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Mechanistic interrogation of combination Bevacizumab/dual PI3K/mTOR inhibitor response in Glioblastoma implementing novel MR and PET imaging biomarkers.

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Abstract

Purpose: Resistance to bevacizumab (BEV) in glioblastoma (GBM) is believed to occur via activation of molecular networks including the mTOR/PI3K pathway. Implementing an MRI/PET molecular imaging biomarker approach, we sought to interrogate response to combining BEV with the mTOR/PI3K inhibitor BEZ235. *Methods:* Tumors were established by orthotopically implanting U87MG-luc2 in mice. Animals were treated with BEZ235 and/or BEV, and imaged using diffusion weighted-MRI, T2 weighted (T2w), and T2* weighted (T2*w) before and following delivery of superparamagnetic iron oxide (SPIO) contrast. Maps for changes in relaxation rates: ΔR2, ΔR2* and apparent diffusion coefficient (ADC) were calculated. Vessel Size Index (VSI) and micro vessel density index (MDI) were derived. 3´-deoxy-3´-[^{18}F]fluorothymidine ([^{18}F]FLT)- and *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine ([^{18}F]FET) PET was further performed and tumor endothelium/proliferation markers assessed by immunohistochemistry. *Results:* Treatment with BEV resulted in a pronounced decrease in tumor volume (T2w MRI). No additive effect on tumour volume was observed in BEV/BEZ235 combination compared with BEV monotherapy. Ki67 proliferation index staining and [^{18}F]FLT uptake studies were used to support observations. Using ΔR2* and ΔR2 values respectively, BEZ235 + BEV combination significantly reduced tumor microvessel volume in comparison to BEV alone. Decreased MDI was further observed in the combination group; supported by von Willebrand Factor (vWF) immunohistochemistry. We observed decreased [^{18}F]FET uptake following BEV, but failed to observe further reduced [^{18}F]FET uptake in the combination cohort. vWF IHC analysis showed mean tumor vessel size increased in all cohorts. *Conclusions:* Assessing MR imaging biomarker parameters together with [^{18}F]FET and [^{18}F]FLT PET, informed drug combination mechanism of action and provided clues as to potential clinical response. Translation of a BEZ35/BEV combination regimen could support reduction of peritumoral edema obviating the requirement for steroids. Implementing hypothesis driven molecular imaging studies facilitates the interrogation of

53 drug response in the pre-clinic. These data may more accurately predict the clinical
54 potential of novel therapeutic approaches in oncology.
55
56 **Keywords:** Glioblastoma, bevacizumab, BEZ235, T2w-MRI, PET
57

58 **Introduction**

59 GBM is the most common primary adult brain tumor. Treatment remains a challenge with
60 median overall survival just 14.6 months [1]. GBMs are highly vascularized, ostensibly
61 representing an attractive target for anti-angiogenic therapies. Clinical trials with BEV, a
62 monoclonal antibody against vascular endothelial growth factor (VEGF) in recurrent GBM,
63 have shown benefit in response rate (28 % - 35 %) and 6 month progression free survival
64 (29 % - 43 %) [2]. Nevertheless, BEV does not improve overall survival, nor does it
65 improve survival when delivered in conjunction with the "Stupp" protocol [3]. Tumor
66 relapse occurs in the majority of patients, characterized by local and distant infiltration [4].
67 Moreover, in a limited number of reports BEV has been shown to enhance GBM invasion
68 [5], which may be facilitated via upregulation of several pathways. Most recently, the MET
69 pathway has been implicated [6]. However, previous work has further suggested a role for
70 BEV-enhanced invasion via PI3K AKT signaling [7]. Moreover, several studies suggest
71 that the EGFR/PTEN/Akt/mTOR signaling pathway is activated in many cancers including
72 GBM. In this pathway, amplification of the gene encoding the EGFR occurs commonly in
73 GBM, leading to activation of downstream kinases including PI3K, Akt, and mTOR with
74 EGFR overexpression occurring in c.60 % of primary GBMs [8]. PI3K, AKT and mTOR,
75 an effector of PI3K, control several cellular functions critical for tumorigenesis, including
76 proliferation, apoptosis and motility [9]. Thus, we hypothesized that treatment with a
77 PI3K/AKT/mTOR inhibitor might work synergistically with BEV to improve outcomes
78 (tumor growth, volume, survival) in the GBM pre-clinical setting. Novartis
79 Pharmaceuticals have developed BEZ235, an imidazo[4,5-c]quinoline derivative and dual
80 PI3K/mTOR inhibitor currently under investigation for the treatment of solid tumours [10].
81 BEZ235 inhibits VEGF secretion *in vitro* [9]. The anti-tumor activity of BEZ235, in
82 combination with the VEGF inhibitor sorafenib was shown as superior to BEZ235 or
83 sorafenib alone in a renal cell carcinoma study [11].

