SUPPORTING INFORMATION

*In vivo* bioorthogonal chemistry enables local hydrogel and systemic pro-drug to treat soft tissue sarcoma.

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Materials and Methods

Doxorubicin was purchased from LC Laboratories (Woburn, MA), cat.# D-4000. All other chemicals were purchased from Krackeler Scientific and used without further purification. Chromatographic purifications were conducted using SiliaSphere™ spherical silica gel 5µm, 60 Å silica gel (Silicycle). Thin layer chromatography (TLC) was performed on SiliaPlate™ silica gel TLC plates (250 µm thickness) purchased from Silicycle. Preparative TLC was performed on SiliaPlate™ silica gel TLC plates (1000 µm thickness). Analytical HPLC was performed using Phenomenex Kinetex 2.6u XB-C18 100 Å analytical column (50 x 2.1 mm). COSTAR® Spin-X spin columns (0.22 µm Cellulose Acetate), purchased from Fisher Scientific (cat# 07-200-385), were used for kinetic experiment. ¹H and ¹³C NMR spectroscopy was performed on a Bruker NMR at 400 (¹H), 100 (¹³C) MHz. All ¹³C NMR spectra were proton decoupled. Fluorescence microscopy experiments were carried out using Zeiss LSM 710 Pascal laser confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Image acquisition and analyses were performed using Zeiss ZEN 2012 Confocal Microscopy Software (Release 2.02). The MTT reagent was purchased from Sigma-Aldrich, cat.# M5655. Ultrapure (UP) MVG sodium alginate, a medium-viscosity (>200 mPa s) sodium alginate where a minimum of 60% of the monomer units are guluronate, was purchased from ProNova BioPharma ASA (Lysaker, Norway). All in-vivo fluorophore imaging was done with an Ivis Spectrum from Perkin Elmer (Waltham, MA).

Cell culture. HT1080 cells were purchased from ATCC (cat.# CCL-121), and propagated in Dulbecco’s modified Eagle’s medium (DMEM; Meditech, Inc. Corning, Manassas, VA) containing 5% fetal bovine serum (FBS; HyClone, Logan Utah), supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Tech Corp., Grand Island, NY) at 37°C in a 5% CO₂ incubator.

Synthesis of Alginate hydrogel modified with tetrazine. Each gram of UP MVG alginate was combined with 176 µmoles of (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine (Tz-Me–amine) under standard carbodiimide chemistry conditions as previously described in Royzen, M.; Mejia Oneto, J. M. PCT/US2015/020718, WO2015139025 A1. The alginate product was purified by dialysis against deionized water containing decreasing salt concentrations for 4 days, frozen and lyophilized for 5–10 days until dry. A 2.5% alginate solution was obtained by adding ddH2O. Covalent modification of alginate was confirmed through ¹H-NMR and IR studies (see Figure S3 and S4). The same protocol without the tetrazine addition was used for the construction of control alginate gels.
**Synthesis of Doxorubicin pro-drug.** Doxorubicin was conjugated with *trans*-cyclooctene as described by: Versteegen, R. M. *et. al.*, Angew. Chem. Int. Ed. 2013, 52, 14112-14116. The spectra from $^1$H NMR (CDCl$_3$) and high resolution mass spectrometry matched the published data (Figure S5 and S6).

**Cytotoxicity Assay.** The colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), assay was used to evaluate the cytotoxicities of doxorubicin and doxorubicin pro-drug. **Day one:** using 96-well format, plated ~100 cells/well in 100 µL DMEM and incubated for 24 h. **Day two:** DMEM was removed and the cells were treated with variable concentrations of doxorubicin and doxorubicin pro-drug in 100 µL DMEM for 48 h. **Day four:** the medium was replaced with 100 µL of fresh DMEM and the cells were incubated for 48 h. **Day six:** DMEM was removed and the cells were incubated with 100 µL of MTT solution (0.6 mg/mL in DMEM) per well for 4 h at 37 °C. The MTT solution was then replaced with 100 µL of DMSO containing 4% aqueous ammonia per well to dissolve the purple formazan crystals. After 30 min, the absorbance of each well at 550 nm was recorded using BioTek Synergy HT multi detection microplate reader. Results were averaged from three independent arrays of triplicate experiments.

