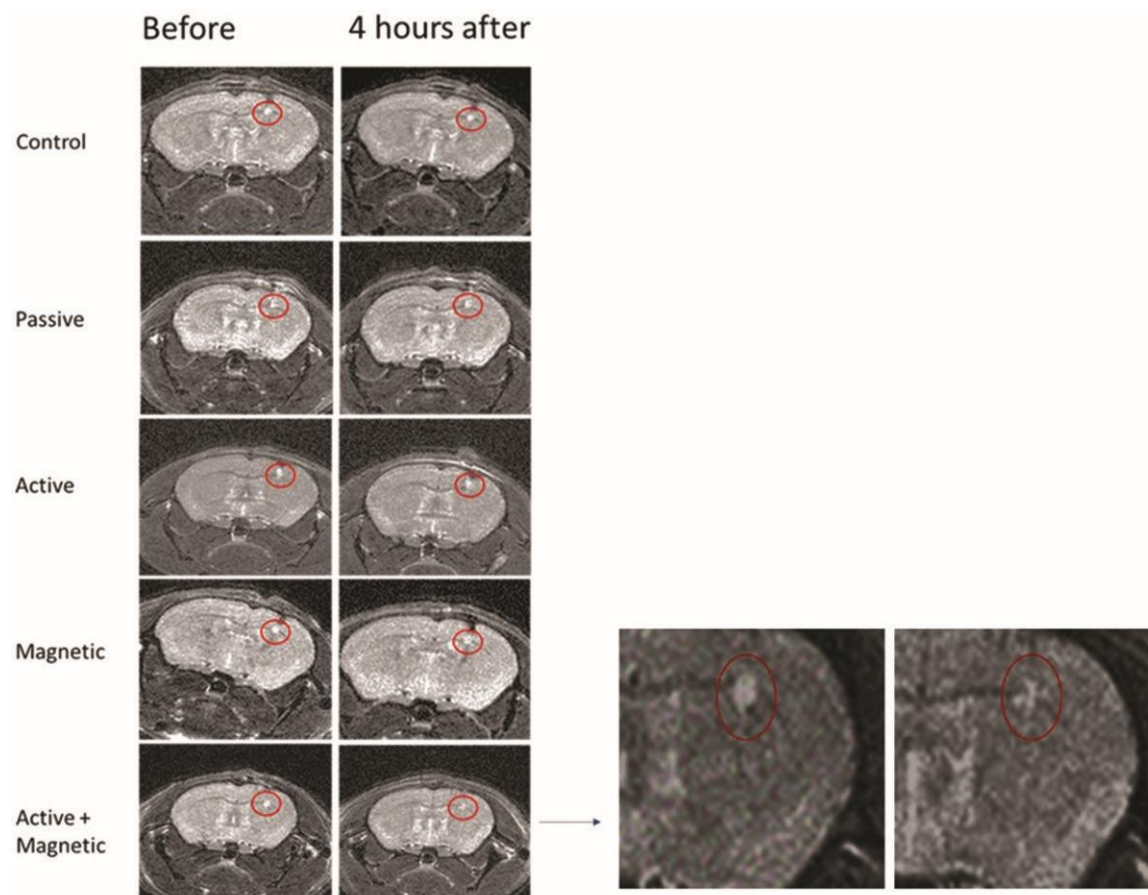
Figure 2. *In vitro* cytotoxicity test using MTT assay. Cytotoxicity of SPIO-NPs, PTX-NPs, PTX/SPIO-NPs and PTX/SPIO-RGD-NPs were tested on U87MG cells at increasing concentrations of PTX. ($n \geq 6$) * $p < .05$, *** $p < .001$.



NON-INVASIVE MRI IMAGING

The BBB disruption of U87MG orthotopic tumour model was previously demonstrated [14]. On day 8, the MRI images were obtained before and 4 h after the first treatment injection (Figure 3). Repositioning of the animals into MRI was required between the pre- and post-treatment scans due to the application of magnetic targeting. We observed that the contrast difference at the tumour site was visually prominent in groups of active β magnetic group and magnetic group indirectly indicating accumulation of higher amounts of NPs in comparison to the other groups ($n \geq 3$). There was no difference in the pre and post-treatment images of the control (saline) group. Further, we plan to evaluate quantitatively and non-invasively the accumulation of NPs in the tumour site using R2 maps.

Figure 3. Non-invasive MRI imaging of NPs accumulation on day 8 at the tumour site before and 4 h after treatment injection ($n \geq 5$). We could observe the contrast difference in the MRI images more pronounced in the groups of magnetic targeting and active + magnetic targeting groups in comparison to the other groups.



