Granzyme B functionalized nanoparticles targeting membrane Hsp70-positive tumors for multimodal cancer theranostics

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Abstract

Functionalized superparamagnetic iron oxide nanoparticles (SPIONs) have emerged as potential clinical tools for cancer theranostics. Membrane-bound 70 kDa heat shock protein (mHsp70) is ubiquitously expressed on the cell membrane of various tumor types but not normal cells and therefore provides a tumor-specific target. The serine protease Granzyme B (GrB) that is produced as an effector molecule by activated T and NK cells has been shown to specifically target mHsp70 on tumor cells. Following binding to Hsp70, GrB is rapidly internalized into tumor cells. Herein, we demonstrate that GrB functionalized SPIONs act as a contrast enhancement agent for magnetic resonance imaging (MRI) and induce specific tumor cell apoptosis. Combinatorial regimens employing stereotactic radiotherapy and/or magnetic targeting have been found to further enhance the therapeutic efficacy of GrB-SPIONs in different tumor mouse models.

1. Introduction

Functionalized nanoparticles are emerging as a novel class of therapeutics for diagnosis and therapy of cancer. Due to their physico-chemical properties, nanoparticles can be applied for multimodal approaches including early detection and monitoring of tumor responses, delivery of anti-cancer drugs and boosting the efficacy of existing therapies (e.g., radiotherapy, thermotherapy, etc)[1,2]. Among others, superparamagnetic iron oxide nanoparticles (SPIONs) possess several unique features such as magnetism, low cytotoxicity, high contrast for magnetic resonance imaging (MRI) that qualifies them for targeted, multimodal anti-cancer therapies as demonstrated in different preclinical models[3–5]. The decoration of the nanoparticle surface with tumor-targeting molecules increases their specificity towards the tumor and thereby improves their diagnostic and therapeutic (i.e., theranostic) potential[6].

Besides its intracellular localization, the 72 kDa heat shock protein Hsp70 (HSPA1A), has been found to be expressed on the membrane of a large variety of different tumor cells, but not on corresponding normal cells[7–9]. The tumor-specific membrane localization of Hsp70 in non-stressed tumor cells could be attributed to an interaction of Hsp70 with the tumor-specific glycosphingolipids such as globoyltriaosylceramide (Gb3) which are present in lipid rafts of tumor but not normal cells[10]. As shown previously, upon environmental stress, such as hypoxia, Hsp70 associates with phosphatidylserine (PS) in tumor cells[11]. Subsequent studies have confirmed a direct interaction of Hsp70 with Gb3 and PS *in vitro*[10, 12–14]. By using atomic force microscopy (AFM) an association of low Hsp70 concentrations with planar lipid bilayers containing 20% dipalmitoyl phosphatidylserine (DPPS) was detected which in the presence of cholesterol supports the formation and bursting of Hsp70 containing membrane vesicles[15]. This finding is in line with the proposed non-classical vesicular export pathway of Hsp70 form viable tumor cells via endolysosomal pathways[16].

Although high mHsp70 expression densities correlate with tumor aggressiveness and therapy resistance, the protein also serves as a tumor-specific target for diagnosis and therapy. At present, fluorescence-, radionuclide- and magnetic nanoparticle-labeled[17] Hsp70-targeting tools including full length antibody cmHsp70.1[18,19], Fab fragment[20–22] and tumor penetrating peptide TPP[20,23] have been successfully applied for fluorescence, positron emission tomography (PET), computed tomography (CT) and magnetic resonance (MR) tumor imaging in preclinical models. Therapeutically, Hsp70-targeting nanoparticles loaded with anti-survivin miRNA have been successfully used for radiosensitization of glioblastoma cells *in vitro*[24].

By affinity chromatography Gross et al. demonstrated that the serine protease Granzyme B (GrB) derived from lysates of activated NK cells specifically binds to a sequence of Hsp70 (TKDNNLLGRFELSG, aa$^{450–463}$) that mediates oligomerization of Hsp70 and is exposed on the cell surface of mHsp70 positive tumor cells[25]. Following binding to mHsp70, GrB is specifically taken up into the cytosol and thereby induces apoptosis in a perforin-independent manner[26]. Therefore, we speculate that mHsp70 serves as an entry port for the apoptosis inducing enzyme GrB into mHsp70-

positive tumor cells. Based on these findings, in the current study we functionalized the surface of SPIONs with GrB for a tumor-specific targeting of nanocarriers and for inducing tumor apoptosis. Moreover, these GrB-SPIONs were assessed as a novel MR contrast agent for the detection of intracranial glioblastoma with a highly sensitive MR scanner in syngeneic GL261 mouse and C6 Wistar rat models. Anti-tumor efficacy of GrB-SPIONs was comparatively evaluated after repeated intravenous injections, prolonged administration with a peristaltic iPRECIO® programmable infusion pump as a single or combined therapy together with radiotherapy or magnetic targeting in orthotopic U87 glioblastoma and H1339 lung xenograft mouse models with brain metastases.

2. Results

2.1. Physico-chemical properties of the GrB-SPIONs magnetic conjugates

Dextran-coated superparamagnetic nanoparticles constituted the hydrodynamic size of 43.2 ± 1.2 nm and Z-potential of 12.8 mV (**Figure 1a–c**). Subsequent decoration of the particle surface with Granzyme B (GrB) increased the size to 44.5 ± 2.3 nm and decreased the Z-potential to –17.2 mV. The SPION structure and composition were examined by X-ray diffraction (XRD) at the DRON-3M dif-fractometer (Cu K_α line was used). **Figure S1** presents the room temperature XRD intensity as a function of diffraction angle and evidences single crystalline phase of magnetite. The diffraction peaks broaden, mainly, due to a finite size of the coherent scattering region. This allows one to evaluate mean size of the crystallinity region[27], giving the estimation 8.7(1.3) nm. Relaxometry NMR measurements of the spin-lattice relaxation of the non-coated and GrB-SPIONs demonstrated significant increase in relaxation rates (i.e., R_2 and R_2*), that indicated the MR contrast enhancement properties of the nanoparticles (**Figure 1d**). Comparison of the magnetic conjugates to the commercially available agent Chemicell® clearly demonstrated higher contrast properties of the GrB-SPIONs. Non-linear magnetic response (NLR) measurements with a registration of the second harmonic of magnetization M_2, showed that both, SPIONs and GrB-SPIONs kept the superparamagnetic properties of the particles in the single domain state (**Figure S2a, S2b**). The K_d

of recombinant GrB to Hsp70 was 15.6 ± 2.3 nmol/L as determined by MST measurements (**Figure S3**). GrB-conjugated SPIONs but not non-coated SPIONs bind soluble Hsp70 as demonstrated by using dynamic light scattering (DLS) method (**Figure 1e**). In the presence of soluble Hsp70 (0.50 μg/L) magnetic relaxometry analysis of the GrB-SPIONs showed changes in proton-proton relaxation (**Figure 1f**). To assess the specific binding of the conjugates to Hsp70, temporal measurements of the T2 relaxation times were performed. Due to the formation of GrB-SPIONs-Hsp70 clusters the T2 transverse time of relaxation increased nearly 2-fold in the magnetic field of the NMR spectrometer (7.1 T) after addition of Hsp70 to GrB-SPIONs there was (**Figure 1f**). MR contrast enhancing properties of GrB-SPION conjugates were confirmed by R_2 relaxometry measurements after incorporation into the cytosol of human U87 glioblastoma cells embedded in 1% agarose gel (**Figure S1c)**. Even after 24 h the intracellularly accumulated particles exhibited a strong MR signal as recorded by T2-weighted, gradient echo (FLASH) scanning regimens (**Figure S1d**). Concomitantly, a drop in the T2 values was detectable from 24.3 ± 2.0 ms (control) to 14.9 ± 2.3 ms in the SPION group and to 6.5 ± 1.3 ms in the GrB-SPION group (**Figure S1e**).

