



Title	Eribulin improves tumor oxygenation demonstrated by F-18-DiFA hypoxia imaging, leading to radio-sensitization in human cancer xenograft models
Author(s)	Bo, Tomoki; Yasui, Hironobu; Shiga, Tohru; Shibata, Yuki; Fujimoto, Masaki; Suzuki, Motofumi; Higashikawa, Kei; Miyamoto, Naoki; Inanami, Osamu; Kuge, Yuji
Citation	European Journal of Nuclear Medicine and Molecular Imaging, 49(3), 821-833 https://doi.org/10.1007/s00259-021-05544-4
Issue Date	2021-09-01
Doc URL	http://hdl.handle.net/2115/86668
Rights	This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature 's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/10.1007/s00259-021-05544-4
Type	article (author version)
File Information	Manuscript_for HUSCUP.pdf



[Instructions for use](#)

1 **Eribulin improves tumor oxygenation demonstrated by ¹⁸F-DiFA hypoxia imaging,**
2 **leading to radio-sensitization in human cancer xenograft models**

3

4 Tomoki Bo^{1, 2}, Hironobu Yasui^{1, 3}, Tohru Shiga⁴, Yuki Shibata³, Masaki Fujimoto¹,
5 Motofumi Suzuki⁵, Kei Higashikawa³, Naoki Miyamoto⁶, Osamu Inanami¹, and Yuji
6 Kuge³

7 ¹ Laboratory of Radiation Biology, Department of Applied Veterinary Science, Faculty of
8 Veterinary Medicine, Hokkaido University, Sapporo, Japan

9 ² Laboratory Animal Center, Institute for Promotion of Medical Science Research,
10 Faculty of Medicine, Yamagata University, Yamagata, Japan

11 ³ Central Institute of Isotope Science, Hokkaido University, Sapporo, Japan

12 ⁴ Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University,
13 Sapporo, Japan

14 ⁵ Laboratory of Bioanalysis and Molecular Imaging, Graduate School of Pharmaceutical
15 Science, Hokkaido University, Sapporo, Japan

16 ⁶ Division of Quantum Science and Engineering, Faculty of Engineering, Hokkaido
17 University, Sapporo, Japan

18

19 **Author responsible for correspondence:** Hironobu Yasui, Ph.D., D.V.M.

20 Address: Laboratory of Radiation Biology, Department of Applied Veterinary Science,
21 Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo,
22 Hokkaido, 060-0818, Japan

23 Tel: +81-11-706-5236 E-mail: yassan@vetmed.hokudai.ac.jp

24

25 **Abstract**

26 **Purpose:** Eribulin, an inhibitor of microtubule dynamics, is known to show antitumor
27 effects through its remodeling activity in the tumor vasculature. However, the extent to
28 which the improvement of tumor hypoxia by eribulin affects radio-sensitivity remains
29 unclear. We utilized 1-(2,2-dihydroxymethyl-3-¹⁸F-fluoropropyl)-2-nitroimidazole (¹⁸F-
30 DiFA), a new PET probe for hypoxia, to investigate the effects of eribulin on tumor
31 hypoxia and evaluate the radio-sensitivity during eribulin treatment.

32 **Methods:** Mice bearing human breast cancer MDA-MB-231 cells or human lung cancer
33 NCI-H1975 cells were administered a single dose of eribulin. After administration, mice
34 were injected with ¹⁸F-DiFA and pimonidazole, and tumor hypoxia regions were
35 analyzed. For the group that received combined treatment with radiation, ¹⁸F-DiFA
36 PET/CT imaging was performed before tumors were locally X-irradiated. Tumor size
37 was measured every other day after irradiation.

38 **Results:** Eribulin significantly reduced ¹⁸F-DiFA accumulation levels in a dose-
39 dependent manner. Furthermore, the reduction in ¹⁸F-DiFA accumulation levels by
40 eribulin was most significant 7 days after treatment. These results were also supported
41 by reduction of the pimonidazole-positive hypoxic region. The combined treatment
42 showed significant retardation of tumor growth in comparison with the control, radiation-
43 alone, and drug-alone groups. Importantly, tumor growth after irradiation was inversely
44 correlated with ¹⁸F-DiFA accumulation.

45 **Conclusion:** These results demonstrated that ¹⁸F-DiFA PET/CT clearly detected
46 eribulin-induced tumor oxygenation and that eribulin efficiently enhanced the antitumor
47 activity of radiation by improving tumor oxygenation.

48 **(221 / 250 words)**

49 **Key words:** Eribulin, ¹⁸F-DiFA, Hypoxia, Radiation, PET/CT imaging

50

51 **Declarations**

52 **Funding**

53 This work was supported, in part, by the Japan Agency for Medical Research and
54 Development (AMED) and by the KAKENHI, Japan (No. 18H02757 [T.S.],) provided
55 by the Japan Society for the Promotion of Science.

56 **Competing interests**

57 The authors declare no competing interests.

58 **Availability of data and material**

59 The datasets during the current study available from the corresponding author on
60 reasonable request.

61 **Code availability**

62 Not applicable

63 **Author contribution**

64 Hironobu Yasui, Tohru Shiga and Tomoki Bo designed and performed the experiments,
65 analyzed data and wrote the manuscript. Yuki Shibata, Masaki Fujimoto, Motofumi
66 Suzuki and Kei Higashikawa performed the experiments and analyzed the data. Naoki
67 Miyamoto adjusted a linear accelerator (CLINAC 6EX) and performed irradiation
68 experiments. Osamu Inanami and Yuji Kuge designed experiments, discussed the data
69 and revised the manuscript.

70 **Ethics approval**

71 All animal experiments were approved by the Laboratory Animal Care and Use
72 Committee of Hokkaido University and performed in accordance with the Guidelines for

73 Animal Experiments at the Graduate School of Medicine, Hokkaido University.

74 **Consent for publication**

75 All authors have read the paper and provided consent for publication.

76 **Introduction**

77 Breast cancer is the most common invasive cancer in women. In 2019, it accounted for
78 30% of all new cancer cases and was the second-most frequent cause of death in women
79 in the United States (1). Breast cancer treatments differ according to the staging at
80 diagnosis. For stage I or II cases, breast-conserving surgery with adjuvant radiation and
81 mastectomy are performed, while almost half of stage IV cases involve radiation or
82 chemotherapy. The five-year survival rate of stage I patients was more than 90%, whereas
83 that of stage IV patients was less than 30% (2).

84 Tumor hypoxia is thought to be an important factor in resistance to therapy. Using HIF-
85 1α as a marker for hypoxia, approximately 25%-40% of invasive breast cancer samples
86 have been shown to be hypoxic (3). Reoxygenation by MnO_2 nanoparticles has been
87 shown to enhance radio-sensitivity in breast cancer xenograft models (4), suggesting that
88 tumor oxygenation may improve the prognosis of breast cancer patients undergoing
89 radiation therapy.

90 Eribulin, a synthetic analog of halichondrin B, is a non-taxane synthetic microtubule
91 dynamics inhibitor originally isolated from the marine sponge *Halichondria okadai* (5).
92 Eribulin binds microtubule ends to inhibit microtubule polymerization, leading to
93 irreversible mitotic blockage and apoptotic cell death (6,7). It is clinically used in many
94 countries for advanced or metastatic breast cancer, and is currently undergoing clinical
95 trials in a variety of human cancer types (8-13). However, eribulin has been reported to
96 show some adverse effects, although the problems are comparatively manageable in
97 comparison with those caused by other anti-tumor drugs (11). Eribulin also has potential
98 uses for vascular remodeling and reversal of the epithelial-to-mesenchymal (EMT)
99 transition at relatively low concentrations (14-16). Tumor vascular remodeling by eribulin

100 improves tumor perfusion and oxygenation, which enhances the delivery of
101 chemotherapeutic drugs and the sensitivity to radiotherapy. Recent studies have shown
102 that eribulin sensitized several human cancer models to anti-tumor drugs, including non-
103 small cell lung cancer, breast cancer, and glioblastoma (17-19). Funahashi *et al.* showed
104 that eribulin increased tumor perfusion, which enhanced the anti-tumor activity of
105 capecitabine in a breast cancer model (19). Combined administration of radiation with
106 eribulin has been shown to significantly extend the survival of mice bearing glioblastomas
107 (20). However, there is no evidence that assesses the level of tumor oxygenation and its
108 influence on the anti-tumor effect of radiation after eribulin treatment.

109 ¹⁸F-fluoromisonidazole (¹⁸F-FMISO) is the most widely used hypoxia-imaging PET
110 probe for detection of the tumor hypoxic region in clinical diagnosis (21,22). ¹⁸F-FMISO
111 allows specific visualization of tumor hypoxic regions using PET/CT. Zhao *et al.* revealed
112 that eribulin eliminated the tumor hypoxia detected by ¹⁸F-FMISO, indicating that
113 PET/CT imaging is useful for detecting eribulin-induced resolution of hypoxia (14).
114 However, visualization of hypoxic regions using this probe takes relatively longer periods
115 of time because of the lipophilicity of the probe (23). Recently, 1-(2,2-dihydroxymethyl-
116 3-[¹⁸F]-fluoro-propyl)-2-nitroimidazole (¹⁸F-DiFA), a newly developed probe, has been
117 found to show lower lipophilicity and is rapidly cleared via the urinary system (24,25).
118 Watanabe *et al.* showed that ¹⁸F-DiFA is well tolerated, and its radiation dose is
119 comparable to those of other hypoxia tracers, such as ¹⁸F-FMISO (26). In this study, we
120 determined the effectiveness of tumor oxygenation by eribulin by using ¹⁸F-DiFA
121 PET/CT and determined the effect of tumor oxygenation by eribulin on radiosensitivity
122 in a human breast cancer MDA-MB-231 xenograft model.

123

124 **Materials and methods**

125 **Human tumor xenograft models**

126 Human breast cancer MDA-MB-231 cells and human lung adenocarcinoma NCI-H1975
127 cells were obtained from American Type Culture Collection (Manassas, VA) and
128 maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal
129 bovine serum (CELLect®, MP Biomedicals, Santa Ana, CA). Female BALB/c athymic
130 nude mice aged 6 weeks were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The
131 animals were initially anesthetized with 3%–4% isoflurane in air and maintained via
132 spontaneous ventilation with 2% isoflurane in air. An MDA-MB-231 or NCI-H1975 cell
133 suspension (5×10^6 cells) diluted in 0.1 mL of PBS was subcutaneously inoculated into
134 the right mammary fat pad or right shoulder of each mouse using a 26 G syringe,
135 respectively. When the tumor volumes reached 300–500 mm³, mice were used for the
136 PET imaging study. All experiments and animal surgical procedures were approved by
137 the Laboratory Animal Care and Use Committee of Hokkaido University and performed
138 in accordance with the Guidelines for Animal Experiments at the Graduate School of
139 Medicine, Hokkaido University.

