Serial Monitoring of Human Systemic and Xenograft Models of Leukemia Using a Novel Vascular Disrupting Agent

Miriam Benezra[1], PhD; Evan Phillips[1], BSc; Derya Tilki[2], MD; Bi-Sen Ding[2], Ph.D.; Jason Butler[2], PhD; Konstantin Dobrenkov[1], MD; Bronwyn Siim[3], PhD; David Chaplin[3], PhD; Shahin Rafii[2], MD; Sina Rabbany[2,4*], PhD; and Michelle S. Bradbury[1*], MD, PhD.

[1]Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, NY, 10065;

[2]Howard Hughes Medical Institute, Ansary Stem Cell Institute and Department of Genetic Medicine, Weill Cornell Medical College, New York, New York 10065

[3]OXiGENE, Inc., South San Francisco, CA 94080

[4]Bioengineering Program, Hofstra University, Hempstead NY 11549

Supplementary Materials and Methods

Cell culture

Human promyelocytic leukemia (HL-60) cells were kindly provided by the lab of S. Rafii (Weill-Cornell Medical College, New York, NY). HL-60 cells were maintained in culture in RPMI supplemented with 10% fetal bovine serum (FBS, Core Media Preparation Facility, Memorial Sloan-Kettering Cancer Center, New York, NY) at 37°C until use.

Vector production and viral transduction

Lentiviruses were produced by transient transfection of 293T cells with vectors expressing either *luc+* or green fluorescence protein (*GFP+*) reporter genes, under the control of the constitutive phosphoglycerate kinase (PGK) promoter, as previously described[3]. HL-60 cells (10^5 cells/well, 24 well plate), seeded in 0.5 mL RPMI medium and supplemented with 10% FBS, were double-transduced *in vitro* (MOI=1) and polybrene (4 µg/mL, Sigma, St. Louis, MO). Twenty-four hours later, 0.5 ml of medium supplemented with 10 % FBS was added, and *GFP+* expression was evaluated in live transduced cells using a Zeiss Axiovert 200M inverted microscope and excitation (480/25) and emission (525/40) filters (Zeiss, Thornwood, NY). Additional cell viability measurements were performed over a 96 hour interval for double transduced and non-transduced HL-60 cells using a Vi-Cell automated cell viability analyzer/imager (Beckman Coulter, Inc., Fullerton, CA). Each experiment was performed in triplicate.

In vitro optical assays

Serial dilutions of *luc+*/*GFP+* HL-60 cells, as well as *luc-/GFP-* precursors, were seeded in 24-well tissue culture plates (1.0×10^4 – 5.0×10^5 cells/well) in triplicate for assessment of *GFP* and *luc+* expression using flow cytometry (FACSCalibur, Becton Dickinson, Mountain View,

CA) and BLI (IVIS 200 Optical Imaging System, Xenogen, Alameda, CA), respectively. For BLI studies, firefly D-luciferin potassium salt (150 μg/mL, Caliper, Hopkinton, MA) was added to wells just prior to assay and plates were placed in a light-tight specimen chamber mounted with the CCD camera, and having a field of view set at 20 cm above the sample shelf. Gray scale photographic images and bioluminescence color images were superimposed using the Living Image™ V.2.11 software overlay (Xenogen). For treatment studies, *luc*+/GFP+ HL-60 cells (10^5 cells/well) were incubated in triplicate with OXi4503 (CA1, 5-500 nM) for 72-hrs prior to assessing *luc*+ expression and cell viability using a Vi-Cell series viability analyzer.

Imaging analyses were performed using FlowJo software (Tree Star, Ashland, OR) to assess *GFP* expression and IGOR imaging software (V.4.02A, WaveMetrics, Lake Oswego, OR) to evaluate *luc* expression. ROIs were manually drawn over each well to determine the number of *GFP+* events or mean total flux (p/s ± SD) as a function of cell number. These were drawn so as to yield a standard deviation (SD) of the maximum signal of no greater than 20%; this corresponded to a region over the uniform central portion of the signal focus. No calibration or background subtraction procedures were used to determine maximum photon flux estimates. Correlation of measured flux values with cell number and *GFP* expression levels was performed using linear regression analysis.