2.2. GrB-SPIONs target mHsp70-positive tumor cells and induce apoptosis

Internalization of the nanoparticles was assessed in U87, C6, GL261, LN229, H1339 and B16 cells that were tested as mHsp70-positive by flow cytometry using cmHsp70.1 mAb[28,29]. Representative views of the uptake of SPIONs and GrB-SPIONs into U87 cells are shown in **Figure 2a**. After a co-incubation period of 24 h non-conjugated SPIONs were incorporated into the cytosol but not in the nucleus (**Figure 2a**). The uptake of GrB-SPIONs into U87 cells was much more pronounced compared to that of non-conjugated SPIONs as shown by reflective laser scanning. Subsequent TEM analysis demonstrated that GrB-SPIONs presented as electron dense particles in endosomes and the cytosol of C6 and U87 cells (**Figure 2b**). Co-staining of GrB-SPIONs (green) with endosomal (EEA1, Rab5, Rab7) and lysosomal (LAMP1) markers indicated an endosomal uptake pathway of the nanoparticles (**Figure 2c**). The assessment of the iron content revealed a nearly two-fold increased

uptake of GrB-SPIONs compared to non-conjugated SPIONs in all cell types (**Figure 2d**). As a control, mHsp70-negative L929 fibroblasts did not incorporate GrB-SPIONs into the cytosol (**Figure S4**). As was demonstrated earlier ionizing radiation can enhance the mHsp70 expression density[17]. After a single irradiation of the cells with 4 Gy the uptake of GrB-SPIONs, but not that of SPIONs was further enhanced (**Figure 2d,e**). Application of the blocking anti-Hsp70 antibodies BRM-22a subsequently reduced the internalization of the magnetic conjugates indicating the role of mHsp70 for the uptake of GrB-SPIONs (**Figure 2e**) The higher accumulation of GrB-SPIONs compared to SPIONs into tumor cells was confirmed by an increase of the side scattering (SSC) but not forward scattering (FSC) parameters, and an increased mean fluorescence intensity signal when FITC-conjugated GrB-SPIONs were used, as determined by flow cytometry (**Figure 2f**). Cell irradiation further increased SSC parameters of tumor cells and incorporation of FITC-labeled GrB-SPIONs. Toxicity studies demonstrated that up to a Fe concentration of 100 µg/mL SPIONs did not induce significant apoptosis, as determined by Annexin V staining in any of the cell lines. In contrast, GrB-SPIONs initiated apoptosis already at a concentration of 50 µg/mL (**Figure 3**). Similar apoptosis induction was observed when purified recombinant GrB was used (data not shown).

2.3. GrB-SPIONs accumulate in the tumor and increase MR contrast enhancement

In a first set of experiments the capacity of fluorescence labeled GrB to accumulate in tumor tissues was assessed. Light sheet microscopy studies of the GL261 and U87 tumors clearly demonstrated the highly vascularized architecture of the tumor (**Figure S5**). GrB-Alexa680 was intravenously administered into the tail vein of C57Bl/6 mice with orthotopic GL261 glioblastoma (**Figure S6**). An accumulation of GrB-Alexa680 and cmHsp70.1-Alexa680, but not BSA-Alexa680 within the tumor was determined by fluorescence intraoperative tumor imaging 24 h after injection. The tumor-to-normal brain ratio of GrB was 3.6-fold for GrB in GL261 glioma tissue of the mice. Intriguingly, the signal intensity of the labeled GrB was similar to that of anti-Hsp70 antibody (cmHsp70.1-Alexa680) that was employed as a positive control for mHsp70-targeting *in vivo*.

For MR contrast enhancement GrB was coupled to superparamagnetic particles that are known as a MR negative contrast agent. Following i.v. injection of GrB-SPIONs a series of T1-, T2-weighted images and Flash in the coronal plane were analyzed (**Figure 4a**). Injection of non-conjugated SPIONs induced the appearance of the 'dark' hypotensive zones throughout the tumor tissue (T2-weighted images) that was more prominent in images obtained in the gradient-echo regimen. Administration of GrB-SPIONs further increased the contrast of the tumor. The analyses of the T2* map images demonstrated the reduction of the T2 values in the SPIONs-treated group of animals as compared to control, of 29.9 ± 5.0 and 20.8 ± 1.4 ms, respectively ($P<0.001$). When GrB-SPIONs were applied a further reduction in the T2 values to 13.19 ± 2.1 ms was observed. Biodistribution analysis of the GrB-SPIONs accumulation in the U87 glioblastoma over the period of 7 days demonstrated the presence of the nanoparticles in the tumor (**Figure S7**). Subsequent histological analysis confirmed the retention of the nanoparticles inside the U87 cells (**Figure 4b**). GrB-SPIONs are visualized as green dots within the cytosol of Hsp70-positive glioma cells. To assess the influence of ionizing irradiation on the uptake of GrB-SPIONs tumors of mice were irradiated with a single dose of 10 Gy (**Figure 4c**). Following irradiation an increased accumulation of GrB-SPIONs was detected in orthotopic U87 tumors (**Figure 4a**) that corresponded to the increased mHsp70 expression density (**Figure 4b**) and a drop in the T2 values from 13.2 ± 2.1 to 7.1 ± 1.5 ms (**Figure 4e,d**).

Magnetic targeting of the nanoparticles *in vivo* with a magnet placed on top of orthotopic U87 glioblastoma in NMRU nu/nu mice and C6 glioma in Wistar rats drastically enhanced the accumulation of nanoparticles to the location of the magnet (**Figure 4g**). T2 values obtained from the MSME sequence decreased nearly 4-fold (3.7 ± 1.3 ms) as compared to the GrB-SPIONs (**Figure 4e**). Biodistribution studies, employing highly sensitive non-linear response measurements (NLR-M_2), confirmed the retention of the particles in the U87 mouse glioblastoma and C6 rat gliomas (**Figure 4f, Figures S8-10**). A similar MR contrast enhancement by GrB-SPIONs was observed in an orthotopic, brain metastasizing human lung tumor H1339 (SCLC) model (**Figure 5a**). After i.v. injection GrB-SPIONs showed a stronger accumulation in brain metastases and primary lung tumors,

as determined by a decrease in the T2 values, compared to non-conjugated SPIONs (**Figure 5b**). The best accumulation of GrB-SPIONs is observed in primary lung tumors and brain metastases after CT guided high-precision irradiation of the tumors with a single dose of 10 Gy (**Figure 5b**). The injection of purified recombinant GrB revealed T2 values that were comparable to that of the PBS control (**Figure 5b**).