84 Herein we sought to assess the effect of combining BEZ235+BEV in a pre-clinical GBM
85 model. Moreover, as an ongoing challenge exists to accurately monitor patients' response
86 to BEV, we further sought to investigate novel imaging biomarkers towards mechanistic
87 interrogation of treatment. As recently described [12], ADC, T2 and T2* mapping
88 following delivery of SPIO contrast were implemented to assess blood volume, vessel
89 density and vessel size. [^{18}F]FET PET was implemented to assess vessel amino acid
90 transport and [^{18}F]FLT PET to monitor cell proliferation. Thus, the following *in vivo*
91 response biomarkers were assessed: (1) tumor volume as estimated by T2w MRI (2) tumor
92 vessel density and size assessed using T2 and T2* MRI following delivery of SPIO
93 contrast, and (3) tumor cell proliferation assessed using FLT and FET-PET.

94

95 **Material & Methods**

96 *Tissue Culture*

97 Authenticated U87MG-luc2 cells were obtained from Caliper Life Science (A PerkinElmer
98 Company, Hopkinton, MA, USA). Cells were cultured in Eagle's Minimum Essential
99 Medium (EMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % heat-
100 inactivated fetal bovine serum (FBS), 1 % L-glutamine (2 mM), 1 %
101 penicillin/streptomycin (50 units/ml), all from Sigma-Aldrich (St. Louis, MO, USA). Note:

102

103 *Animal Experiments*

104 All experiments were in accordance with the German Law on the Care and Use of
105 Laboratory Animals and approved by the Landesamt für Natur, Umwelt und
106 Verbraucherschutz Nordrhein-Westfalen (LANUV). GBM cells were stereotactically
107 implanted into the right striatum of female NMRI (Charles River) nude mice. Animals
108 were anaesthetized with Ketamine/Xylazine (20 mg/kg each) and placed in a stereotactic
109 frame. A midline incision was made, and a burr hole drilled in the skull. 1.0 x 10^5 U87MG-

110 luc2 cells in 2 µl of plain DMEM were injected with a 10 µl Hamilton syringe at the
111 following coordinates: lateral -2.0 mm, dorsoventral -2.5 mm, using the bregma as a
112 reference. Bioluminescence imaging of luciferase positive cells was initially used as a
113 surrogate marker of tumour growth when establishing the orthotopic model and defining
114 tumour growth kinetics.
115
116 *Imaging and Treatment Protocols*
117 Animals were imaged at baseline, at 3 weeks (immediately prior to treatment) and at week
118 6. Treatment time-points were selected based on U87-luc orthotopic growth kinetics
119 established prior to commencing the current study using Bioluminescence Imaging as a
120 surrogate marker of tumour growth. Specifically, animals were imaged using 3´-deoxy-3´-
121 [^{18}F]fluorothymidine ([^{18}F]FLT)- and *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine ([^{18}F]FET)- PET
122 and MRI (diffusion weighted-MRI, T2w and T2*w before and following delivery of SPIO
123 contrast). Mice were randomized into four groups: Control (vehicle), BEZ235 alone (45
124 mg/kg po, daily 5/7, 2/7 off), BEV alone (10 mg/kg ip, alternate days 12/7), and
125 Combination (45 mg/kg po, daily 5/7, 2/7 off; 10 mg/kg ip, alternate days 12/7) (n=7
126 animals per group). Post-treatment imaging was performed at week 6.
127
128 *MRI*
129 Experiments were performed on a 9.4 T small animal scanner with 20 cm bore size with a
130 mouse brain surface coil (Bio-Spec 94/20; Bruker BioSpin MRI GmbH, Germany). The
131 system was operated with ParaVision 5.1 software (Bruker BioSpin MRI GmbH,
132 Germany). Mice were anaesthetized with isoflurane (5 % induction, 1-1.5 % maintenance,
133 DeltaSelect; Dreieich, Germany) in compressed air/O_2 (70:30, 1 L/min). Body temperature
134 and respiration rate were continuously monitored. An i.v. catheter (27 G) was introduced
135 into one tail vein. The animal was placed prone in the animal cradle with the head fixed by
136 bite bar, nose cone and earplugs. We obtained T2w anatomical images with a fast spin echo

