

Supplemental Data

Synergy and growth delay determination

To determine the degree to which the combination of CYT-6091 and radiation had synergistic effects on tumor growth delay, the following formula was applied: observed growth delay from combination treatment / [(observed growth delay from treatment 1) + (observed growth delay from treatment 2)]. A ratio >1 indicates a synergistic (greater than additive) effect¹. Growth delay in this equation was calculated by subtracting the average time for control tumors to grow 2 or 4-fold change (where applicable) in volume from the time required for treated tumors to increase in volume by 2 or 4-fold from the first day of treatment (Supplemental Table 1 & Table 2).

Quantification of red blood cells (RBCs) and hypoxia

To assess the extent of hemorrhaging within 4T1 tumors we quantified RBCs in tissue sections using Image J software. In brief, all the slides (both control tissues, treated and untreated tumor sections) were H&E stained and analyzed using Olympus IX71 microscope equipped with a DP72 digital color camera (20× objective). All camera and microscope parameters were the same for all the samples. A minimum of 3 images were obtained per individual tumor in the PNZ zone and in the surrounding peripheral tumor tissues. In order to differentiate between RBCs and stained tissues we developed automated Image J macro. First, Image J software separated red, green and blue channels; blue channel was discarded, while red image was subtracted from the green one to enhance RBC contrast by reducing intensity of pink/purple regions. The resulting image was converted into a B&W image using a pre-selected threshold further applied for all the images throughout the data analysis. The resulting image accurately reflected presence of RBCs within the H&E stained tissue sections as was confirmed by comparison of B&W and original photos. The original H&E acted as a guide to identify PNZ, necrotic and viable tissue regions (Supplemental Figure 2). The hemorrhaging was quantified as a “corrected

mean", i.e. mean intensity for the selected sample areas. The "corrected means" were reported in the paper as mean \pm SE (Figure 4C).

Hypoxia staining was performed as described previously². Hematoxylin counter stain was applied, distinguishing necrotic regions from surrounding viable regions in untreated control tumors. Perinecrotic regions were analyzed by approximating the necrotic and viable tissue interface and analyzing 100 μ m on either side with the volume analyzed approximating $2 \times 10^5 \mu\text{m}^2$. A total of 14 areas were analyzed for the perinecrotic region and 19 for the viable region from 3 tumors.

Statistical Analysis

RBC extravasation was statistically analyzed using a one-way ANOVA with post-hoc Dunn's multiple comparison test, $p < 0.05$. Tumor tissue hypoxia analysis was performed with a two-way ANOVA with post-hoc Sidak's multiple comparison test, $p < 0.05$.

Custom Image J macro:

```
run("RGB Stack");
```

```
run("Stack to Images");
```

```
selectWindow("Blue");
```

```
close();
```

```
selectWindow("Green");
```

```
imageCalculator("Subtract create", "Red", "Green");
```

```
wait(1000);
```

```
selectWindow("Result of Red");
```

```
setAutoThreshold("Default");
```

```
run("Threshold...");
```

```
setThreshold(0, 72);
```

```
setOption("BlackBackground", false);
```

```
run("Convert to Mask");
```

```
run("Invert LUT");
```

Supplemental Tables. Growth-delay and synergistic determination of single-dose and fractionated ionizing radiation therapy combined with CYT-6091.

Supplemental Figure 1. Combined radiation and CYT-6091 therapy results in widespread vascular hemorrhaging with unaffected tumor rim in SCCVII tumors.

Representative H&E images from treatment groups at time of tissue harvest. At day 1, tumor tissue from the combined therapy group displays marked tumor specific vascular hemorrhaging with unaffected rim typical of VDAs.

Supplemental Figure 2. Quantification of RBC extravasation.

Flow diagram of RBC extracted image from an H&E image.

Literature Cited

1. Dings RP, Van Laar ES, Webber J, et al. Ovarian tumor growth regression using a combination of vascular targeting agents anginex or topomimetic 0118 and the chemotherapeutic irifolven. *Cancer Lett.* 2008;265: 270-280.
2. Przybyla BD, Shafirstein G, Koonce NA, Webber JS, Griffin RJ. Conductive thermal ablation of 4T1 murine breast carcinoma reduces severe hypoxia in surviving tumour. *Int J Hyperthermia.* 2012;28: 156-162.

