Ultrasound Activated Nano TiO_2 Loaded With Temozolomide Paves the Way for Resection of Chemo-resistant Glioblastoma Multiforme

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Abstract

Background

Glioblastoma Multiforme (GBM) is one the most daunting issue to modern therapeutics, with a higher mortality rate post-diagnosis. Temozolomide (TMZ) is the only available treatment; however, the frequent resistance leaves the oncologists at the dead end. Therefore, new approaches to circumvent the GBM are highly desired. In this contribution, we have employed TiO_2 nanosticks loaded with TMZ as nanomedicine for TMZ resistant GBM resection.

Results

The ultrasonication triple action effect could highly facilitate the tumor ablation by enhancing the TiO_2 nanosticks traversing across BBB, releasing the TMZ payload from TiO_2 nanosticks and Reactive Oxygen Species (ROS) generation from TiO_2 nanosticks within GBM milieu. The tumor ablation was confirmed by MTT and Annexin(v)-PI assays, apoptotic proteins expression via western blot and ROS level detection *in vitro*, whereas tumor volume, weight, survival rate, and relative photon flux in the xenograft and orthoptic TMZ resistant GBM murine models as *in vivo*.

Conclusion

We found this nanomedicine-based ultrasound modality highly efficient in GBM treatment and is of future clinical application value due to employment of already FDA approved techniques and nanomedicine.

Background

Glioblastoma is a major type of glioma that primarily affects the central nervous system and known with highly engraved prognosis and postdiagnosis patient survival for less than 15 months(Xu, Chen et al. 2017, Patel, Fisher et al. 2019). Temozolomide (TMZ) is the only Food and Drug Administration (FDA) approved therapeutic agent for GBM that could merely add several months to the survival of patients and is mostly used as adjuvant therapy after surgical resection of the tumor(Chamberlain 2010). In addition, the suboptimal concentration of TMZ at the tumor site, frequent development of chemoresistance and the blood-brain barrier (BBB) selective amenability, are some of the major bottlenecks in complete resection of GBM(Haar, Hebbar et al. 2012, Casals, Gusta et al. 2017, Bahadur, Sahu et al. 2019).

The BBB is a physiological barrier comprised of endothelial cells having tight junctions, basal membrane, and podocytes of astrocytes(Pardridge 2007). The primary mandate of BBB is the central nervous system homeostasis and protection against potentially toxic substances. The BBB is only amenable to small molecules (i.e., <400 Da size and <9 hydrogen bonding, CO_2, O_2, Alcohol, and Glucose etc.)(Abbott, Rönnbäck et al. 2006) and it constrain 98% of all other biochemicals and drugs that may be efficient

therapeutic agents in other parts of the body(Mitragotri 2013). Therefore, modalities that increase the BBB amenability for the cure of CNS ailments are highly valued. For instance, the focused ultrasound has been reported to open the BBB via micro/nanobubbles formation(Bing, Hong et al. 2018, Wu, Aurup et al. 2018) and allow maximum drug accumulation in the brain. Recently, Liu et al. employed focused ultrasound to reversibly break the BBB and enhance the TMZ localization in GBM from 6.98 to 19 ng/mg(Liu, Huang et al. 2014). Likewise, during the phase I clinical trial, Lipsman et al. employed focused ultrasound to open BBB in Alzheimer's disease (AD) patients that consequently lowered the β amyloid plaques and tau protein aggregates resulting in AD's amelioration(Lipsman, Meng et al. 2018).

The nanotechnology-based approaches have also been more efficient in drug delivery to targeted tissues (viz brain) as compared to free drugs(Srikanth and Kessler 2012, Mi, Shao et al. 2016, Rehman 2020, Younas Iqbal, Wang et al. 2020). For instance, nanoscale TiO_2 has been reported with promising biomedical applications and higher biocompatibility that has already been recognized by FDA(Rehman, Zhao et al. 2016, Youssef, Vanderesse et al. 2017). The TiO_2 nanosticks provide a large surface area and efficient scaffold for drug delivery due to their porous nature. Moreover, upon excitation with ultrasound waves, the nanoscale TiO_2 can produce reactive oxygen species (ROS), including $\cdot OH$, H_2O_2, HO_2, $^1O_2$, and O_2^-, which can efficiently induce apoptosis in the tumor by interfering with cellular signaling pathways(Zhao, Ur Rehman et al. 2015). Recently, Harada et al. reported that nanoscale TiO_2 could generate the singlet oxygen ($^1O_2$) within HeLa cells and thus exert cytotoxic effects leading to apoptosis(Harada, Ono et al. 2013). Similarly, Deepagan et al. used Au-TiO_2 nanocomposites to ablate tumor via sonodynamic therapy (Deepagan, You et al. 2016), whereas Ninomiya et al. used only TiO_2 nanoparticles to arrest the growth of the HepG2 cells by 46% after sonication(Ninomiya, Noda et al. 2014).

To establish a robust nano-drug delivery system that could efficiently deliver the therapeutic cargo to GBM across the BBB and could also resect the TMZ-resistant GBM, we established the TiO_2 nanosticks based drug delivery system for TMZ delivery to GBM. Moreover, the ultrasound triple action trigger could open the BBB and further added to the release of TMZ within tumor milieu, and also generated the ROS for the re-sensitization of GBM to TMZ.

Results

Nanomedicine properties

The SEM confirmed elongated morphology of TiO_2 nanosticks **(Fig. 1 a)**, whereas the DLS analyzed the average hydrodynamic size of 210±23 nm size **(Fig. 1 b)**. The TEM releveled the pours nature of the TiO_2 nanosticks that played an essential role in the drug loading, as shown in **Fig. 1b inset**. The zeta potential of TiO_2 nanosticks was 15±0.89, which shows good dispersibility of the as-prepared nanomedicine **(supporting Fig. 1)**.