Tissue processing

Mice were euthanized with 5% CO_2 prior to excising the major organs and tissues (liver, spleen, sternum, lung, spine, femur, and brain). Excised tissues were fixed in 10% formalin or 4% paraformaldehyde (PFA) overnight. For immunofluorescence (IF), tissues were permeabilized for 10-min with 0.5% Triton X-100, and washed with PBS prior to paraffin embedding. Bone specimens were additionally decalcified (1 L, 2 days) in Decalcifier I

(Surgipath, Richmond, IL) prior to embedding. For immunohistochemistry (IHC), formalin-fixed tissues were transferred to 70% ethanol (48 h), embedded in paraffin, serially sectioned (5 μm sections), and slide-mounted. Tissues were deparaffinized in xylene (25°C, 3 h) and passed through graded alcohols (25°C, 4.5 h) prior to H&E (hematoxylin and eosin) reagent and GFP antibody staining.

Tissue immunofluorescence and immunohistochemistry

For IF, tissue specimens (10–30 mm) were blocked with 10% donkey serum/0.1% Triton X-100 and incubated in primary antibody: anti-VEGFR3 mAb (mF4-31C1, 10 μg/ml, ImClone Systems, New York, NY) or anti-VE-cadherin pAb (2 μg/ml). After incubation (1 μg/ml) in fluorophore-conjugated secondary antibodies (Jackson IR, West Grove, PA), sections were counterstained with TO-PRO-3 (Molecular Probes, Carlsbad, CA) or Hoechst. For detection of VE-cadherin, CD31, and VEGFR3, paraffin sections were antigen-retrieved using Target Retrieval Solution (DAKO, Carpinteria, CA). For IHC, tissue sections were incubated in 0.1% hydrogen peroxide in PBS (15 min), washed with PBS, microwaved in 10 mM citrate buffer (15 min) to unmask antigenic epitopes, cooled, and washed with distilled water. Sections were subsequently blocked with 10% normal goat serum (MP Biomedicals, Solon, OH) in 2% BSA-PBS diluent (30 min, humid chamber), and additionally incubated with Avidin and Biotin blocking reagent. Polyclonal rabbit anti-GFP (1:100, Millipore, Billerica, MA) in 2% BSA-PBS was applied to slides, followed by multiple washings and application of biotinylated anti-rabbit IgG secondary antibody (1:1000, Vector Labs, Burlingame, CA). After rinsing, Avidin-Biotin Complex Elite (Vector Labs) was applied for detection, followed by 3,3-diaminobenzidine (Sigma). H&E staining was performed on sequential tissue sections per standard protocols. In addition, TUNEL assays were performed on paraffin sections per routine protocols[32].

Image acquisition and quantitative analysis of tissue *GFP* expression

Immunofluorescence images were captured on an AxioVert LSM 510 Meta confocal microscope (Zeiss). For quantitative analysis of *GFP* immunohistochemistry, sections were scanned using MIRAX SCAN (Zeiss) for high-resolution viewing and automated cell counting. MetaMorph Offline software (Molecular Devices, Downingtown, PA) was used to perform leukemic cell counts on *GFP*-stained sections. Three non-consecutive tissue sections from the liver, spleen, lung, spine, and femur were analyzed at high power magnification (i.e., 40x) using five randomly selected high power fields; in the femur and spine, marrow parenchyma was examined. Two measurements were made per field based on user-defined color thresholds: the number of pixels for *GFP*-stained cells and the sum of the pixels for *GFP* (brown) and hematoxylin counter-stained (blue) cells. The ratio of these numbers, averaged over all high power fields and all sections, yielded quantitative measures of the %*GFP+* cells in all tissues for untreated (n=4) and treated (n=4) mice. Manual cell counting of tissues was performed in those cases where excess background staining was observed (i.e., spine, femur, spleen), and the *%GFP+* cells quantified.