140

141 **Radiopharmaceutical and reagent**

142 ¹⁸F-DiFA was obtained from the Hokkaido University Hospital Cyclotron Facility
143 (Sapporo, Japan), which was synthesized as previously described (25). Eribulin mesylate
144 (E7389; Halaven) was kindly provided by Eisai Co., Ltd. (Tokyo, Japan). The
145 Hypoxyprobe™-1 Omni kit consisting of pimonidazole and anti-pimonidazole antibody
146 was purchased from Natural Pharmacia International Inc. (Burlington, MA, USA). Anti-
147 CD31 and anti- α -smooth muscle actin (α -SMA) were obtained from Abcam (Cambridge,

148 UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

149

150 **¹⁸F-DiFA PET/CT studies**

151 For MDA-MB-231 xenografts, mice in the PET imaging study group were randomly
152 divided into eribulin treatment and non-treatment groups. Mice were intraperitoneally
153 treated with a single dose of eribulin (0.3, 1.0 mg/kg) or saline (control) 1, 3, and 7 days
154 before PET/CT imaging. For NCI-H1975 xenografts, the same mice were sequentially
155 scanned for PET imaging before, 4 days, and 7 days after eribulin treatment (1.0 mg/kg).
156 The mice were anesthetized with 1.0%–1.5% isoflurane and injected with 10 MBq of ¹⁸F-
157 DiFA into the tail vein. The mice were placed on a heating sheet in a small animal PET/CT
158 scanner (Inveon; Siemens Medical Solutions USA Inc., Knoxville, TN, USA) in a supine
159 position 1 h after the injection of ¹⁸F-DiFA. PET and CT were performed for 20 and 15
160 min to capture images, respectively. Anesthesia was maintained with 1.0%–1.5%
161 isoflurane. In some experiments, the mice were breathing a carbogen gas (95% oxygen +
162 5% CO₂, AIR WATER Inc., Tokyo, Japan) 10 min before ¹⁸F-DiFA injection until the end
163 of PET/CT imaging. The images were reconstructed and corrected for attenuation and
164 scatter using the Fourier rebinning algorithm and filtered back projection with the ramp
165 filter cut-off at the Nyquist frequency. The image matrix was 128 × 128 × 159, resulting
166 in a voxel size of 0.776 × 0.776 × 0.796 mm³. The spatial resolution of the reconstructed
167 images was 1.63 mm at full-width at half-maximum (27). Images were analyzed using
168 the Inveon Research Workplace 4.2. A three-dimensional ROI was manually defined for
169 the tumor in each mouse and delineated with the threshold of 10 percentile of SUV_{max}
170 to exclude the necrotic regions. We calculate the SUV_{mean} in ROIs and used it as a
171 parameter of the hypoxic status.

172

173 **Immunohistochemistry**

174 To detect the hypoxic region in the tumor histologically, pimonidazole was administered
175 to mice at 60 mg/kg intravenously 1 h before sacrifice. Excised tumors were fixed with
176 4% buffered formaldehyde, embedded in paraffin, and sectioned at 6- μ m thickness. After
177 antigen retrieval and blocking of non-specific binding, slides were probed with anti-
178 pimonidazole, anti-CD31, and anti- α -SMA (1:200). The slides were then incubated with
179 Alexa Fluor 555 anti-mouse or Alexa Fluor 488 anti-rabbit IgG (both 1:2000, Invitrogen,
180 Carlsbad, CA) secondary antibodies. Images were acquired using a fluorescence
181 microscope (BZ-X700; Keyence, Tokyo, Japan) and a fluorescence microscope (BX61;
182 Olympus, Tokyo, Japan). For quantitative analysis of hypoxia, the percentage of the
183 pimonidazole-positive area in an entire cross-section was determined using ImageJ
184 software (Java version 1.6.0; National Institutes of Health, Bethesda, MD, USA). For the
185 quantitative analysis of microvessel density, intratumoral CD31- and α -SMA-positive
186 areas were calculated using ImageJ software. A total of >10 fields per section were
187 randomly analyzed.

188

189 **X-ray irradiation**

190 The time-schedule diagrams for the combination treatment of eribulin with X-irradiation
191 and PET/CT imaging are presented in Fig. 5A and 7A. For the combination therapy, 7
192 days after the eribulin treatment (1.0 mg/kg), tumor-bearing mice were imaged by
193 PET/CT, followed by local X-irradiation using a linear accelerator (CLINAC 6EX; Varian
194 Medical Systems, Palo Alto, CA) at a dose of 10 Gy (dose rate, 2.19 Gy/min). To test the
195 effect of the postradiation treatment of eribulin on tumor growth, PET/CT imaging and

196 subsequent X-irradiation were performed, followed by the administration of eribulin to
197 NCI-H1975-bearing mice. In some experiments, mice were X-irradiated while breathing
198 carbogen gas. The prescribed dose was defined as the isocenter. Tumor size was measured
199 every other day after irradiation. When the tumor volumes reached 1,500 mm³, mice were
200 sacrificed.

201

202 **Statistical analysis**

203 Multiple comparisons were performed using the Tukey–Kramer test. The therapeutic
204 effects of eribulin, X-ray irradiation, and combination treatment were statistically
205 evaluated using repeated-measures two-way ANOVA. Survival rates after irradiation
206 were estimated using the Kaplan–Meier method. Comparisons between groups were
207 performed using the log-rank test. Significance was assumed at $P < 0.05$.

208 **Results**

209 **Eribulin improves tumor oxygenation in a dose-dependent manner**

210 To determine the appropriate dosage of eribulin for tumor oxygenation, MDA-MB-231-
211 bearing mice were treated with a single dose of 0.3 and 1.0 mg/kg eribulin. Three days
212 after the administration, mice were injected with ¹⁸F-DiFA and pimonidazole, and
213 intratumoral hypoxia levels were examined by PET/CT imaging and histological analysis.
214 No significant changes in tumor volume and body weight were observed between the
215 control and eribulin-treated groups (data not shown). Intratumoral ¹⁸F-DiFA
216 accumulation was less observed in eribulin-treated mice than in control mice. (Fig. 1A
217 and 1B). The tumor/muscle ratio, which was calculated using SUV_{mean}, revealed that
218 1.0 mg/kg eribulin significantly reduced intratumoral ¹⁸F-DiFA accumulation. These
219 results were also supported by the reduction of the pimonidazole-positive hypoxic region
220 (Fig. 2A). In addition, immunohistochemical analysis of CD31, a vascular endothelial
221 marker, showed that eribulin treatment led to marked changes in CD31-expressing
222 microvessels in comparison with control; stained areas became more frequent and the
223 number of large vascular structures increased (Fig. 2B). Quantitative analysis
224 demonstrated that eribulin significantly increased the microvessel area (%) (Fig. 2B(b)).
225 These results indicate that eribulin improves tumor oxygenation by remodeling the tumor
226 vasculature in a dose-dependent manner.

227

228 **Eribulin shows maximal effectiveness for tumor oxygenation seven days after**
229 **administration**

230 To determine the time point at which eribulin treatment shows the optimal effects on
231 tumor oxygenation, tumor-bearing mice treated with a single dose of 1.0 mg/kg eribulin

232 were imaged by PET/CT 1, 3, and 7 days after the administration. In the experiments of
233 MDA-MB-231 tumor, we performed a comparative assessment between the different
234 groups: Control, Day 1, Day 3, and Day 7 (Fig. 3A). As a result, the reduction of the ^{18}F -
235 DiFA accumulation levels by eribulin was most significant 7 days after treatment (Fig.
236 3A(b)). In addition, to reveal the effect of eribulin on the same individual, we PET/CT
237 scanned the mice before, 4 days, and 7 days after eribulin administration using NCI-
238 H1975-bearing mice (Fig. 3B). As shown in Fig. 3B(b), in most mice, the tumor/muscle
239 ratio calculated from SUVmean had significantly decreased with time after eribulin
240 treatment. In parallel with the findings of ^{18}F -DiFA PET imaging, a decrease in the
241 hypoxic region of MDA-MB-231 tumor was also observed from Day 3 in
242 immunohistochemical analysis with pimonidazole. (Fig. 4A). To evaluate tumor
243 vasculature remodeling precisely, we performed immunohistochemical analysis of CD31
244 and α -SMA (Fig. 4B). As shown in Fig. 4B(a), CD31-stained tumor microvessels were
245 increased by eribulin. Furthermore, CD31 and α -SMA double-positive vessels were
246 frequently observed in the tumor sections 7 days after eribulin treatment (high
247 magnification photograph of Day 7). Quantitative analysis showed that eribulin
248 significantly increased the microvessel area at 3 and 7 days after treatment (Fig. 4B(b),
249 left panel). On the other hand, eribulin tended to increase the α -SMA-positive area up to
250 7 days after treatment, although the increase was not significant (Fig. 4(b), right panel).
251 These results indicate that tumor oxygenation showed the maximal improvement 7 days
252 after eribulin treatment in the MDA-MB-231 mouse xenograft model.

253

254 **Tumor oxygenation by eribulin significantly delayed tumor growth after irradiation**

255 To examine the effect of tumor oxygenation by eribulin on radiation therapy, we
256 conducted combination therapy with radiation in MDA-MB-231 tumor (Fig. 5A). Before
257 X-irradiation, we examined tumor hypoxia levels by PET/CT imaging and confirmed that
258 the eribulin-treated tumors were oxygenated (Fig. 5B). The tumors were then locally X-
259 irradiated at a dose of 10 Gy. With the most effective protocol for tumor oxygenation by
260 eribulin, significant retardation of tumor growth was observed in comparison with the
261 control, radiation-alone, and drug-alone groups (Fig. 5C). Furthermore, the combination
262 therapy significantly increased the survival rate up to 50 days after treatment in
263 comparison with the control (Fig. 5D). These results suggested that eribulin efficiently
264 enhanced the antitumor activity of radiation by improving tumor oxygenation. To further
265 verify the effect of eribulin-induced tumor oxygenation on the antitumor activity of
266 radiation, we investigated the correlation between the tumor oxygenation level estimated
267 by PET imaging and the antitumor activity of radiation. In Fig. 6, the data for the
268 combination group and X-ray only group show a clear distinction; the combination
269 treatment significantly prolonged the number of days required for doubling the size of the
270 tumor. Furthermore, tumor hypoxia level inversely correlated with tumor growth after
271 irradiation ($R^2 = 0.8119$), suggesting that eribulin-induced tumor oxygenation is a critical
272 factor for radio-sensitization.