2.4. *In vivo* anti-tumor efficacy of GrB-SPIONs alone or in combination with radiotherapy and magnetic targeting

The effects of GrB-SPIONs in comparison to SPIONs and GrB alone were firstly assessed with respect to tumor growth delay in an orthotopic GL261 glioma model (**Figure S11**). A single intratumoral (i.t.) injection of GrB-SPIONs and SPIONs, but not PBS or GrB, resulted in significant decrease in the T2 values ($p<0.001$), as shown by MR imaging (**Figure S11a**). With respect to tumor responses no effects were seen after administration of PBS and SPIONs, but, GrB and even more pronounced GrB-SPIONS induced a significantly delayed the tumor growth, as shown by MR imaging and volumetrics, which corresponds to an increase in active caspase 3 in IHC glioma sections (**Figure S11c**). The presence of iron after administration of SPIONs and GrB-SPIONs was confirmed by a Berliner Blau staining of the tumor sections after i.t. injection.

The therapeutic potential of a systemic administration of GrB-SPIONs was evaluated in o.t. xenograft H1339 lung cancer model with and without brain metastases and U87 glioma mouse models (**Figure 6a-d; Tables S2-6**). Three repeated i.v. injections of GrB-SPIONs on days 5, 7 and 9 after tumor implantation resulted in a significantly prolonged overall survival (OS) compared to animals treated with PBS, or non-conjugated SPIONs in a xenograft H1339 lung tumor model, H1339 lung tumor model with brain metastases (**Figure 6a**) and U87 glioma model (**Figure 6b**). The anti-tumor efficacy of GrB alone was lower than that of GrB-SPIONs in all tumor models (**Figure 6a,b**) Intriguingly, the survival rate of immunodeficient tumor-bearing mice after treatment with GrB-SPIONs (H1339: 39.17 ± 11.13; H1339 with brain metastases: 19.33 ± 6.98 days; U87: 31.7 ± 12.67 days) was

significantly longer compared to those treated with free GrB (H1339: 30.0 ± 6.6; H1339 with brain metastases: 14.5 ± 3.99 days; U87: 23.6 ± 7.72 days) (P<0.001, **Figure 6a,b**)). No significant differences were observed between the control groups of mice treated with PBS or non-conjugated SPIONs with median survival rates of 13.17 ± 2.99 and 14.83 ± 4.31 days for H1337 tumors, 10.17 ± 1.94 and 9.17 ± 2.4 days for H1339 tumors with brain metastases and 13.6 ± 1.9 and 13.1 ± 2.96 days, for U87 tumors (P>0.05).

When GrB conjugates and purified GrB were administered via iPRECIO® programmable infusion pump SMP-200 into the jugular vein for a period of 10 days the median survival rates further increased up to 37.6 ± 5.37 and 33.6 ± 7.57 days, respectively for mice with o.t. U87 gliomas. Moreover, at the end of the follow-up period of 40 days 5(6) mice were still alive (**Figure 6c**).

A combination of the nanoparticle treatment with a single radiation dose (10 Gy) significantly prolonged the survival rate of the mice with U87 tumors up to that 36.8 ± 12.8 days in the GrB-SPIONs group (P<0.001) (**Figure 6d**).

Magnetic targeting (MT) employing NdFeB ferromagnets alone also demonstrated a therapeutic efficacy in the GrB-SPIONs group in the model of orthotopic C6 glioblastoma in rats – 36.67 ± 10.54 days (**Figure 6e; Table S7**). A combined treatment of rats with a single radiation dose of 10 Gy and systemic GrB-SPIONs injections (i.v.) increased the survival rates up to 41.17 ± 9.39 days and thus was significantly higher than any of the single therapy regimens (**Figure 6f; Table S8**). Subsequent IHC studies demonstrated high apoptosis rates (active caspase 3) in mice treated with GrB or with GrB-SPIONs (**Figure 6g**).

3. Discussion

Therapeutic applications of nanocarriers for drug delivery show promising results in various types of cancers[30,31]. However, a main limitation for the development of nanotherapies is the lack of adequate (addressable) markers that allow a high-affinity binding and tumor-specific targeting and uptake. In the present study mHsp70 was exploited for the development of theranostic nanoparticles conjugated

to the apoptosis inducing serine protease Granzyme B. Since Hsp70 is ubiquitously expressed on the cell surface of a large variety of cancers (e.g., primary glioblastomas, breast cancer, leukemia, colon cancer, gastric cancer, squamous cell carcinoma, low rectal cancer), mHsp70 provides a universal tumor target[9, 32–37]. Beside other anti-Hsp70 targeting agents (e.g., monoclonal antibodies, Fab-fragments, anticalins, peptides), the serine protease Granzyme B has been found to bind to Hsp70 with a high affinity (K_d 15.6 ± 2.3 nmol/L, **Figure S3**). After binding, GrB gets internalized into tumor cells via Hsp70 translocation and elicits pro-apoptotic activity specifically in mHsp70-positive tumor cells[25]. This is the first report analyzing the theranostic capacity of GrB-functionalized SPIONs in immunocompetent syngeneic rodent (rat C6 glioma, mouse GL261 glioma) and immunodeficient human xenograft (glioblastoma U87, small cell lung cancer H1339) mouse models. Hsp70 specificity of GrB-SPIONs was demonstrated in mHsp70-negative L929 fibroblasts showing neither uptake nor apoptosis induction after co-incubation with GrB-SPIONs.

Anti-tumor efficacy of GrB-SPIONs conjugates as a single therapy regime was firstly demonstrated in a syngeneic o.t. GL261 glioma model after intratumoral injection (**Figure S11**). Delayed tumor progression (as measured by MR imaging) was attributed to the pro-apoptotic activity of GrB. Although *in vitro* studies indicated a pro-apoptotic activity of non-conjugated SPIONs (**Figure 3**), that might be mediated by reactive oxygen species (ROS)[38–40], *in vivo* application of these SPIONs did not induce significant apoptosis (IHC staining for caspase 3) in tumors. Subsequent systemic administrations of GrB-SPIONs as a monotherapy resulted in an increased overall survival of mice with o.t. U87 glioblastoma and H1339 small cell lung cancer (**Figure 6**). In addition, a GrB-SPION therapy alone was even effective in advanced, late-stage H1339 small lung cancer model with intracranial metastasis. Compared to the PBS and SPION control groups, overall survival increased nearly two-fold after treatment with GrB-SPIONs (**Figure 6**). Notably, the use of a programmable mini-pump iPrecio® system for a long-term delivery of GrB-SPIONs via the external jugular vein induced long-term survival in 5 out of six animals. This indicates that a prolonged administration period of GrB-SPIONs nanoparticles results in beneficial tumor outcome. In comparison to a

monotherapeutic regimen a combination consisting of a systemic GrB-SPION therapy and radiotherapy has been found to further increase the survival of U87 glioma-bearing animals ($P<0.001$) (**Figure 6**). These findings are of particular interest with respect to the clinical application of nanotherapeutics which will be applied in patients with mHsp70 positive tumors in combination with radiochemotherapy. Previously, it has been shown that radio- and/or chemotherapy, anti-inflammatory agents (e.g., celecoxib, rofecoxib, acetyl-salicylic acid), insulin-sensitizer pioglitazone are able to increase the expression of mHsp70 on tumor cells[17, 41–44]. Sublethal single dose irradiation of the tumor cells increased the upregulation of mHsp70 on the cell surface and thereby enhances the uptake of GrB-SPIONs. By blocking studies using anti-Hsp70 monoclonal antibodies, the Hsp70 dependency of the uptake could be confirmed (**Figure 2**). Elevated mHsp70 expression levels increased the retention of GrB-SPIONs in U87 glioblastoma and thereby resulted in a MR contrast enhancement of tumors in T2-weighted and gradient echo images (further proved by immunofluorescence studies and NLR-M_2 biodistribution analysis) (**Figure 4d**). The efficacy of tumor detection by GrB-SPIONs was comparable to that of other MR negative targeted magnetic agents including SPION-IL1Ra[45], IONP-RGD[46], EGFRvIIIAb-IONPs[47]. The presence of the serine protease GrB on SPIONs has been shown to promote tumor cell death (IHC staining for caspase 3) and thereby results in an increased overall survival of the animals (**Figure 6; Figure S11**). The therapeutic efficacy of GrB-SPIONs was found to be significantly higher as compared to the previously reported data including the intra-tumoral convection-enhanced delivery (CED) of EGFRvIIIAb-IONPs[47].