**In vitro DOX release from alginate.** A 2.5% w/w solution of alginate hydrogel (200 µL) was placed inside of a spin column and treated with 30 µL doxorubicin pro-drug (14 nmol). After 30 min, the supernatant was collected after a quick centrifugation at 6,000 RPM and the hydrogel was resuspended in PBS (30 µL). The supernatants were collected after 4 hours, 24 hours, and once daily for 7 more days, while the hydrogel was resuspended in fresh PBS (30 µL). The supernatant fractions were analyzed by HPLC.

**Control cohorts for Inhibition of HT1080 Xenograft Growth.** The exact same protocol as described in the paper was used, except that mice were separated into three additional cohorts: (i) an unmodified alginate gel was placed near the tumor and the mice were treated with 7 µmoles/kg of doxorubicin pro-drug every other day for 4 doses, (ii) HMT gel was placed near the tumor side and the cohort was treated only with vehicle, (iii) after tumor implantation the mice were not given any further interventions to monitor the natural progression of the disease. No statistical significant differences were detected between any of these cohorts (Figure S2).

**Animal Studies.** All studies involving animals were approved by the appropriate Institutional Animal Care and Use Committee before initiation. All animals used in efficacy studies were allowed to acclimate for at least 1 wk in the animal facilities before experimentation. Animals were exposed to a 12-h light/dark cycle and received food and water ad libitum through the studies.

**Inhibition of HT1080 Xenograft Growth.** Tumors were created by injecting 2.5 x 10$^5$ HT1080 cells (American Type Culture Collection) combined with Matrigel (BD Biosciences) to a total volume of 100 µL (50 µL PBS and 50 µL Matrigel) into the flank region of 5-7-wk-old NCR:nu/nu male mice (Charles River Laboratories). Eighteen days following tumor inoculation, mice were separated into two cohorts with the same median tumor size and a similar tumor size distribution (Fig. 3B). 100 µg of 2.5% w/w HMT was injection by palpation next to the tumor site, being careful of not perforating the capsule. The intravenous therapy consisted of either
maximum tolerable dose of doxorubicin (3 doses of 14 µmoles/kg every 4 days) or doxorubicin pro-drug (14 µmoles/kg daily for 10 days). Throughout the study, tumor area was measured twice per week with digital calipers. The mice were euthanized when tumors reached 2000 mm$^3$ endpoint as prescribed by our institutional animal protocol. Mice that died or had to be euthanized before completion of the experiment were excluded from subsequent time points in the analysis.

**Reticulocyte measurements.** Two mice per condition were anesthetized with isoflurane anesthesia 3 days after the last treatment during the nadir of reticulocyte decrease. Blood was collected by cardiac puncture in a syringe containing lithium heparin to prevent coagulation. RBC and reticulocyte concentrations were determined the same day using a DEXX ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Inc.).

**Statistical Analyses.** Data are expressed as means ± SEM, unless otherwise noted. Unpaired t tests were used to make comparisons of continuous values between groups. Unadjusted P values are reported for pairwise comparisons when an overall difference was detected.
Figure S1. Dose-response curve of HT1080 cells treated with different concentrations of doxorubicin (A) and doxorubicin pro-drug (B) as measured by the MTT assay. Cells were treated with the drug or the pro-drug for 48 h, followed by additional 48 h in DMEM, prior to measuring their viability. The 50% growth inhibitory concentration (IC$_{50}$) values determined from these data are doxorubicin: 0.018 µM; doxorubicin: 1.02 µM.