273 To evaluate the radiosensitizing effect of eribulin on multiple cancer types, we tested the
274 combination therapy in the lung cancer NCI-H1975 xenograft. As shown in the diagram
275 of Fig. 7A, we also examined the regimens for the postradiation treatment of eribulin and
276 for X-irradiation under carbogen-breathing instead of eribulin. Similar to the MDA-MB-
277 231, eribulin treatment significantly decreased the tumor/muscle ratio of ^{18}F -DiFA (Fig.
278 7B). Although the suppression level of ^{18}F -DiFA accumulation was weak compared to

279 eribulin, carbogen breathing also significantly improved the oxygenation status (Fig. 7B).
280 The combination of eribulin pretreatment with radiation showed the most efficient anti-
281 tumor effect on tumor growth (Fig. 7C). Post-treatment of eribulin also enhanced the
282 radiation effect on tumor growth, but the suppression level was weaker than that of pre-
283 treatment regimen. Indeed, carbogen breathing could not show the apparent
284 radiosensitizing effect. Finally, our results showed a significant prolongation of survival
285 in all treatment groups compared to control. However, only the combination group with
286 eribulin pretreatment with radiation showed significant improvement of survival rate
287 compared to the radiation group (Fig. 7D).
288
289

290 **Discussion**

291 Hypoxia is known to be associated with cancer resistance to radiotherapy and
292 chemotherapy (28-30). Although eribulin has been shown to increase tumor perfusion and
293 induce tumor reoxygenation, there is no evidence assessing the level of tumor
294 oxygenation and its influence on the anti-tumor effect of radiation after eribulin treatment.
295 We conducted a dose-response and time-course analysis using ^{18}F -DiFA PET/CT imaging
296 to determine an optimal eribulin treatment protocol for tumor oxygenation when used in
297 combination with radiation therapy. As shown in Fig. 1, eribulin significantly reduced
298 intratumoral ^{18}F -DiFA accumulation in a dose-dependent manner, as shown in a previous
299 study using ^{18}F -FMISO (14). Eribulin treatment also reduced pimonidazole-positive areas
300 (Fig. 2A). These results demonstrate that ^{18}F -DiFA PET/CT imaging is a useful tool to
301 detect tumor oxygenation, and that eribulin improves tumor hypoxic conditions in a dose-
302 dependent manner. Compared to the rate of decrease in pimonidazole-positive area after
303 eribulin treatment, that in tumor/muscle ratio of ^{18}F -DiFA is smaller. This difference
304 between the T/M ratio and pimonidazole positive region may be accounted for by the
305 limitation of PET hypoxia imaging as it reflects not only hypoxic region but also tissue
306 perfusion (31). Because tumor tissue is often hypoperfused, probe accumulation was
307 influenced by both hypoxia and the limiting probe delivery. If eribulin only promotes
308 vascular remodeling, the suppressive effect on ^{18}F -DiFA accumulation would be
309 relatively weak compared to the effects on pimonidazole and CD31 intensities. In the
310 other report with ^{18}F -DiFA imaging and an angiogenesis inhibitor, trastuzumab, the
311 suppression level of SUV by drug treatment is apparently weak compared to that of
312 pimonidazole-indicated hypoxic region (32). We next performed a time-course analysis
313 of eribulin-induced oxygenation (Figs. 3 and 4). Tumor hypoxia was most significantly

314 improved 7 days after eribulin treatment. Immunostaining of CD31 showed that eribulin
315 significantly increased the number of tumor vessels as time passed. In addition, eribulin
316 seemed to increase the number of mature vessels that were CD31- and α -SMA-double-
317 positive, although the α -SMA-positive area did not significantly increase in eribulin-
318 treated sections. These results suggest that eribulin promotes new blood vessel formation,
319 resulting in tumor oxygenation. This effect of eribulin on tumor vasculature environment
320 is thought to be vascular remodeling, but not vascular normalization. Generally, vascular
321 normalization is defined as the temporal phenomenon with a decrease of immature blood
322 vessels and improvement of vascular function induced by the suppression of VEGF
323 signaling. As Ito K. et al. also discussed (33), the vascular remodeling induced by eribulin
324 should provide a long-lasting effect with an increased number of tumor vessels, which
325 differs from vascular normalization through the use of other antiangiogenesis inhibitors
326 such as bevacizumab. The data we obtained from the MDA-MB-231 tumor coincide with
327 this result. Conversely, Miki S. et al. demonstrated the opposite effect: a significant
328 decrease of tumor vasculature in xenografted mouse brain tumor tissue (20). The reason
329 for this inconsistency is unknown, but it may be due to the different tumor origin
330 (glioblastoma or other tumors) or the different implanted site (intracranial brain or
331 subcutaneously tissue).

332 To determine whether the antitumor effect of eribulin is truly due to the changes in
333 tumor oxygenation, we examined the effects of post-radiation treatment of eribulin and
334 carbogen breathing on NCI-H1975 tumors. As shown in Fig. 7C and D, post-radiation
335 treatment of eribulin also significantly suppressed the tumor growth and survival rate
336 compared to the control, but not significantly compared to the radiation group, suggesting
337 that the abrogation of the hypoxic region through eribulin pretreatment is necessary to

338 enhance the effect of radiation. On the other hand, carbogen breathing could not
339 sufficiently improve the oxygen status compared to the eribulin treatment (Fig. 7B), hence
340 we did not observe an enhancement of the radiation effect on tumor growth (Figs. 7C and
341 D). This suggests that the vascular remodeling by eribulin pretreatment most efficiently
342 improved tumor oxygenation enough to obtain the radiosensitization effect.

343 Tumor hypoxia should be considered for radiation therapy since it is thought to be an
344 important factor in resistance to radiation (31). Prasad et al. showed that tumor
345 oxygenation by MnO₂ nanoparticles enhanced the anti-cancer activity of radiation
346 therapy (4). Thus, the tumor oxygenation by eribulin was expected to enhance the effect
347 of radiation therapy. Miki et al. showed that eribulin increased radio-sensitivity in
348 glioblastoma xenograft mouse models (20). We also demonstrated that the combination
349 treatment with eribulin and radiation significantly delayed tumor growth and extended
350 survival (Figs. 5 and 7), suggesting that the combined administration of radiation with
351 eribulin may serve as a novel therapeutic strategy. Furthermore, we showed that ¹⁸F-DiFA
352 accumulation was inversely correlated with tumor growth after irradiation. This result
353 strongly suggests that tumor hypoxia contributes to radio-resistance, and that eribulin-
354 induced tumor oxygenation is a critical factor for radio-sensitization. These observations
355 are important for radiation therapy.

356 Eribulin has been reported to increase tumor oxygen saturation in advanced breast
357 cancer patients (15). Furthermore, ¹⁸F-DiFA has already been tested in clinical patients,
358 and it achieved better contrast imaging of tumor hypoxia with a shorter waiting time in
359 comparison with ¹⁸F-FMISO, although it showed these findings via the same mechanism
360 (26). On the basis of these observations, ¹⁸F-DiFA may be useful to predict the treatment
361 response of radiation therapy in clinical patients undergoing eribulin treatment.

362

363 **Conclusion**

364 Eribulin treatment improved tumor oxygenation by vascular remodeling associated with
365 increased microvessels, resulting in radio-sensitization in human breast and lung cancer
366 models. The eribulin-induced tumor oxygenation level correlated with the antitumor
367 activity of radiation. These results demonstrated that eribulin-induced tumor oxygenation
368 is a critical factor for radio-sensitization. Moreover, detection of tumor hypoxia with ¹⁸F-
369 DiFA PET/CT may be an important clinical indicator for estimating the efficiency of
370 radiation therapy in patients undergoing eribulin treatment. We expect that eribulin will
371 be a potent drug for tumor radiation therapy by improving tumor reoxygenation.

372

373 **Acknowledgements**

374 We thank Eisai Co., Ltd. for providing eribulin.

375

376

377 **References**

- 378 **1.** Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* 2019;69:7-
379 34.
- 380 **2.** Desantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al.
381 Breast cancer statistics, 2019. *CA Cancer J Clin.* 2019;69:438-51.
- 382 **3.** Lundgren K, Holm C, Landberg G. Hypoxia and breast cancer: prognostic and
383 therapeutic implications. *Cell Mol Life Sci.* 2007;64:3233–3247.
- 384 **4.** Prasad P, Gordijo CR, Abbasi AZ, Maeda A, Ip A, Rauth AM, et al. Multifunctional
385 albumin-MnO₂ nanoparticles modulate solid tumor microenvironment by attenuating
386 hypoxia, acidosis, vascular endothelial growth factor and enhance radiation response.
387 *ACS Nano.* 2014;8:3202–3212.
- 388 **5.** Uemura D, Takahashi K, Yamamoto T, Katayama C, Tanaka J, Okumura Y, et al.
389 Norhalichondrin A: an antitumor polyether macrolide from a marine sponge. *J Am*
390 *Chem Soc.* 1985;107:4796–4798.
- 391 **6.** Towle MJ, Salvato KA, Wels BF, Aalfs KK, Zheng W, Seletsky BM, et al. Eribulin
392 induces irreversible mitotic blockade: implications of cell-based pharmacodynamics
393 for in vivo efficacy under intermittent dosing conditions. *Cancer Res.* 2011;71:496–
394 505.
- 395 **7.** Kuznetsov G, Towle MJ, Cheng H, Kawamura T, TenDyke K, Liu D, et al. Induction
396 of morphological and biochemical apoptosis following prolonged mitotic blockage
397 by halichondrin b macrocyclic ketone analog E7389. *Cancer Res.* 2004;64:5760–
398 5766.
- 399 **8.** Swami U, Chaudhary I, Ghalib MH, Goel S. Eribulin—A review of preclinical and
400 clinical studies. *Crit Rev Oncol Hematol.* 2012;81:163–184.

