Paclitaxel Loaded Magnetic Nanoparticles Induce In vivo Tumor Growth Inhibition in a Rat Glioblastoma Model

Ramulu Chintala1, Fahima Dilnawaz2, Manas Panigrahi3, Sanjeeb K Sahoo2, Phanithi Prakash Babu1*

1Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India
2Laboratory of Nano-medicine, Institute of Life Sciences, Bhubaneswar-751023, India
3Department of Neurosurgery, Krishna Institute of Medical Science, Secunderabad -500003. India

ABSTRACT
Glioblastoma multiforme (GBM) is one of the most common and lethal diseases and the current therapeutic options remain sparse. The magnetic nanoparticles (MNPs) based drug delivery approach for the cancer treatment has become one of the promising options to achieve controlled drug delivery to target tumor. In the present study the efficacy of paclitaxel loaded glyceryl monooleate (PAC-GMO)-MNPs was evaluated by administering it in a glioblastoma induced rat model. The results of H&E and immunohistochemistry (IHC) suggest that the treatment with PAC-GMO-MNPs significantly reduced or inhibit the tumor growth as compared to the native drug and may be the potential vehicle for efficiently treating GBM.

Keywords: Glioma, paclitaxel, Magnetic nano particles.

INTRODUCTION
GBM is one of the most aggressive type of primary brain tumors with a low survival rate1,2. However, effective treatment is so far not available due to complex nature of the tumor environment. The presence of Blood brain barrier (BBB) limits the effective entry of a majority of chemotherapeutic drugs, and thus severely restricts therapy for glioma. Moreover, these tumor cells are resistant to chemotherapeutic drugs largely due to multidrug resistance (MDR). Conventional methods are applied to deliver different drug molecules to the brain tissues, such as osmotic disruption of the BBB, intraventricular drug injection or local therapy/implants3-5. Nanotechnology based drug delivery approach is one of the upcoming approach for the treatment of brain tumor and neurodegenerative diseases, as the particulate system can cross the BBB with adsorption on the capillary walls, opening of the tight junctions and transcytosis6,7. Therefore, nanocarriers with appropriate surface functionalization, can be effectively used to transport various kinds of molecules across the BBB7,8. Nanoparticulate drug delivery to the brain preferably depends upon the coating of various polysorbates, as it can adsorb the apolipoprotein E from the blood stream onto the nanoparticle surface, and in turn mimic as Low density lipoprotein (LDL) particles, that interacts with the receptors of LDL leading to the uptake by endothelial lining of the BBB4. Many polysorbates as coating materials have been able to cross BBB and are evaluated for the efficacy of brain targeting, out of which polysorbate 80 illustrates better brain targeting effect4. MNPs based drug delivery approach offers the potential treatment due to the biocompatibility, biodegradability, non-toxicity, and the high-level accumulation in the target tissue or organ9. The other advantage in case of MNPs for brain delivery is that significant amount of doses can be accumulated at the tumor site with the aid of an external magnet10. Exploiting this advantage, many investigators have shown vivid examples of drug targeting to its specific sites through magnetic material11,12. Paclitaxel is a chemotherapeutic drug for the treatment of wide range of cancers including brain tumor. However, it is unable to traverse the BBB and attain a therapeutic dose in brain tissues as it is associated with efflux transporters p-glycoprotein (P-gp)13. Recently, researchers adapted diverse approaches for targeting brain tumors as these tumors utilize enormous amount of natural fatty acids for their growth and energy. Therefore, tumors were targeted with natural fatty acid and its synthetic derivatives14. Using prodrug approach, Ke et al. conjugated linoleic acid (CLA) to paclitaxel which showed higher anti tumor activity in brain tumor bearing rats than that of native paclitaxel15. In our previous study we have reported, different hydrophobic and hydrophilic drugs can be entrapped inside the polymeric cages GMO-MNPs and its potential in drug delivery16,17. In another study, we have also demonstrated significant amount of paclitaxel loaded GMO-MNPs can be transported to the brain tissues as compared to native paclitaxel in healthy rat under physiologic condition18. In the present study GMO coated MNPs are very small in size and have the potential ability...
to accumulate inside the tumor tissues by crossing the BBB, and hence the therapeutic efficacy of the paclitaxel loaded GMO-MNPs was evaluated for GBM after systemic administration in a rat glioma model.