***IN VIVO* ANTI-TUMOUR EFFICACY**

The *in vivo* anti-tumour efficacy was analysed by following tumour volume by MRI and survival rates of the orthotopic glioma-bearing mice administered NPs with different targeting strategies.

The tumour progression (Figure 4(A,B)) was analysed by evaluating tumour volumes from ROI of MRI images obtained on day 7 and day 28 (immediately post last dose administration). On day 7, the tumour volume of all the groups was $\sim 0.2 \text{ mm}^3$. By day 28, the tumour progression was higher in control (saline) group with tumour volume $\sim 3 \text{ mm}^3$, whereas the tumour volumes in all the treated groups were in between 1 and 2 mm^3 . The magnetic and active + magnetic groups showed the lower tumour volumes of $\sim 1 \text{ mm}^3$. Intra and intertumoral heterogeneity is one of the characteristic features of GBM [40] which could evidently explain the varied degree of the therapeutic responses towards the administered NPs and the

employed strategies. The acquired images showed that the tumour progression was significantly less in all the treated groups.

Figure 4. (A) Follow up MRI images of tumour progression on day 7 and day 28. (B) Tumour progression and volumes analysed from ROI of MRI images obtained on day 7 and day 28 ($n \geq 5$). (C) Kaplan–Meier survival graph showing the anti-tumour efficacy of different targeting strategies ($n \geq 5$).