- 401 **9.** Cortes J, O'Shaughnessy J, Loesch D, Blum JL, Vahdat LT, Petrakova K, et al. Eribulin
402 monotherapy versus treatment of physician's choice in patients with metastatic breast
403 cancer (EMBRACE): a phase 3 open-label randomised study. *Lancet*. 2011;377:914–
404 923.
- 405 **10.** Donoghue M, Lemery SJ, Yuan W, He K, Sridhara R, Shord S, et al. Eribulin mesylate
406 for the treatment of patients with refractory metastatic breast cancer: use of a
407 "Physician's Choice" control arm in a randomized approval trial. *Clin Cancer Res*.
408 2012;18:1496–1505.
- 409 **11.** Gamucci T, Michelotti A, Pizzuti L, Mentuccia L, Landucci E, Sperduti I, et al.
410 Eribulin mesylate in pretreated breast cancer patients: a multicenter retrospective
411 observational study. *J Cancer*. 2014;5:320–327.
- 412 **12.** Schöffski P, Chawla S, Maki RG, Italiano A, Gelderblom H, Choy E, et al. Eribulin
413 versus dacarbazine in previously treated patients with advanced liposarcoma or
414 leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. *Lancet*.
415 2016;387:1629–1637.
- 416 **13.** Pean E, Klaar S, Berglund EG, Salmonson T, Borregaard J, Hofland KF, et al. The
417 European Medicines Agency Review of Eribulin for the Treatment of Patients with
418 Locally Advanced or Metastatic Breast Cancer: Summary of the Scientific
419 Assessment of the Committee for Medicinal Products for Human Use. *Clin Cancer*
420 *Res*. 2012;18:4491–4497.
- 421 **14.** Zhao S, Yu W, Ukon N, Tan C, Nishijima K-I, Shimizu Y, et al. Elimination of tumor
422 hypoxia by eribulin demonstrated by 18F-FMISO hypoxia imaging in human tumor
423 xenograft models. *EJNMMI Res*. 2019;9:1–10.
- 424 **15.** Ueda S, Saeki T, Takeuchi H, Shigekawa T, Yamane T, Kuji I, et al. In vivo imaging

- 425 of eribulin-induced reoxygenation in advanced breast cancer patients: a comparison
426 to bevacizumab. *Br J Cancer*. 2016;114:1212–1218.
- 427 **16.** Yoshida T, Ozawa Y, Kimura T, Sato Y, Kuznetsov G, Xu S, et al. Eribulin mesilate
428 suppresses experimental metastasis of breast cancer cells by reversing phenotype
429 from epithelial–mesenchymal transition (EMT) to mesenchymal–epithelial
430 transition (MET) states. *Br J Cancer*. 2014;110:1497–1505.
- 431 **17.** Mok TS, Geater SL, Iannotti N, Thongprasert S, Spira A, Smith D, et al. Randomized
432 phase II study of two intercalated combinations of eribulin mesylate and erlotinib in
433 patients with previously treated advanced non-small-cell lung cancer. *Ann Oncol*.
434 2014;25:1578–1584.
- 435 **18.** Lee JS, Yost SE, Blanchard S, Schmolze D, Yin HH, Pillai R, et al. Phase I clinical
436 trial of the combination of eribulin and everolimus in patients with metastatic triple-
437 negative breast cancer. *Breast Cancer Res*. 2019;21:1–3.
- 438 **19.** Funahashi Y, Okamoto K, Adachi Y, Semba T, Uesugi M, Ozawa Y, et al. Eribulin
439 mesylate reduces tumor microenvironment abnormality by vascular remodeling in
440 preclinical human breast cancer models. *Cancer Sci*. 2014;105:1334–1342.
- 441 **20.** Miki S, Imamichi S, Fujimori H, Tomiyama A, Fujimoto K, Satomi K, et al.
442 Concomitant administration of radiation with eribulin improves the survival of mice
443 harboring intracerebral glioblastoma. *Cancer Sci*. 2018;109:2275–2285.
- 444 **21.** Troost EGC, Laverman P, Philippens MEP, Lok J, Van Der Kogel AJ, Oyen WJG, et
445 al. Correlation of [18F]FMISO autoradiography and pimonidazole
446 immunohistochemistry in human head and neck carcinoma xenografts. *Eur J Nucl*
447 *Med Mol Imaging*. 2008;35:1803–1811.
- 448 **22.** Toyonaga T, Hirata K, Shiga T, Nagara T. Players of ‘hypoxia orchestra’ – what is the

- 449 role of FMISO? *Eur J Nucl Med Mol Imaging*. 2017;44:1679–1681.
- 450 **23.** Chung J-K, Chang YS, Lee YJ, Kim YJ, Jeong JM, Lee DS, et al. The effect of tumor
451 size on F-18-labeled fluorodeoxyglucose and fluoroerythronitroimidazole uptake in
452 a murine sarcoma model. *Ann Nucl Med*. 1999;13:303–308.
- 453 **24.** Nakata N, Kiriū M, Okumura Y, Zhao S, Nishijima K, Shiga T, et al. Comparative
454 evaluation of [18F]DiFA and its analogs as novel hypoxia positron emission
455 tomography and [18F]FMISO as the standard. *Nucl Med Biol*. 2019;70:39–45.
- 456 **25.** Shimizu Y, Zhao S, Yasui H, Nishijima K-I, Matsumoto H, Shiga T, et al. A novel
457 PET probe “[18F]DiFA” accumulates in hypoxic region via glutathione conjugation
458 following reductive metabolism. *Mol Imaging Biol*. 2019;21:122–129.
- 459 **26.** Watanabe S, Shiga T, Hirata K, Magota K, Okamoto S, Toyonaga T, et al.
460 Biodistribution and radiation dosimetry of the novel hypoxia PET probe [18F]DiFA
461 and comparison with [18F]FMISO. *EJNMMI Res*. 2019;9:1–11.
- 462 **27.** Ukon N, Zhao S, Yu W, Shimizu Y, Nishijima K-I, Kubo N, et al. Dynamic PET
463 evaluation of elevated FLT level after sorafenib treatment in mice bearing human
464 renal cell carcinoma xenograft. *EJNMMI Res*. 2016;6:1–8.
- 465 **28.** Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer*.
466 2011;11:393–410.
- 467 **29.** Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome.
468 *Cancer Metastasis Rev*. 2007;26:225–239.
- 469 **30.** Horsman MR, Mortensen LS, Petersen JB, Busk M, Overgaard J. Imaging hypoxia to
470 improve radiotherapy outcome. *Nat Rev Clin Oncol*. 2012;9:674–687.
- 471 **31.** Fleming IN, Manavaki R, Blower PJ, West C, Williams KJ, Harris AL, Domarkas J,
472 Lord S, Baldry C, Gilbert FJ. Imaging tumour hypoxia with positron emission

- 473 tomography. *Brit J Cancer*. 2015;112:238–50
- 474 **32.** Sorace AG, Syed AK, Barnes SL, Quarles CC, Sanchez V, Kang H, Yankeelov TE.
475 Quantitative [¹⁸F]FMISO PET Imaging Shows Reduction of Hypoxia Following
476 Trastuzumab in a Murine Model of HER2+ Breast Cancer. *Mol Imaging Biol*.
477 2017;19:130–137.
- 478 **33.** Ito K, Hamamichi S, Abe T, Akagi T, Shirota H, Kawano S, Asano M, Asano O, Yokoi
479 A, Matsui J, Umeda IO, Fujii H. Antitumor effects of eribulin depend on modulation
480 of the tumor microenvironment by vascular remodeling in mouse models. *Cancer*
481 *Sci*. 2017;108:2273–2280.
- 482 **34.** Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts
483 radiation response in advanced squamous cell carcinoma of the head and neck.
484 *Radiother Oncol*. 1996;41:31–39.

485 **Figure legends**

486 **Figure 1.**

487 Dose-dependent analysis of the effects of eribulin on intratumoral ^{18}F -DiFA accumulation
488 in MDA-MB-231 tumors. Mice were intraperitoneally administered a single dose of
489 eribulin (0.3 or 1.0 mg/kg). Intratumoral ^{18}F -DiFA accumulation levels were examined
490 by PET/CT imaging three days after administration. (A) Representative images of ^{18}F -
491 DiFA PET/CT. (B) Quantitative analysis of intratumoral accumulation levels of ^{18}F -DiFA.
492 The dashed lines show the tumor regions. Data are expressed as the mean \pm standard error
493 of 5 animals per group. *: $p < 0.05$.

494

495 **Figure 2.**

496 Dose-dependent analysis of the effects of eribulin on the intratumoral pimonidazole- (A)
497 and CD31-positive areas (B) in MDA-MB-231 tumors. Mice were intraperitoneally
498 injected with pimonidazole (60 mg/kg) 1 h before sacrifice. A(a) Representative images
499 of immunohistochemical staining for pimonidazole. A(b) Quantitative analysis of the
500 pimonidazole-positive area in tumor tissue. B(a) Representative images of
501 immunohistochemical staining for CD31. B(b) Quantitative analysis of the CD31-
502 positive area in tumor tissue. Data are expressed as the mean \pm standard error. *: $p < 0.05$,
503 **: $p < 0.01$.

504

505 **Figure 3.**

506 Time-course analysis of the effects of eribulin on intratumoral ^{18}F -DiFA accumulation in
507 MDA-MB-231 (A) and NCI-H1975 (B) tumors. Mice were intraperitoneally
508 administered a single dose of eribulin (1.0 mg/kg). In MDA-MB-231 tumors,

509 intratumoral ^{18}F -DiFA accumulation levels were examined in different treatment groups
510 by PET/CT imaging 1, 3, 7 days after the administration. A(a) Representative images of
511 ^{18}F -DiFA PET. A(b) Quantitative analysis of intratumoral accumulation levels of ^{18}F -
512 DiFA. For NCI-H1975 xenografts, the same mice were sequentially scanned for PET
513 imaging before, 4, and 7 days after eribulin treatment (1.0 mg/kg). B(a) Representative
514 images of ^{18}F -DiFA PET (upper panels; axial images, lower panels; coronal images). A(b)
515 Quantitative analysis of intratumoral accumulation levels of ^{18}F -DiFA. Each line
516 represents the temporal change of each mouse. The dashed lines show the tumor regions.
517 Data are expressed as the mean \pm standard error for five animals per group. *: $p < 0.05$,
518 **: $p < 0.01$.

519

520 **Figure 4.**

521 Time-course analysis of the effects of eribulin on intratumoral pimonidazole- (A), CD31-
522 and α -SMA-positive areas (B). Mice were intraperitoneally injected with pimonidazole
523 (60 mg/kg) 1 h before sacrifice. A(a) Representative images of immunohistochemical
524 staining for pimonidazole. A(b) Quantitative analysis of the pimonidazole-positive area
525 in tumor tissue. B(a) Representative images of immunohistochemical staining for CD31
526 and α -SMA. Right panel of Day 7 shows the representative image of the co-localization
527 of CD31 and α -SMA. B(b) Quantitative analysis of the CD31-positive area (left) and the
528 α -SMA-positive area (right) in tumor tissue. Data are expressed as the mean \pm standard
529 error. **: $p < 0.01$. n.s.: not significant.

530

531 **Figure 5.**

532 The combination of eribulin and X-ray irradiation causes significant retardation of tumor

533 growth in MDA-MB-231 tumors. For the combined treatment with radiation, 7 days after
534 the eribulin treatment (1.0 mg/kg), tumors were locally X-irradiated at a dose of 10 Gy
535 after ^{18}F -DiFA PET/CT imaging. The tumor size was measured every other day after
536 irradiation. RT; radiation, Eri; eribulin. (A) The diagram for the treatment regimen. (B)
537 Quantitative analysis of intratumoral accumulation levels of ^{18}F -DiFA. (C) Tumor
538 volumes were monitored every other day after irradiation. (D) Kaplan–Meier survival
539 curves of tumor-bearing mice treated with eribulin and irradiation. When the tumor
540 volumes reached 1,000 mm³, mice were sacrificed. Data are expressed as the mean \pm
541 standard error for 5–7 animals per group. *: $p < 0.05$, **: $p < 0.01$.