Magnetic targeting employing external NdFeB ferromagnets has been shown to modify the localization of GrB-SPIONs inside C6 glioblastoma of rats (**Figure 5**; **Figure S9, S10**). We could demonstrate an up to 12.3-fold local accumulation of GrB-SPIONs inside the glioblastoma. Furthermore, combination of a single dose (10 Gy) irradiation with magnetic targeting dramatically enhanced the accumulation of the targeted particles within the tumor. Previously Chertok et al. reported successful application of magnetic targeting (0.4 T, 30 min) of non-conjugated Chemicell®

magnetic nanoparticles using 9L glioma bearing rats with the 9.6-fold glioma selectivity of nanoparticle retention[48]. Although subsequent carotid administration provided 1.8-fold increase in nanoparticle accumulation in glioma the translation of this approach into clinical practice would be limited due to potential surgical complications[49]. Application of nanocarries conjugated with GrB that are targeting mHsp70-positive tumors via clinically relevant systemic route with external magnetic field targeting proved to be more effective as compared to the reported data by Chertok et al.[48,49]. Presumably application of powerful electromagnets could increase the retention of particles inside the tumor[50].

4. Conclusion.

The presented study demonstrated that systemically administered mHsp70-targeted GrB-SPIONs magnetic conjugates can be employed for both MR imaging of tumors and tumor killing which enhances overall survival of tumor-bearing mice. Single-agent therapy with GrB-SPIONs achieved an impressive survival benefit, but combined applications of functionalized nanoparticles with radiotherapy and magnetic targeting exhibited the best therapeutic outcome. Continuous long-term administration of GrB conjugates via the programmable subcutaneously implanted mini-pump system iPrecio® further enhanced the therapeutic benefit of GrB-SPIONs. Moreover, significant therapeutic efficacy was also achieved in immunodeficient animal models (xenograft small lung cancer H1339 with brain metastasis and advanced U87 brain tumors) with a high tumor burden. These findings indicate that even in immunosuppressed patients i.e. after radiochemotherapy, a GrB-SPION therapy might still be able to elicit anti-tumor effects. Together with the finding that a lipHsp70 ELISA[51] allows the stratification of patients with mHsp70 tumors based on a simple blood test, the present findings might justify further clinical development of mHsp70-targeted therapies that are based on GrB-SPIONs in combination with radiochemotherapy.

5. Experimental Section

5.1. Synthesis of the GrB superparamagnetic nanocarriers

Superparamagnetic iron oxide nanoparticles (SPIONs) were prepared from salt solutions $FeSO_4$ and $FeCl_3$ by co-precipitation as was described earlier[52]. During the process of ultrasound application dextran (10 kDa; Sigma-Aldrich) was added to the nanosuspension for prevention of sedimantation. For functionalization of dextran-coated SPIONs recombinant human Granzyme B (GrB) or bovine serum albumn (BSA) (Sigma, USA) were applied. The dextran coating of the synthesized nanoparticles was cross-linked with epichlorohydrin and aminated. Activated by carbodiimide aminated-dextran was coupled to the carboxyl groups of proteins. The hydrodynamic size and electrophoretic properties of the nanoparticles were estimated employing a Zetasizer Nnao (Malvern Instruments, UK) and transmission electron microscopy (TEM) using a JEOL-2000 microscope (Jeol, Japan). For analysis of the specific interaction of GrB-SPIONs with recombinant human Hsp70 the nanoparticles (Fe concentration of 150 μg/mL) were co-incubated with protein. Following 4 hours the size of the clusters GrB-SPIONs-Hsp70 was analyzed with the help of DLS. To precisely assess the binding of the Hsp70 to the magnetic conjugates temporal T_2 relaxation times were estimated when GrB-SPIONs were co-incubated in the presence of Hsp70 in the magnetic field of the NMR spectrometer CXP-300 (Bruker, Germany). NMR relaxivity studies were performed to estimate R_1, R_2 and R_2* coefficients in the magnetic field of 7.1 T. To assess the superparamagnetic properties of the nanosuspenison higly sensitive non-linear magnetic response (NLR-M_2) measurements were performed.

5.2. Granzyme B production and purification

Human recombinant GrB was produced and purified from transfected human embryonal kidney cells (HEK293) as described previously[53]. Briefly, following purification of inactive GrB on a nickel column utilizing the (His)(6) tag the protein was digested employing enterokinase with subsequent purification by heparin affinity chromatography.

5.3. Microscale thermophoresis measurements of K_d

Binding affinities of GrB to purified Hsp70 were measured by microscale thermophoresis (MST) analysis. Gradual thermophoretic changes of GrB in a concentration range of 0.035 to 2.000 nmol/L was tested against 100 nmol/L FITC-labeled human recombinant Hsp70 protein (in temperature range 20–22°C) employing the Monolith NT (NanoTemper). As control FITC-labeled bovine serum albumin (BSA) (Sigma-Aldrich, USA) was applied.

5.4. Cells

Human tumor cell lines (LN229 (glioblastoma), U87 (glioblastoma), HeLa (cervix carcinoma), H1339 (SCLC)) and animal cell lines (C6 (rat glioma), GL261 (glioma)) were provided by Tumorbank Deutsches Krebsforschungszentrum (Heidelberg, Germany). Cells were cultured in RMPI-1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin) at 37 °C in 5% CO_2-incubator. For experiments single cell suspension was obtained using Trypsin-0.53 mM EDTA. All cell lines were tested for the expression of mHsp70 by employing flow cytometry and confocal microscopy.

5.5. Colorimetric ferrozine-based assay

Ferrozine assay was employed for estimation of Fe concetration of nanoparticles incorporated into the cells as described previously[54]. Following co-incubation of the tumor cells for 24 h with non-conjugated or GrB-SPIONs (Fe concentration of 100 µg/mL) cells were washed with PBS and subsequently dissociated by iron-releasing agent (1.4 M HCl, 4.5% (w/v) $KMnO_4$) for 2 h at 60°C. Iron concentration was measured following adding iron-detection reagent (1 M ascorbic acid, 2.5 M ammonium acetate, 6.5 mM neocuproine, and 6.5 M ferrozine) on microplate reader (at 595 nm).