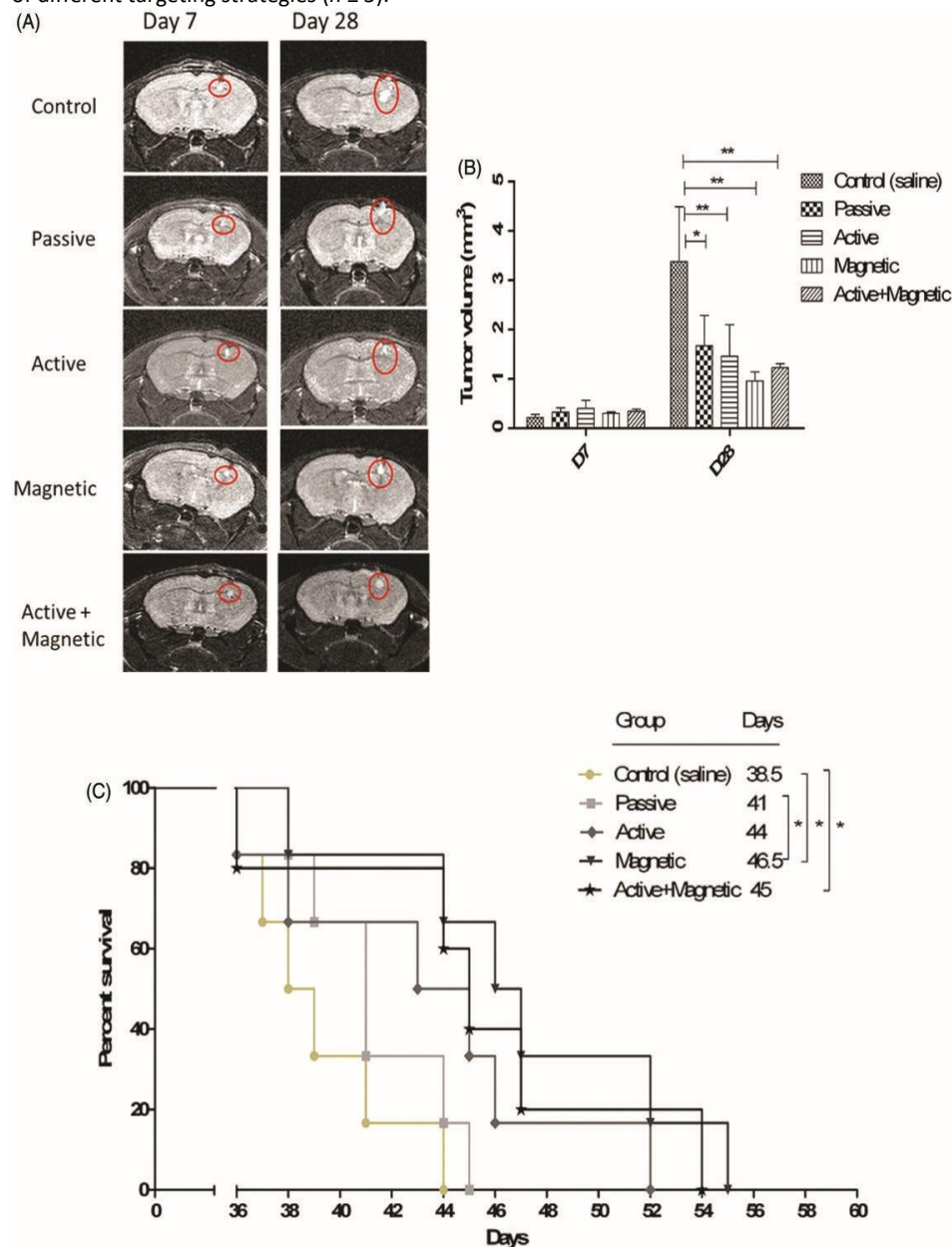
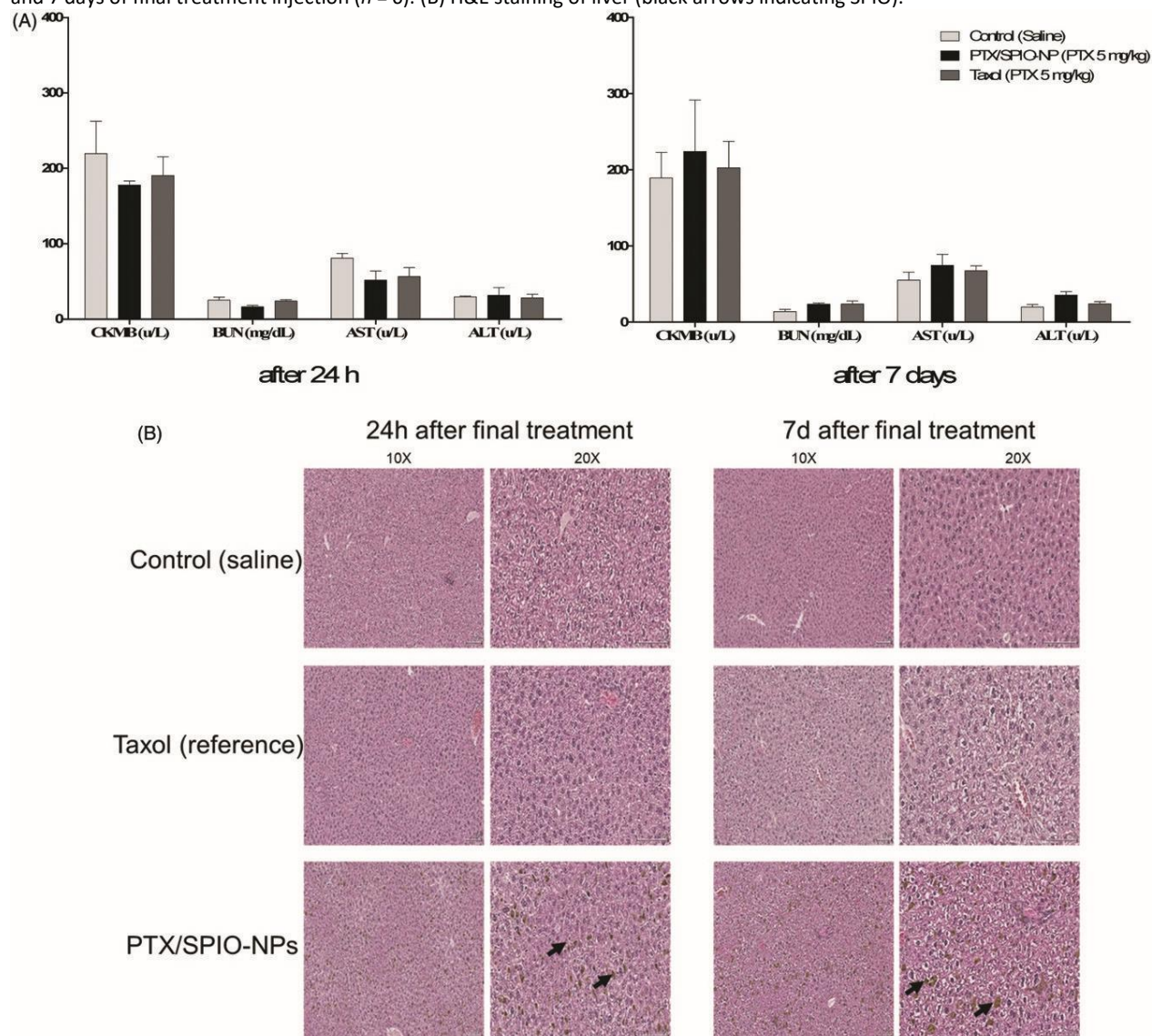