Statistical methods

For all *in vitro* and *in vivo* studies, correlation analyses of total flux values and %*GFP+* cells were performed using a least squares regression algorithm. Pair-wise comparisons (Holm-Sidak method) of non-transduced HL-60 xenograft mice receiving fixed doses of OXi4503, CA4P, Ara-C and saline or a range of OXi4503 doses (i.e., 2.5–75 mg/kg) were evaluated using Kaplan-Meier survival analysis, with $p<0.05$ considered statistically significant. This analysis approach was additionally used to evaluate overall survival between cohorts of mice systemically inoculated with *luc+/GFP+* or non-transduced HL-60 cells. For HL-60 xenografts, differences in

tumor volumes among treated and untreated cohorts were tested for statistical significance 1 week after each administration of the drug using the one-tailed Mann-Whitney U test ($p<0.05$ considered statistically significant). Total BLI flux and tissue-specific *GFP+* expression changes in treated *vs* untreated *luc+*/*GFP+* systemic models were additionally evaluated using the one-tailed Mann-Whitney U test 13 days post-inoculation.

Supplementary Table 1

Time-dependent BLI Signal Changes in Untreated and Treated Tissue Specimens

Tissue/Organ	Treatment Interval (days)	ΔS, UT	ΔS, T
Femur	7-10	0.13	6.67
	10-13	0.67	0.77
	7-13	0.09	5.26
Spine	7-10	0.09	2.94
	10-13	0.38	0.67
	7-13	0.04	2.0
Liver	7-10	0.12	0.26
	10-13	0.17	0.48
	7-13	0.02	0.12
Lung	7-10	0.04	5.26
	10-13	0.37	0.83
	7-13	0.01	4.35

Supplementary Figure legends

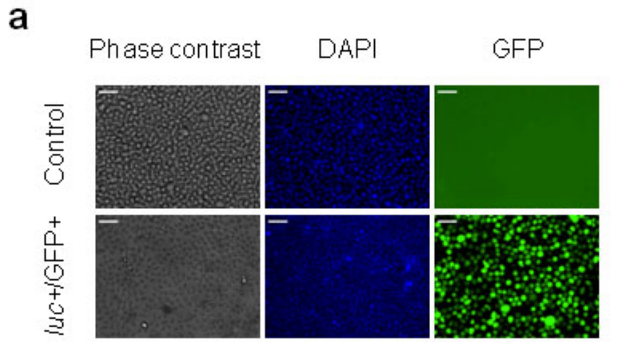
Supplementary Figure 1. *In vitro* characterization of *GFP*+ and *luc*+ expressing HL-60 cells. (a) Phase contrast (left) and native *GFP* expression (right) in doubly transduced HL-60 cells with DAPI counterstaining (center) were observed on an Axiovert 200M inverted microscope with excitation (480/25) and emission (525/40) filters (Zeiss). Room temperature. Scale bar = 50 µm. (b) *In vitro* BLI images of *luc*- (I) and *luc*+ (II) cells. Correlation between BLI photon counts (flux, p/s) and pre-determined numbers of *luc*+ HL-60 cells (R=0.993, n=3). Inset shows the correlation between *luc*+ and *GFP*+ cells (R=0.93, n=3, cell range 6.2×10^4 - 5×10^5); SD < 2% of mean signal. (c) Flow cytometry for *GFP* expression in *luc*+/*GFP*+ transduced HL-60 cells (bottom panel) against a non-transduced population of HL-60 cells (top panel). Each point represents the mean ± s.d. of three replicates. (d) Viability and growth (%) of HL-60 cells over 96 h for *luc+/GFP+* and non-transduced cell populations.