5.6. Assessment of nanoparticles incorporation into cells

5.6.1. *Confocal microscopy analysis*

Accumulation of the nanoparticles in tumor cells was analyzed employing confocal microscopy. U87, C6, B16, H1339 cells (1×10^6 cells/mL) were allowed to settle on glass slides coated by poly-L-lysine. Cells were co-incubated with SPIONs or conjugated GrB-SPIONs (both at Fe concentration 100 μg/mL) for 1, 3, 6, 12 and 24 hours at 37°C. Following incubation cells were washed with PBS and fixed in 0.5% PFA. Nuclei were stained with Hoechst 33342 (ThermoFisher Scientific, USA) or DAPI. Glasses were mounted in DAKO fluorescent mounting medium (Dako North America Inc., USA) and subsequently analyzed employing Leica TCS SP8 confocal system (Leica Microsystems, Heidelberg, Germany). To assess the pathway for nanoparticles uptake U87 or C6 cells were additionally stained by FITC conjugated mouse antibody against EEA1 (Abcam), PE conjugated mouse antibodies against Rab5 and Rab7 (Abcam), FITC conjugated mouse antibody against LAMP1 (Abcam), FITC conjugated mouse monoclonal antibodies cmHsp70.1 against membrane-bound Hsp70 (MultiImmune, Germany), LysoTracker (Invitrogen) to analyze the lysosomes. To assess the role of the membrane-bound Hsp70 in the internalization of the GrB-SPIONs U87 cells prior to co-incubation with nanoparticles were treated with mouse monoclonal antibodies BRM-22a against Hsp70 (Abcam, USA). Images were processed using Photoshop 7.0 software (Adobe Software, Palo Alto, CA).

5.6.2. *Transmission electron microscopy*

C6 and U87 cells were co-incubated with SPIONs or GrB-SPIONs (both at Fe concentration 100 μg/mL) for 24 hours. Following incubation cells were washed with PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at 4°C, postfixed for 1 hour in 1% aqueous OsO_4, dehydrated and subsequently embedded in Epon and Araldite. Sections were analyzed using Zeiss Libra 120 electron microscope.

5.6.3. *Flow cytometry analysis*

Incorporation of the nanoparticles into the tumor cells was additionally analyzed employing Cytomics FC500 (BeckmanCoulter, USA) flow cytometer. Tumor cells (C6, U87, LN229, HeLa, B16, GL261)

were co-incubated with SPIONs or GrB-SPIONs (both at 100 μg/mL) for 24 hours, washed by PBS and assessed by using a flow cytometer. Measured side scattering (SSC) and forward scattering (FSC) were utilized to assess the nanoparticles retention. Mean fluorescence intensity (MFI) was employed for assessment of FITC labeled nanoparticles incorporation into cells.

5.6.4. *Cytotoxicity assay*

The Annexin V assay kit (Abcam, USA) was employed for the detection of cell apoptosis according to the manufacture's protocol. Tumor cells (Gl261, C6, U87, H1339, B16, HeLa) were co-incubated with SPIONs or GrB-SPIONs magnetic conjugates (0.1, 0.5, 1.0, 5.0, 10, 50, 100 μg/mL) for 24 hours and then assessed employing Cytomics FC500 (BeckmanCoulter, USA) flow cytometer.

5.6.5. *Irradiation of cells*

Cells were irradiated at 0.5, 1, 2 and 4 Gy in a culture flask employing RS225A Gulmay X-ray research system (Gulmay Medical Ltd., UK).

5.7. Animals

Female C57Bl/6 mice, NMRI nu/nu mice, SCID mice 8–10 weeks old were purchased from Charles River (Sulzfeld, Germany) or The Jackson Laboratory (Bar Harbor, USA). Male Wistar rats weighing 280–300 g were purchased from the animal nursery "Rappolovo" of the Russian Academy of Medical Sciences (RAMN) (Rappolovo, Russia). All mice and rats were kept and bred under specific pathogen-free conditions in accordance with the guidelines of the Federation of European Laboratory Science Association (FELASA). All animal experiments were performed in compliance with both European Union and German law and approved by local authorities (Regierungspräsidium Oberbayern 55.2-1-55-2532-75-2015). Rats experiments have been approved by the ethical committee of First Pavlov State Medical University of St.Petersburg (St. Petersburg, Russia).

5.8. *In vivo* tumor models

5.8.1. *Orthotopic C6 glioblastoma model*

Following anesthesia of rats with ketamine (87 mg/kg) and xylazine (13 mg/kg) animals were mounted into stereotactic frame (David Kopf Instruments, Tujundra, CA). C6 cells (1×10^6 cells/mL) in 10 μl were injected into *nucl.caudatus dexter*.

5.8.2. *Orthotopic U87 and GL261 glioblastoma models*

Mice C57Bl/6 or NMRI nu/nu were anesthetized with i.p. injection of fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg) mixture and fixed in stereotactic frame. A total of 6×10^4 cells (U87 cells for NMRI nu/nu mice; GL261 cells for C57Bl/6 mice) were injected at a depth 3.0 mm (2.0 mm lateral and 1.2 mm posterior to the bregma). To visualize the tumor cells prior to implantation U87 cells were stained by CellMask^{TM} Deep Red plasma membrane staining (Invitrogen, USA).

5.8.3. *Orthotopic human H1339 small cell lung tumor and brain metastasis model*

After anesthesia of NMRI nu/nu mice 100 μl H1339 single cell suspension (5×10^6 cells/mL) was percutaneously inoculated in the upper margin of the sixth rib on the right anterior axillary line into the right lung (5 mm depth). For obtaining the brain metastasis H1339 cells (10 μl) were injected via the internal carotid artery.

5.9. Magnetic targeting of nanoparticles

Magnetic field strenght of about 320 mT for targeting SPIONs of GrB-SPIONs conjugates in the model of C6 glioblastoma in rats was achieved by employing 9 mm in diameter NdFeB cylindrical ferromagnet (K&J Magnetics, Plumsteadville, PA, USA).

5.10. Pump surgery

Implantable peristaltic pumps (iPrecio® model SMP-200, Tokyo, Japan) were employed for the continuous systemic delivery of nanoparticles via the right external jugular vein. NMRI nu/nu mice

were implanted with a subcutaneous mini-pump into the midscapular region on the 5th day following intracranial injection of U87 glioblastoma cells. PBS, GrB, SPIONs or GrB-SPIONs were infused through the catheter for 10 days (at a constant flow rate of 5 μl/hr).

5.11. Irradiation of animals

The irradiation of the implanted tumors was performed stereotactically using X-ray beams on the Small Animal Radiation Research Platform (SARRP) (XSTRAHL Ltd., Camberley, UK). MRI guided tumor irradiation of the anesthesized animals was performed employing 225 kV (peak) X-ray beam at a dose rate of 2.5 Gy min^{-1} with a collimator 5.0 × 5.0 mm. The single dose irradiation constituted 10 Gy.

5.12. Magnetic resonance imaging

Accumulation of the nanoparticles in the tumor site was assessed employing a high-field MRI scanner (11.0 T) (Bruker, Germany) with a custom small animal head coils. MR scans were performed in the following regimens: gradient echo (FLASH) images (TR/TE 350/5.4 ms, slice thickness 1.0 mm, flip angle 40°, Field of View (FoV) 3.0 × 3.0 cm), RARE-T1 images (flip angle 180°, TR/TE 1500/7.5 ms, slice thinckness 1.0 mm), TurboRARE-T2 images (flip angle 180°, TR/TE 4200/36 ms, slice thickness 1.0 mm). To quantify the T2 values (mean and standard deviation) that correlate to the amount of nanoparticles retention in the tumor multi-slice multi-echo (MSME) sequences were employed to generate T_2* maps over the tumor regions. Software package Paravision 3.1 (Bruker BioSpin GmbH, Rheinstetten, Germany) was applied for analysis of T_2 maps.

