

1 **Changes in the tumor microenvironment and treatment outcome in glioblastoma: A pilot**
2 **study.**

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35 **Running title:** TME targeted therapy in GBM

36

37 **Abstract:**

38 Glioblastoma (GBM) is a hypervascular and aggressive primary malignant tumor of the central
39 nervous system. Recent investigations showed that traditional therapies along with antiangiogenic
40 therapies failed due to the development of post-therapy resistant and recurrent GBM. Our
41 investigations show that there are changes in the cellular and metabolic compositions in the tumor
42 microenvironment (TME). It can be said that tumor cell-directed therapies are ineffective and we
43 need to rethink how to treat GBM.

44 We hypothesize that the composition of TME-associated cells will be different based on the
45 therapy and therapeutic agents, and TME-targeting therapy will be better to decrease recurrence
46 and improve survival. Therefore, the purpose of this study is to determine the changes in the TME
47 in respect of T-cell population, M1 and M2 macrophage polarization status, and MDSC population
48 following different treatments in a syngeneic model of GBM. In addition to these parameters,
49 tumor growth and survival were also studied following different treatments.

50 The results showed that changes in the TME-associated cells were dependent on the therapeutic
51 agents and the TME-targeting therapy improved the survival of the GBM bearing animals.

52 The current GBM therapies should be revisited to add agents to prevent the accumulation of bone
53 marrow-derived cells in the TME or to prevent the effect of immune-suppressive myeloid cells in
54 causing alternative neovascularization, the revival of glioma stem cells, and recurrence. Instead of
55 concurrent therapy, a sequential strategy would be best to target TME-associated cells.

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57 **Keywords:** Glioblastoma (GBM), Tumor microenvironment (TME), TME-associated cells,
58 Radiation, Temozolomide, Myeloid cells, check-point inhibitor, arachidonic acid metabolites.

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74 **Introduction:**

75 Even with current treatment strategies and the addition of expensive immunotherapies or
76 antiangiogenic therapies, the prognosis of glioblastoma (GBM) is dismal (1-3). GBM is a very
77 hypervascular and invasive malignant tumor. So much so that, current treatments consisting of
78 surgery, radiation and chemotherapies with or without adjuvant still show no hope to patients (4-
79 6). Interestingly, recent investigations demonstrated that traditional therapies along with newer
80 antiangiogenic therapies are changing the cellular as well as the metabolic compositions of the
81 tumor microenvironment (**TME**) tremendously (7-11). Therefore, newer treatment strategies
82 targeting TME should be considered along with targeting tumor cells in GBM.

83 The TME is composed of tumor cells, stromal cells, cells from the bone marrow, and the
84 extracellular matrix (12). Except for a few cell types, such as normal epithelial cells, myoepithelial
85 cells, dendritic cells, M1 macrophages, N1 neutrophils and CD8 T-cells, most of the stromal and
86 bone marrow-derived cells promote tumor growth and metastasis (10, 11, 13-15). In fact, platelets
87 have also been shown to promote tumor growth (16-19). Therefore, it is imperative to include
88 targeting tumor-associated cells in the current standard regimen of therapies for malignant tumors
89 such as GBM. However, there have been limited investigations done to understand the changes in
90 the TME following standard as well as experimental therapies in GBM.

91 Tumor induction and evolution is driven by the interplay between stromal and immune cells within
92 the TME. Tumor-associated macrophages (TAM), a critical component of the TME, have a
93 differential function in respect to tumor growth and metastasis (20-22). TAM recruitment,
94 localization, and phenotypes are regulated by the tumor-secreted factors at the hypoxic areas of
95 the tumor (23, 24). Depending on the stimuli, macrophages undergo a series of functional
96 reprogramming as described by two different polarization states, known as M1 and M2 (24, 25).
97 Phenotypically, M1 macrophages express high levels of major histocompatibility complex
98 class II (MHC II), the CD68 marker, and co-stimulatory molecules CD80 and CD86. On the
99 other hand, M2 macrophages express high levels of MHC II, CD163, CD206/MRC1, Arg-1
100 (mouse only) and others. In the TME, classically activated macrophages, also known as M1
101 macrophages, are activated by tumor-derived cytokines such as granulocyte monocyte colony
102 stimulating factor (GM-CSF), interferon- γ , and tumor necrosis factor (TNF). These M1
103 macrophages play an important role as inducer and effector cells in polarized type 1 helper T cell
104 (Th1) responses. These Th1 cells drive cellular immunity to eliminate cancerous cells. To
105 accomplish Th1 activation, M1 macrophages produce high amounts of IL-12 and IL-23, and low
106 amounts of IL-10, reactive oxygen and nitrogen species, and IL-1 β , TNF, and IL-6 inflammatory
107 cytokines (25, 26). M1 macrophages also release anti-tumor chemokines and chemokines such as
108 CXCL-9 and CXCL-10 that attract Th1 cells, (27-29). Th1 cells drive cellular immunity to
109 eliminate cancerous cells. On the other hand, M2-polarized macrophages, also known as
110 alternatively activated macrophages are induced by IL-4, IL-13, IL-21 and IL-33 cytokines in the
111 TME (30, 31). M2 macrophages release high levels of IL-10 and, transforming growth factor-beta
112 (TGF- β) and low levels of IL-12 and IL-23 (type 2 cytokines). M2 macrophages also produce
113 CCL-17, CCL-22, and CCL-24 chemokines that regulate the recruitment of Tregs, Th2,
114 eosinophils, and basophils (type-2 pathway) in tumors (27, 29). The Th2 response is associated
115 with the anti-inflammatory and immunosuppressive microenvironment, which promotes tumor
116 growth.

117 Recent investigations including our own indicated the involvement of myeloid-derived suppressor
118 cells (MDSCs) in the primary as well as metastatic TME (32-36). MDSCs are a heterogeneous

119 population of immature myeloid cells, generated from bone marrow hematopoietic precursor cells
120 that fail to undergo terminal differentiation to mature monocytes or granulocytes. They are divided
121 broadly into monocytic (CD11b+/Gr1+/Ly6C+) and granulocytic (CD11b+/Gr1+/Ly6G+) (37-
122 39). During tumor progression, MDSCs are greatly expanded and they exhibit remarkable
123 immunosuppressive and tumorigenic activities. They are directly implicated in the escalation of
124 tumor metastases by partaking in the epithelial-mesenchymal transition (EMT) and, tumor cell
125 invasion, while also promoting angiogenesis and formation of the pre-metastatic niche (13, 33,
126 34). MDSCs were demonstrated to promote tumor invasion and metastasis by two mechanisms:
127 (i) increasing production of multiple matrix metalloproteinases (MMPs) that degrade the extra-
128 cellular matrix and chemokines that establish a pre-metastatic milieu (40, 41), and (ii) merging
129 with tumor cells (42, 43).