542

543 **Figure 6.**

544 ^{18}F -DiFA accumulation levels inversely correlated with tumor growth after irradiation in
545 MDA-MB-231 tumors. Pearson's correlation analysis was performed to analyze the
546 correlation between the level of ^{18}F -DiFA accumulation levels and the required number
547 of days to double the size of the tumor. RT; radiation. R^2 ; Pearson's correlation coefficient.
548 **: $p < 0.01$, significant between tumor/muscle ratio of RT and eribulin + RT.

549

550 **Figure 7.**

551 The combination of eribulin and X-ray irradiation causes significant retardation of tumor
552 growth in NCI-H1975 tumors. For the combined treatment with radiation, 7 days after
553 the eribulin treatment (1.0 mg/kg), tumors were locally X-irradiated at a dose of 10 Gy
554 after ^{18}F -DiFA PET/CT imaging. The other combination group were X-irradiated,
555 followed by the eribulin treatment. Instead of eribulin treatment, some mice received
556 PET/CT imaging and X-irradiation under carbogen gas breathing. The tumor size was

557 measured every other day after irradiation. RT; radiation, Carb; carbogen, Eri; eribulin.
558 (A) The diagram for the treatment regimen. (B) Quantitative analysis of intratumoral
559 accumulation levels of ^{18}F -DiFA. (C) Tumor volumes were monitored every other day
560 after irradiation. (D) Kaplan–Meier survival curves of tumor-bearing mice treated with
561 eribulin and irradiation. When the tumor volumes reached $1,500 \text{ mm}^3$, mice were
562 sacrificed. Data are expressed as the mean \pm standard error for 5–6 animals per group. *:
563 $p < 0.05$, **: $p < 0.01$ vs. Control. ††: $p < 0.01$. n.s.: not significant vs. radiation (RT)
564 group.

565

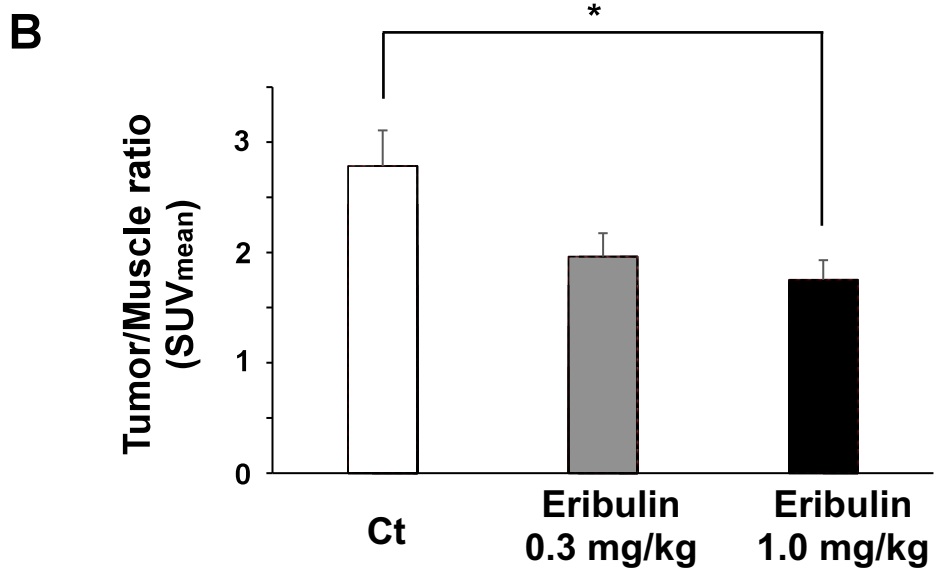
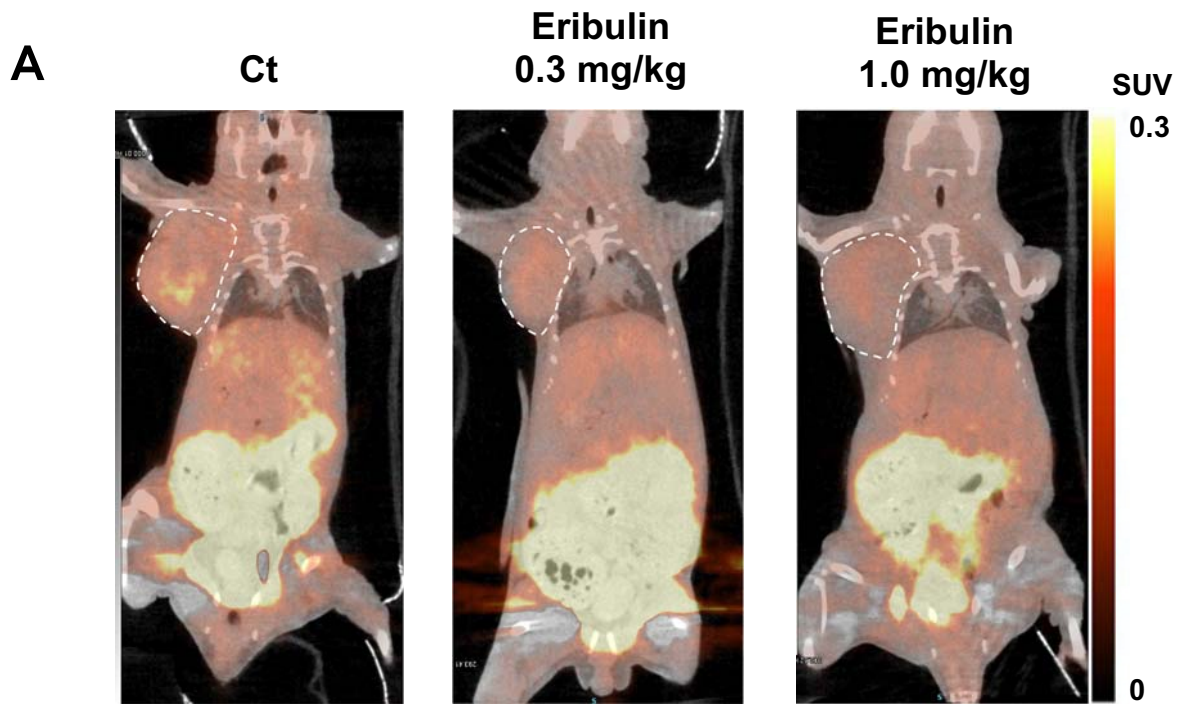


Figure 1. Bo et al.