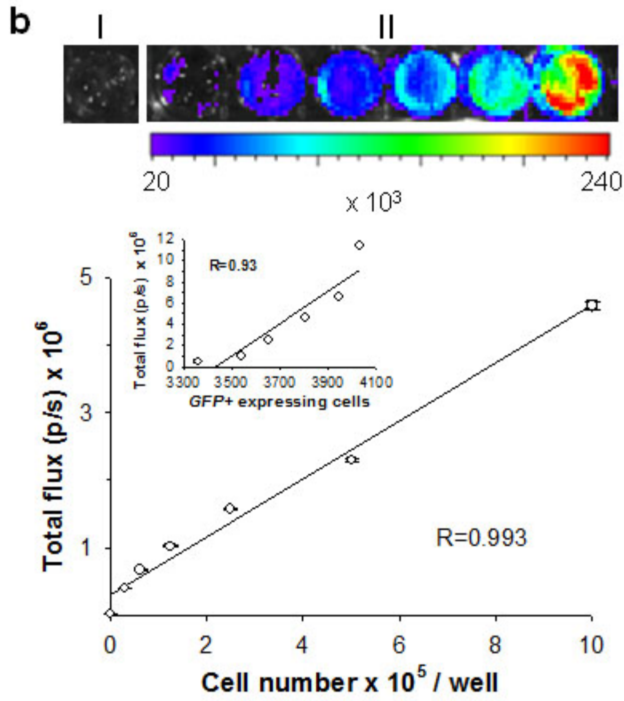
Supplementary Figure 2. OXi4503 mechanism of action and HL-60 viability as a function of VDA concentration. (a) Schematic illustrating the mechanism of action of OXi4503 following *in vivo* activation. (b) BLI flux and *luc*+/GFP+ HL-60 cell number for serial dilutions of CA1 in media supplemented with 10% serum. Inset shows BLI images of wells with serial dilutions of CA1 (0 – 500 nM). Color scale represents total flux values (p/s).

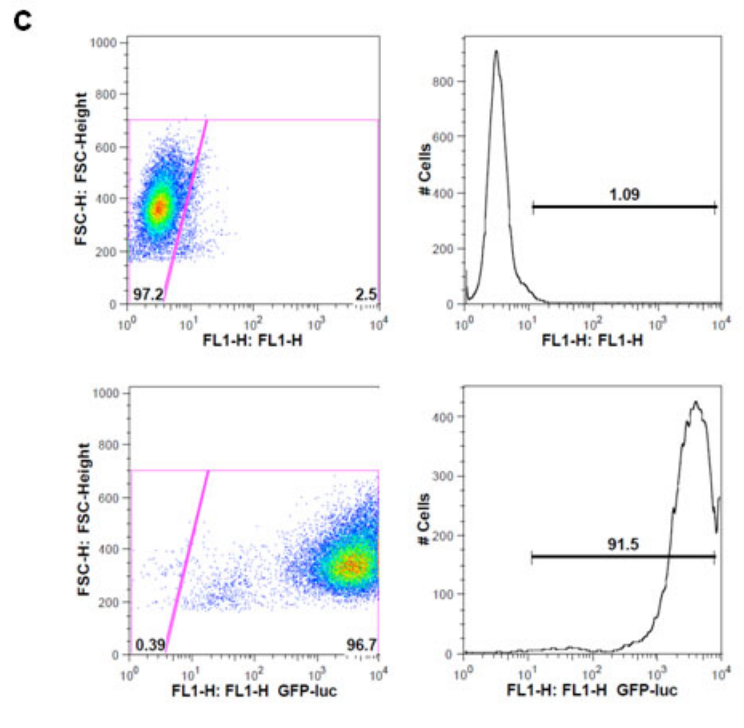
Supplementary Figure 3. Overall survival studies in a systemic model. Time-dependent changes in survival following administration of double-dose OXi4503 (25 mg/kg) to mice systemically inoculated with *luc+/GFP+* HL-60 cells or non-transduced control HL-60 cells.