130 From the above discussion, it is obvious that TME-associated bone marrow-derived cells are
131 important in treatment resistance, invasion and metastasis. Therefore, the purpose of this study is
132 to determine the changes in the TME in respect of T-cell population, M1 and M2 macrophage
133 polarization status, and MDSC population following different treatments in a syngeneic model of
134 GBM. In addition to these parameters, tumor growth and survival were also studied following
135 different treatments. In this study, we have used the following agents: a drug that alters
136 hydroxylase pathways of arachidonic acid metabolism (HET0016 and its different analogs),
137 colony stimulating factor 1 receptor (CSF1R) inhibitor (GW2580), anti PD-1 (program death)
138 antibody, CXCR2 receptor blockers (Navarixin and SB225002), temozolomide (TMZ),
139 irradiation, VEGFR2 receptor tyrosine kinase inhibitor (Vatalanib), and conditional CSF1R
140 knockout mice plus different treatments.

141

142 **Materials and methods:**

143 **Ethics statement:** All the experiments were performed according to the National Institutes of
144 Health (NIH) guidelines and regulations. The Institutional Animal Care and Use Committee
145 (IACUC) of Augusta University (protocol #2014–0625) approved all the experimental procedures.
146 All animals were kept under regular barrier conditions at room temperature with exposure to light
147 for 12 hours and dark for 12 hours. Food and water were offered ad libitum. All efforts were made
148 to ameliorate the suffering of animals. CO₂ with a secondary method was used to euthanize
149 animals for tissue collection.

150 **Materials:** HP β CD (2-hydroxy Propyl- β -Cyclodextrin) was purchased from Sigma-Aldrich (St.
151 Louis, MO), cell culture media was from Thermo Scientific (Waltham, MA), and fetal bovine
152 serum was purchased from Hyclone (Logan, Utah). HET0016 was made by Dr. Levedyeva in the
153 Department of Chemistry, Augusta University with a purity of more than 97%. Cell culture grade
154 DMSO was purchased from Fischer Scientific (PA). We made the complex of HET0016 plus
155 HP β CD as per our previous publication (8). VEGFR2 tyrosine kinase inhibitor (Vatalanib) and
156 colony stimulating factor 1 receptor (CSF1R) inhibitor (GW2580) were purchased from LC
157 Laboratories, Woburn, MA. SB225002 (CXCR2 inhibitor) was purchased from Selleckchem,
158 Houston, TX. Navarixin was purchased from MedKoo bioscience Inc, Morrisville, NC. All flow
159 antibodies are from Bio Legend, San Diego, CA. All antibodies for western blotting,
160 immunohistochemistry, and immunofluorescence were purchased from Santa Cruz (total-CXCR2
161 and anti-GAPDH), R&D systems (anti-hCXCR2), Thermo Scientific (anti-Laminin), and Sigma

162 Aldrich (β -actin and FITC-conjugated tomato lectin). All culture media were purchased from
163 Corning and GE Healthcare Life Sciences.

164 **Tumor cells and orthotopic animal model of GBM:** To determine the *in vivo* effect of different
165 treatments, orthotopic GBM models using syngeneic GL261 cells in wild type and CSF1R
166 conditional knockout C57BL/6 mice were prepared according to our published methods (8, 10, 11,
167 44). In short, luciferase positive GL261 cells were grown in standard growth media (RPMI-1640
168 plus 10% FBS) and collected in serum-free media on the day of implantation. After preparation
169 and drilling a hole at 2.25 mm (athymic nude mice) to the right and 2 mm posterior to the bregma,
170 taking care not to penetrate the dura, a 10 μ L Hamilton syringe with a 26G-needle containing
171 tumor cells (10,000) in a volume of 3 μ L was lowered to a depth of 4 mm and then raised to a depth
172 of 3 mm. During and after the injection, a careful note was made for any reflux from the injection
173 site. After completing the injection, we waited 2-3 minutes before withdrawing the needle 1 mm
174 at a time in a stepwise manner. The surgical hole was sealed with bone wax. Finally, the skull was
175 swabbed with betadine before suturing the skin (45-47). There were at least three animals in each
176 group of treatment. Tumor growth was determined by optical imaging (bioluminescence imaging
177 after injecting luciferin) on days 8, 15 and 22. For flow cytometry of tumor-associated cells,
178 animals were euthanized on day 22 after the last optical imaging. Both male and female animals
179 were used.

180 **Treatments:** All treatments were started on day 8 following tumor implantation and continued for
181 two weeks. The following treatment groups were used to determine the TME associated T-cells,
182 different macrophages, MDSCs present by flow cytometry; 1) vehicle, 2) HET0016 complexed
183 with HP β CD at 10mg/kg/day for 5 days/week, intravenous (IV), 3) GW2580, 160mg/kg/day
184 3day/week, oral, 4) temozolomide (TMZ) 50mg/kg/day, 3days/week, oral, 5) Vatalanib
185 50mg/kg/day, 5 days/week, oral, 6) Navarixin, 10mg/kg/day, 5 days/week, intraperitoneal (IP), 7)
186 anti-PD-1 antibody, 200 μ g/dose, 2 doses/week, IP, 8) image guided radiation therapy,
187 10Gy/dose/week for two weeks, 9) combined HET0016 plus GW2580, 10) combined HET0016
188 plus GW2580 plus anti- PD-1 antibody.

