Supplemental Material

Cells lines

PC-3 (wild-type) and PC-3/PSMA+ cells were maintained in F-12K medium supplemented with 2 mM of L-glutamine and 10% fetal bovine serum (FBS) or 0.2% human albumin. PC-3/PSMA+ cells were supplemented also with 6 μg/mL of puromycin. LNCaP cells were maintained in RPMI 1640 medium supplemented with HEPES and 10% FBS. All cell lines were maintained under standard conditions in a water/saturated atmosphere of 5%CO_2.

In vitro therapy studies

Cell permeabilization was achieved by using radiotherapy, chemotherapy (etoposide) and anti-androgen hormonal therapy (flutamide) regimens. Approximately 1×10^6 cells were cultured in 100-mm culture dishes (Corning, Inc. NY) for flow cytometry experiments (Fig. 1 and Suppl. Fig. 1). 1×10^5 cells were seeded in 4-well chambers (Lab-Tek, Naperville, IL) for microscopy experiments (Fig. 2 and Suppl. Fig. 2). 24 h after seeding, cells were cultured in the corresponding medium containing 0.2% human albumin instead of 10% Fetal Bovine Serum (FBS). 48 h after seeding, PC-3/PSMA+ cells received etoposide (150 or 300 μM, dissolved in 10 μL DMSO, SigmaAldrich, St. Louis, MO). Control cells were treated with the vehicle only (DMSO). LNCaP cells were exposed to 20 Gy by using an XRAD 320 (Precision X-Ray, Inc.) at a dose rate of 117.5 cGy/min. Cells not exposed to radiation therapy were used as a control. In separate experiments, LNCaP cells were treated with flutamide (10, 50 and 100 μM, dissolved in 15 μL ethanol; Sigma Aldrich, St. Louis, MO). Cells exposed to the vehicle only were used as control. Additional experiments with LNCaP cells exposed to 0.2% sodium azide (Sigma Aldrich, St. Louis, MO) were performed. Each experiment was conducted in triplicate. At 24, 48, 72, 96 and 120 h time points post-treatment floating dying/dead cells were collected from the culture medium and adherent cells harvested by trypsinization. The two fractions were mixed to reconstitute the total population and prepared for FC or microscopy.

Flow cytometry (FC)

Approximately 2.5×10^5 cells were stained for flow cytometry evaluation by using 0.06 μg of the mAb J591 conjugated to allophycocyanin (APC) (Invitrogen, Carlsbad, CA, USA) and 1.8 μg of the mAb 7E11 conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Cells were incubated with both antibodies in 5% Fetal Calf Serum (FCS) for 30 min. at room

temperature. Cells were washed twice with 5% FCS and 5 μL of 7-amino-actinomycin-D (7AAD; BD Bioscience San Jose, CA) was added to assess permeabilization of dying cells. FC analysis was performed after 15 min. incubation by using a FACS Calibur flow cytometer, (Becton Dickson, San Jose, CA). Single (7AAD) and double staining (7AAD and 7E11) were also performed as control.

Fluorescence microscopy

7E11 was applied at the concentration 5 μg/mL in phosphate buffered saline (PBS) on treated cells for 45 min at room temperature. After washing with PBS, cells were fixed with 10% buffered formaldehyde for 10 min. The first immunofluorescent reaction was completed by applying donkey anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen Carlsbad, CA) for 1h at room temperature. After blocking with 5% Normal Goat Serum (NGS), cleaved Caspase-3 antibody (Cell Signaling Danvers, MA) was applied overnight at 4ºC. After washing, goat anti-rabbit IgG antibody conjugated with Alexa Fluor 555 (Invitrogen, Carlsbad, CA) was applied to complete the second staining. Mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, Inc Burlingame, CA) was used for nuclei staining. Slides were mounted with a coverslip and imaged by using a Zeiss Axiovert Imaging (Carl Zeiss, Inc. Thornwood, NY, USA) or a Nikon TiE inverted microscope (Nikon Instruments Inc., Melville, NY, USA). Images were acquired by using the DAPI, TRITC and FITC filter sets. Additional Z-stacks were obtained for 3D reconstruction of multi-stained samples on cell cultures (see supplemental movie).