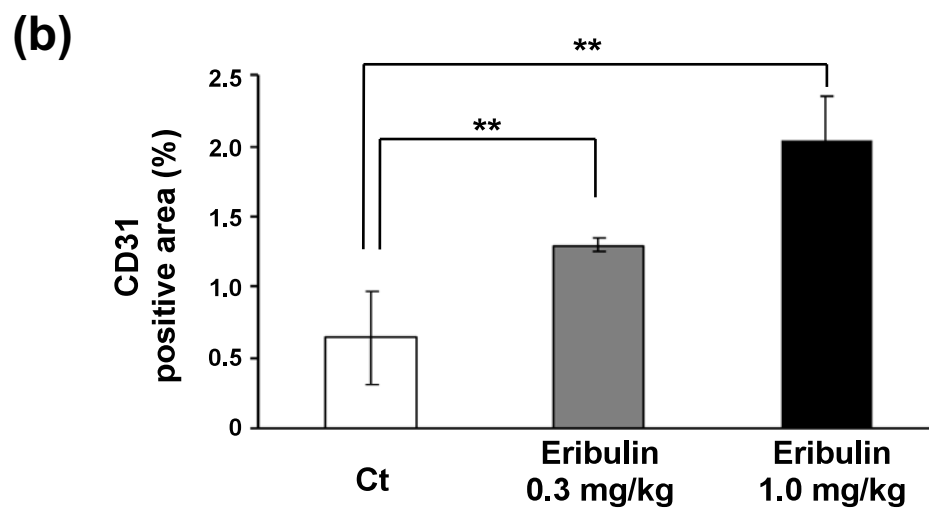
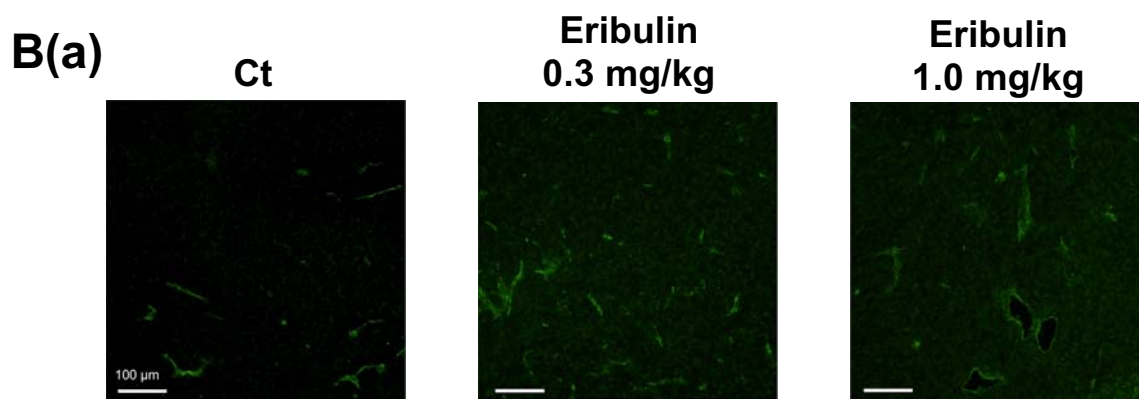
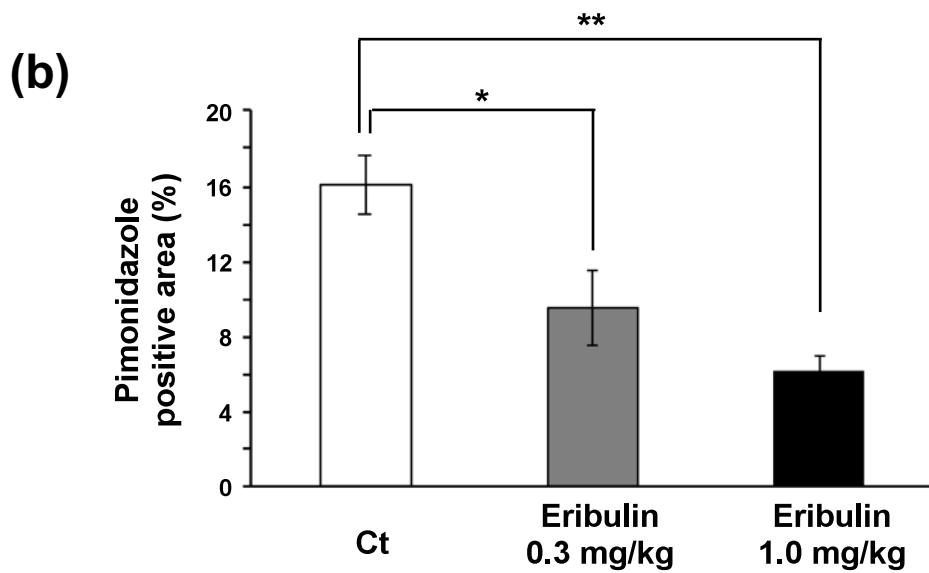
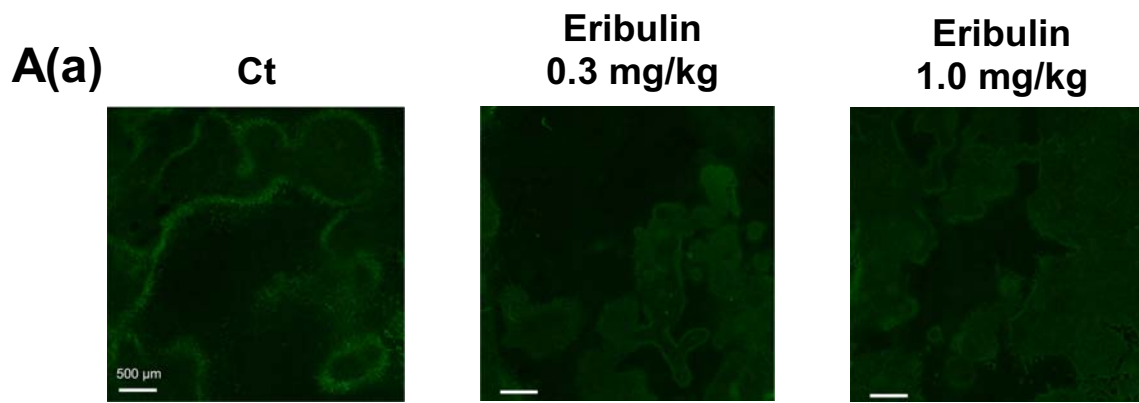
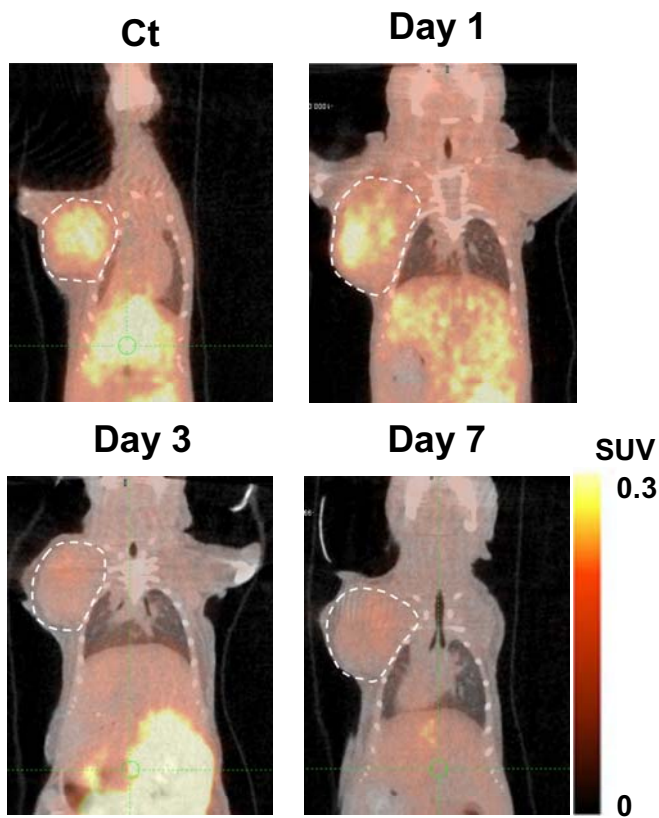
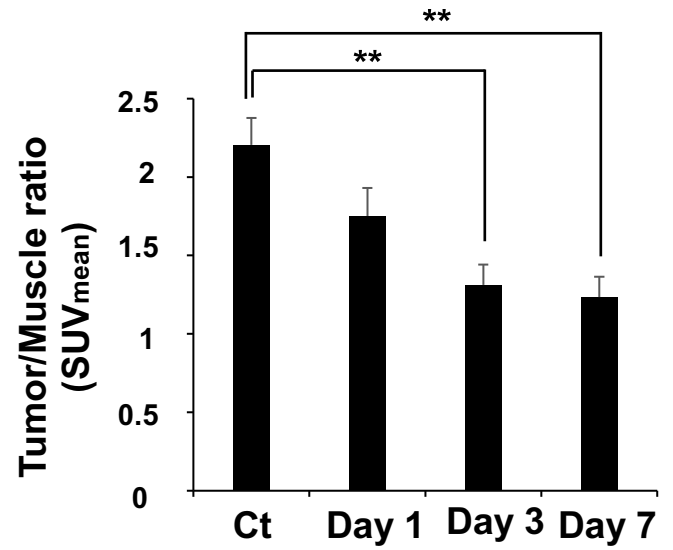


Figure 2. Bo et al.

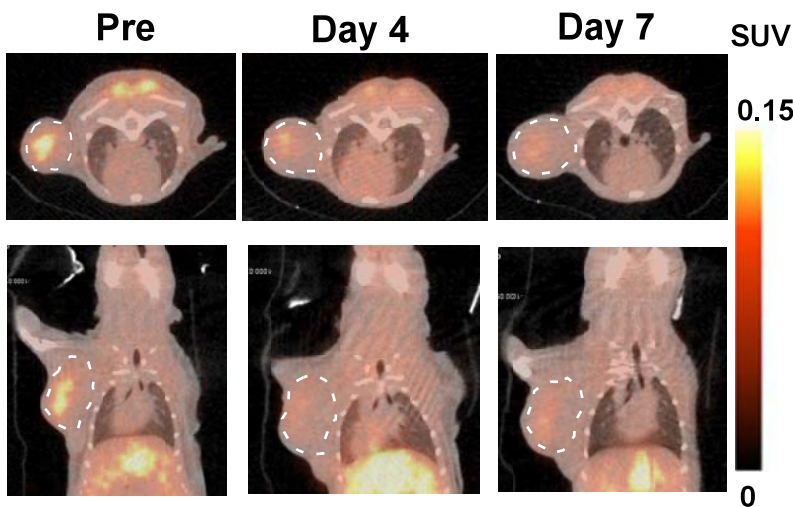
A (a) MDA-MB231



(b)



B (a) NCI-H1975



(b)

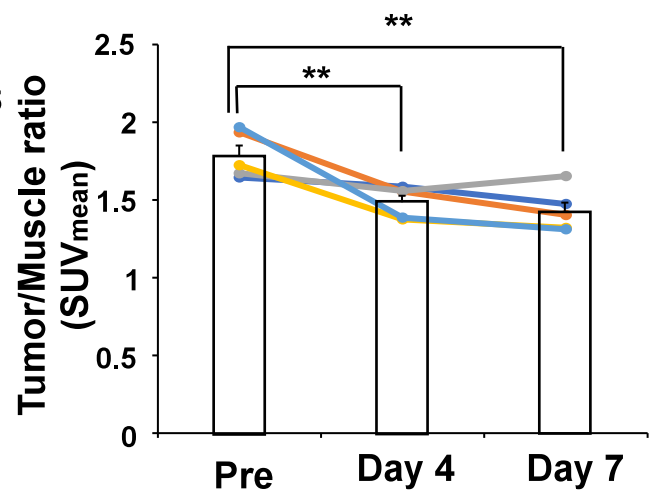


Figure 3. Bo et al.