189 **Making of a conditional knockout mouse model of bone marrow-derived CSF1R+ myeloid**
190 **cells:** Heterozygous CSF1R_{flox/wt}/MX1-Cre⁺ male was mated with a heterozygous CSF1R_{flox/wt}/MX1-Cre⁺
191 female to achieve 25% of the progeny with homozygous CSF1R_{flox/flox}/MX1-Cre⁺ (knockout) genotype in bone marrow cells. Other progeny was wild-type CSF1R_{wt/wt}/MX1-Cre⁺ (25%) and heterozygous CSF1R_{flox/wt}/MX1-Cre⁺ (50%) genotypes. After repeated cross-breeding, we have generated a colony of CSF1R_{flox/flox}/Cre⁺ (knockout). These animals are healthy and are being used for breeding. Analysis of myeloid cells in the peripheral blood before and after injection of polyinosinic-polycytidylic acid (poly-IC) showed bone marrow-specific depletion of CSF1R⁺ cells (**Figure 1**). These animals (male and female) were used to generate GL261 derived syngeneic GBM after depletion of bone marrow-derived myeloid cells and then treated with HET0016 or anti PD-1 antibody alone or in combination or with CXCR2 antagonist SB225002 (10mg/kg/day 5 days/week, IP) for two weeks.

201 **Determination of bone marrow-derived cells in the TME:** Following euthanasia, animals were
202 perfused with ice-cold PBS and the right brain containing GBM was collected, passed through 40-
203 micron mesh and a single-cell suspension was made. Similarly, spleens were collected, passed
204 through 40micron mesh and a single-cell suspension was made. Before adding panels of antibody
205 cocktail, non-specific uptake of the antibody was blocked by adding recommended blocker. The
206 population of the following cells were determined by a Accuri C6 flow cytometer from cells

207 collected from tumors and spleen; CD45+/CD4+, CD45+/CD8+, CD45+/CD11B+/Gr1+/Ly6C+,
208 CD45+/CD11B+/Gr1+/Ly6G+, CD45+/CD86+/CD80+, AND CD45+/CD206+. The findings
209 were compared among all the treatment groups.

210 **Determination of tumor growth:** Bioluminescent imaging was used to determine the tumor
211 growth following different treatments. All animals underwent imaging following IP injection of
212 luciferin (150mg/kg). Images were obtained from all animals on days 8, 15 and 22. Photon density
213 (photon/sec/mm²) was determined by drawing an irregular region of interest to cover the tumor
214 area. The findings were compared among all the treatment groups.

215 **Determination of survival:** Groups of animals were also used to determine the survival following
216 different TME targeted therapies. All animals were routinely observed 2-3 times a week to assess
217 the wellbeing as well as body weight. The animals were followed up until they become moribund
218 or fulfill the criteria for euthanasia as per the approved IACUC protocols. The findings were
219 compared among all the treatment groups.

220 **Statistical analysis:** Quantitative data were expressed as mean ± standard error of the mean (SEM)
221 unless otherwise stated. For the flow-cytometric studies, we used ordinary one-way analysis of
222 variance (ANOVA) followed by multiple comparisons using Dunnett's multiple comparisons test.
223 For BLI (optical imaging) data, the general framework of analyses included two-way ANOVA
224 followed by either Tukey's or Sidak's multiple comparisons. We analyzed the survival of the animals
225 following different treatments. Log-rank test (Mantel-Cox) was applied to determine the significance
226 of differences among the groups. A P value of 0.05 was considered significant.

227

228 **Results:**

229 In this study, we successfully developed CSF1R conditional knockout mouse. These conditional
230 knock out mice showed homozygous CSF1R^{fl^{ox}/fl^{ox}}/MX1-Cre+ (knockout) genotype (**Figure 1A**).
231 Compared to wild type mice, conditional knockout mice showed a significant dose-dependent
232 decrease in CD45+CSF1R+ cells following two weeks of treatments with poly-IC. There was
233 almost 80% decrease of CSF1R+ cells in the peripheral blood (**Figure 1B**). Wild type mice treated
234 with poly-IC did not show any significant difference in CD45+CSF1R+ cells (**Figure 1C**). Both
235 wild type (control) and knockout mice (after two weeks' of treatments with poly-IC) received
236 intracranial implantation of syngeneic GL261 glioblastoma. On day 8 of tumor implantation,
237 groups of animals received either vehicle or SB225002 for two weeks. All animals underwent
238 optical imaging pre and post-treatment. Photon intensities were determined to measure tumor
239 growth. Wild type control animals showed significantly increased tumor growth (**Figure 1D**)
240 which is indicated by a 10-fold increase in the photon intensity (**Figure 1E**). On the other hand,
241 both wild type (control) treated with SB225002 and knockout mice showed significantly decreased
242 tumor growth at week 3, indicating the involvement of CSF1R+ cells in the TME. It is also known
243 that the CXCR2 antagonist can inhibit the function of myeloid cells by blocking the interaction of
244 CXCR2 and IL-8 (48-50). Tumor-associated CD45+CD11b+CD86+ and CD45+CD11b+CD206+
245 cells were determined following treatment with SB225002 in wild type animals. Both cell types
246 were significantly decreased following the treatments (**Figure 1F**). T-cells and MDSC populations
247 showed no significant difference between the treated and untreated wild type animals.

248 Both wild type and CSF1R knockout mice received different treatments that target tumor cells or
249 tumor-associated cells. All treatments were for two weeks and the treatment was started on day 8

250 of orthotopic tumor implantation. On day 22 following last optical imaging, animals were
251 euthanized and the tumors were collected for flow cytometry to determine the population of T-
252 cells (CD4, CD8), CD11b+ cells, macrophages (M1 and M2), and MDSCs (Ly6C and Ly6G). To
253 our surprise, CD4, CD8, CD11b, and Ly6G positive cells significantly increased in tumors treated
254 with TMZ (**Figure 2**). On the other hand, different cellular populations were significantly
255 decreased in post-radiation tumors. All other treatments that targeted tumor-associated myeloid
256 cells or checkpoint showed increased accumulation of CD4 and CD8 cells in the tumors but
257 myeloid cell populations including MDSCs, CD11b+ cells, and macrophages showed insignificant
258 changes in the TME compared to that of control and Vatalanib treated tumors (**Figure 3**).