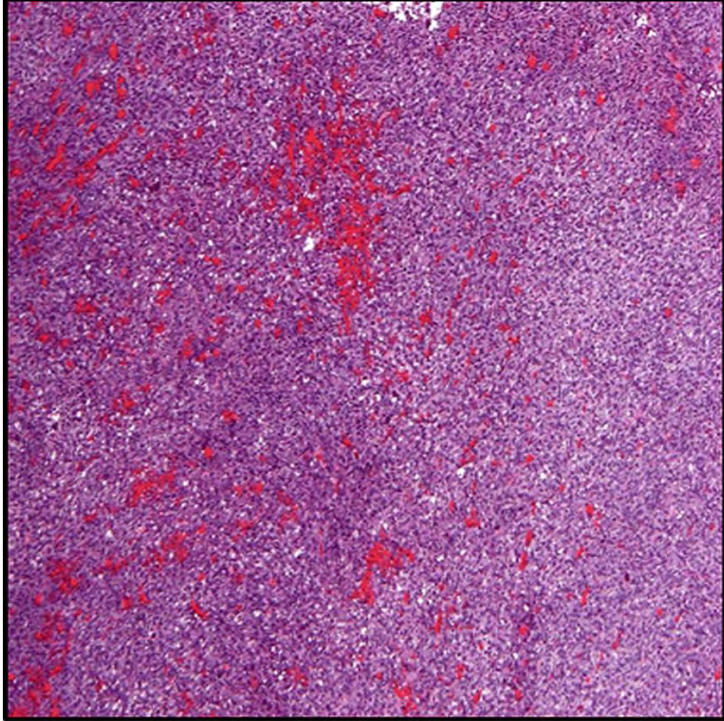
Table 1 Single-dose radiotherapy

Growth delay		Combination treatment		Synergy >1	
Fold-change	CYT-6091	Radiation	Expected	Observed	Ratio
2	0	4	4	7	1.75
4	0	4	4	11	2.75

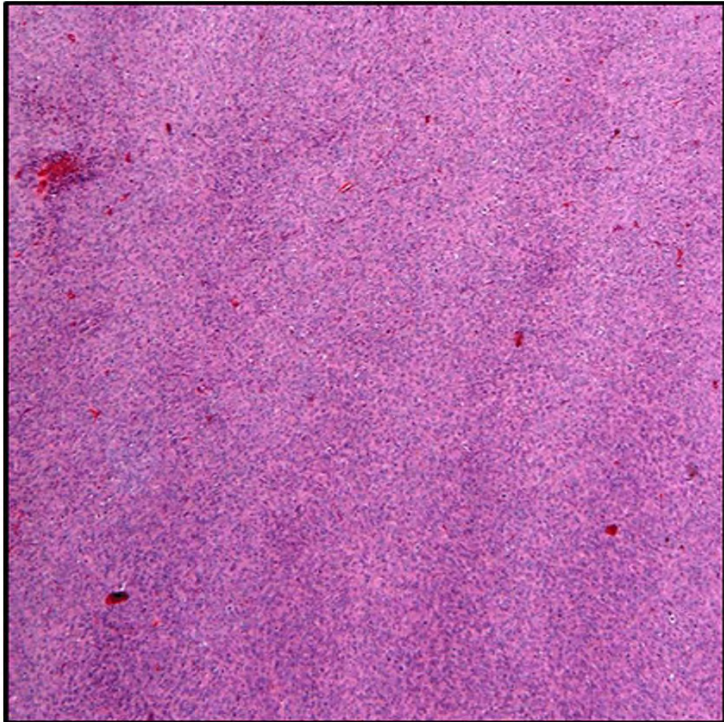
Table 2 Fractionated radiotherapy

Growth delay		Combination treatment		Synergy >1	
Fold-change	CYT-6091	Radiation	Expected	Observed	Ratio
2	0	4	4	17	4.25

Control



Radiation



Combination