Drug loading/release ability

The TiO_2 nanosticks were mixed with TMZ and incubated overnight at various ratios of 1:1, 2:1, 3:1, respectively. It was observed that 3:1 (TiO_2 nanosticks: TMZ) could produce the highest loading of drugs, i.e., 35±3.06%, followed by 31±2.98 and 28±3.43 percent for 2:1 and 1:1 respectively **(Fig. 1c)**. During the drug release study, it was found that after 36 hours, 53±7.48% of the drug was released, and no further significant increase was observed until 48 hours **(Fig. 1d).**

After the ultra-sonication, the TMZ release from TiO_2 nanosticks was evaluated. It was observed that after a one-hour time point, the sonication could trigger 18±4.89 % of TMZ release as compared to the non-sonicated that could only 8±4.21%, indicating that sonication could trigger TMZ release 2 x higher than non-sonicated in a given time **(Fig. 1e).**

In vitro anticancer effect

MTT assay was performed to see the anticancer effect of ultrasound triggered TiO_2-TMZ nanomedicine. It was observed that TiO_2-TMZ could induce higher cell apoptosis 52.0±13.01% at 10 µl of nanomedicine treatment as compared to TiO_2 alone that remained around 22±9.90% at various consternations, in U251-TMZ resistant cells **(Fig. 2a, b)**. The TiO_2-TMZ and TiO_2 alone treatment could also induce apoptosis in TMZ sensitive GBM cells (U87) **(supporting Fig. 2)**. Meanwhile, the non-sonicated nanomedicine could not exert any significant cytotoxic effects in U251-TMZ resistant cells, i.e., cell viability remained above 90% **(supporting Fig. 3).**

The western blot analysis was performed for apoptosis-related vital proteins expressions (P53 and Bcl-2) in TMZ resistant GBM cells after treatment with TiO_2-TMZ and TiO_2 alone, post sonication. It was observed that TiO_2-TMZ could produce a higher expression of p53, which is essential for apoptosis induction, whereas the Bcl-2 expression was significantly lowered as compared to TiO_2 treated cells. The Bcl-2 is known for its anti-apoptosis function, and its down regualtion indicates the apoptosis induction **(Fig. 2c)**.

The annexin(v)-PI assay was also performed for apoptosis induction in the U251-TMZ resistant cells. The flow cytometry data revealed that ultrasound activated TiO_2-TMZ could produce higher apoptosis as compared to TiO_2 nanosticks alone and non-sonicated treated groups **(Fig. 2d, Supporting Fig. 4).**

Oxidative stress generated by nanomedicine

The DCFDA was used as a ROS marker of the oxidative stress in U251-TMZ resistant cells after treatment with TiO_2-TMZ and TiO_2 in sonicated, and non-sonicated group. The confocal fluorescence microscopy revealed that TiO_2-TMZ could generate a significantly higher number of ROS as compared to non-sonicated and TiO_2 alone **(Fig. 3 a, b supporting 5)**. Besides, the ROS intensity was also evaluated via flow cytometry analysis that also exhibited higher fluorescence in TiO_2-TMZ treated group after

sonication **(Fig. 3 c)**. Meanwhile, the SOD level was also examined in the treated cells. The SOD level was significantly lowered in the TiO_2-TMZ treated group after sonication as compared to non- sonicated one **(Fig. 3d)**.

In vitro BBB crossing ability

The BBB crossing ability of TMZ-TiO_2 nanomedicine pre and post sonication was evaluated via the transwell BBB model **(Fig. 4a)**. The EDS data revealed that TiO_2 nanosticks could readily cross the BBB after sonication and were uptaken by the U251-TMZ resistant cells, as shown in **Fig. 4b, c**. Moreover, the TiO_2 nanosticks were loaded with Doxorubicin as a model drug having fluorescence properties. When the TiO_2-Dox and free Dox treatment was given to the transwell BBB model, the sonication could boost the TiO_2-Dox BBB traversing, which was evidenced by the fluorescence intensity in the confocal fluorescence micrographs **(Fig. d).**

Animal model survival post-therapy

The subcutaneous xenograft mice model was initially prepared with TMZ resistant glioblastoma cells and divided into three groups of TiO_2-TMZ, TiO_2, and control. After relevant treatments and sonication, it was observed that TiO_2-TMZ could circumvent the tumor growth and significantly lower the tumor volume as compared to other TiO_2 alone and PBS treated groups **(Fig. 5 a, b).** The tumor weight was significantly lowered in TMZ-TiO_2 treated groups, i.e., 0.1±0.23 g, as compared to TiO_2 (0.81±0.44) and PBS group (2.0=-0.89) after sonication **(Fig. 5 c).** Likewise, the body weight gain was higher in TMZ-TiO_2 treated group as compared to TiO_2 and control **(Fig. 5 d).**

Similarly, the tumor volume was also lower in the TIO_2-TMZ treated models are compared to other treated groups **(supporting Fig. 6).** The biodistribution data of vital body organs showed higher TMZ accumulation in the liver, i.e., the elimination route for the TMZ from the body, followed by the tumor and brain **(Fig. 5 e).** The vital organ histopathology also revealed no pathological lesions after treatment and sonication that vouch for the biocompatibility and inertness of the as-prepared nanomedicine **(supporting Fig. 7).**

The orthotopic TMZ resistant GBM tumor expressing luciferase was prepared and treated with TMZ-TiO_2 at day ten till day 21 on alternate days, as shown in the experimental layout **(Fig. 5f)**. Meanwhile, every next day of treatment, mice were imaged for bioluminescence, which was directly proportional to the tumor volume **(Fig. 5g)**. The relative photon flux data showed that TiO_2-TMZ after sonication could circumvent the tumor volume more efficiently than other treated groups **(Fig. 5h).** The animal treated with TMZ-TiO_2 had the highest survival rate (56 days) post-therapy as compared to other groups **(Fig. 5i)**.

Discussion

GBM is one of the fatal brain diseases. Its complete resection is still a challenge with only TMZ as available chemotherapeutic agent. In addition, the frequent resistance development to TMZ has further complicated GBM cure(Casals, Gusta et al. 2017). In this contribution, we have employed ultrasound activated TiO_2 nanosticks loaded with TMZ to resect the TMZ resistant GBM *in vitro* as well as *in vivo*. The ultrasound trigger has a triple action effect aiding to GBM resection by (i) opening BBB, (ii) releasing TMZ payload from TiO_2 nanosticks, and (iii) ROS generation from TiO_2 nanosticks that helps in re-sensitization of GBM to TMZ(You, Deepagan et al. 2016). Previously, the nanoscale TiO_2 sonication at 1.0 MHz frequency has been reported with efficient intratumor ROS generation that inhibited cell viability and tumor growth(Harada, Ogawa et al. 2011). Herein, we have employed 1.5 MHz frequency ultrasound that could efficiently penetrate the skull bones of orthotopic GBM models. The typical diagnostic and materials application ultrasound are ranged from 1-10 MHz frequency(Silva, Ferreira et al. 2011). Hence, the employed ultrasound modality is in line with therapeutic ultrasound *in vogue*.