259 All animals that were followed for survival and euthanized on day 22 to determine the TME
260 associated cells also underwent optical imaging before treatment and at one and two weeks after
261 treatments. The dose of luciferin and exposure time were kept identical for every animal at each
262 time point. Then the photon intensity (intensity/sec/mm²) was determined by making an irregular
263 region of interest encircling the tumors at each time point. **Figure 4** shows the tumor growth
264 following different treatments. Tumor in all therapy groups except in Vatalanib treated animals,
265 were stable following 1 week of treatments and there was no significant difference compared to
266 that of vehicle-treated animals. However, Vatalanib treated animals showed significantly increased
267 photon intensity indicating tumor growth following 1 week of treatments. Tumor growths were
268 substantially increased in vehicle, Vatalanib, and TMZ treated animals following 2 weeks of
269 therapy indicating the development of resistance in TMZ group. All other groups showed increased
270 tumor growth but were significantly slower than that of vehicle, Vatalanib, or TMZ treated
271 animals. It should be noted that the animals that received TME-associated cell-directed therapy
272 showed significantly lower tumor growth 2 weeks following treatments. The animals that receive
273 antiangiogenic (Vatalanib) and tumor cell-targeted (TMZ) therapy exhibited rebound tumor
274 growth at 2 weeks of treatments.

275 We instituted different treatments targeting both tumor cells and the tumor microenvironment
276 including arachidonic acid metabolisms and anti-depressant (selective serotonin reuptake inhibitor
277 (SSRI), fluoxetine) drugs alone or in combination with TMZ. We also used a very high dose of
278 HET0016 (50mg/kg/day). Usual dose of HET0016 is 10mg/kg/day. All treatments significantly
279 increased the survival of animals bearing syngeneic GL261 GBM (**Figure 5A**). The most
280 significantly increased survival was observed in animals' groups that were treated with TMZ,
281 HET0016, TMZ+HET0016, and with a HET analog. Although Navarixin (IL-8CXCR2 axis
282 blocker) increased the survival of the animals, the addition of TMZ did not improve survival
283 (**Figure 5B**).

284

285 **Discussion:**

286 GBM is a devastating malignant tumor of the central nervous system. Once diagnosed it becomes
287 a death sentence to patients within 15 months (51-54). Currently, surgical resection followed by
288 radiation and TMZ therapies is the standard of care for GBM patients (55). With these extensive
289 therapies, almost all patients show therapy resistance and recurrence of GBM (56). To address
290 resistance and recurrence, clinicians have adopted antiangiogenic therapies in recurrent GBM.
291 These treatments decrease the formation of new blood vessels and decrease edema, thus reducing
292 the dose of corticosteroids needed after therapy (57, 58). Additionally, advanced immunotherapy
293 and targeted therapies have been instituted (59). However, early reports demonstrated that these

294 are non-effective treatment strategies (10, 45, 60-65). Investigations from our lab indicated that
295 most of the instituted therapies mobilized bone-marrow cells to the sites of GBM and orchestrated
296 therapy resistance (10, 11). Our results showed that antiangiogenic therapies initiate alternate
297 vascularization pathways and eventually increased neovascularization in therapy-resistant GBM
298 (7, 45, 66). We found that angiogenic and vasculogenic myeloid cells accumulated at GBM sites
299 following therapies(11, 65). Furthermore, we reported the process of vascular mimicry in which
300 GBM cells transdifferentiate into glioma stem cells that can then form functional blood vessels (7,
301 67). All of these results support our conclusion that the possible changes occurring in the TME
302 following standard or investigational treatments in GBM have not been properly studied. This
303 includes both changes in TME associated cells as well as the changes that occur in the metabolic
304 cascade of TME associated cells. In this pilot study, we aimed to investigate these changes. To
305 accomplish this, we used standard therapies (radiation and TMZ) as well as agents that targeted
306 TME associated cells (CSF1R inhibitor GW2580 to target myeloid cells, IL-8-CXCR2 antagonists
307 Navarixin and SB225002 to target stem cells causing vascular mimicry, anti-PD1 antibody
308 targeting immune suppressive molecules) and different metabolic pathways (HET0016 and its
309 analog to target CYP4A-20-HETE axis of arachidonic acid metabolisms, fluoxetine to target
310 serotonin reuptake). Following therapies, we determined the changes in the composition of TME-
311 associated cells and the survival benefit of the therapeutic agents alone or in combination with
312 TMZ.

313 Our results clearly demonstrated the importance of TME associated CSF1R positive cells. Animals
314 treated with GW2580 and conditional knockout animals (CSF1R knockout) showed a decreased
315 number of myeloid cells in the TME, whereas TMZ therapy increased the population of myeloid
316 cells in the treated GBM. Previously, our reported results, as well as results from different
317 investigators, have proven the importance of myeloid cells in developing therapy resistance in
318 GBM and other cancers (11, 13, 15, 68-70). Myeloid cells, such as macrophages and MDSCs,
319 produce an immunosuppressive microenvironment that promotes tumor growth. Following
320 chemotherapy, macrophage differentiation is altered to promote the production of cancer-
321 supporting M2 macrophages in the TME (71). Chemotherapy has also been shown to promote
322 macrophage aggregation, thus facilitating cathepsin protease B- and S- mediated therapy resistance
323 (72). Some chemotherapeutic agents activate MDSCs to produce IL-1 β . This leads to the secretion
324 of IL-17 by CD4⁺ T-cells (73). Additionally, MDSCs have been shown to partake in the epithelial-
325 mesenchymal transition, increase the production of multiple matrix metalloproteinases, and merge
326 tumor cells (71-73). Therefore, the addition of myeloid cell blockage could mitigate these
327 mechanisms of resistance. However, it is to note that, previous investigations also indicated the
328 development of resistance following long-term therapy using CSF1R inhibitors (74, 75). This
329 indicates the importance of sequential or intermittent therapy targeting GBM TME associated cells
330 following or in between standard therapies for GBM.

331 To our surprise, we noticed a decreased accumulation of T-cells as well as different myeloid cell
332 populations in the TME following radiation therapies. This decreased accumulation of T-cells may
333 be due to the disruption of intact blood vessels that act as a delivery system of T-cells to the tumor
334 site. This disruption is likely caused by radiation therapy-induced necrosis in tumors leading to
335 tumor cell death. Therefore, most tumor recurrence in post-radiation GBM occurs from the
336 periphery of the irradiated areas where a few cells may have survived the radiation injury. Our
337 previous studies showed that the addition of HET0016 (blocker of CYP4A-20-HETE axis of
338 arachidonic acid metabolisms) improved the survival of animals bearing patient-derived xenograft
339 (PDX) GBM following 30Gy of radiotherapy (8). HET0016 is known to inhibit tumor and

340 endothelial cell (EC) proliferation, EC migration, and prevent neovascularization including
341 vascular mimicry (44, 67, 76). Although we have not tested agents that prevent the repair of DNA
342 damage, the addition of PARP inhibitor may also help prevent the recurrence of GBM following
343 radiotherapy (77, 78). However, in contrast to HET0016, PARP inhibitor has a very narrow
344 therapeutic window and causes severe toxicity (77). Therefore, adding an inhibitor of arachidonic
345 acid metabolic pathways may be useful in preventing the recurrence of post-radiation GBM.