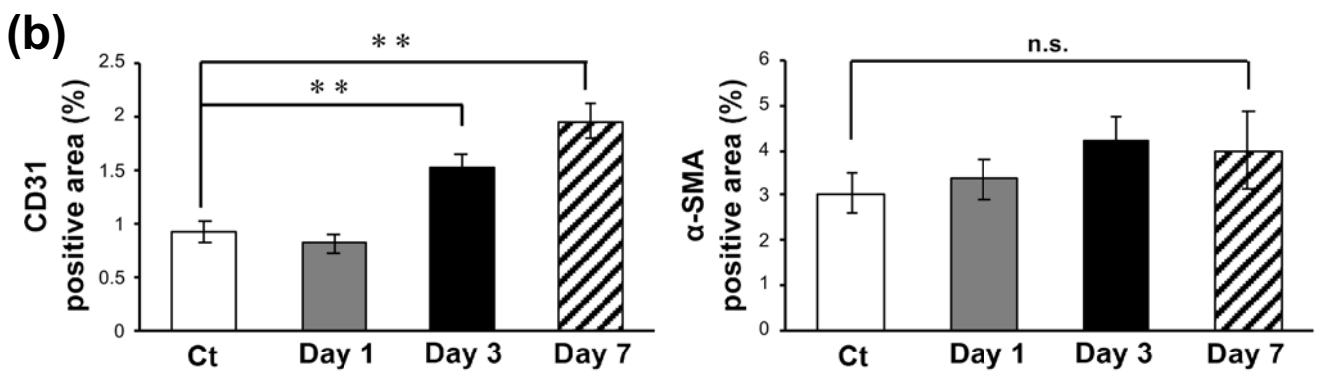
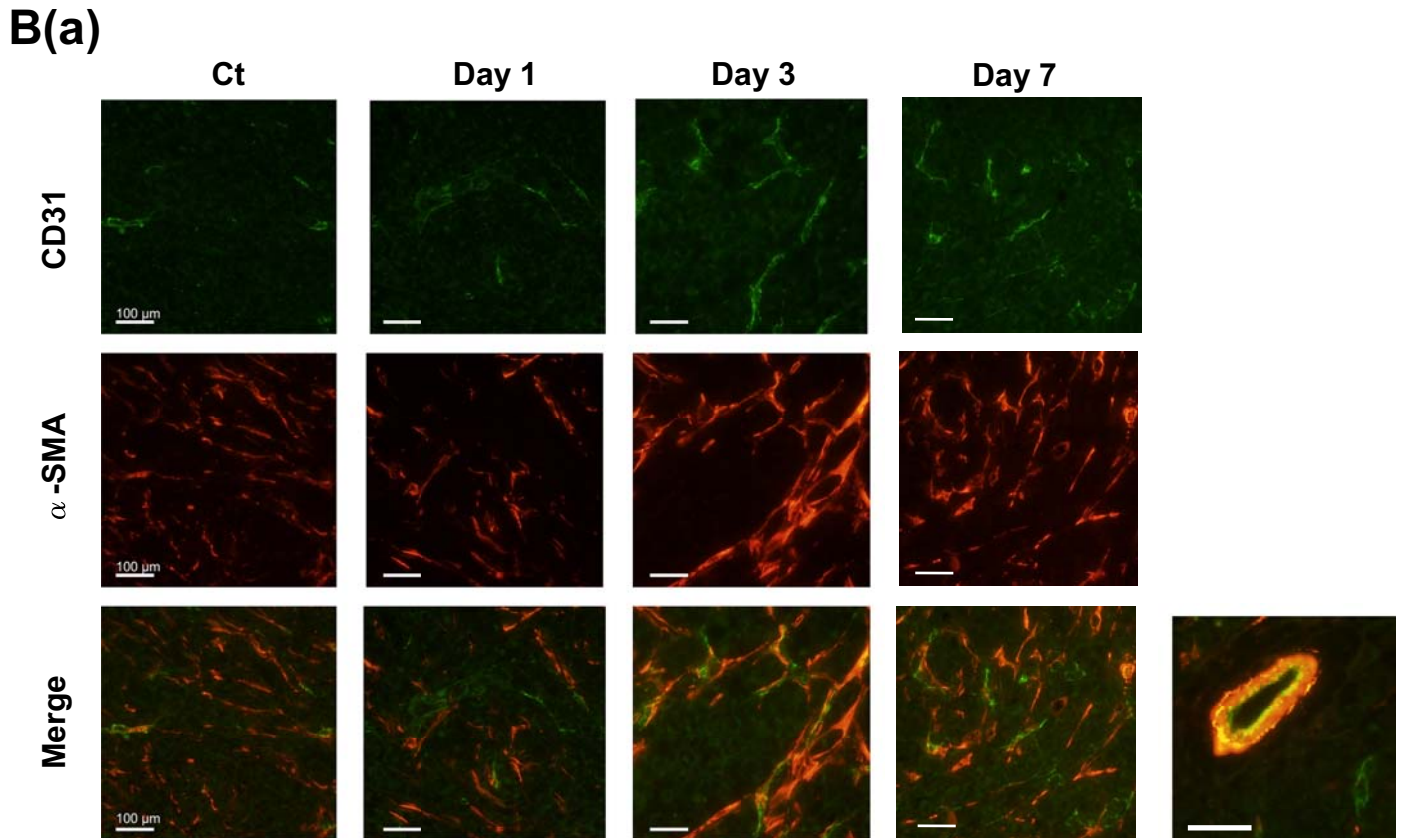
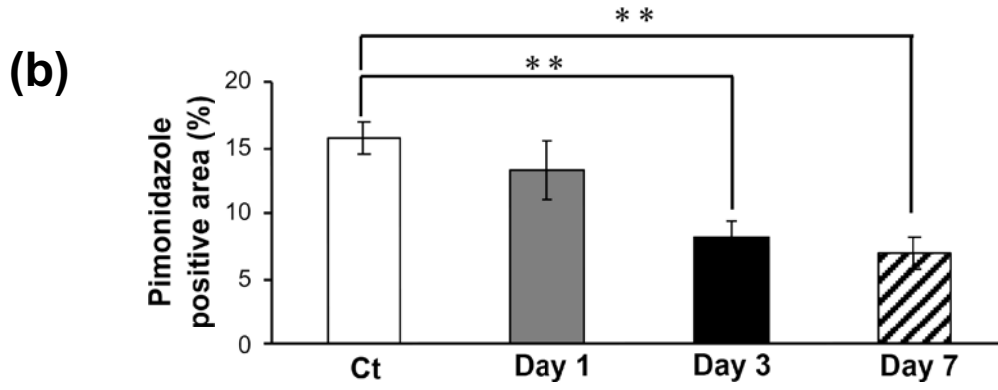
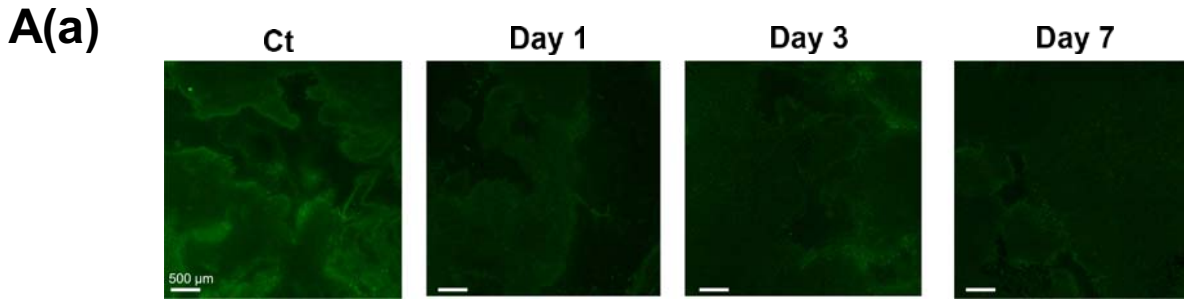


Figure 4. Bo et al.

A MDA-MB231

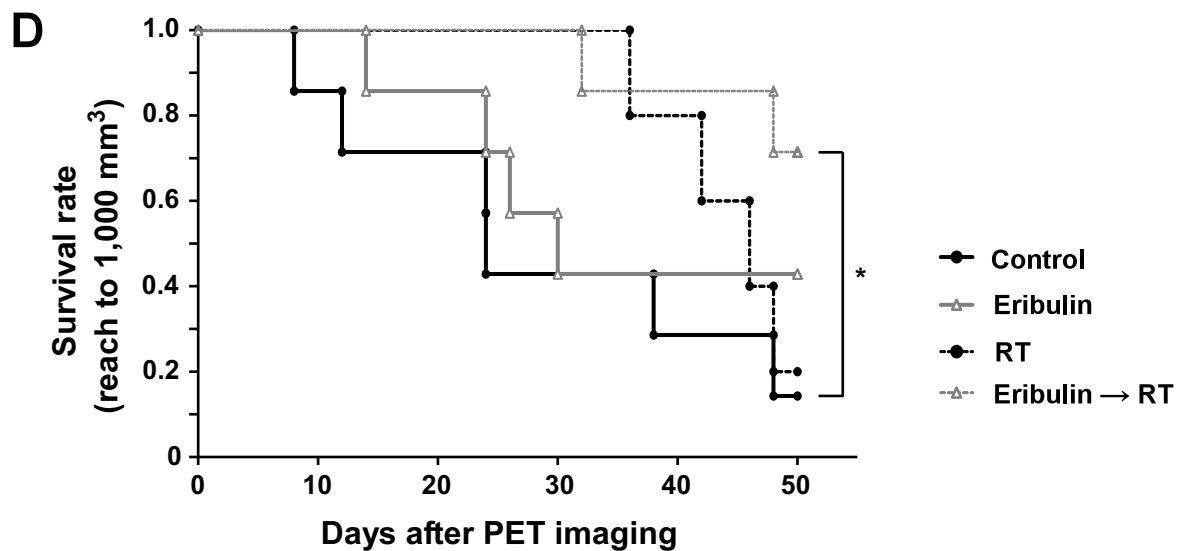
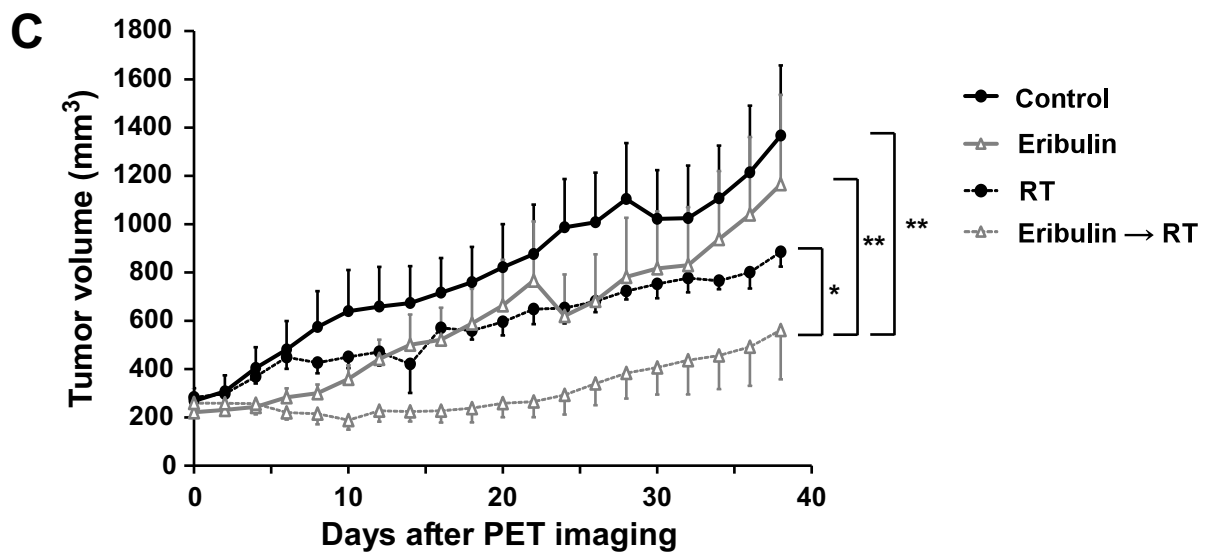
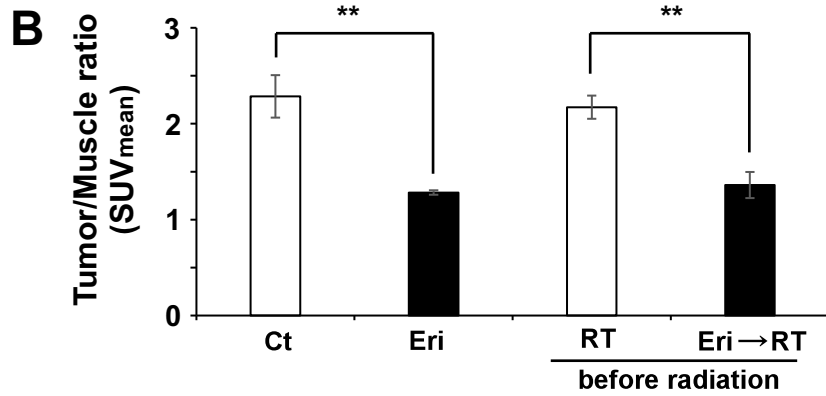
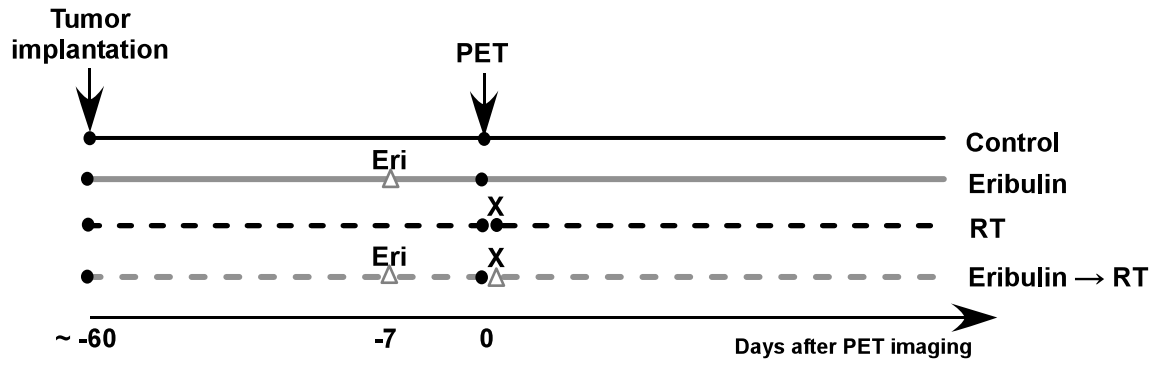