The TiO_2 nanosticks can upload maximum TMZ at a ratio of 3:1. The TMZ has been adsorbed on the porous surface of TiO_2 nanosticks after overnight incubation, the same as previously reported(Rehman, Zhao et al. 2016). Interestingly, it was observed that ultrasonication could trigger the drug release much faster than non-sonicated TiO_2. Likewise, Shi et al. loaded Docetaxel on the mesoporous TiO_2 nanoparticles for the treatment of cancer via sonodynamic therapy(Shi, Chen et al. 2015). It was observed that Docetaxel loaded TiO_2 could significantly deliver and release the drug in tumor site after sonication and exhibited excellent anticancer efficacy. Moreover, the TMZ has a natural ability to cross the BBB, as earlier reported(Brun, Carrière et al. 2012). However, when the ultrasound is employed to the BBB, the nanomedicine traversing becomes faster with maximum drug bioavailability in brain tissue.

This study has used two-step ultrasonication in orthotopic animal models; the first one is to open the BBB for nanomedicine traversing that ensures maximum drug accumulation within the brain tissue. The second sonication ensures the swift releases of TMZ from TiO_2 nanosticks and also start ROS generation. The ultrasound activated TiO_2 can generate OH, $^1O_2$, H_2O_2, etc. that interferes with cell signal pathways and thus initiates apoptosis or necrosis within the target tissue(Ninomiya, Ogino et al. 2012). In this study, when TMZ resistant GBM cells were treated with as prepared nanomedicine, the sonication effect could produce less than 50% cell viability.

Moreover, the critical apoptosis markers (P53 and Bcl-2) were significantly influenced by the sonicated nanomedicine. Earlier Zhang et al. reported that TMZ induces O^6-methylguanine production that is involved in AMP-activated protein kinase (AMPK) activation via an elevated level of ROS generated. The AMPK is involved in the GBM apoptosis induction via p53 upregulation. Meanwhile, by inhibition of mTOR complex 1, the Bcl-2 protein expression is downregulated, which is further inducing TMZ mediated pro-apoptotic effect(Zhang, Wang et al. 2010). Likewise, Kim et al. also reported the tumor targeting p53 nano drug delivering system with enhanced sensitization of chemoresistant GBM to TMZ therapy(Kim, Rait et al. 2015). In analogy to TMZ, the TiO_2 also generates ROS that results in AMPK activation and apoptosis induction in TMZ resistant GBM. The sonoluminescence is considered a critical phenomenon

to generate ROS from nanoscale TiO_2(Hu, Fan et al. 2015), that on the one hand, induces apoptosis in the tumor(You, Deepagan et al. 2016) and on the other hand destroy the tumor vasculature endothelial layer or blood stasis via platelet aggregation(Borsig, Wong et al. 2001, Volanti, Gloire et al. 2004).

In a recent study, Feng et al., employed the NAMPT inhibitors (FK866, CHS828) along with TMZ to sensitize the GBM cells to TMZ(Feng, Yan et al. 2016). In addition to other factors, these NAMPT inhibitors could elevate the ROS level and reduced the SOD and total antioxidants activity in GBM cells that could sensitize the GBM cells to TMZ. Likewise, Seyfrid et al. employed Smac mimetic (BV6) along with TMZ to sensitize the GBM to chemotherapy(Seyfrid, Marschall et al. 2016). The combination therapy of BV6-TMZ could orchestrate the ROS generation in the mitochondria and cytosolic contents of GBM. These ROS could then activate the pro-apoptotic factors viz Bax protein upregulation to resect GBM. It has been investigated that TMZ could induce cytoprotective autophagy in GBM cells, thus leading to compromised TMZ sensitivity. However, when mitochondria transport chain inhibitors are combined with TMZ as adjuvant therapy, the autophagic cell death is mediated by ROS(Chen, McMillan-Ward et al. 2007), and GBM sensitivity to TMZ is significantly augmented(Yan, Xu et al. 2016). Our findings are also in agreement with the aforementioned results suggesting that elevated ROS level and decreased antioxidant level (i.e., SOD) ensures the re-sensitization of GBM to TMZ. Thus, apoptosis is initiated in the GBM.

Conclusion

In summary, we report that TiO_2 nanosticks cloud significantly delivers the TMZ payload to brain milieu across the BBB and resect the TMZ-resistant GBM. The ultrasonication triple action effect could open the BBB, release the TMZ from TiO_2 nanosticks, and mimic the ROS generation within GBM. The generated ROS enhances the tumor sensitivity to TMZ that resulted in apoptosis induction and tumor growth arrest *in vitro* as well as in xenograft and orthotopic TMZ resistant GBM models. The reported modality is novel and reported for the first time (as per our knowledge).

Methods

All the chemicals used in this study were experimental grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA), otherwise mentioned. The Milli-Q deionized water with -18mΩ/cm^2 was used to prepare chemical reagents. HyClone Inc USA, provided the cell culture media and reagents, whereas the cell culture flasks were purchased from Nest biotechnologies Wuxi, China. The TiO_2 nanosticks were kindly provided by Dr. Xiao Hua Lu, College of Engineering, Nanjing University of Technology, Nanjing China.

All the animals were provided with pallet feed and water ad libitum in an environmentally controlled house. All the experiments were performed under the guidelines of Henan University animal welfare committee.

Materials Characterization

The TiO_2 nanosticks were morphologically characterized by Scanning and transmission electron microscopy (Jeol, the USA, and JEM-2010HT Japan, respectively). The size and zeta potential of materials was performed by using Zetasizer Nano (Malvern Pananalytical Ltd.). The Energy Dispersive X-ray Spectroscopy (EDS) was performed by SEM to see cell uptake of TiO_2 nanosticks after crossing BBB *in vitro*.