5.13. Biodistribution analysis of GrB-SPIONs

The biodistribution of non-conjugated and GrB-SPIONs was assessed in C6 and U87 glioblastoma models using highly sensitive method of nonlinear longitudinal response (NLR-M_2) a weak *ac* magnetic field $h(t) = h \cdot \sin(\omega t)$ (the frequency $f = \omega/2\pi$ = 15.7 MHz and h = 13.8 Oe) in parallel to it

steady field H employing the home-made installation described earlier[55,56]. The signals from fragments of different organs and tissues of experimental rats (control and after injection of nanocarriers) were recorded. In case of magnetic nanoparticles (MNP) the simultaneously registered phase components Re$M_2(H)$, Im$M_2(H)$ of the response are characterized by a presence of extremes in a weak field region of their H-dependence. The latter is due to the large average magnetic moments of MNPs, $m \sim 10^5\ \mu_B$ that is followed by large nonlinearity of magnetization on field dependence, $M(H)$, in a weak fields leading to extremes in Re$M_2(H) \propto \partial^2 M/\partial H^2$ and Im$M_2(H) \propto (\partial M/\partial H)\cdot(\partial \Gamma/\partial H)$, Γ being the rate of magnetic relaxation. When MNPs are inside some organ or tissue of experimental animal the environmental paramagnetic species with linear dependence M on H reveal negligibly small response $\propto \partial^2 M/\partial H^2$ with linear dependence on field as well in accordance with symmetry properties of M_2. Thus, MNPs signal is easily separated from paramagnetic background even in critical paramagnetic region of ferromagnetic materials. The amplitude of extremum in ReM_2 component of MNPs signal is proportional to their concentration in the sample under study, other parameters of MNP response being the same. This is used to obtain the relative MNP concentration in samples from different organ and tissues of experimental rats for characterization of their biodistribution. The important feature for characterization of MNP magnetic state (single-/multi-domain) and regime (superparamagnetic(SPM)/blocking) is field hysteresis of $M_2(H)$ response. In accordance with M_2 symmetry properties its observation indicates the presence in the sample of regions, possessing by ferromagnetic moment. In case of MNPs the presence of H-hysteresis evidences a deviation from SPM regime. For convenience of H-hysteresis control the dc field H was scanned back-and-forth symmetrically within ±300 Oe with the period of scan $1/F_{sc}$ = 0.125 – 4 s, both, real and imaginary, components of the signal being simultaneously recorded. The hysteresis depends on F_{sc} and temperature, hysteresis-free SPM regime arises above blocking temperature T_B [57]. The regime of magnetic behavior of NPs and H-hysteresis can be transformed under effect of environment giving some qualitative information about the latter. The amplitude h = 13.8 Oe of the ac field ensured the condition $M_2 \propto h^2$, so expression for second order susceptibility can be used for

semiquantitative analysis of results. Sample temperature was stabilized by flow thermostat, using evaporated N_2. The integral sensitivity of M_2 registration constituted 10^{-10} emu[55].

5.14. Treatment protocols

Therapeutic potential of the GrB-SPIONs magnetic conjugates was at first assessed in the orthotopic model of GL261 in C57/Bl6 mice. On the 6th day following the cells implantation animals randomly devided into four groups (6 animals each): (1) injection of PBS; (2) GrB (1.0 mg/kg); (3) SPIONs (5 mg/kg); (4) GrB-SPIONs (5 mg/kg). Following anesthesia animals were intratumorally injected with 2.5 μl of PBS or GrB or nanosuspensions. Glioblastoma progression was assessed before the infusion and on days 10, 15 and 20 employing MR scanner (11.0 T) (Bruker, Germany). T2- and T1-weighted images were used for tumor volumetrics analysis by measuring the cross-sectional areas on each MR image and multiplying their sum by the slice thickness.

The efficacy of the systemic intravenous (i.v.) administration of the nanopasrticles was further analyzed in the orthotopic model of glioblastoma U87 in NMRI nu/nu mice. On the 5th following tumor implantation mice were devided into four groups (10 animals each): (1) injection of PBS; (2) GrB (1.0 mg/kg); (3) SPIONs (5 mg/kg); (4) GrB-SPIONs (5 mg/kg). Injections were i.v. performed on day 5, 7 and 9 after the tumor implantation. Additionally, to assess the effect of continuous administration of GrB (n=5) or GrB-SPIONs (n=5) the animals were implanted with iPrecio® mini-pump on day 5 after tumor injection. The infusion continued over the period of 10 days. Additonally therapeutic potency was assessed in the orthotopic model of human small cell lung cancer H1339 in NMRI nu/nu mice. On day 5 following intrathoracic injection of H1339 cells animals were randomly divided into four groups (6 animals each) as follows: 1) injection of PBS; (2) GrB (1.0 mg/kg); (3) SPIONs (5 mg/kg); (4) GrB-SPIONs (5 mg/kg). Injections were i.v. performed on day 5, 7 and 9 after the tumor implantation. To assess the potency of GrB magnetic conjugates against advanced, later-stage tumors the orthotopic H1339 model with brain metastasis (intracarotid injection of H1339 cells)

was employed. The animals from four groups as decsribed earlier received single-agent therapy on day 5, 7 and 9.

For analysis of the combined treatment with radiothepy and nanoparticles the U87 glioma-bearing mice were devided into four groups (6 animals each) on the 5th day after tumor implantation: (1) injection of PBS; (2) single dose (10 Gy) irradiation; (3) SPIONs (5 mg/kg) with single dose (10 Gy) irradiation; and (4) GrB-SPIONs (5 mg/kg) with single dose (10 Gy) irradiation. Nanoparticles were i.v. administered on day 5, 7 and 9 after tumor implantation. Single dose (10 Gy) stereotactical irradiation employing SARRP machine was perfomed on day 5.

Evaluation of the magnetic targeting alone or in combination with radiotherapy was performed in the orthotopic model of C6 glioblastoma in Wistar rats. On the 5th day following C6 cells injection animals were devided into three groups (6 animals each) as follows: (1) injection of PBS; (2) SPIONs (5 mg/kg), and (3) GrB-SPIONs (5 mg/kg). Nanoparticles injection were i.v. performed on days 5, 7 and 9. Magnetic field was adjusted (for animals from all three groups) at 320 mT for 1 hour on the day 5 following the i.v. injection of nanocarriers. In the second set of this experiment single dose (10 Gy) was introduced to the animals treated with PBS, SPIONs or GrB-SPIONs and magnetic field.

5.15. Histological analysis

Following fixation of the tumors in the 10% PFA tissues were transferred into a Tissue-Tek® compound and frozen and kept at –80°C. Cryosections 5 – 7 μm mounted on SuperFrost Plus slides (ThermoFischer Scientific, USA) were stained with Hoechst 33342 and FITC labeled monolconal antibodies cmHsp70.1 against Hsp70. Nanoparticles were detected by reflective laser scanning using a Leica TCS SP8 confocal system (Leica Microsystems, Heidelberg, Germany). For analysis of the tumor vasculature of the GL261 and U87 tumors ultramicroscopic analysis of cleared tissues was performed following i.v. administration of AlexaFluor 750–labeled lectin. Animal brains were fixated in Paxgene Tissue Fix (PreAnalytix), cleared employing the iDISCO mehtod and assesed by light sheet ultramicroscopy (LaVision Biotec).