MATERIALS AND METHODS

Materials

Iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) 99%, Iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) pure granulated, 99%, ammonium hydroxide, 3-aminopropyl triethoxysilane (saline) were purchased from (Sigma Aldrich, St Louis, MO, USA), and acetonitrile of HPLC grade was procured from E-Merck, India. Alcohol, Xylene, Acetone and other chemicals, inorganic salts used were of analytical grade from (SRL chemicals and Qualigens Fine Chemicals, Mumbai, India). Glycercyl monooleate (GMO) was obtained from Eastman Chemical Company (Memphis, TN, USA). Paclitaxel was purchased from Shaanxi Schiphar Biotech Pvt Ltd, China. Milli Q water purged with nitrogen (N$_2$) gas was used in all steps involved in the synthesis and formulation of magnetic nanoparticles. Stereotaxic apparatus and infusion pump purchased from Stoelting Co. IL, USA.

Preparation of drug loaded magnetic nanoparticles

The preparation of PAC-GMO-MNPs were done following our previous published protocol [16]. In brief, 0.1 M Fe (III) dissolved in 50 ml N$_2$ purged water and 0.1 M Fe (II) dissolved in 50 ml N$_2$ purged water from which, 15 ml of Fe (III) and 7.5 ml of Fe (II) were mixed and heated at 80°C for 10 min under constant stirring with a magnetic stirrer in N$_2$ atmosphere. To that 1.5 ml of ammonium hydroxide (14.5 M) was added drop wise and thereafter, to that, 100 % w/w GMO was added and stirred for 20 min in N$_2$ atmosphere. Finally the precipitate was cooled to room temperature and then washed with ethyl acetate and acetone in the ratio of 70:30 (v/v) twice and finally with N$_2$ purged water, at 20,000 rpm for 20 min at 10°C (Sigma centrifuge, 3-16PK, Osterode, Germany).

The obtained pellets dispersed in Milli Q water, was frozen at – 80 °C and lyophilized with a lyophilizer (LABCONCO Corporation, USA) for two days. For loading of the drug, 100 mg of MNPs particles were dispersed in 10 ml of nitrogen purged Milli Q water, sonicated (VC505, Vibra-Cell, Sonics, Newton, USA) for 1 min at 30 % amplitude and left for stirring. Thereafter, 10 mg paclitaxel (dissolved in ~1 ml acetonitrile) was added drop wise to the dispersion and left for overnight stirring. Next day, the particles were washed with N$_2$ purged Milli Q water for 3 times at 20,000 rpm for 20 min at 10°C (Sigma centrifuge, 3-16PK, Osterode, Germany) and the pellets were lyophilized as stated above and the obtained lyophilized powder was used for further experimental studies.

Physicochemical characterization

The three dimensional surface topology of the paclitaxel loaded magnetic nanoparticles (PAC-GMO-MNPs) was observed by Atomic Force Microscopy (AFM) (JPK Nanowizard II, JPK instrument, Bouchestrasse, Berlin, Germany). For this, a drop of PAC-GMO-MNPs solution in distilled water (~ 1mg/ml) was dropped over freshly cleaved mica and left for 5 min, air dried at room temperature and mounted on microscope scanner and imaged on a contact mode having a frequency of 13 kHz and scan speed of 1 Hz. The amounts of encapsulated paclitaxel in MNPs were estimated by the reverse phase isocratic mode of high performance liquid chromatography (RP-HPLC) as reported by us previously [16]. Briefly, lyophilized PAC-GMO-MNPs was dissolved in acetonitrile (~1 mg/ml), sonicated for 1 min in an ice bath and kept in the shaker at 15°C and 150 rpm (Innova 43 Newbrunswick Scientific, Eppendorf, Germany) for 24 h for proper dissolution of the particulate formulations and complete release of the entrapped drug. After that, the samples were removed and centrifuged at 13,800 rpm for 10 min at 10°C (Sigma centrifuge, 1-15 K, Germany). The supernatant was collected and from this 500 µl was taken out for analysis using (WATERS™ 600 RP-HPLC system. 20 µl of supernatant was injected manually in the injection port, quantified at a flow rate of 1 ml/min with mobile phase of acetonitrile: water, (80: 20 v/v) with a quaternary pump (M 600E) at 25 °C, using C18 column (Nova-Pak, 150 x 4.6 mm, internal diameter) at 228 nm using UV-VIS absorbance detector (M2489). The standard curve of paclitaxel was prepared under identical conditions and the entrapment efficiency was calculated as reported earlier [16].