Nanomedicine preparation

The TiO_2 nanosticks and TMZ were dissolved in the deionized distilled water to obtain the final concentration of 0.5 mg/ml and 0.15 mg/ml, respectively. The two solutions were then mixed and kept at room temperature on a magnetic stirrer for overnight rotation. Then the solution was centrifuged at 8000 X g for 10 minutes to remove unbound TMZ from TiO_2 nanosticks. The TiO_2 nanosticks were then washed with deionized distilled water and used in further experiments.

Drug loading and releases

The TiO_2 nanosticks and TMZ were dissolved in deionized water and then mixed at the various ratios of 1:1, 2:1, and 3:1. The TMZ concentration was kept constant, whereas the TiO_2 nanosticks ratio was variable. The TiO_2-TMZ mixture was kept on rotation for overnight at room temperature and then centrifuged at 8000 X g to remove untrapped TMZ. The TMZ absorption value was determined at 327 nm to determine the TMZ uploading value on TiO_2 nanosticks.

The release kinetics of TiO_2-TMZ nanomedicine were performed at 37 ^oC in PBS. At various time points of 0.5, 1, 2, 4, 8, 12, 16, 24, 36, 48, and 72 hours the centrifugation was performed (8000 X g), and TMZ concentration was measured by taking absorbance value at 327 nm. For sonication effect on TMZ releases, at a time point of 1, 4, 12, 24 and 48 hours, after incubation, the sonication was performed, i.e., 1.0 Watt/cm^2, 1.5 MHz frequency with 50% intensity by intellect mobile ultrasound (Chattanooga®, GLOBAL DJO HEADQUARTERS 2900 Lake Vista Drive Dallas, TX 75067) and then reading for TMZ concentration was performed.

Cell culture experiments

The U87 cell line of glioblastoma and b.End3 cell line of endothelial cells were provided by the Chinese Academy of Sciences, Shanghai, China. The U251 cells were TMZ resistant and transfected with luciferase were obtained from iCell Biosience Inc., Shanghai China. The cells were cultured in the 25 cm^2 tissue culture flasks in DMEM high glucose medium supplemented with 10% FBS and 1% penicillin-streptomycin solution under standard incubation conditions of 95% relative humidity at 37 ^oC temperature, in the presence of 5 % CO_2. When the confluency reached 90%, the cells were trypsinized with 0.25% trypsin containing EDTA and were further utilized for downstream experiments.

Western blot

The western blot technique was employed to evaluate the apoptotic protein biomarkers (Bcl-2 and p53) and housekeeping genes β-actin by the procedure mentioned earlier(Haney, Klyachko et al. 2015). The Bcl-2 and p53 primary rabbit anti-human antibodies were provided by Biolegend and diluted to a concentration of 1:5000, whereas secondary antibodies (IRDye 800CW goat anti-rabbit secondary antibodies) were provided by LI-COR Biosciences U.S. The protein bands, were visualized by Odessey® Clx western blot scanner (LI-COR Biosciences - U.S.) and processed by image studio software.

Flow cytometry for Apoptosis

U251-TMZ resistant cells were cultured in 10 cm Petri dishes for 24 hours under standard conditions. The cells were divided into five groups, i.e., PBS, TiO_2 only, TiO_2-TMZ only, TiO_2 sonicated and TiO_2-TMZ sonicated, and treatment was given for another 12 hours. Meanwhile, after one hour of inoculation, the two groups, i.e., TiO_2 sonicated and TiO_2-TMZ sonicated, were sonicated for one minute. Afterward, the cells were trypsinized and treated with Annexin(v)-PI reagent (Beyotime Biotech Inc.) for cell apoptosis evaluation according to manufacturer instructions. The cells were then immediately analyzed through the BD FACSAriea flow cytometer for apoptosis detection.

MTT assay for cytotoxicity

U251-TMZ resistant cells were cultured in 96 well plates at an equal concentration of 1 $x10^3$ cell per mL for 24 hours. The cells were treated with TiO_2 only, TiO_2-TMZ, sonicated, and non-sonicated groups, whereas the control group was treated with PBS for 24 hours with concertation from 1 to 10 µg of nanomedicine per well. The ultrasound group was sonicated for one minute after one hour of nanomedicine inoculation by sonication procedure mentioned earlier. Afterward, 20 µl of 5 mg/mL of MTT solution was added into each well and incubated for 4 hours. Then the medium was discarded, and 200 µl of DMSO was added to each well and further incubated for 10 minutes at room temperature. The plates were then subject to an optical density (OD) reading at 492 nm wavelength by ELISA microplate reader (SpectraMax® i3x, Molecular Devices, LLC).

The following formula evaluated the cell viability:

Cell viability (%) = OD value of treatment/OD value of Control X 100

Confocal microscopy

The U251-TMZ resistant cells were cultured for 24 hours on the confocal microscopy Petri dishes provided with lens at bottom. The cells were then divided into two main groups, i.e., sonicated and non-sonicated. These groups were further divided into TiO_2, TiO_2-TMZ, and PBS. Each group was inoculated with corresponding nanomedicine @ 10 mg/mL and further incubated for 12 hours; meanwhile, the sonicated group was ultrasound treated one-hour post nanomedicine inoculation. The cells were then

treated with 2',7'-dichlorodihydrofluorescin diacetate (DCFDA) reagent (Invitrogen^{TM}) 1% solution for one hour. Afterward, the cells were washed with PBS 3 times and added DAPI solution 1:1000 for five minutes to stain the cell nucleus. Again, after washing, the cells were fixed with paraformaldehyde 4% for 10 minutes. Then washed again with PBS and imaged under a confocal microscope (Zeiss LSM 880) at the FITC channel, whereas the cell nucleus was imaged at DAPI channel.

For quantitative ROS analysis, the DCFDA treated medium from cells was collected after one hour of incubation, and fluorescence was measured at 488 nm wavelength by using SpectraMax® i3x, Molecular Devices, LLC.