346 Previously, we have reported the effectiveness of HET0016 in controlling GBM and breast cancer
347 (8, 32). However, we had not yet reported TME-associated cells present following the treatment
348 of HET0016. In this study, HET0016 treatment exhibited a similar phenomenon to that of myeloid
349 cell-targeted therapies. It showed an increased T-cell population in the TME compared to that of
350 vehicle and Vatalanib treated GBM. There was also a tendency to decrease immunosuppressive
351 myeloid cell populations in the TME. Additionally, treatments using HET0016 and its analog
352 showed significantly improved survival which corroborates with our previous reports (8). Our
353 ongoing investigations show that the CYP4A-20-HETE pathway is active not only in tumor cells
354 but also in TME associated myeloid cells (data not shown). Inhibition of 20-HETE increases the
355 cytotoxic T-cells population in *in vitro* studies (manuscript under preparation). Details of
356 HET0016 mediated therapies and its mechanisms are discussed in our previous reports (8).
357 Therefore, we propose that the use of an inhibitor of the cytochrome P450 γ -hydroxylase pathway
358 of arachidonic acid metabolisms may be used as an agent to target post-therapy GBM to prevent
359 recurrence.

360 **In conclusion:** current GBM therapies should be revisited to add agents to prevent the
361 accumulation of bone marrow-derived cells in the TME or to prevent the effect of immune-
362 suppressive myeloid cells in causing alternative neovascularization, the revival of glioma stem
363 cells, and recurrence. Instead of concurrent therapy, a sequential strategy would be best to target
364 TME associated cells.

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370 imaging (CIFSA) for acquiring optical images.

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380 **Author Contributions Statement:**

381 Sehar Ali: Analyze the flow cytometry and optical image data. She also helped writing the
382 manuscript.

383 Thaiz F Borin: Tumor cell propagation, tumor implantation and acquisition of flow cytometry
384 data. She edited the manuscript.

385 Raziye Piranlioglu: Tumor cell propagation, tumor implantation and acquisition of flow
386 cytometry data.

387 Roxan Ara: Help acquiring optical images and analysis

388 Iryna Lebedyeva: Synthesize HET0016 and its analog. Helped editing the manuscript.

389 Kartik Angara: Initiated the experiments using CXCR2 antagonist and Vatalanib treatments

390 Bhagelu R Achyut: Helped making the transgenic CSF1R knockout animals and conducted initial
391 optimization of Poly-IC injection and depletion of CSF1R+ cells. He also helped the interpretation
392 of TME data.

393 Ali S. Arbab: Conceived the hypothesis, design the experiments and provided the funds. He edited
394 the manuscript.

395 Mohammad H Rashid: Helped Sehar Ali to analyze the data, interpreted the results, maintaining
396 CSF1R knockout animal colony, tumor implantation, treating animals, acquisition of flow
397 cytometry data, preparing graphs and wrote the manuscript.

398

399 **Conflict of Interest Statement:** *None*

400

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402 intramural grant program at Augusta University to Ali S. Arbab.

403 **Contribution to the Field Statement:** Glioblastoma (GBM) is a devastating primary brain cancer.
404 Current treatments that use surgery, chemotherapy and radiotherapy do not increase the survival
405 of the patient. Almost all patients with GBM die with 15 months of diagnosis. GBM is also a tumor
406 with many blood vessels, therefore, clinician started using anti-neovascular agents. However,
407 recent reports indicated that all these treatments caused therapy resistance and enhance alternative
408 neovascularization due to mobilization and accumulation of cells derived from patients' bone
409 marrow. These mobilized bone marrow cells accumulate in the GBM microenvironment and
410 initiate an environment that is immunosuppressive and increase tumor cell invasion causing
411 recurrent tumors. There is a movement of rethinking of therapy strategies in GBM. Investigators
412 started using immunotherapy to change the microenvironment, however, early results are not
413 encouraging. We hypothesize that agents that target GBM microenvironment should be included
414 along with standard therapies either concurrently or sequentially. In this studies we showed the
415 changes in GBM microenvironment following different therapies and showed the improvement of
416 survival in mouse model following GBM microenvironment targeting therapies.

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643 **Figure legends:**

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645 **Figure 1: CSF1R conditional knockout mouse and GBM development.** (A) Agarose gel
646 electrophoresis showing homozygous CSF1R^{flox/flox}/MX1-Cre+ (knockout) genotype. (B) Flow-
647 cytometric analysis of peripheral blood cells from conditional knock out mice showed a significant dose-
648 dependent decrease in CD45+CSF1R+ cells following two weeks of treatments with poly-IC. (C)
649 Flow-cytometric analysis of peripheral blood cells from wild type mice did not show any significant
650 difference in CD45+CSF1R+ cells following two weeks of treatments with poly-IC. (D and E)
651 Optical images and quantified photon intensities of pre and post-treatment (either vehicle or
652 SB225002) showed significantly increased tumor growth in the vehicle-treated wild type animals
653 after 3 weeks. Knock out animals treated with either vehicle or SB225002 and wild type animals
654 treated with SB225002 did not show any significant tumor growth after 3 weeks. (F) Flow-
655 cytometric analysis showing significantly decreased tumor-associated CD45+CD11b+CD86+ and
656 CD45+CD11b+CD206+ cells.

657 **Figure 2: Flow cytometric analysis of T-cells and myeloid cell populations in wild type and**
658 **knockout animals.** There was a significant increase in CD4, CD8, CD11b, and Ly6G positive
659 cells in tumors treated with TMZ (red arrows) while irradiation caused a significant reduction
660 (black arrows) in different cellular populations compared to control group. All other treatments
661 showed increased infiltration of CD4 and CD8 T-cells but insignificant changes in MDSCs,
662 CD11b populations.

663

664 **Figure 3: Flowcytometric analysis of M1 and M2 macrophage populations.** Treatment with
665 Navarixin and GW2580 increased the macrophage population insignificantly, and all other
666 treatments changed the macrophage population inconsequentially.