Cell culture

C6 rat glioma cell lines were procured from National Centre for Cell Science (NCCS), Pune, India. Cells were grown in RPMI media (Gibco, Invitrogen Corp) containing 10% FBS (Invitrogen Corp), 1 % antibiotics i.e., penicillin G (Gibco, Invitrogen Corp) and 100 mg/ml streptomycin (Gibco, Invitrogen Corp) and maintained in CO$_2$ incubator at 5% CO$_2$ (MCO-19M. Sanyo). Healthy 80 % confluent C6 cells were trypsinized and pellet collected, cell count done with haemocytometer.

Development of rat glioblastoma model and treatment

Male Wistar rats (3 months) were procured from Mahaveer agencies, Hyderabad, India. Rats were housed for one week for acclimatization prior to stereotaxic surgery. Rats were deeply anesthetized with ketamine and xylazine (100
mg/kg: 10 mg/kg respectively), after anesthesia animals were fixed on the stereotaxic frame with help of ear bars. Using a surgical blade insertion was made on head and skull was exposed. With the following stereotaxic coordinates (depth 6mm, 0.5 mm posterior, 3mm lateral from bregma), two million C6 cells in a volume of 5µl were implanted in striatum of rat. After implantation of cells skull hole was filled with dental cement and animal was removed from apparatus. 7th day of post-surgery rats were randomly divided into 4 groups, each group having four rats. Group -I received PBS, Group –II received GMO-MNPs alone (vehicle), the Group –III received native PAC and the Group –IV received PAC-GMO-MNPs. Each rat received dose of drug 3 mg/Kg body weight either in native form or equivalent concentration of drug encapsulated in MNPs formulations. Every alternative day from 7th day post-surgery onwards up to 13th day total 4 doses were administered intravenously. On 18th day, rats were sacrificed by deep anesthesia and perfused with normal saline and 4 % paraformaldehyde (PFA). Thereafter, rats were sacrificed and brains were collected in 4 % PFA. Thereafter, brains were processed in graded sucrose solution. Tumor bearing regions of the brain slice were prepared with the help of rat brain matrix and used for sectioning on saline coated slides with Leica cryostat microtome. All animal experiments were performed after institutional ethical committee (IAEC) University of Hyderabad, Hyderabad permission.

Haematoxylin and eosin staining
Sections were stained with haematoxylin and eosin by the following protocol. Sections were rehydrated with graded alcohol (100%, 95%, 70% and deionized water each step for 3 min) then stained with haematoxylin (Fisher Scientific) for 1min and washing with deionizing, tap water for 3 min. later stained with eosin (Fisher Scientific) for 45 sec, then dehydrated with graded alcohol (70 %, 95 % and 100 % for 3 min each), later sections were cleared in xylene for 3 min, mounted with DPX mount (Fine-Chem...

Figure 2: A) Representative pictures of H&E Histological section of glioma rats brains, where tumor regions are identified as predominate cell density with blue color which are shown in circle. Smaller tumor area were observed in PAC-GMO-MNPs group than Native-PAC and Control group. B) Control, Native-PAC and PAC-GMO-MNPs groups, each group animals tumor areas were measured with Image J software and plotted as graph. These three groups tumor areas statistical difference were analyzed by ANOVA all pairwise comparison with Student-newman-keules, where PAC-GMO-MNPs and Native-PAC groups showing significant level smaller tumor areas compare to Control group (*** P<0.001; ** P 0.001 respectively;). The difference between Native-PAC and PAC-GMO-MNPs treated groups also found significant level difference (P 0.033).
Pvt. Ltd) and observed under microscope (Olympus, CKX41, microscope digital camera, Japan).

**Measurement of tumor area**

Tumor bearing region serial haematoxylin and eosin (H&E) stained sections were prepared from each rat brain and analyzed for the cross-sectional area that contained the largest diameter of the tumor and these tumor areas were measured with Image J software.