In vitro BBB model preparation

The endothelial cells were cultured on the transwell membrane (Millicell®, 100 nm pore size) until the complete cell confluency was achieved, and the cells' conductivity resistance become above 300 ohms. Then U251-TMZ resistant cells were cultured on the lower chamber of the 12 well plate containing round glass coverslips and treatment of (Doxorubicin) DOX, TiO_2-DOX, and PBS was given to sonicated and non-sonicated group @ 10 mg/mL of corresponding nanomedicine. After one hour, the sonicated group was ultrasound treated as mentioned above and incubated under standard incubation conditions with 100 rotations per minute (to generate sufficient shear force) for 12 hours. Later the cell culture medium was removed and washed with PBS, then added 1:1000 DAPI solution for 5 minutes to stain the cell nucleus. Afterward, the cells were washed with PBS and fixed with paraformaldehyde 4% for 10 minutes. The glass coverslips were removed from the 12 well plates and mounted on the glass slide to observe under a confocal microscope.

SOD activity

The U251-TMZ resistant cells were cultured in six-well plates. When the cell confluence reached 90%, they were divided into two groups, i.e., sonicated and non-sonicated. Both groups were separately treated with TiO_2 and TiO_2-TMZ @ 10 mg/mL, whereas the PBS group was kept as control. The sonicated group after one hour was treated with ultrasound, as mentioned above. After overnight incubation, the Superoxide Dismutase level was determined in each group from the cell lysate by the commercially available kit (SOD Assay Kit- WST, Dojindo Molecular Technologies, Inc.) following the manufacture instructions. Microplate reader was used to read the optical density value at 450 nm wavelength.

Xenograft and orthotopic animal models preparation

Luciferin expressing U251-TMZ resistant cells were cultured in the 75 cm^2 culture flasks. After attainting 90% confluency, the cells were trypsinized (0.25% trypsin), and collected in pellet form. These cells were then injected to BALB/C athymic nude mice @ 3 x10^6 cells subcutaneously to form tumors. After two weeks, a palpable size of tumor was noticed. These mice were then used for further experiments, whereas few were euthanized to obtain the tumor for orthotopic model preparation.

The tumor was chopped to small size in normal saline and injected to a mouse brain that was exposed by skull puncture under general anesthesia of isoflurane. The skull bone was then sealed with a bone sealant (CP Medical), and the wound was sealed via tissue adhesive glue (Vetbond^{TM}, 3M, Japan). On day 3, mice were imaged for bioluminescence by IVIS-Lumina Series III, PerkinElmer Inc, after injection of D-luciferin solution (5 mg/mL). The successful models showing bioluminescence were further selected for experimental trials.

Animal treatment

The subcutaneous xenograft GBM TMZ resistant mice were divided into three groups (i.e., TiO_2, TMZ-TiO_2, and a control group treated with PBS. N= 5x3). At day 21 of the first implant, the mice were treated with as-prepared TiO_2 and TMZ solution @ dose rate of 200 µl, either separately or in combination, every alternate day for 15 days. Thirty minutes post-injection, ultrasound therapy was given at the tumor site. Then mice were euthanized, and vital organs (liver, kidney, spleen, lungs, brain) and tumor were removed and further analyzed.

The three groups of orthotopic mice were injected with the nanomedicine (200 µl) on day 10 to 22, every alternate day. Post-injection every 10 minutes, followed by 30 minutes, the ultrasound therapy at the head region was performed (N=7x3). Meanwhile, the mice were imaged for bioluminescence every next day of treatment during the experimental trial, and the relative photon flux value was recorded. Animal survival was also recorded daily.

Histopathology

The mice's vital organs were collected and preserved in the 10% formalin solution. Then paraffin embedding technique was used to prepare 3 µm slides and stained them with Hematoxylin & Eosin, and observed under an Olympus fluorescence microscope for histopathological lesions observations.

Statistical analysis

All the data was initially recorded in the MS-Excel and then subjected to analysis of variance (ANOVA) by using statistical software named SPSS version 18 (SPSS Inc. Chicago Illinois USA). All results presented were in mean ± standard deviation (SD). The probability value < 0.05 was considered as statistically significant.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data sets generated and/or analysis during this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests

Funding

Not applicable

Authors' contributions

FU Rehman and MA Rauf designed and conducted the experiments. S Shaikh and A Qambrani performed the cell experiments. P Muhammad and S Hanif preformed the animal experiments. FU Rehman also analyzed the data and prepared the manuscript.