The accumulation of the nanoparticles was additionally assessed in the formalin-fixed paraffin-embedded (FFPE) specimes employing Prussian blue (Berliner blau) staining. The sections were immersed for 20 min in staining solution (20% hydrochloric acid and 10% potassium ferrocyanide solution mixture, ratio 1 : 1) and counterstained using nuclear fast red for 5 min. Sections were additionaly H&E stained. For IHC analysis, 5 μm-thick sections cut from FFPE blocks were deparaffinized, xylene dehydrated, graded with a series of ethanol (absolute, 95%, 80%, and 50%), and washed in PBS with tween-20 (PBST). Sections were incubated with the primary antibodies anti-cleaved caspase-3 (1:150 dilution; Abcam, USA) or antibodies against Granzyme B (1:200 dilution; Abcam, USA) overnight at 4°C. Sections were then washed with PBST and incubated with secondary goat anti-rabbit antibodies for 1 hour. Following washing with PBST sections were processed with sterptavidin peroxidase at room temperature for 30 min, rinsed with water and developed employing DAB kit (Dako North America Inc., CA, USA). Subsequently sections were counterstained with hematoxylin, dehydrated and mounted.

5.16. Statistics

The comparative survival of the tumor-bearing animals was assessed with Kaplan-Meier curves that are based on the Kaplan-Meier estimator. All such estimates were computed with corresponding confidence intervals. The Kaplan-Meier estimator is a non-parametric statistic that accommodates right-censoring in the data. When the means of the groups of two continuous variables were compared, the parametric Student's *t*-test was employed. Variances between groups were considered to be equal, and degrees of freedom for such tests were computed accordingly. The significance level for all tests was alpha = 0.05, and all confidence intervals are reported at the 95% level. All p-values reported for all t-tests are two-sided. When comparing multiple groups, each of which had so few observations that standard parametric assumptions could not be validated, the Kruskal-Wallis test, which is a non-parametric analog to the one-way ANOVA test, was applied. The Krukal-Wallis test analyzes the differences in ranks between groups, rather than the difference in means. Depending on

the test, either Statistica Version 9.2 for Windows or the R programming language was run for all tests.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The study was conceived and designed by MS and GM. MS, SS, BN, LY, YM, RT, SE, WS, EP, AM, PT, OG, VR, KC, WK, OS, KS conducted and analyzed the experiments. EP supported statistical analysis. MS and GM wrote the manuscript with valuable comments from OG, VR, and KC.

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Figure legends

Figure 1. Physico-chemical characterization of GrB-SPIONs. (a) Transmission electron microscopy image of the obtained GrB magnetic conjugates. Scale bar, 50 nm. **(b)** Hydrodynamic size of the non-conjugated SPIONs and GrB-SPIONs. **(c)** Zeta-potential (mV) for the SPIONs and GrB-SPIONs. **(d)** R_1, R_2, and R_2* magnetic relaxation times of water protons in nanodispersion of SPIONs and GrB-SPIONs. Ferrocin was used as a positive control. **(e)** Dynamic light scattering (DLS) analysis for the GrB-SPIONs co-incubated with BSA or Hsp70 for 4 hours. **(f)** Magnetic relaxation ("switch") assay for GrB-SPIONs following co-incubation with Hsp70.

Figure 2. Cellular interactions of GrB-SPION magnetic conjugates. (a) Confocal microscopy images of the U87 glioblastoma cells co-incubated for 24 hours with SPIONs or GrB-SPIONs (Fe concentration 100 μg/mL). DAPI was applied for nuclei staining (blue). Magnetic nanoparticles were detected employing reflective laser scanning (green). Scale bar, 5 μm. **(b)** Electron microscopy images of the U87 and C6 cells following co-incubation with GrB-SPIONs (Fe concentration 100 μg/mL) for 24 hours. Incorporated nanoparticles are pointed by red arrows. **(c)** Immunofluorescence images of the U87 cells stained for EEA1, Rab5, Rab7, and LAMP1 (all shown in red) following co-

incubation with GrB-SPIONs (green) for 24 hours. Scale bar, 5 μm. **(d)** Colorimetric ferrozine-based analysis of the nanoparticles uptake by tumor cells before and 24 hours following single dose (4 Gy) irradiation. Data are presented as M ± SD from three different experiments. **(e)** Immunofluorescence confocal microscopy images of the U87 cells co-stained for Hsp70 before and after 4 Gy irradiation and co-incubation with SPIONs or GrB-SPIONs for 24 hours. Additionally blocking monoclonal antibodies (BRM-22a) against Hsp70 were applied prior to co-incubation with GrB-SPIONs conjugates. Scale bar, 4 μm. **(f)** Side scattering (SSC), forward scattering (FSC) and Mean fluorescence intensity (MFI) for tumor cells floowing co-incubation with SPIONs or GrB-SPIONs for 24 hours. M ± SD was calculated from the three different experiments.

Figure 3. Cytotoxicity activity of GrB-SPIONs. Annexin V assay was employed for the detection of the tumor cell apoptosis following co-incubation with SPIONs or GrB-SPIONs at various Fe concentrations for 24 hours. Data are presented as M ± SD from three different experiments.

Figure 4. Tumor targeting of GrB-SPIONs. (a) Representative magnetic resonance images (T1- and T2-weighted, gradient echo (Flash)) of the orthotopic U87 glioblastoma obtained in coronal plane for mice treated with PBS, SPIONs or GrB-SPIONs. Tumor is delineated by red dotted line. **(b)** Confocal immunofluorescence images of the U87 glioblastoma from animals treated with PBS, SPIONs or GrB-SPIONs. Nuclei were stained by DAPI (blue). FITC-conjugated monoclonal cmHsp70.1 antibodies against Hsp70 were applied for the detection of protein (red). Nanoparticles were detected by reflective laser scanning (green). Cell membrane was stained by CellMask^{TM} Deep Red plasma membrane staining (dark blue). Scale bar, 15 μm. **(c)** Dose planning for single dose (10 Gy) irradiation of the orthotopic U87 glioblastoma in NMRI nu/nu mice. **(d)** Representative magnetic resonance images of U87 glioblastoma and corresponding confocal microscopy images of the glioma tissue following single dose irradiation and i.v. injection of GrB-SPIONs. **(e)** T2 values (ms) obtained from the T2* maps of the animals treated with PBS, SPIONs, GrB-SPIONs, and following combination with single dose irradiation or magnetic targeting. Data are presented as M ± SD. **(f)** NLR-M_2 biodistribution analysis of nanoparticles in the o.t. U87 model following administration of

SPIONs, GrB-SPIONs or administration of GrB conjugates after single dose irradiation. **(g)** Representative magnetic resonance images and confocal immunofluorescence staining of the orthotopic C6 glioma following magnetic targeting of GrB-SPIONs. Hypointesity regions in the tumor are pointed by red solid arrows.

Figure 5. Targeting of the small cell lung cancer H1339 with GrB-SPIONs, *in vivo*. (a) Dose planning for single dose (10 Gy) irradiation of the o.t. H1339 tumor and brain metastasis. **(b)** T2 values (ms) deducted from T2* maps of animals treated with PBS, GrB, SPIONs, GrB-SPIONs and combination of GrB conjugates with single dose (10 Gy) irradiation.