**Immunohistochemistry**

5μ thickness sections were used for immunohistochemistry analysis, prior to IHC staining tissue sections were dried for 1 hr at 60°C. Sections were rehydrated with graded alcohol and water. Antigen retrieval step was performed for 18 min (6 min x 3 times) with Tris-EDTA buffer (pH 9). Indigenous peroxidase was inhibited with peroxidase blocker as suggested by DAKO company IHC kit K0679 protocol. All Primary antibodies (Anti-Ki67 #AB9260, Anti-VEGF# 05-443, Nestin # MAB353, GFAP# AB5541 were purchased from MILLIPORE and Cleaved Caspase-3 Asp175, Bcl-2, Bax antibodies were procured from Cell Signaling Technology) were used in 1:100 dilutions and incubated for 1 h at room temperature, after primary incubation sections were washed with Tris-Buffered Saline (TBS), Tris-Buffered Saline Tween (TBST) and TBS for 15 min (5 min X 3 times). Then sections were incubated with biotinylated link and streptavidin (5 min X 3 times). Then sections were incubated with Tris-Buffered Saline and mounted with Distyrene Plasticizer Xylene (DPX) mounting media and observed in Olympus light microscope attached with camera.

**Statistical analysis**

Statistical analysis was performed with Sigma plot 11.

**RESULTS AND DISCUSSION**

GBM, is one of the devastating cancers having high mortality rate because it faces unique challenge in comparison to other cancer types19. As GBM tumor tissues are protected externally by the skull, the treatment options primarily include surgery, radiation therapy, chemotherapy and its combinational approach. Further the treatment of GBM has been particularly ineffective due to the existence of BBB along with the blood tumor barrier (BTB) that prevents the entry and accumulation of drugs to tumors at clinically effective concentrations by avoiding nonspecific toxicity remains a great challenge. In this regard, nanotechnology based drug delivery approach has brought a paradigm shift in the diagnosis and treatment of glioma21. Currently much emphasis has been given to MNPs based drug delivery system to achieve selective targeting towards the intended site and augment the therapeutic efficiency and avoid the off-target tissue toxicity5. MNPs as drug delivery system are unique approach for the GBM therapy. MNPs are illustrating significant potentiality as a drug delivery vehicle and represent a unique treatment option for complicated brain cancer. Paclitaxel is one of the most efficient anticancer drugs for the treatment of varieties of cancers i.e. breast, ovarian, lungs, kaposi sarcoma brain etc. Earlier our group has reported the higher cytotoxic activity of paclitaxel loaded-GMO-MNPs in the *in vitro* studies16. The formulated PAC-GMO-MNPs showed ~ 90% entrapment efficiency as estimated by RP-HPLC. The surface morphology of PAC-GMO-MNPs was exemplified to be having smooth and rounded topography as demonstrated by the AFM (Fig 1).

GBM animal models are developed in order to understand the disease progression, possible interference and therapeutic down regulation of tumor progression22. In this particular study the therapeutic efficacy of PAC-GMO-MNPs were evaluated using glioma animal model. Here the Pac-GMO-MNPs treated group illustrated significant smaller tumors as compared to Native-PAC and control (vehicle) treated group (Fig 2 A). This initial result suggested the efficacy of delivery of paclitaxel to the tumor when it is loaded into the GMO-MNPs. Apart from that, the measurement of tumor area by imageJ software depicted the reduction of tumor area in PAC-GMO-MNPs treated rats than that of native-PAC which further corroborated the effectiveness of the drug loaded MNPs formulation (Fig. 2 B).