667

668 **Figure 4: Bioluminescent image-based analysis of tumor growth.** All animals underwent
669 optical imaging to monitor tumor growth before starting the treatment (day 8 post-inoculation), 1
670 week, and 2 weeks after treatment. There was no significant difference between all treatment
671 groups compared to that of vehicle-treated animals after 1 week of treatment except the Vatanallib
672 treated group that showed significant tumor growth. Following 2 weeks of treatment, tumor
673 growths were substantially increased in the vehicle, Vatalanib, and TMZ treated animals. All other
674 groups showed increased tumor growth but were significantly slower than the above-mentioned
675 groups.

676

677 **Figure 5: Survival studies showing improved survival following the use of TME targeting**
678 **agents.** (A and B) Kaplan-Meier curve showing significantly increased survival in animal groups
679 treated with TMZ, HET0016, TMZ+HET0016, and with a HET analog. Although Navarixin)
680 increased survival, the addition of TMZ with it did not improve the outcome.

Fig 2

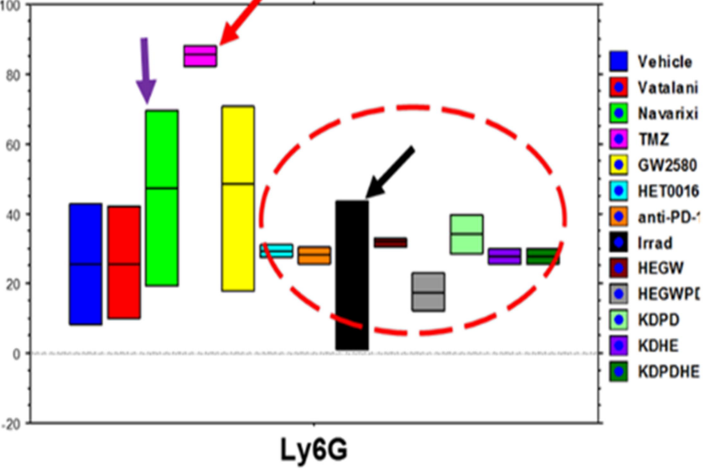
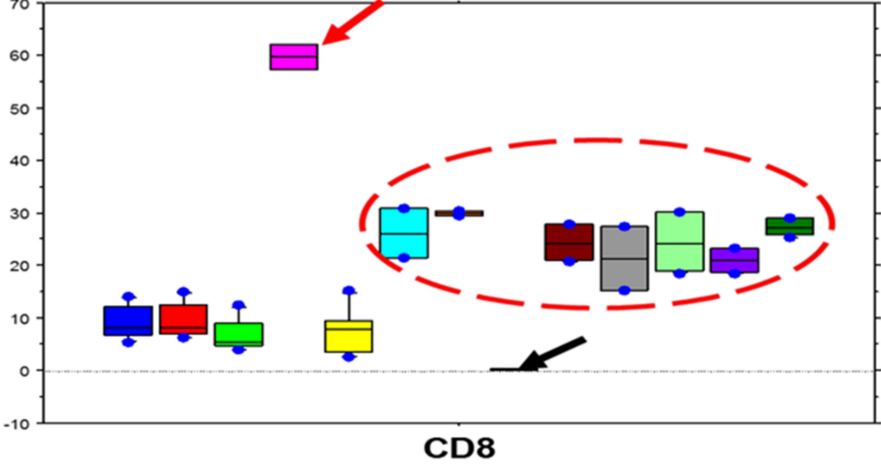
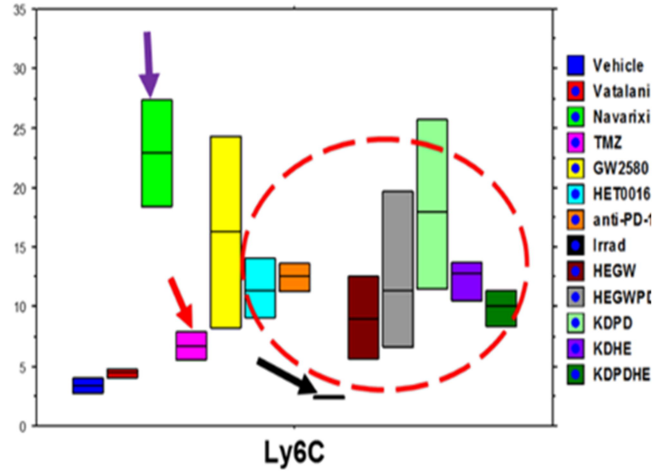
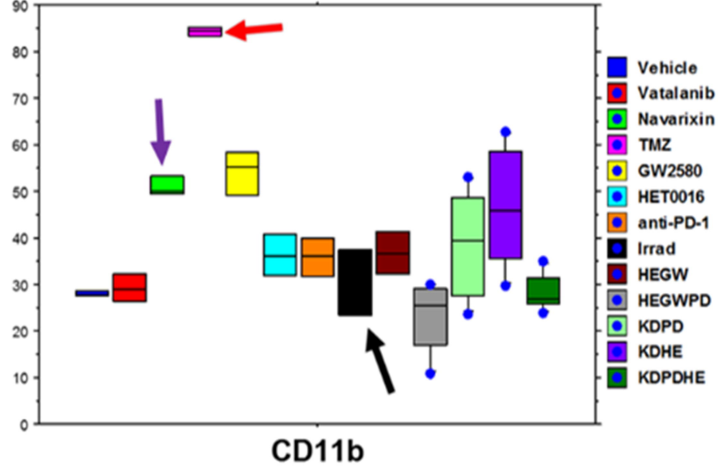
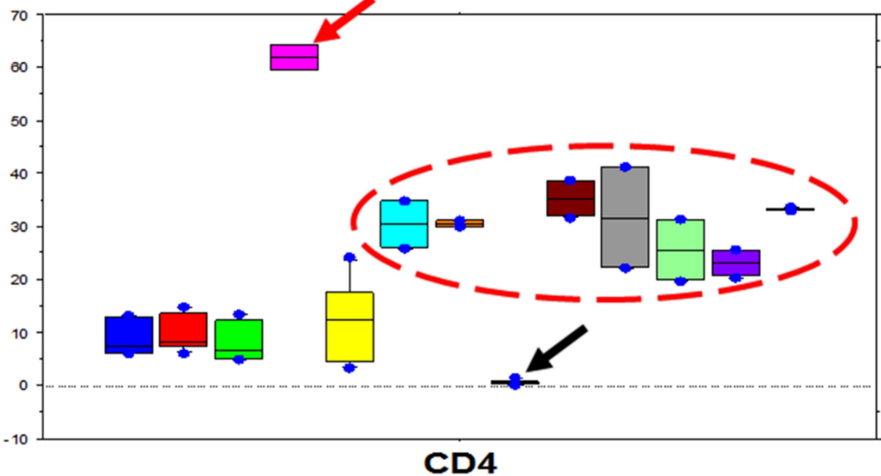
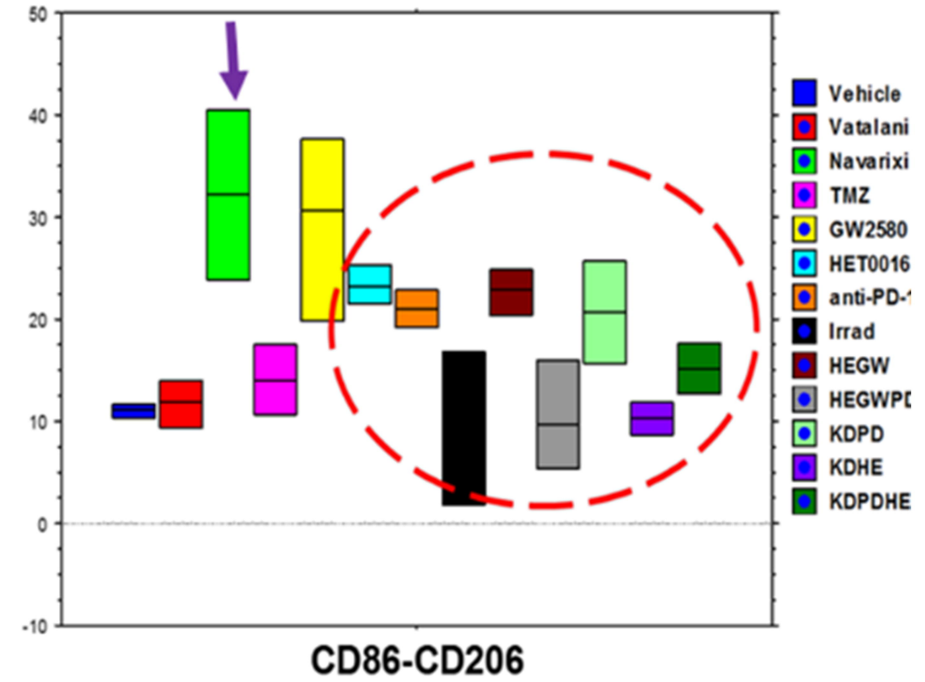
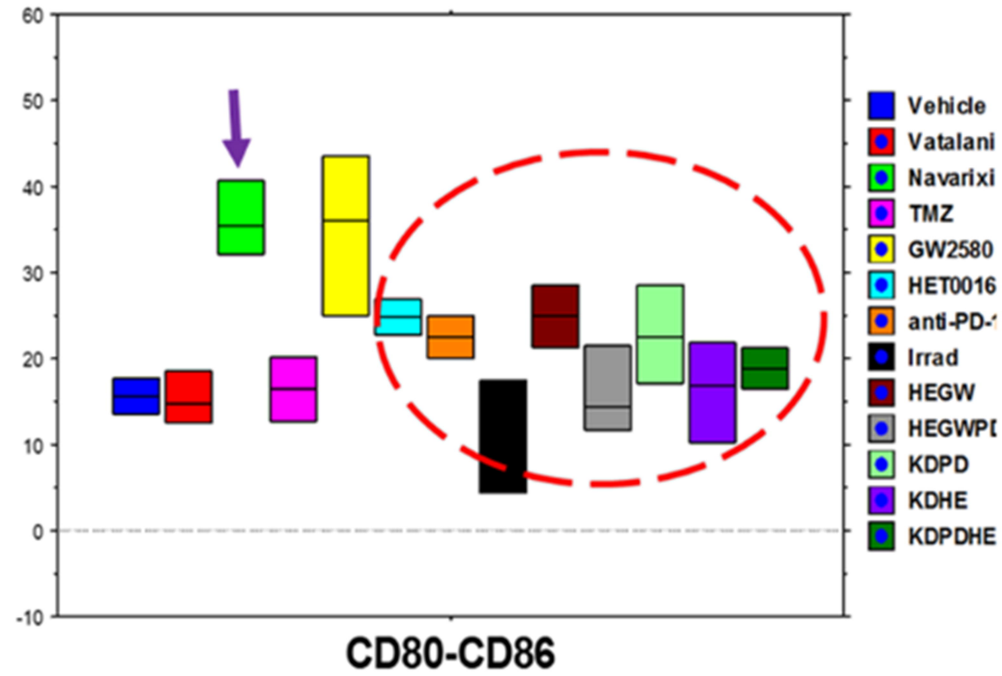