Figure S2. Negative controls for therapeutic effect of doxorubicin pro-drug in a xenograft model of soft tissue sarcoma. NCR/nu:nu mice were injected with human HT-1080 fibrosarcoma cells at day 0. Tumors were then injected with HMT and started on intravenous doses of either doxorubicin pro-drug or a maximum tolerable dose of doxorubicin. Tumor sizes were monitored for more than 16 weeks (n=5-10). The control groups included (i) no intervention, (ii) regular alginate implantation and treatment with doxorubicin pro-drug, (iii)
HMT implantation with vehicle treatment. No significant difference was noted between any of the control groups.

**Figure S3.** Fluorescently labeled TCO compounds that were used to study stability of HMT and *in vivo* properties of the ‘catch and release’ system.
Figure S4. Functional assay to determine stability of HMT in PBS over 14 days in 37 °C. A. Chemical reaction and protocol. B. Comparison of reacted TCO-NR-F in alginate control vs HMT at different time points.

HMT and unmodified alginate 2% (w/w) gels were challenged with non releasable fluorescently labeled TCO to determine the functional amount of tetrazines that remain active after incubation in PBS at 37 °C for different time periods (0, 2, 3, 14 days). In short, the hydrogels were prepared as outlined above for in-vitro analysis. Disks of hydrogel (50 mg) were placed in well plates with 1 mL of PBS. Then the plates were maintained at a 37 °C incubator until the period was over. Then, the hydrogels were challenged with 50 nmoles of a solution of TCO-NR-F for 90 min in a shaker. The resulting supernatant (approximately 1 mL) was transferred to another well plate leaving the hydrogel behind. The radiance of the supernatant in each well plate was measured via an IVIS Spectrum. The data are average ± SEM, n=3. P values were determined by unpaired t-test. These data suggests that more than 70% of the tetrazine moieties remain stable and reactive after 14 days.
Figure S5. Functional assay to determine stability of HMT in cell lysate. Activity of HMT treated with cell lysate for 3, 6, and 10 days relative to the untreated HMT (day 0).

HMT and was challenged with non-releasable fluoresceinlabeled TCO to determine the functional amount of tetrazines that remain active after incubation in cell lysate at 37 ºC for different time periods (3, 6, 10 days). In short, the hydrogels were prepared as outlined above for in-vitro analysis. Disks of hydrogel (50 mg) were placed in spin columns containing 200 µL of cell lysate (MDA-MB-231 cells). The spin columns were maintained at a 37 ºC incubator until the period was over. Then, the cell lysate was removed by centrifugation and HMT was washed with H2O (3x200 µL). HMT was challenged with 50 nmoles of an aqueous solution of TCO-NR-Fl for 90 min in a shaker. The resulting supernatant was collected after centrifugation and analyzed by HPLC. The data (n=1 per time point) suggest that 73% of the tetrazine moieties remain stable and reactive even after 10 days of incubation in cell lysate.
Figure S6. NMR spectrum of the hydrogel modified tetrazine (HMT). The peaks at 8.4 and 7.6 ppm correspond to the aryl protons of the tetrazine group, while the broad multiplet 4.3-3.6 ppm corresponds to the polyalginate protons.
Figure S7. IR spectrum of the hydrogel modified tetrazine (HMT).
Figure S8. $^1$H NMR spectrum of the doxorubicin pro-drug. The spectrum matches the one previously reported by Robillard and co-workers. [Versteegen, R. M. et. al., Angew. Chem. Int. Ed. 2013, 52, 14112-14116]
Figure S9. High resolution ESI-MS spectrum of the doxorubicin pro-drug. The major observed peak corresponds to the expected value for the sodium adduct of the doxorubicin pro-drug.
Figure S10. Synthesis of TCO-NR-F1

Dissolved 2-((Z)-cyclooct-2-enyloxy)-N-(2-aminoethyl)acetamide (50.0 mg, 0.221 mmol) and fluorescein-NHS ester (105 mg, 0.221 mmol) in DMF (5 mL). Added triethylamine (60 µL, 0.442 mmol) and stirred at rt for 18 h. Evaporated the solvent under high vacuum and redissolved the reaction mixture in methanol. Purified by preparatory thin layer chromatography using 1:9 MeOH:CH$_2$Cl$_2$ mixture as mobile phase.