Figure 5. Bo *et al.*

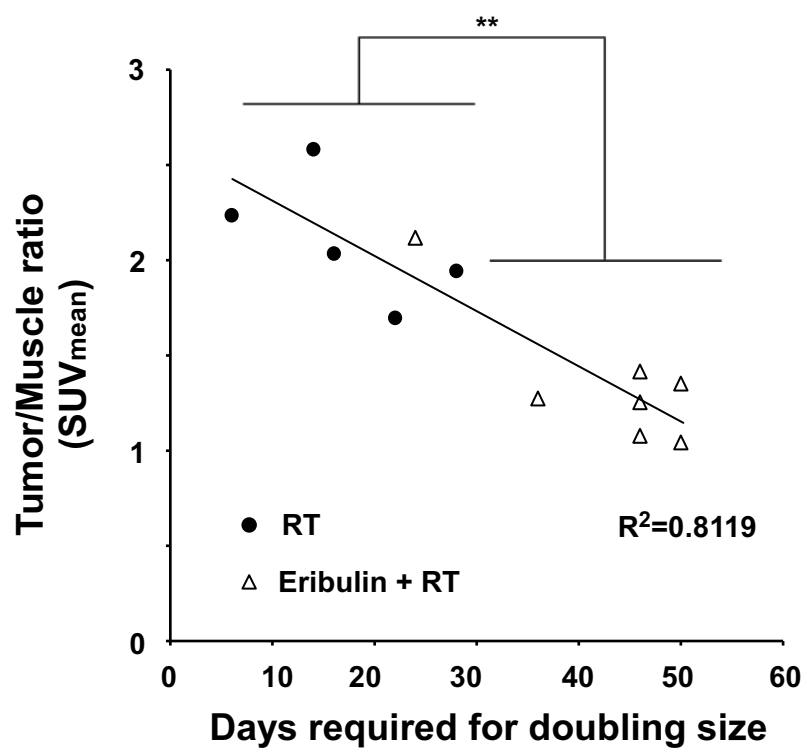


Figure 6. Bo *et al.*

A NCI-H1975

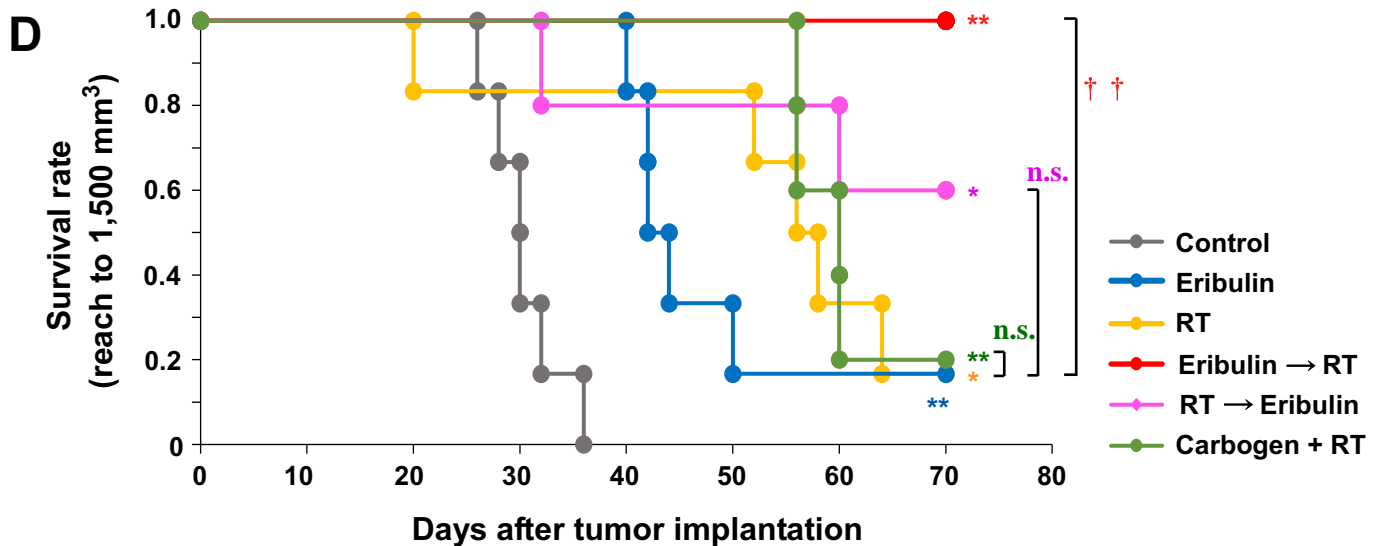
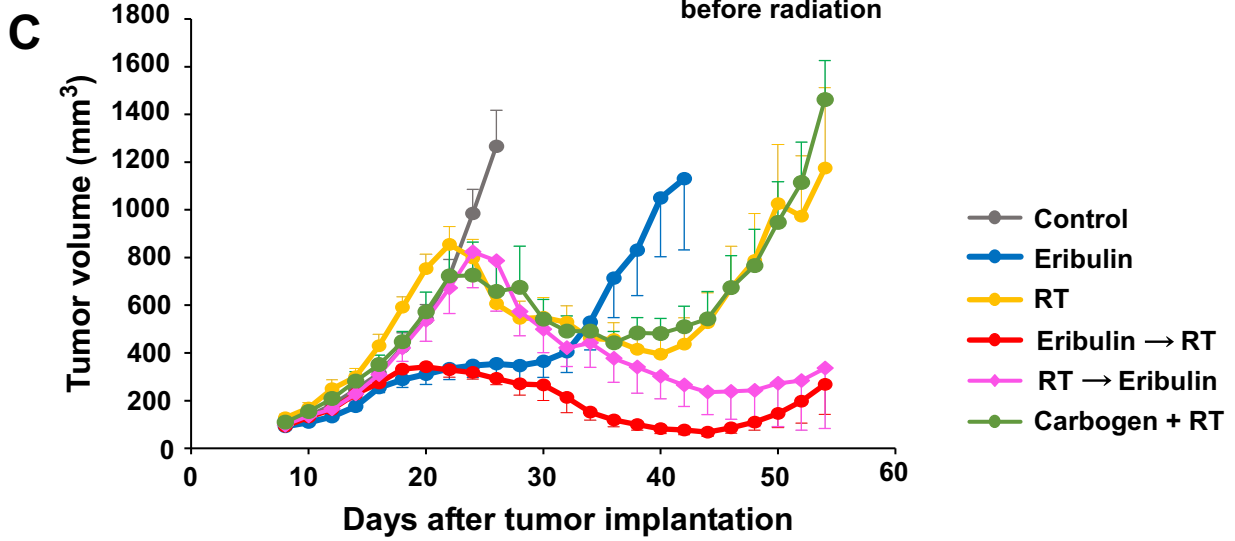
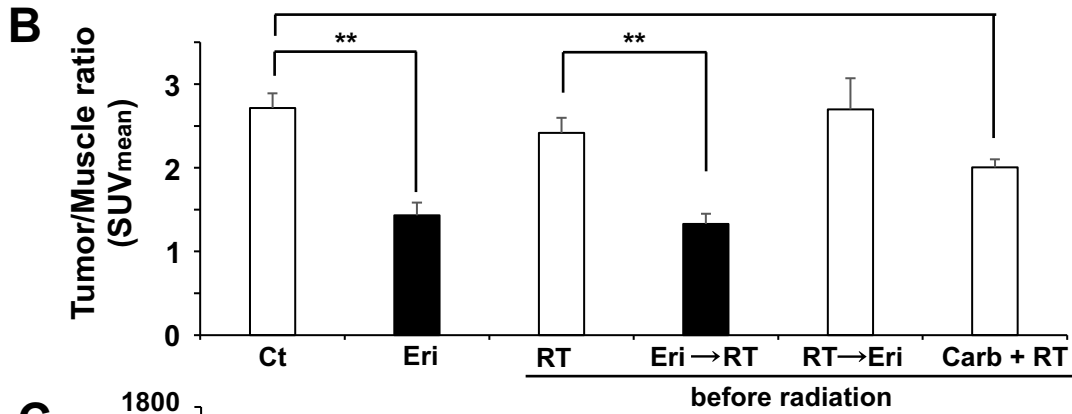
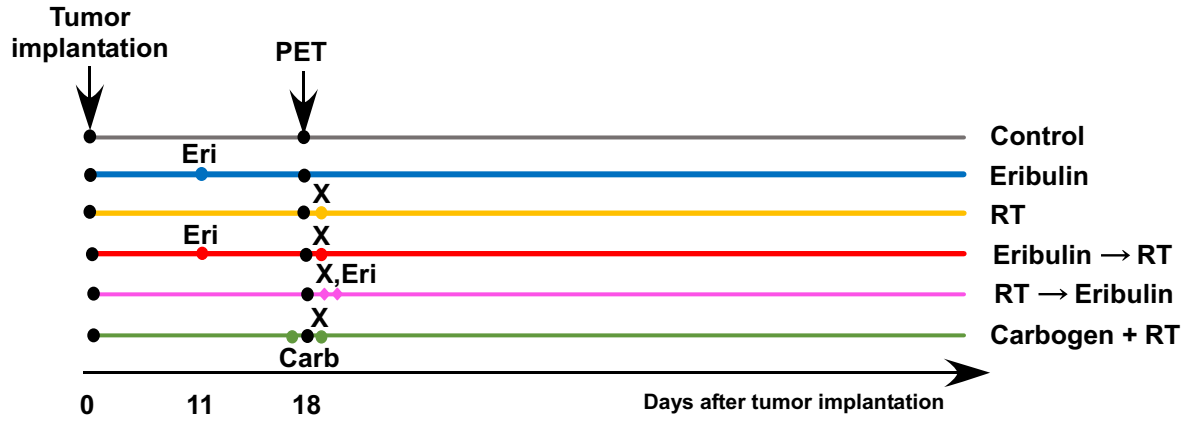


Figure 7. Bo *et al.*