137 sequence in the sagittal, axial, and coronal imaging plane: TR = 2000 ms, TE = 50 ms,
138 Rapid Acquisition with Relaxation Enhancement (RARE) factor 8, 8 averages (coronal 12),
139 field of view 1.6 cm, matrix 128 x 128 (coronal 192), slice thickness 0.75 mm, resolution
140 125 x 125 (coronal 83 µm²). Tumor blood volume, tumor microvessel volume, tumor
141 microvessel density and tumor vessel size, were determined by acquiring ADC maps and
142 ΔT2 and ΔT2* maps before and after i.v. injection of very small superparamagnetic iron
143 oxide particles (VSOP, C184, Ferropharm, Teltow, Germany 30 mg Fe/kg). C184 is coated
144 with citrate and has a sphere size of 8 nm and a core size of 4 nm. Protocol parameters
145 were adapted as previously described [12, 13]. Multi Slice Multi Echo (MSME) spin echo
146 sequence images for T2 mapping and multiple gradient echo (MGE) gradient echo
147 sequence MR images for T2* mapping were obtained with the same geometry (FOV 16
148 mm², matrix 64², slice thickness 0.3 mm). MSME was acquired with Repetition Time (TR)
149 = 5000 ms and 10 echoes, Echo Time (TE) = 10.9, 21.8, 32.7, 43.6, 54.5, 65.4, 76.3, 87.2,
150 98.1, 109 ms. MGE was acquired with TR = 1400 ms and 10 echoes, TE = 4, 8, 12, 16, 20,
151 24, 28, 32 ms with a 60° hermite pulse. Post contrast image acquisition was delayed by 3
152 min. Data for an apparent diffusion coefficient (ADC) map with the same geometry were
153 additionally acquired before contrast agent application, with a diffusion-weighted Echo
154 Planar Imaging (EPI) protocol (TR/TE 7500/18.4 ms) with b = 0, 300, 800 s/mm2. Total
155 scan time was approximately 45 minutes per animal.
156
157 *PET*
158 Mice were anaesthetized with isoflurane (4 % induction, 2 % maintenance DeltaSelect;
159 Dreieich, Germany) in O_2, and one lateral tail vein catheter was positioned using a 27 G
160 needle connected to a 15 cm polyethylene catheter tubing. Twelve MBq of 3´-deoxy-3´-
161 [^{18}F]fluorothymidine ([^{18}F]FLT) or *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine ([^{18}F]FET) were
162 injected as a bolus (100 µl of [^{18}F]FLT /[^{18}F]FET solution flushed with 100 µl saline) via
163 the tail vein, and subsequent PET scanning was performed. PET tracer specific

164 radioactivity obtained with the synthesis module employed was 30-70 GBq/µmol [^{18}F-]FET

165 was used to image amino acid transport, and [^{18}F]FLT was used to image actively

166 proliferating cells. PET experiments were carried out using a high resolution (spatial

167 resolution of 0.7 mm using iterative EM reconstruction including resolution recovery)

168 small animal scanner (32 module quadHIDAC, Oxford Positron Systems Ltd., Oxford, UK)

169 with uniform spatial resolution (< 1 mm) over a large cylindrical field (165 mm diameter,

170 280 mm axial length) [14]. List-mode data were acquired for 20 min starting 70 min after

171 tracer injection.