Chemistry

All chemicals, unless otherwise stated, were purchased from Sigma Aldrich (St. Louis, MO) and were used as received. Water (>18.2 MΩ.cm at 25 ºC, Milli-Q, Millipore, Billerica, MA) was purified by passing through a 10-cm column of chelex resin (Bio-Rad Laboratories, Hercules, CA) at a flow rate <1.0 mL/min. All instruments were calibrated and maintained in accordance with previously reported routine quality-control procedures. Radioactivity measurements were made by using a Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ) with a calibration factor of 465 for ^{89}Zr. For accurate quantification of radioactivities, experimental samples were counted for 1 min. on a calibrated Perkin Elmer (Waltham, MA) Automatic Wizard^2 Gamma Counter by using a dynamic energy window of 800–1000 keV for ^{89}Zr (909 keV emission). ^{89}Zr-radiolabeling reactions were monitored by using silica-gel impregnated glass-fiber instant thin-layer chromatography (ITLC-SG) paper (Pall Corp., East

Hills, NY) and analyzed on a radio-TLC plate reader (Bioscan System 200 Imaging Scanner coupled to a Bioscan Autochanger 1000 (Bioscan Inc., Washington, DC, using Win-Scan Radio-TLC software version 2.2). Solvent systems included diethylene triamine pentaacetic acid in water (DTPA, 50 mM, pH7) and phosphate buffered saline (PBS).

Synthesis of N-succinyldesferrioxamine B (N-succDFO). DFO mesylate (0.508 g, 0.77 mmol, Calbiochem, Spring Valley, CA) was dissolved in pyridine (7.5 mL) and reacted with excess succinic anhydride (1.704 g, 0.017 mol) at room temperature for 24 h. The white suspension was then poured into NaOH(aq.) (120 mL, 0.015 mol dm^{-3}) and stirred at room temperature for 16 h. The colorless solution was adjusted to pH2 by the addition of 12 mol dm^{-3} HCl and cooled with stirring at 4 ºC for 2 h. The white precipitate was collected by filtration, washed with copious amounts of HCl (0.01 mol dm^{-3}) then water and dried *in vacuo* to give the *N*-succinyldesferrioxamine B (*N*-succDFO) as a white microcrystalline solid (0.306 g, 4.75×10^{-4} mol, 62%). HRMS-ES^+: Calc. for $[C_{29}H_{52}N_6O_{11} + H^+] = 661.3772$; found 661.3760 ($[M + H^+] = 100\%$).

Preparation of [Fe(N-succDFO-TFP)] activated ester. *N*-succDFO (9.0 mg, 14 µmol) was suspended in 3.0 mL sterile saline and the pH adjusted to 6.5 with 0.1 M Na_2CO_3(aq.) (50 – 75 µL in chelex purified water). Then a solution of $FeCl_3 \cdot 6H_2O$ [4.0 mg, 15 µmol, 300 µL of 0.1 M HCl(aq.)] was added. Upon addition of the $FeCl_3$(aq.) the reaction mixture changed from colorless to deep orange due to the intense electronic absorption band of [Fe(DFO)] with a peak at 430 nm ($\varepsilon_{430} = 2216 \pm 49$ mol^{-1} dm^3 cm^{-1}). After stirring the reaction at room temperature for 1 h, a solution of 2,3,5,6-tetrafluorophenol (TFP, 300 µL, 36 µmol, 1.2 mol dm^{-3} in chelex purified MeCN; SigmaAldrich) was added to the reaction followed by addition of solid *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC, 120 mg, 0.63 mmol, SigmaAldrich). The reaction mixture (pH 6.5) was then stirred at room temperature for 1 h before the [Fe(*N*-succDFO-TFP)] product was purified by use of a C-18 Light Sep-pak cartridge (Waters, Milford, MA). The reaction mixture was loaded onto a pre-activated (6 mL MeCN, 10 mL H_2O) C-18 cartridge, washed with copious amounts of water (>40 mL), and eluted with 1.5 mL MeCN. The final [Fe(*N*-succDFO-TFP)] solution had a concentration of approximately 9.8 mM. The [Fe(*N*-succDFO-TFP)] solution can be stored for 24 h at 4 ºC but the most efficient conjugation reactions were achieved by using fresh preparations.