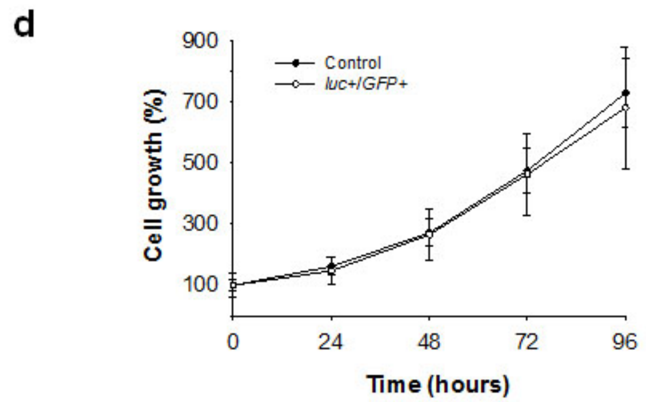
Supplementary Figure 4. Correlative *GFP*+ immunohistochemistry and H&E tissue staining. (a, b) Representative low power images of *GFP+* expressing leukemic cells (brown) from untreated (a) and treated (b) liver, lung, spine, femur, and splenic tissues. Normal cells

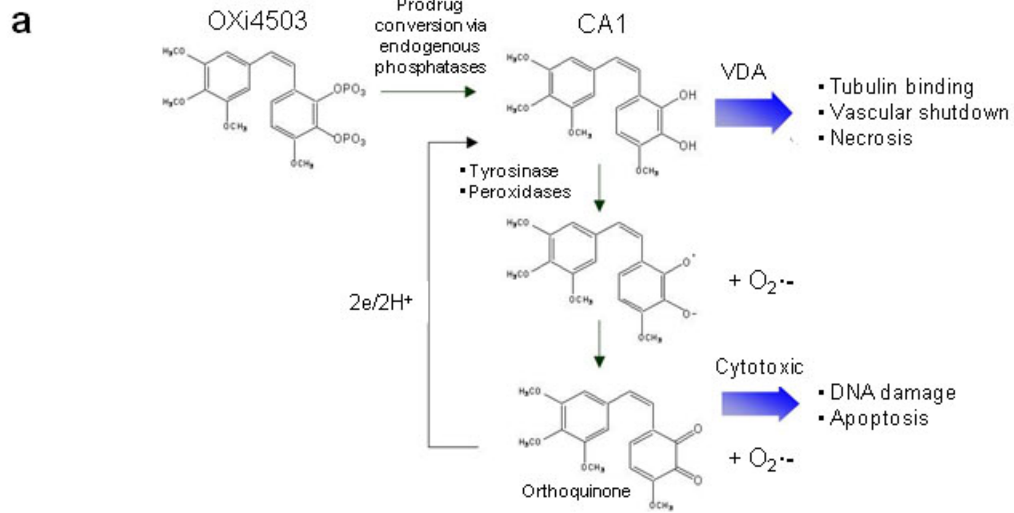
(blue) are hematoxylin-stained. (c, d) Representative sequential H&E-stained sections from untreated (c) and treated (d) mice. Scale bar = 50 μm.