Figure 4(C) shows the survival graph of *in vivo* antitumor efficacy of PTX/SPIO-NPs and PTX/SPIO-RGD-NPs with different targeting strategies. The median survival time of control (saline) group was 38.5 days, passive targeting (PTX/SPIO-NPs) 41 days and magnetic targeting (PTX/SPIO-NPs) 46.5 days while the

median survival of active targeting (PTX/SPIO-RGD-NPs) was 44 days and the combination of active + magnetic targeting was 45 days. At the given doses and in the tested conditions, the magnetic targeting strategy resulted in significant survival of mice compared to control (saline) and passive targeting groups. The result is consistent with our previous report [14]. Inclusion of RGD for active targeting exerted no additional therapeutic effect when tested in combination with the magnetic strategy. However, it showed a higher response than the passive and control (saline) groups.

Figure 5. (A) *In vivo* toxicity evaluation of 6 doses of PTX/SPIO-NPs via biochemical analysis (AST, ALT, BUN and CKMB) after 24 h and 7 days of final treatment injection ($n = 6$). (B) H&E staining of liver (black arrows indicating SPIO).



IN VIVO SAFETY EVALUATION

We have estimated different biochemical parameters such as AST, ALT, BUN and CKMB, to assess potential toxicity on liver, kidney and heart. The results are presented in comparison with that of Taxol (reference) (Figure 5(A)). The full dosage regimen (6 doses) did not induce any significant effect on liver, kidney and heart: there were no significant elevations of biochemical parameters observed within groups after 1 and 7 days. No significant loss in the body weights observed (Supplementary data S4). From our previously reported biodistribution data, the major amounts of administered NPs were accumulated in the liver tissue. Therefore, we analysed the liver tissue. PTX/SPIO-NPs elicited accumulation of SPIO in Kupffer cells and to lesser extent in hepatocytes after 1 and 7 days. In comparison to control (saline) group and Taxol, the tissue samples of PTX/SPIO-NPs group did not show necrosis and inflammation (Figure 5(B)). The histopathological observations were corroborating the biochemical analyses indicating no significant toxicity caused by the NPs in comparison to Taxol.

Discussion

Therapy for GBM is highly challenging, due to its aggressiveness, infiltration into surrounding normal brain tissue and recurrence. Surgical debulking of tumour is not possible in all cases. Consequently, there is need for a realistic approach of targeting GBM more selectively and less invasively by exploiting the abnormalities or differences in the permeability and vasculature of tumour in contrast to the normal brain to improve the amount of drug reaching the GBM. We hypothesised that magnetic and active targeting of PTX/SPIO-NPs would enhance drug delivery to GBM and aimed to compare these different targeting strategies in an orthotopic GBM model.

We have prepared PTX/SPIO-NPs and PTX/SPIO-RGD-NPs with size ranging between 230 and 255 nm and with zeta potential values less than - 15 mV which were reported as relatively stable [41]. The encapsulation efficiency and drug loading were found to be ~ 30% and ~ 1%, respectively. The formulations made of PLGA are usually associated with a poor drug loading [7]. Nevertheless, despite low encapsulation efficiency of PTX, our nanoformulations with PTX were able to deliver therapeutic dose of PTX (5 mg/kg in 150 μ l) U87MG orthotopic nude mouse. Typically, NPs between 20 and 400 nm can accumulate in tumour tissues via EPR effect (passive targeting) [16,17,33,42,43]. Though EPR effect has been highly debated and is controversial, clinical use of various approved nanomedicines for instance, Doxil[®], Abraxane[®] relies on EPR effect [44]. Consequently, the PTX/SPIO-NPs could benefit from the EPR effect. Hence, passive targeting was used as a baseline/control for other targeting strategies. Even if we observed traceable amounts of NPs in the brain, passive targeting did not result in significant prolongation of survival period [14]. This could be due to the fact that insufficient quantities of NPs reach the tumour site.

SPIO in the formulation is exploited for its dual properties as superparamagnetic agent for magnetic targeting and as a T₂ contrast agent for non-invasive imaging with MRI. Magnetic targeting has shown better accumulation of NPs at tumour site via applied external magnetic field in our *ex vivo* biodistribution

study [14]. This was followed by significant inhibition of tumour progression and prolonged *in vivo* efficacy compared to passive targeting and control (saline) groups. However, magnetic targeting could result in more efficacy in regions close to the applied external magnetic field and be limited by distance between the targeted site and applied magnet, time and strength of applied magnetic field and inadequate magnetic gradients [23,27].