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Figures

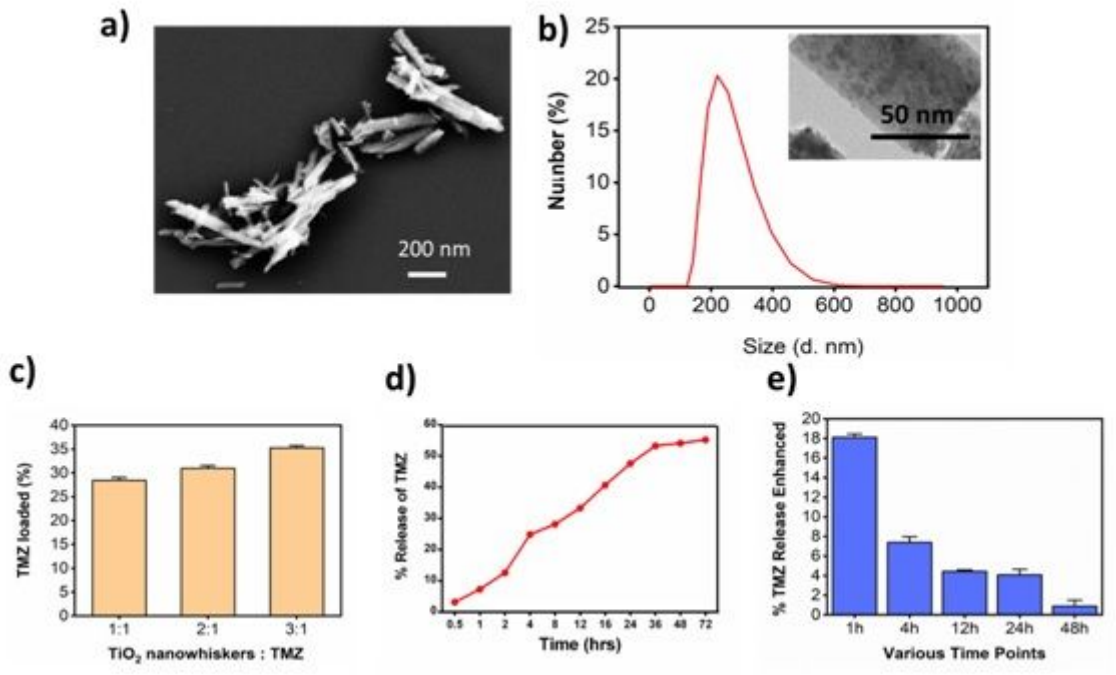

Figure 1

TiO2 nanosticks characterization and drug loading ability. a) Scanning Electron Microscope (SEM) micrograph of TiO2 nanosticks. b) size distribution of TiO2 nanosticks. The inset Transmission Electron micrograph showing the porous nature of the TiO2 nanosticks. c) TMZ loading ability on TiO2 nanosticks at different ratios. d) in vitro release of TMZ from TiO2 nanosticks at various time points at 37 oC. e) Ultrasonication effect on the release of TMZ from TiO2 nanosticks at various time points. (N=3)

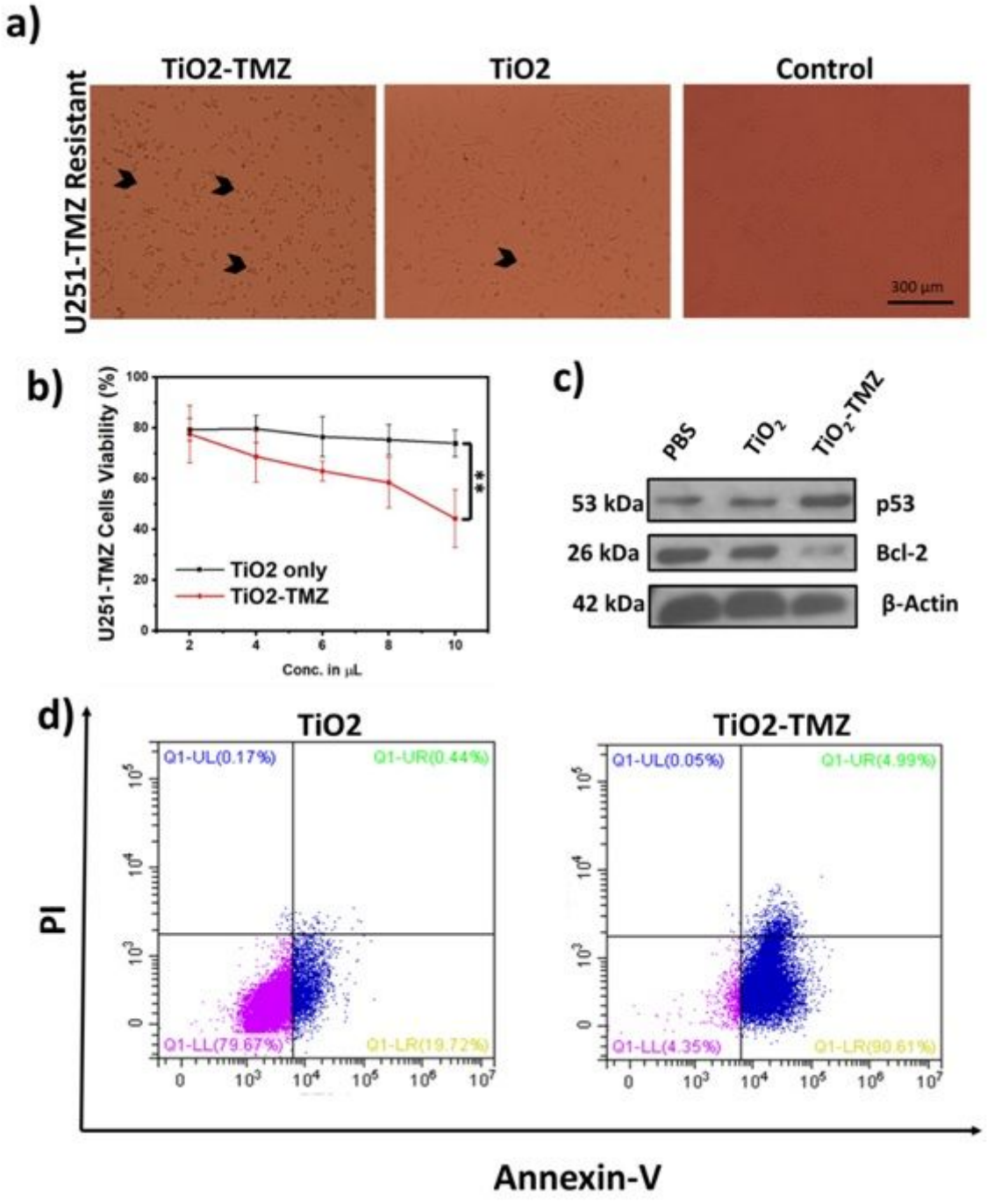

Figure 2

Effect of ultrasound activated TiO2 nanosticks with TMZ on glioblastoma (GBM). a) Bright-field micrographs showing the anticancer effect of ultrasound activated TiO2 nanosticks with TMZ on TMZ resistant GBM cells. The back arrowhead indicated the apoptotic cells' round morphology after treatment b) MTT assay for cell viability of TMZ resistant GBM cells after sonication. ** shows a highly significant difference between the two groups d) western blot data showing apoptosis effect of TiO2 nanosticks loaded with TMZ on TMZ resistant GBM cells after sonication. e) Annexin-V PI assay apoptosis data of

U251-TMZ resistant cells after treatment with TiO2 nanosticks loaded with TMZ and ultrasound activated. (N=3)