Figure 6. Therapeutic efficacy of GrB-SPIONs in preclinical models. The survival curves of the tumor-bearing animals were analyzed employing the Kaplan-Meier method. **(a)** Kaplan-Meier survival curves for the animals treated with PBS, GrB, SPIONs or GrB-SPIONs. Additionally GrB-SPIONs were assessed in the advanced stage H1339 model with brain metastasis. **(b)** GrB magnetic conjugates were administered as single-agent therapy in the o.t. model of U87 glioblastoma as series of i.v. injections or via subcutaneous implanted peristaltic iPrecio® mini-pump **(c)**. Subsequently i.v. injected GrB-SPIONs were combined with single dose (10 Gy) irradiation **(d)**. The feasibility of GrB-SPIONs magnetic targeting employing NdFeB ferromagnets was assessed in the o.t. C6 glioblastoma model in Wistar rats. Nanoparticles were i.v. administered in combination with magnetic targeting **(e)** or with subsequent single dose (10 Gy) irradiation **(f)**. **(g)** Analysis of the U87 glioblastoma for iron (Berliner Blau stainin) and IHC for granzyme B and caspase 3 following treatment by PBS, GrB, SPIONs or GrB-SPIONs. Scale bar, 25 μm.

Supplementary Figures

Figure S1. The X-ray diffraction (XRD) intensity *v*s diffraction angle for magnetite superparamagnetic iron oxide nanoparticles (SPIONs) at room temperature. The marks at the bottom indicate nominal reflections: the upper and lower sets are for magnetite (Fe_3O_4) and hematite (Fe_2O_3), respectively.

Figure S2. Biophysical characterization of GrB-SPIONs. (a) Principle of nonlinear longitudinal response M_2 measurements. **(b)** Second harmonic magentic response measurements of GrB-SPIONs to a weak *ac* field. **(c)** Relaxation R_2 measurements of GrB-SPIONs in various systems (2% agarose gel, 5% human serum albumin (HAS) solution, and H_2O distilled water). **(d)** Representative magnetic resonance images (T1- and T2-weighted, gradient echo (Flash)) of the U87 cells in 1% agarose gel following co-incubation with SPIONs or GrB-SPIONs for 24 hours. **(e)** T2 values (ms) deducted from T2* map images of the U87 cells co-incubated with SPIONs or GrB-SPIONs for 24 hours. Data are presented as M ± SD.

Figure S3. Microscale thermophoresis measurements of GrB K_d for Hsp70 binding.

Figure S4. Confocal microscopy images of the human U87 glioblastoma cells and L929 fibroblasts co-incubated with GrB-SPIONs for 24 hours. Nanoparticles were detected employing reflective laser scanning (green). Lysomes were stained with LysoTracker dye (red). Nuclei stained with Hoechst33342 (blue). Scale bar, 4 μm.

Figure S5. Representative light sheet microscopical views of human U87 glioblastoma 15 minutes after intravenous injection of 200 μg of Lectin-[AF750] (white signals).

Figure S6. Targeting of the orthotopic GL261 glioblastoma with GrB or cmHsp70.1 monoclonal antibodies. GrB, cmHsp70.1 and BSA (as negative control) were conjugated with Alexa680. Following 24 hours of intravenous administration of labeled agents epifluorescence images of the brain tissues were obtained. Signal intenstity (arb. units) was measured in the tumor region and normal brain tissues. Data are presented as M ± SD.

Figure S7. Biodistribution of the GrB-SPIONs following intravenous injection 24, 48 hours and 7 days in the model of the orthotopic U87 glioblastoma in the NMRI nu/nu mice.

Figure S8. Amplitudes of the Re$M_2(H)$ signals for the samples from non-treated NMRI nu/nu animals. The presented values are $[(ReM_{2max}^{dir} - ReM_{2min}^{inv})/2]\cdot 10^8 \propto C_{NPs}$

Figure S9. Biodistribution of the magnetic nanoparticles following intravenous administration as a single-agent or in combination with single dose (10 Gy) radiotherapy and/or magnetic

targeting. Images are presented for brain, C6 glioblastoma, spleen, pancreas, and heart tissues.

Figure S10. Biodistribution of GrB-SPIONs after i.v. administration as a single-agent or in combination with a single irradiation dose (10 Gy) and/or magnetic targeting. Images are presented for liver, kidney, lung, muscle, and skin tissues.

Figure S11. Therapeutic activity of intra-tumorally injected GrB-SPIONs in orthotopic GL261 glioblastoma mouse models. (a) Representative magnetic resonance images (gradient echo (Flash), T2-weighted, T2* map) with corresponding T2 values (ms) for C57/Bl6 mice treated with PBS, GrB, SPIONs or GrB-SPIONs. Data for T2 values are presented as M ± SD. **(b)** MR tumor volumetrics measurements of the GL261 glioblastoma in the tumor-bearing animals treated with PBS, GrB, SPIONs or GrB-SPIONs. MR images were obtained on day 5, 10, 15 and 20 following tumor cells implantation. **(c)** Berliner blau staining of the GL261 glioblastoma for detection of the iron. IHC of the tumor sections stained for GrB and caspase 3. Scale bar, 100 μm.

Table S1. Amplitudes of the Re$M_2(H)$ signals for the samples from animals treated with PBS, GrB, SPIONs or GrB-SPIONs in orthotopic U87 glioma mouse models.. The presented values are $[(ReM_{2max}^{dir} - ReM_{2min}^{inv})/2]\cdot 10^8 \propto C_{NPs}$

Table S2. Survival of animals with o.t. small cell lung cancer H1339 afterthree i.v. injections of PBS, GrB, SPIONs or GrB-SPIONs (6 animals per group). Data are presented as M ± SD.

Table S3. Survival of animals with o.t. small cell lung cancer H1339 and brain metastasis afterthree i.v. injections of PBS, GrB, SPIONs or GrB-SPIONs (6 animals per group). Data are presented as M ± SD.

Table S4. Survival of animals with o.t. U87 glioblastoma treated with three i.v. injections of PBS, GrB, SPIONs or GrB-SPIONs (10 animals per group). Data are presented as M ± SD.

Table S5. Survival of animals with o.t. U87 glioblastoma treated with three i.v. injections of PBS, GrB, SPIONs or GrB-SPIONs and GrB and GrB-SPIONs employing peristaltic mini-pump iPrecio® system (5 animals per group). Data are presented as M ± SD.

Table S6. Survival of animals with o.t. U87 glioblastoma treated with three i.v. injections of PBS, GrB, SPIONs or GrB-SPIONs in combination with single dose (10 Gy) irradiation (6 animals per group). Data are presented as M ± SD.

Table S7. Survival of Wistar rats with o.t. C6 glioblastoma treated with three i.v. injections of PBS, SPIONs or GrB-SPIONs in combination with external magnetic targeting (6 animals per group). Data are presented as M ± SD.

Table S8. Survival of Wistar rats with o.t. C6 glioblastoma treated with three i.v. injections of PBS, SPIONs or GrB-SPIONs in combination with external magnetic targeting and single dose (10 Gy) irradiation (6 animals per group). Data are presented as M ± SD.

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70-kDa heat shock protein (Hsp70) is expressed on membrane of tumor cells, but not on corresponding normal cells and thus could be exploited for development of theranostic agents. Serine protease Granzyme B conjugated with superparamagnetic iron oxide nanoparticles (GrB-SPIONs) is shown to specifically target tumors. Combination of nanoparticles with radiotherapy and/or magnetic targeting significantly enhances the therapeutic potential of the nanocarriers.

Keywords: superparamagnetic nanoparticles, granzyme B, brain tumors, magnetic targeting, radiotherapy

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Title: Granzyme B functionalized nanoparticles targeting membrane Hsp70-positive tumors for multimodal cancer theranostics