Yield = 51 mg (39.5%).

$^1$H NMR (CD$_3$OD, 400 MHz) δ 8.46 (s, 1H), 8.21 (d, $J = 8.2$ Hz, 1H), 7.65 (bs, 1H), 7.28 (d, $J = 8.2$ Hz, 1H), 6.69 (d, $J = 2.7$ Hz, 2H), 6.58-6.50 (m, 4H), 5.46-5.44 (m, 2H), 3.91 (d, $J = 5.5$ Hz, 2H), 3.65-3.59 (m, 5H), 2.67 (s, 1H), 2.32-2.26 (m, 2H), 2.32-2.26 (m, 2H), 2.20-2.13 (m, 1H), 2.08 (d, $J = 4.2$ Hz, 1H), 1.96-1.92 (m, 1H), 1.79-1.67 (m, 3H), 1.54-1.46 (m, 1), 1.30-1.25 (m, 1H), 1.23-1.15 (m, 1H).

$^{13}$C NMR (CD$_3$OD, 100 MHz) δ 173.60, 170.67, 168.69, 154.21, 137.21, 137.66, 136.66, 136.75, 132.53, 130.30, 125.89, 125.09, 113.84, 110.98, 103.79, 77.56, 69.38, 41.31, 40.75, 40.07, 39.94, 35.50, 33.58, 30.82, 29.05.

HRMS (ESI-MS) $m/z$: calcd. for C$_{33}$H$_{32}$N$_2$O$_8$ [M+ H$^+$] 585.2237; found 585.2183.
The Rhodamine –NHS ester was synthesized as described by:
Brunet, A.; Aslam, T.; Bradley, M. Bioorg. Med. Chem. Lett. 2014, 24, 3186-3188.

Dissolved rhodamine-NHS ester (50 mg, 0.095 mmol) and (E)-cyclooct-2-enyl-2-
aminoethylcarbamate (40.0 mg, 0.190 mmol) in CH$_2$Cl$_2$ (5 mL). Added triethylamine (129 µL, 0.95 mmol) and stirred at rt for 18 h. Evaporated the solvent under high vacuum and redissolved the reaction mixture in methanol. Purified by preparatory thin layer chromatography using 7.5:2.5:90 MeOH:Et$_3$N:CH$_2$Cl$_2$ mixture as mobile phase. Yield = 28 mg (47 %)

$^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.54 (s, 1H), 8.04 (d, $J$ = 8.2 Hz, 1H), 7.34 (d, $J$ = 8.2 Hz, 1H), 7.23 (d, $J$ = 9.6 Hz, 1H), 6.99 (dd, $J_1$ = 2.7 Hz, $J_2$ = 9.5 Hz, 1H), 6.89 (d, $J$ = 2.8 Hz, 1H), 5.85 (t, $J$ = 13.7 Hz, 1H), 5.55 (d, $J$ = 16.4 Hz, 1H), 5.26 (s, 1H), 3.67-3.36 (m, 4H), 3.32-3.21 (m, 9H), 2.93-2.77 (m, 6H), 2.49-2.36 (m, 1H), 2.10-1.79 (m, 5H), 1.78-1.41 (m, 4H), 1.39-1.25 (m, 1H), 1.22-1.06 (m, 10H), 0.93-0.79 (m, 1H).

$^{13}$C NMR (CD$_3$OD, 100 MHz) $\delta$ 172.47, 169.41, 161.68, 159.08, 158.76, 142.06, 137.19, 132.96, 132.70, 130.90, 129.85, 129.63, 115.09, 114.86, 97.53, 76.85, 75.38, 41.96, 41.78, 41.01, 37.18, 36.92, 30.21, 25.35.

HRMS (ESI) $m/z$: calcd. for C$_{36}$H$_{41}$N$_4$O$_6$ [M+1]$^+$ 625.3026; found 625.2976.
Figure S12. NMR spectra of TCO-NR-F1.
Figure S13. NMR spectra of TCO-R-Rh.