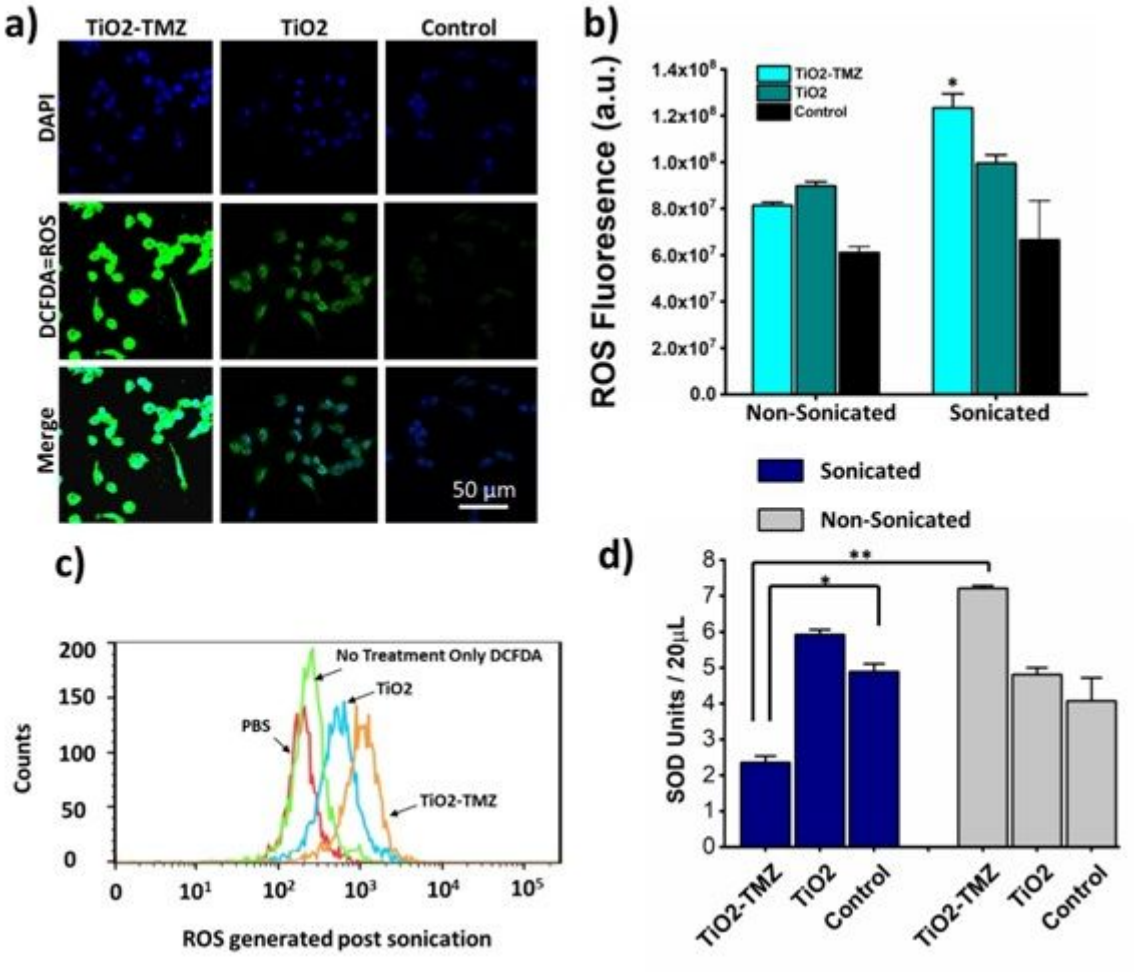

Figure 3

Oxidative stress generated by TiO2 nanosticks loaded with TMZ on TMZ resistant GBM. a) DCFDA (2',7'-Dichlorofluorescin Diacetate) treated TMZ resistant cells confocal micrograph after ultrasonication. Herein green color indicates the ROS generated, and the cell nucleus is stained with DAPI. b) Quantitative data of reactive oxygen species (ROS) generated after treatment with TiO2 nanosticks loaded with TMZ. * indicates the significant difference ($p < 0.05$) between TiO2-TMZ sonicated and non-sonicated cells ROS level c) Flow cytometry data of ROS generated by the TiO2-TMZ treatment after sonication. d) TMZ resistant GBM cells' SOD (superoxide dismutase) enzyme activity after treatment with by TiO2 nanosticks loaded with TMZ, post sonication. The * and ** are the probability value < 0.05 and 0.01, respectively. (N=3)