To further validate this study we performed immunohistochemistry (IHC) staining on tissue sections with specific molecular markers related to cell proliferation, angiogenesis and cell death. The IHC analysis were done through specific molecular biomarkers, i.e antibodies that are characteristics of particular cellular events such as proliferation or cell death for the diagnosis of abnormal cells that are found in tumorigenic tissues. Cellular proliferation is the primary feature during gliomagenesis and Ki-67 is one of the classical proliferation markers for GBM. Ki-67 is a nuclear protein expressed during the various phases of the cell cycle widely and hence used as a stable marker of cell proliferation in various types of human tumors, including GBM23. Assessment of cell proliferation was done with a nuclear antigen Ki-67, which is basically expressed in all phases of the cell cycle except the resting G0 phase; therefore, it is a good marker for the proliferating cells24. Control rats have more Ki-67 nucleus stain, than that of native-PAC and PAC-GMO-MNPs. In PAC-GMO-MNP less number of nuclear stained cells were observed, illustrating the growth arrest of cells at G0 phase (Fig. 3). Glial fibrillary acidic protein (GFAP), member of cytoskeletal protein family is widely detected in GBM25. Therefore, GFAP was investigated as tumor marker due to its high expression in astroglial tumors, such as astrocytoma and glioblastoma26. There was no significant difference in the staining of GFAP in control, native-PAC and PAC-GMO-MNPs (Fig. 3). Nestin the intermediate filament protein was another marker for invasiveness evaluated for the neural stem cells and mostly expressed in all GBMs27. The invasiveness of GBM is partly responsible for the shorter survival and possess a critical challenge in the treatment and management of GBM23. High level of nestin expression in control rats indicated invasive nature of the developed glioma, whereas, less intense staining of nestin in native-

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PAC and least staining in PAC-GMO-MNPs indicated the presence of residual glioma cells probably due to tumor involution (Fig. 3). This is inconsistency with another study, wherein Bernadi et al also observed less intense nestin staining in Indomethacin loaded nanocapsules treated rats probably as a result of tumor involution. Angiogenesis in tumors is caused by a number of factors, in which the vascular endothelial growth factor (VEGF) plays an important role and a potential prognostic factor for several types of cancer including GBM. VEGF overexpression in control groups demonstrated intense staining, than that of native-PAC and PAC-GMO-MNPs. Considerable inhibition of angiogenesis in PAC-GMO-MNPs treated groups led to least staining of VEGF, depicting growth inhibition of the tumor (Fig. 3). Administration of PAC-GMO-MNPs resulted in inhibition of proliferation as well as inhibition of angiogenesis which is in agreement with nanoparticulate mediated study conducted by various groups reported the substantial inhibition of angiogenesis using doxorubicin loaded in polysorbate 80-coated poly-butylcyanoacrylate (PBCA) nanoparticles and decreased vessel density in poloxamer 188 coated doxorubicin loaded poly lactide-co-glycolide (PLGA) nanoparticles to GBM rats. The active form of caspase-3 was explored for the detection of apoptotic event. This protease has been implicated as an effector and important marker for initiation of apoptotic event. As evident from Fig. 3 the
control group of rats has less staining of caspase-3 indicating very less cell death. In native-PAC treated groups showed increased immunostaining in more number cells compared to control whereas, in PAC-GMO-MNP treated groups more number of cells were stained intensively for caspase -3 illustrating higher number of cells have undergone cell death (Fig. 3). The apoptosis is mostly regulated by the members of the Bcl-2 protein family. The inactivation of Bax leads to rapid tumor growth and the decrease extent of inactivation leads to spontaneous apoptosis of tumor cells\cite{15}. In our observation control group and native-PAC greater numbers of cells were faintly stained, whereas in PAC-GMO-MNPs greater numbers of cells were intensely stained for Bax suggesting the activation of Bax leading to apoptosis. Whereas, in case of Bcl-2 protein, PAC-GMO-MNPs treated groups were faintly stained than that of native-PAC and control groups, implying the initiation of the apoptotic event. Taken together the intense staining of proapoptotic proteins caspase-3 and Bax protein and less intense staining of anti apoptotic protein Bcl-2 promote cell death significantly in PAC-GMO-MNP treated glioma induced rat model (Fig. 3). Our findings are in line with previous reports of targeted efficacy of surfactant coated drug loaded nanoparticles in the brain against various disease including the GBM\cite{6,7,8,9,10,11,12,13,14,15,16,17,18,19,20}. Nance et al, observed delayed tumor growth of GBM using nanof ormulation of paclitaxel and triggered cell death. Therefore, the developed MNPs have demonstrated enhanced therapeutic efficacy as drug delivery vehicle for MRI monitored magnetic brain barrier by nanoparticles. J Control Release. 2012., 161:264-73.

CONCLUSIONS

Administered PAC-GMO-MNPs with high drug loading capacity demonstrated enhanced therapeutic efficacy as observed in a GBM rat model using H&E and IHC analysis approach as compared to native paclitaxel. Therefore, we envisage that this drug loaded GMO-MNPs could play an essential role in the treatment of chemotherapy.

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