Further, to explore active targeting strategy, the NPs were functionalised with RGD peptide. Grafting of RGD in the formulation specifically increased cellular uptake of NPs by TNF α -activated U87MG and HUVEC cells. This can be attributed to RGD affinity towards $\alpha\beta 3$ integrins. $\alpha\beta 3$ integrins were found to be over expressed on tumour cells and endothelial cells lining the tumour [45]. In addition, TNF- α which is present in tumour microenvironment promotes the expression of $\alpha\beta 3$ integrins [35,46,47]. However, RGD targeting did not enhance PTX cytotoxicity *in vitro* compared to untargeted NPs.

In vitro cytotoxicity test indicates that cytotoxic effects were mainly due to PTX. The same was true for the *in vivo* therapeutic efficacy studies. All the treated groups showed better survival than the control (saline) group. In our previous report, we analysed PTX/SPIO-NPs efficacy using passive and magnetic targeting.

The magnetic targeting resulted in significant tumour accumulation and efficacy with the dosage regimen tested [14]. In the current study, we have tested the efficacy of PTX/SPIO-NPs using active and active + magnetic targeting. From the *in vivo* efficacy results, active targeting showed a trend of improved survival compared to control (saline). Our group has reported that the active + magnetic targeting resulted in better survival of mice than the magnetic targeting in the tested CT26 subcutaneous tumour model [27]. We hypothesised that the same result of CT26 subcutaneous tumour model could also be possible in U87MG orthotopic tumour model. However, the result of U87MG orthotopic tumour model was not in full agreement with the hypothesis. Factors such as (i) difference in tumour models and cell lines used (ii) tumour location and barriers to drug penetration (e.g. BBB for GBM tumours) (iii) size of the tumour (iv) $\alpha\beta 3$ receptors expression on tumour endothelial cells and GBM cells might have contributed to the variant result. $\alpha\beta 3$ receptors play a key role in RGD targeting [48]. GBM shows heterogeneous expression of $\alpha\beta 3$ receptors (ranging from mild to very strong) depending on tumour microenvironment and the rate expression of $\alpha\beta 3$ is also associated with the GBM progression [45]. We suspect that the $\alpha\beta 3$ receptors, which are needed for RGD binding, were not sufficiently either available or expressed in the tested tumour model and affected the outcome of the active targeting strategies. To fully evaluate the effects of active targeting strategies, a thorough understanding of *in vivo* expression of $\alpha\beta 3$ in correlation with tumour progression is needed.

Furthermore, balancing the risks and efficacy of therapy is critical. A strategic way to achieve this balance is by tailoring a proper dosage regimen. Additionally, there is a great need to gain deep knowledge about the fate of NPs *in vivo* and their associated effects at key metabolic organs. PLGA-based NPs are well known for their biocompatibility, but it is necessary to ensure that the developed PTX/SPIO-loaded PLGA nanocarriers do not exhibit major toxicological problems over multiple doses, in particular hepatotoxicity due to high accumulation in the liver [14,49]. The dosage regimen was six injections (doses) administered every other 4th day from the first injection. The blood samples were collected 24 h and 7 days after the

last dose of administration. In our previous reports, we have evaluated the toxicity data after single intravenous injection whereas in the current study we evaluated the toxicity of the full dosage regimen to better understand the overall impact of six doses. For single dose testing, a transient increase in AST levels in serum was observed which could be a general short-term response due to the hepatic accumulation of the injected NPs. On the contrary, for six dose testing, no significant changes were read for AST levels. In comparison with that of Taxol (reference), the multiple doses (6 doses) of PTX/SPIO NP did not show any significant effect on the evaluated biochemical parameters and thereby indicated that there were no major toxic effects on liver, kidney and heart.

Conclusion

The aim of the study was to evaluate different targeting strategies and their efficacy for reaching tumour site and exerting antitumour efficacy of PTX, a potent drug against U87GM cell (IC₅₀ 1 ng/ml). RGD grafting increased NPs uptake in activated U87MG and HUVEC cells due to favoured binding to the overexpressed $\alpha\beta 3$ receptors suggesting that RGD could be a potential targeting strategy to enhance the accumulation of NPs in GBM. However, *in vivo* magnetic targeting strategy resulted in better therapeutic effect than the other groups. RGD functionalisation did not exert any significant add-on effects to the magnetic targeting alone.

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Disclosure statement

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