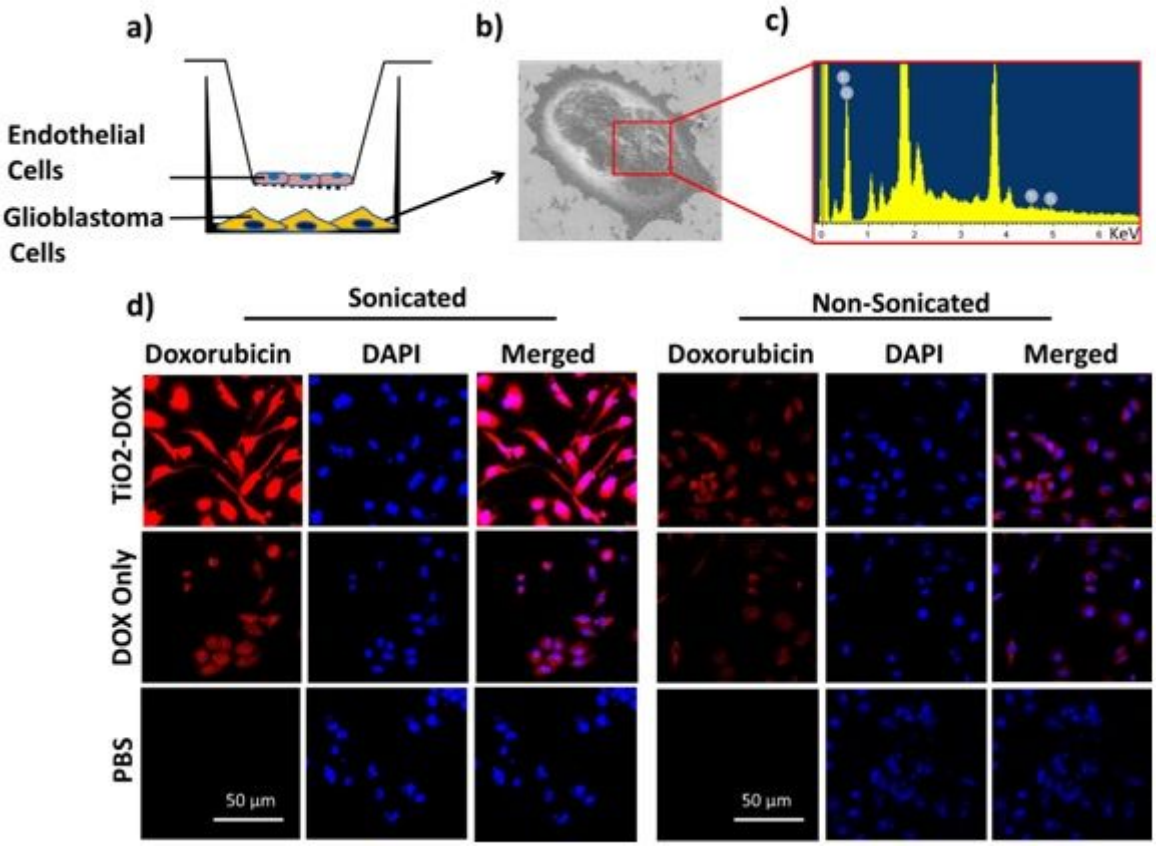

Figure 4

in vitro BBB traversing ability of TiO2 nanosticks loaded with Doxorubicin. a) is the schematic representation of the transwell BBB model. b) SEM micrograph of GBM cell. c) the energy-dispersive X-ray spectroscopy (EDS) data of transwell TiO2 nanosticks up taken by GBM-TMZ resistant cells. d) in vitro BBB confocal fluorescence data of Doxorubicin (Dox) (free and TiO2 nanosticks loaded) with and without sonication (Doxorubicin Em = 595 nm).

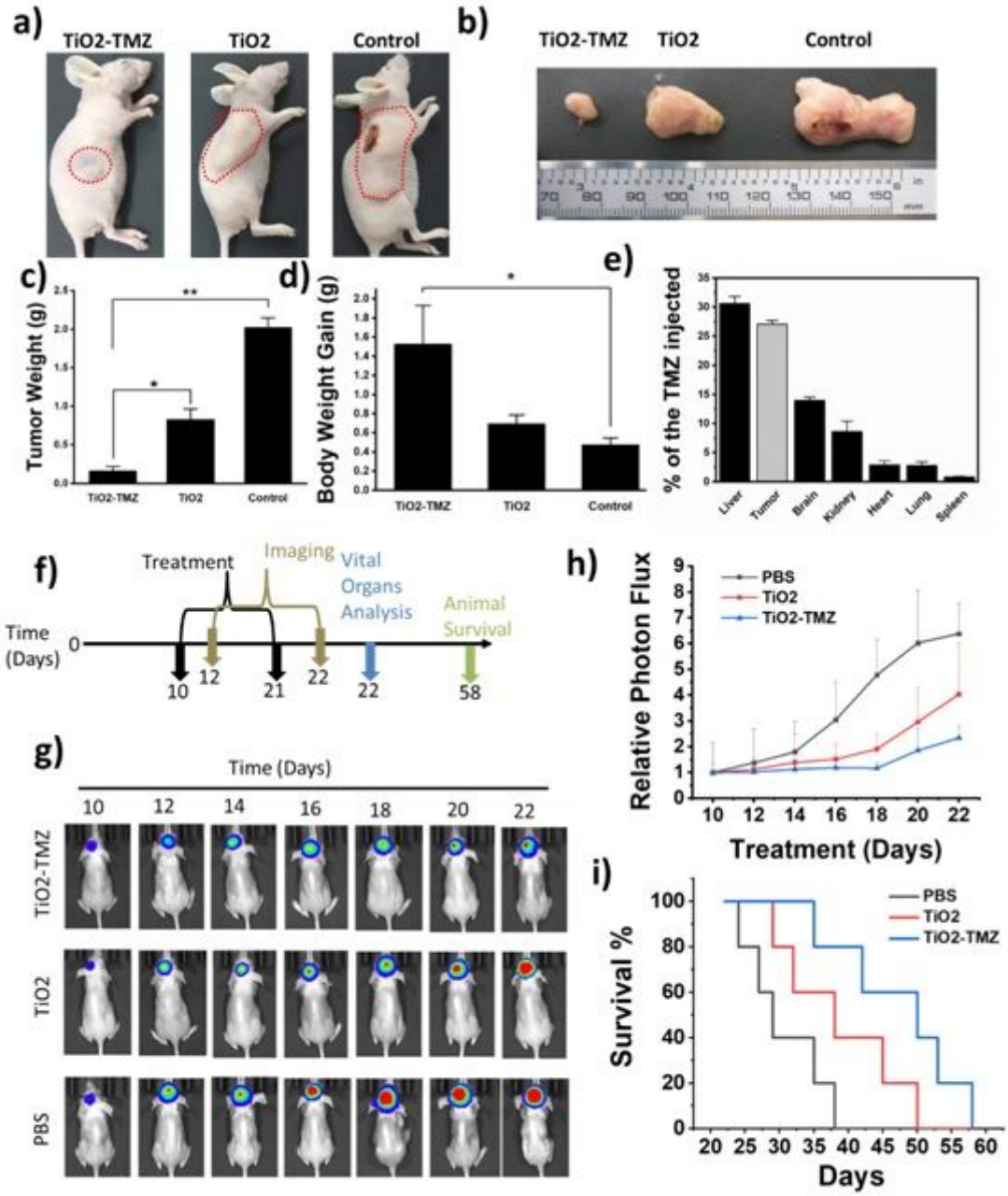

Figure 5

Orthotopic mice treatment TiO2 nanosticks loaded with TMZ. a) is the micrograph of xenograft TMZ resistant GBM model after treatment. The red dotted circle indicates subcutaneous tumor boundaries. (N=15) b) is the tumor volume after treatment and sonication. c) tumor weight and d) body weight gain of various treated xenograft mice models. e) vital organ distribution of TMZ after loading on TiO2 nanosticks. f) is the experimental layout. g) micrograph of TMZ resistant GBM orthotopic mice expressing Luc at various time points after treatment with TiO2 nanosticks loaded with TMZ, activated with ultrasound (N=21). h) relative photon flux representing tumor volumes and i) is the survival of orthotopic mice models after treatment. * is showing $P < 0.05$, whereas ** is $P < 0.01$.

Supplementary Files

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