172

173 *Data Analysis*

174 PET data were reconstructed into a static frame using an iterative reconstruction algorithm.

175 PET and MR images were co-registered using contours of the skull and head of the mice

176 using the software VINCI (http://www.nf.mpg.de/vinci3/) [15]. T2w anatomical MR

177 images were used to delineate the contour of the tumors, to measure their volumes, and to

178 draw the volume-of-interest (VOI). PET images were used to determine radiotracer uptake

179 in the tumor. To calculate tumor-to-background uptake ratios ([^{18}F]FLT and [^{18}F]FET T/B),

180 the tumor [^{18}F]FLT and [^{18}F]FET uptake was divided by the radiotracer uptake in the mirror

181 region of the tumors drawn in the contralateral hemisphere. For MRI data analysis, a

182 Matlab routine (R2010b) was implemented for calculation of ΔR2, ΔR2* and ADC maps.

183 ROI analysis was performed on the slice with the greatest tumor diameter. Vessel

184 parameters, Vessel Size Index (VSI) and micro vessel density index (MDI) were derived as

185 described previously [12]. The ADC map was calculated from diffusion-weighted images

186 with a mono-exponential fit of the signal intensities of the diffusion-weighted images (b =

187 0, 300, 800 s/mm²). □R2* maps were calculated with the second echo (8 ms) by:

188

189
$$\Delta R_2^* = \frac{2}{3}\,\delta\omega\xi_0 = \frac{\ln\left(\frac{GE_{pre}}{GE_{post}}\right)}{TE}$$

190 (1)

191 □R2 maps were determined with the third echo (32.7 ms) by:

192 $$\Delta R_2 = 0.694\delta\omega^{2/3}\xi_0\#^{2/3}ADC^{1/3}R^{-2/3} = \frac{\ln\left(\frac{SE_{pre}}{SE_{post}}\right)}{TE}$$

193 (2)

194 (ξ_0 = blood volume fraction; $\delta\omega = 2\pi$□□B_0 = frequency shift, # = number of 180° pulses; R

195 = Vessel Size Index (VSI) Δ□ = $1x10^{-3}$ = changes in the susceptibility, B_0 = magnitude of

196 the magnetic field).

197

198 NB: ADC maps were acquired before contrast to facilitate subsequent calculation of vessel

199 parameters, taking into account confounding effects of heterogeneous tumor diffusion [12].

200 Micro vessel density index was calculated from:

201

202 $$MDI = 1.327\#^2 \frac{\left(\frac{\Delta R_2}{(\Delta R_2^*)^{2/3}}\right)^3}{ADC}$$

203 (3)

204

205 Vessel size index (VSI) was calculated from:

206 $$VSI = 0.425\#\left(\frac{ADC}{\delta\omega}\right)^{\frac{1}{2}}\left(\frac{\Delta R_2^*}{\Delta R_2}\right)^{\frac{3}{2}}$$

207 Median indices of tumor tissue within the skull were compared between groups.

208

209 *Immunohistochemistry (IHC)*

210 At week 6 (post treatment), tumors were excised, fixed in 4 % paraformaldehyde,

211 embedded in paraffin and cut in 5 µm sections. Following rehydration and heat-induced

212 epitope retrieval for 30 min in citrate buffer (pH 6.0), sections were incubated in peroxidase