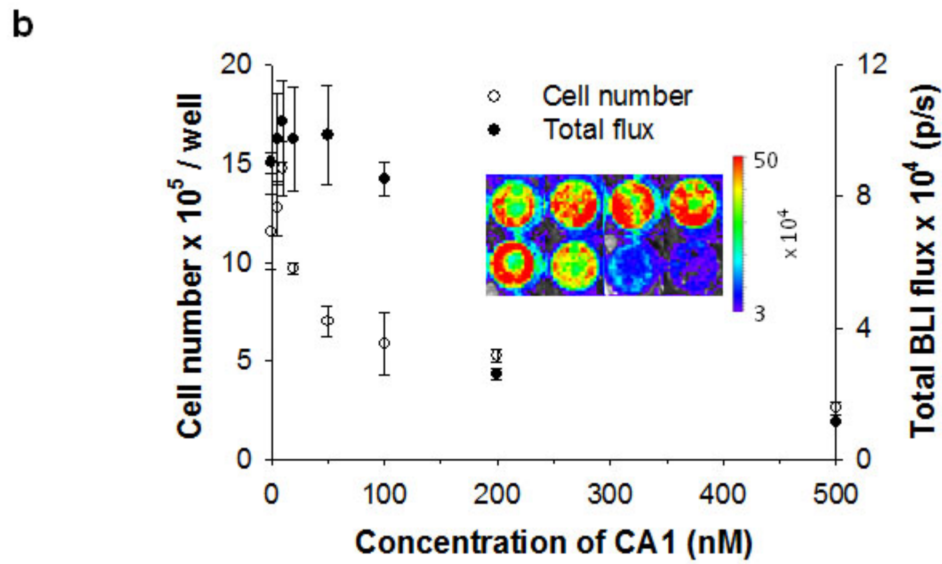

a
Phase contrast
DAPI
GFP
Control
luc+/GFP+

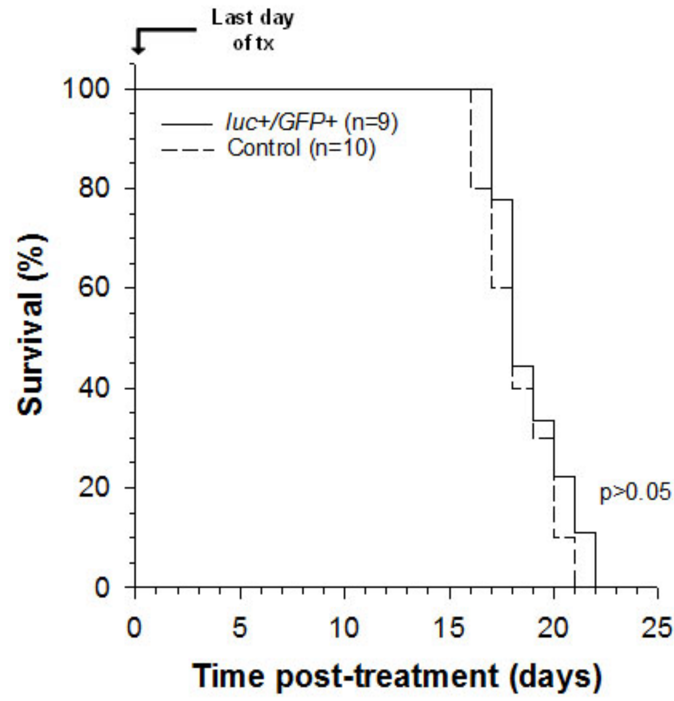

b
I
II
20
240
x 10³
Total flux (p/s) x 10⁶
Cell number x 10⁵ / well
R=0.993
R=0.93
Total flux (p/s) x 10⁶
GFP+ expressing cells
3300 3500 3700 3900 4100