Fig 3



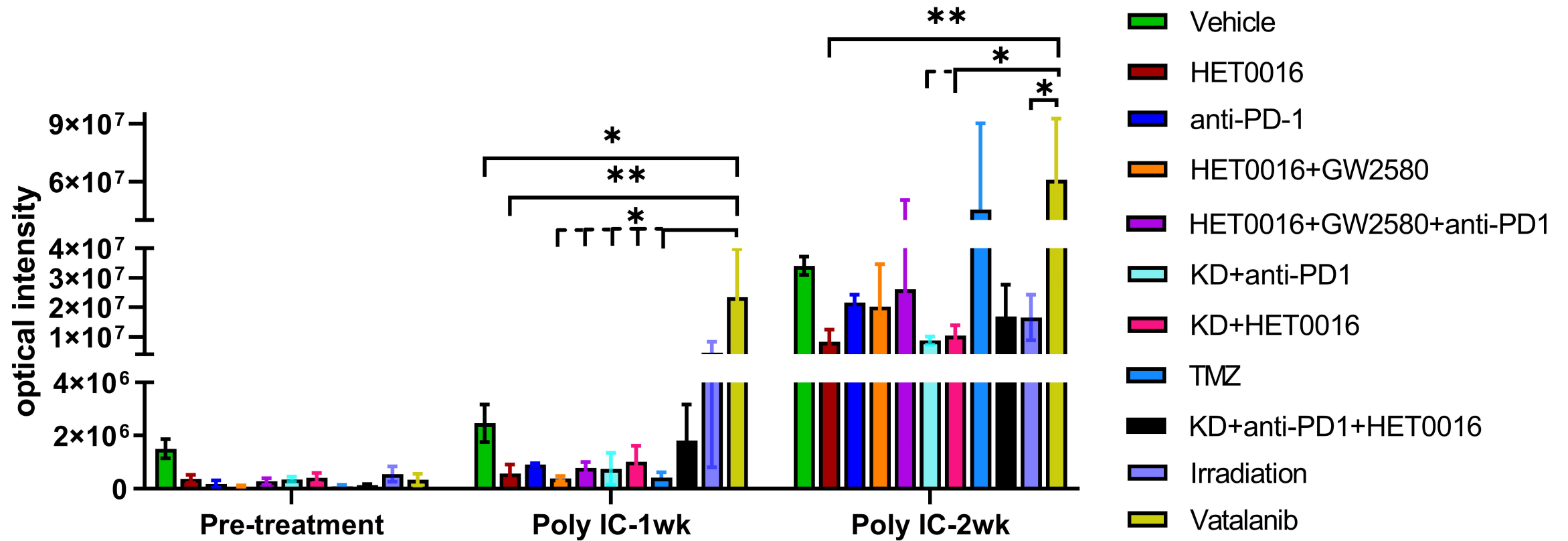
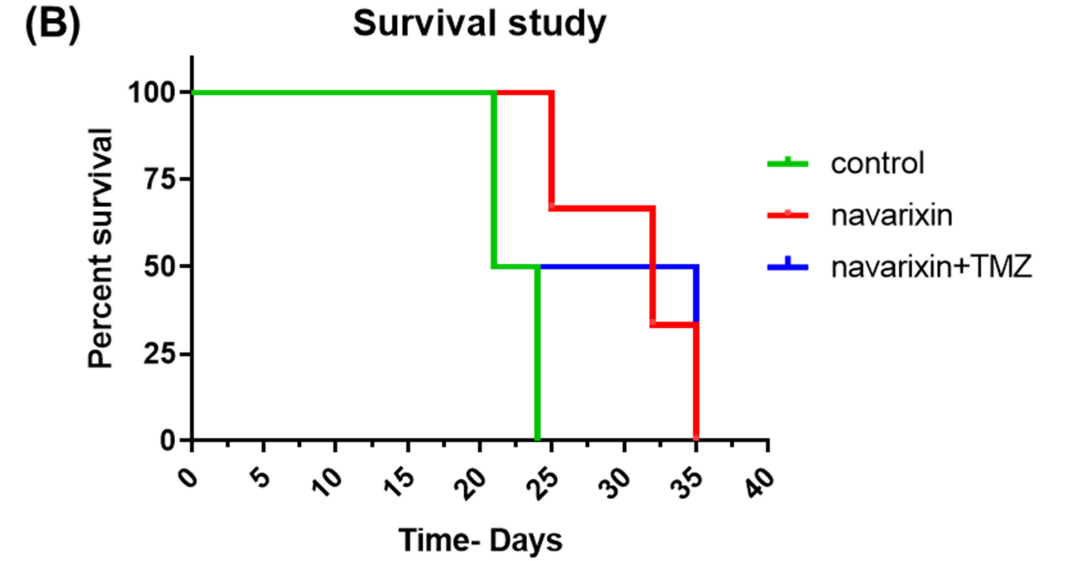
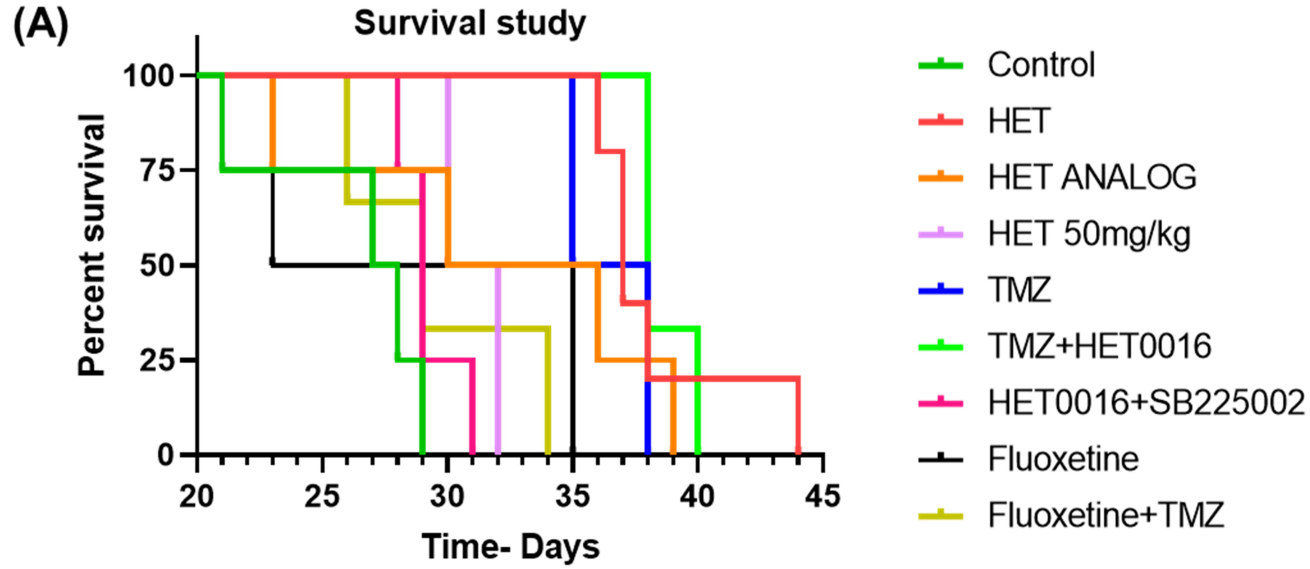


Fig 5



Comparison of Survival Curves-Log-rank (Mantel-Cox) test:

Comparison	Control vs HET0016	Control vs HET 50mg/kg	Control vs TMZ	Control vs HET0016+TMZ
<i>p</i> value	**0.002	*0.04	*0.04	*0.01