blocking solution (S3022; DAKO, Germany) for 5 min and treated with serum-blocking solution for 15 min. Sections were incubated overnight at 4 °C with the rabbit anti-human primary antibodies against the proliferation marker Ki67 (dilution 1:100, ab16667, Abcam) and endothelial marker Von Willebrand factor (vWF, dilution 1:500, DAKO). Labeling of the primary antibody was performed using a commercial avidin-biotin complex detection kit based on a biotinylated anti-rabbit antibody (goat anti-rabbit dilution 1:500, B21078, Invitrogen) according to the manufacturers manual, followed by incubation with 3,3'-diaminobenzidine (DAB, D-5637; Sigma) for 5 min. Sections were counterstained with hematoxylin, dehydrated and mounted using Entellan (Merck, Germany). Ki67-positive cell number and total number of cells per image were quantified manually using cell counter in ImageJ software. Proliferation index was expressed as a percentage of Ki-67-positive cells. Microvessel density was determined using ImageJ software using a grid over lay method. The number of positively stained vessels that crossed the overlaid grid-lines were counted. Vessel size was quantified using Image J with the Analyze Particles plugin.

Statistical Analysis

Statistical analyses were performed using GraphPad (San Diego, CA, USA) and SAS (Cary, NC, USA). Data are represented as median ± standard deviation, unless otherwise stated. Two sample non-parametric median tests were employed as well as Pearson's correlation test, using SigmaStat 3.0 (SPSS, Inc., Chicago, IL, USA). P values < 0.05 were considered to be statistically significant. A summary of statistical methods is included as supplementary material.

Results

Combined treatment with BEV + BEZ235 does not significantly enhance BEV tumor volume inhibitory effect

240 A significant decrease in tumor volume (T2w MRI) was observed 3 weeks following
241 treatment in BEV treated tumors (94% reduction) and in combination treated tumors (97%
242 reduction) ($P < 0.05$) (Figure 1A). [Note: more than one tumor was observed in some
243 animals. A summary of observations is provided (Supplementary Table 1) Thus, where
244 possible, tumors were analyzed separately]. Animals treated with BEZ235 + BEV did not
245 exhibit significantly reduced tumor volume in comparison with animals treated with BEV
246 alone. No significant difference in [^{18}F]FLT uptake was observed when BEV alone and
247 BEV +BEZ235 treated cohorts were compared supporting MRI data (Figure 1B and C).
248 [Note: untreated animals did not survive until the post treatment PET imaging session.]
249 Ki67 staining further indicated that combined BEV/BEZ235 treatment did not reduce tumor
250 proliferation compared to BEV alone. We observed a significant correlation with tumor cell
251 proliferation measured with [^{18}F]FLT PET and Ki67 staining (Supplementary Figure 1D).
252
253 *Combined treatment with BEV + BEZ235 causes decreased total tumor and micro-vessel*
254 *blood volume compared to BEV alone*
255 A significant (47 %) decrease in total tumor blood volume was evident in animals treated
256 with BEV in comparison to untreated animals ($P < 0.05$) (Figure 2A & B). Treatment with
257 combination BEZ235/BEV resulted in a further decreased (51 %) total tumor blood volume
258 when compared with animals treated with BEV alone ($P < 0.05$). Treatment with BEZ235
259 or BEV alone did not alter tumor micro-vessel blood volume (Figure 2A and C).
260 Treatment with combination BEZ235 and BEV caused a significant (57 %) reduction in
261 tumor microvessel blood volume when compared with animals treated with BEV alone ($P <$
262 0.01).
263
264 *Combined treatment effects of BEV + BEZ235 on tumor vessel density (MDI), size (VSI),*
265 *vWF staining and [^{18}F]FET uptake*

266 Tumor vessel density (MDI) was significantly (69 %) reduced in animals treated with BEV
267 alone in comparison with BEZ235 (Figure 3 A and B) ($P < 0.05$). However, tumor vessel
268 density was not reduced further in animals treated with combination BEZ235/BEV when
269 compared with animals treated with BEV alone. We observed a 46 % decrease in [^{18}F]FET
270 uptake in animals treated with BEV alone (non-significant) or in combination with BEZ235
271 (49 % decrease, $P < 0.05$) when compared with animals treated with BEZ235 alone (Figure
272 3 C and D). No significant difference in [^{18}F]FET uptake was observed when BEV alone
273 and BEV + BEZ235 treated cohorts were compared. vWF staining showed a significant
274 MVD decrease in animals treated with BEZ235 + BEV in comparison with animals treated
275 with BEV alone (Figure 3E). We compared MDI with vWF immuno-staining and found a
276 significant correlation between both readouts irrespective of treatment group
277 (Supplementary Figure 1A). [^{18}F]FET uptake also significantly correlated with MDI
278 (Supplementary Figure 1B) and vWF staining irrespective of treatment group
279 (Supplementary Figure 1C). Vessel size was assessed using T2* mapping after SPIO
280 contrast. No effect on tumor vessel size was observed in any treatment cohort (Figure 4 A
281 and B). However, using vWF staining we observed that treatment with BEV alone or in
282 combination with BEZ235 significantly increased tumor vessel size (Figure 4C and D).
283