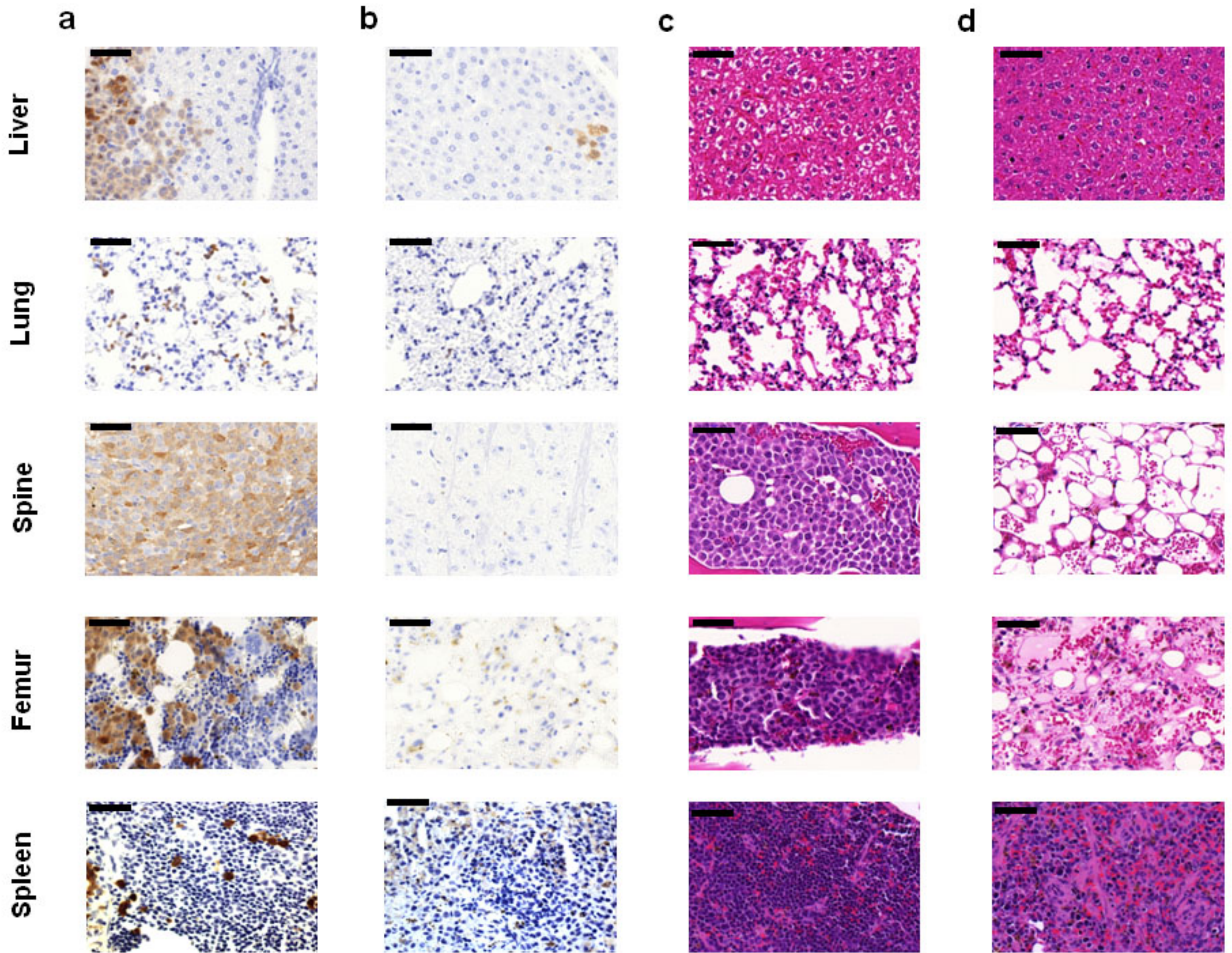

c
FSC-H: FSC-Height
FL1-H: FL1-H
97.2
2.5
Cells
1.09
FL1-H: FL1-H GFP-luc
0.39
96.7
91.5


d
Control
luc+/GFP+
Cell growth (%)
Time (hours)
0 24 48 72 96
100 300 500 700 900


a
OXi4503
Prodrug conversion via endogenous phosphatases
CA1
VDA
▪ Tubulin binding
▪ Vascular shutdown
▪ Necrosis
▪ Tyrosinase
▪ Peroxidases
+ O2•-
2e/2H+
Cytotoxic
▪ DNA damage
▪ Apoptosis
+ O2•-
Orthoquinone


b
Cell number
Total flux
Cell number x 10^5 / well
Total BLI flux x 10^4 (p/s)
Concentration of CA1 (nM)
50
3
x 10^4
0
100
200
300
400
500
20
15
10
5
12
8
4


Last day
of tx
100
80
60
40
20
0
Survival (%)
luc+/GFP+ (n=9)
Control (n=10)
p>0.05
0
5
10
15
20
25
Time post-treatment (days)


a
b
c
d
Liver
Lung
Spine
Femur
Spleen