284 **Discussion**

285 Common alterations in cell signaling networks occur in most GBMs including the
286 PI3K/AKT and MEK/MAPK pathways [16]. These alterations drive GBM hallmarks
287 including invasiveness, proliferation, survival and angiogenesis. Molecular diversity and
288 reliance on multiple signaling pathways may explain why single-agent trials with
289 molecularly targeted therapies have largely failed to demonstrate survival benefit in patient
290 populations [17]. Herein, we have implemented a mechanistic hypothesis-driven multi-
291 modality imaging approach to study the effects of dual targeting VEGF and PI3K/mTOR in
292 an orthotopic model.

293 BEV treatment has been shown to induce normalization of the blood brain barrier, and as a
294 result, to reduce MR contrast enhancement due to a reduction in vessel permeability and
295 perfusion. Nevertheless, while contrast enhancement does not accurately reflect tumor
296 volume or vascular density, DCE-derived parameters may be used as efficient noninvasive
297 biomarkers of response to anti-angiogenic therapies [18]. Indeed, the reference imaging
298 method for non-invasive *in vivo* assessment of anti-angiogenic therapies is Dynamic
299 Contrast-Enhanced MRI (DCE-MRI) [18-21], which provides information on tumour blood
300 volume, extravascular/extracellular space fractions, blood-to-tissue transfer constant and
301 blood flow. However, as angiogenesis is a complex process involving formation of
302 abnormally dilated vessels and proliferation of microvessels, access to additional methods
303 to non-invasively assess the size and density of tumor vessels can provide important
304 additional information. To this end, an additional MR approach was developed to provide a
305 more anatomical description of the tumor vessel network [22]. In this context, MR
306 sequences are based on the ratio of gradient and spin echo relaxation rate changes
307 ($\Delta R*2/\Delta R2$) after injection of an iron oxide-based superparamagnetic contrast agent of
308 high molecular weight. $\Delta R2*/\Delta R2$ increases with increasing vessel size, allowing the
309 calculation of average vessel size within a voxel. A good correlation has been demonstrated
310 between MRI derived vessel size and density index and histology [13, 23].
311 In this study we sought to measure tumor vessel size and density using such dedicated
312 sequences recently implemented by our group and others [12, 13]. Our approach facilitates
313 the assessment of vessel parameters, total and microvessel tumor blood volume, VSI, and
314 MDI from $\Delta R2*$, $\Delta R2$ and ADC maps. These MR sequences may be adapted for clinical
315 translation [24]. We believe that providing anatomical information on tumor vasculature
316 could complement the physiological information provided by DCE-MRI towards a more
317 complete characterization of the tumor angiogenesis process. Unfortunately, due to
318 practical (time) limitations during our experimental pre-clinical imaging procedures it was
319 not feasible to include a DCE-MRI protocol. Furthermore, the effect of Gd injections on

320 VSOP T2/T2* relaxation is as of yet unknown, as is any effect of VSOP on putative DCE-
321 MRI T1 relaxation.
322 Using T2w MRI, we have shown that treatment with BEV alone results in a pronounced
323 decrease in tumor volume. A similar finding has been observed in patients, with effects
324 evident within two weeks of treatment [25]. However, our data suggests that BEV/BEZ235
325 combination does not further decrease GBM tumor volume compared to BEV
326 monotherapy. Thus, it is possible that there is redundancy in the pro-growth signaling
327 pathways inhibited by both BEV and BEZ235. These observations are supported by Ki67
328 proliferation index and [^{18}F]FLT uptake studies. [^{18}F]FLT uptake has been shown to
329 strongly correlate with tumor response following BEZ235 treatment in mouse models of
330 gastric cancer [26] and anaplastic large cell lymphoma [27]. [^{18}F]FLT has been used
331 previously to evaluate response to BEV treatment e.g. in combination with irinotecan [28]
332 and warrants continued assessment as a clinical imaging biomarker of response.
333 Using $\Delta R2^*$ and $\Delta R2$ values respectively, we have shown that BEZ235 + BEV
334 combination significantly reduces GBM tumor blood volume and tumor microvessel
335 volume in comparison to BEV alone, likely due to BEZ235 mediated inhibition of tumor
336 vessel signaling pathways. Most brain tumors over-secrete VEGF, which on binding and
337 signaling though its receptor (VEGFR2) results in angiogenesis, vasculogeneis and
338 abnormal permeabilisation of the tumor vasculature. This hyper-permeability allows fluid
339 to leak from the intravascular space into the brain parenchyma, which causes vasogenic
340 cerebral edema and increased interstitial fluid pressure. Herein we, and elsewhere others
341 [18], have shown that treatment with BEV (which inhibits VEGFA binding to its receptor)
342 reduces GBM tumor vessel permeability. It is possible that synergistic inhibition of
343 VEGFA/VEGFR2 binding (BEV) alongside inhibition of the VEGFR2 downstream
344 PI3K/mTOR pathway (BEZ235) may explain the enhanced reduction in tumor blood
345 volume we observe following combinatorial therapy.

346 BEV has previously been shown to decrease tumor blood volume as measured using
347 gadolinium-based DCE- MRI in a rat model of GBM [7]. Moreover high-grade glioma
348 patients with low tumor blood volume following treatment with BEV, demonstrate
349 improved progression free survival (PFS) compared to patients with high tumor blood
350 volume [29]. We hypothesize that the effect of BEZ35/BEV combination could translate to
351 improved quality of life for GBM patients by supporting reduced peritumoral edema [7,
352 30].
353 Data elucidating BEZ235/BEV effects on tumor blood volume and tumor micro-vessel
354 volume are further supported by observed vessel density reduction in the combination
355 group as measured by vWF staining. A corresponding trend towards decreased MDI was
356 also observed in the combination group. Further studies are warranted to fully unravel the
357 utility of MDI as a novel anti-angiogenic imaging biomarker.
358 We also assessed tumor uptake of the novel tracer [^{18}F]FET PET to monitor therapy
359 response. [^{18}F]FET uptake is driven by large neutral amino acid transport and has been
360 shown to correlate with L-type amino acid transporter expression (predominantly in the
361 tumor cytoplasm and on the vascular endothelium), microvessel density and vessel
362 formation in glioma [31]. [^{18}F]FET uptake has further been evaluated clinically as a
363 prognostic marker of BEV + irinotecan response and has been shown to perform better than
364 Response Assessment in Neuro-Oncology (RANO) criteria towards prediction of treatment
365 failure [32]. As before [32], we observed decreased [^{18}F]FET uptake following BEV, but
366 failed to observe further reduced [^{18}F]FET uptake in the combination cohort. Nevertheless,
367 as [^{18}F]FET also accumulates in actively metabolizing tumor cells, this finding would
368 further support our earlier observations which suggest that the combination regimen fails
369 to elicit enhanced direct tumor response.
370 vWF IHC data suggests that mean tumor vessel size is increased in all treatment cohorts.
371 These data agree with recent studies which showed increased tumor vessel size following
372 BEV treatment in an orthotopic rat spheroid model, suggesting vessel normalization,

373 maturation and pruning of immature vessels following treatment [13]. Surprisingly, VSI
374 data showed no effect in any treatment cohort. IHC derived VSI values have previously
375 been shown to correlate with VSI values derived using the imaging method described here
376 [12]. However, this study did not assess VSI correlations post treatment with anti-
377 angiogenic agents. Further refinement of VSI as a robust imaging biomarker is required to
378 decrease parameter variability.
379 Herein, we have demonstrated the utility of implementing a multi-modality, multi
380 parametric imaging approach towards the mechanistic interrogation of BEZ235/ BEV
381 combination in a murine model of glioblastoma. Assessing novel MRI parameters ($\Delta R2^*$,
382 $\Delta R2$ and ADC maps to derive total and microvessel tumor blood volume, VSI and MDI)
383 together with [^{18}F]FET and [^{18}F]FLT PET has informed drug mechanism of action and
384 further provided clues to potential clinical response; The extensive pre-clinical imaging
385 protocol employed has thus provided important clues as to potential drug limitations (vis a
386 vis potential clinical efficacy) and/or putative benefits. It is possible that translation of a
387 BEZ35/BEV combination regimen to the clinic could further reduce peritumoral edema·
388 obviating the requirement for steroid treatment and improving patient quality of life.
389 Additional pre-clinical studies using larger animal cohorts and clinically relevant orthotopic
390 GBM patient derived xenograft models are warranted to extend on and validate these
391 findings. Nevertheless extensive studies of this kind performed in the pre-clinic are
392 increasingly necessary to refine clinical trial design, to better define the clinical setting
393 where experimental regimens are likely to succeed, and to avoid long and costly clinical
394 studies which may ultimately fail due to lack of response or toxicities. Implementing
395 mechanistic hypothesis driven pre-clinical molecular imaging biomarker studies facilitates
396 robust interrogation of drug response. These data may more accurately predict the true
397 clinical potential of novel therapeutic approaches.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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523

524 **Figure Legends**

525 *Figure 1: Effect of BEZ235/BEV combination on GBM tumor volume and growth*

526 Post-treatment imaging and IHC analyses were performed following 3 weeks of treatment.
527 (A) T2w MRI derived tumor volume. (B) Representative trans-axial [^{18}F]FLT PET images
528 co-registered with MR in the same mouse. (C) Quantification of [^{18}F]FLT uptake. (D, upper
529 panel) Representative micrographs of Ki67 assessment of tumor cell proliferation (20x),
530 (D, lower panel) Quantitation of proliferation index (Ki67 staining). * *P < 0.05, ** P <
531 0.005, *** P < 0.001. Data points within each group represent individual tumors analyzed.

532

533 *Figure 2: Effect of BEZ235/ BEV combination on tumor blood volume and vasculature*

534 (A) Trans-axial co-registered brain image sections for a representative mouse. (B) tumor
535 blood volume and (C) microvessel blood volume,. *P < 0.05. Data points within groups
536 represent individual tumors analyzed

537

538 *Figure 3: Effect of BEZ235/ BEV treatment on GBM vessel density and [18F]FET uptake*

539 (A) Representative trans-axial sections of co-registered images (MDI) of a representative
540 mouse. (B) Quantification of MDI in treatment cohorts. (C) Representative transaxial
541 sections of [^{18}F]FET showing PET uptake co-registered with MR images in the same
542 mouse. (D) Quantification of [^{18}F]FET uptake. (E, upper panel) vWF staining of tumor
543 vessel density (20x). (E, lower panel)) Quantitation of vWF staining *P < 0.05* Data
544 points within groups represent individual tumors analyzed.

545

546 *Figure 4: Effect of BEZ235/ BEV treatment on tumor vessel size*

547 (A) Quantification of VSI in treatment cohorts. (B) vWF staining of tumor vessel size
548 (20x). (C) Quantification of tumor vessel size by vWF staining *P < 0.05* Data points
549 within groups represent individual tumors analyzed.

550

551 *Supplementary Figure 1: Correlation of imaging and IHC data*

552 (A) Correlation of MDI with microvessel density as determined by vWF staining. (B)
553 Correlation of [^{18}F]FET uptake with microvessel density as determined by vWF staining.
554 (C) Correlation of MDI with [^{18}F]FET uptake. (D) Correlation of [^{18}F]FLT uptake, with
555 Ki67 index.