Preparation of DFO-7E11. Murine monoclonal antibody 7E11.C5.9 (2.5 mg/mL, 1.2 mL, 3.0 mg, 20 nmol, MW ~150,000 g mol^{-1}) was added to a centrifuge vial and the pH adjusted to 9.5 – 10.0 by using aliquots of 1.0 M and 0.1 M Na_2CO_3(aq.). Then 6 equivalents of [Fe(N-succDFO-TFP)] (120 nmol, 12.5 μL, 9.8 mM) were added and gently mixed using an automated pipette. The reaction was allowed to react at room temperature without agitation for 1 h. Then 2,5-dihydroxybenzoic acid [gentisic acid, 50 μL, 0.65 mol dm^{-3} in 0.32 M Na_2CO_3(aq.)] was added to the reaction and the pH was adjusted to 3.9 – 4.2 by the addition of 5 to 10 μL aliquots of 0.25 M H_2SO_4(aq.). Then a 10-fold excess of ethylenediaminetetraacetic acid disodium salt with [$EDTA^{2-}.2Na^{+}$(aq.), 0.0674 mol dm^{-3}, 1.35 μmol, 20 μL] with respect to [Fe(N-succDFO-TFP)] was added. The reaction was incubated in a water bath at 38 ºC for 1 h during which time the solution changed from clear yellow to colorless. Subsequent purification led to colorless solutions which were found to have immunoreactive fractions >0.9. The DFO-7E11 was purified by size-exclusion chromatography (Sephadex G-25 M, PD-10, >30 kDa, GE Healthcare; dead-volume = 2.5 mL, eluted with 200 μL fractions of sterile saline).

Radiolabeling of DFO-7E11. Briefly, [^{89}Zr]Zr-oxalate (138.8 MBq, [3.75 mCi]) in 1.0 M oxalic acid (200 μL) was adjusted to pH7.7–8.1 with 1.0 M Na_2CO_3(aq.). CAUTION: Acid neutralization releases CO_2(g) and care should be taken to ensure that no radioactivity escapes the microcentrifuge vial. After CO_2 evolution ceased, DFO-7E11 (200 μL, 3.68 mg/mL [0.736 mg of mAb], in 0.9% sterile saline) was added and the reaction was mixed gently. The reaction was incubated at room temperature for between 1–2 h, and complexation progress was monitored with respect to time by ITLC (DTPA, 50 mM, pH7). After 1 h, crude radiolabeling yields and RCP was >95%. ^{89}Zr-7E11 was purified by using spin-column centrifugation (4 mL volume, >30 kDa, Amicon Ultra-4, Millipore, Billerica, MA; washed with 4×3 mL, sterile saline). The radiochemical purity (RCP) of the final ^{89}Zr-7E11 (typical isolation: 3.32 mCi, 89% radiochemical yield; formulation: pH5.5–6.0; <500 μL; sterile saline) was measured by both radio-ITLC and analytical size-exclusion chromatography (<0.74 MBq [20 μCi], ca. 5–10 μL aliquots) and was found to be >99% in all preparations. In the ITLC experiment ^{89}Zr-7E11 and ^{89}Zr-DFO remain at the baseline (R_f = 0.0), whereas $^{89}Zr^{4+}$(aq.) ions and [^{89}Zr]Zr-DTPA elute with the solvent front (R_f = 1.0). Suppl. Fig. 3A shows typical radio-ITLC chromatograms of the crude and purified ^{89}Zr-7E11 and Suppl. Fig. 3B shows a typical elution profile for the purification of ^{89}Zr-7E11 by using PD-10 size-exclusion chromatography.

Immunoreactivity assays

The immunoreactive fraction of ^{89}Zr-7E11 was assessed by using specific, infinite-antigen excess cellular binding assays similar to the methods described by Lindmo *et al.*[1,2] Permeabilization of the cells was achieved by incubating PC-3/PSMA+ cells with 0.2% sodium azide dissolved in media at 37 ºC for 24 h. Cells not exposed to sodium azide were used as control. Cell staining with 7AAD and 7E11 and subsequent Flow Cytometry (FC) analysis, conducted in accordance with the procedures described in the main text, confirmed that ~90% of cells were apoptotic. PC-3/PSMA+ cells were suspended in micro-centrifuge tubes at concentrations of 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, and 0.5 × 10^6 cells/mL in 500 µL PBS (pH7.4). Aliquots (50 µL, <0.37 kBq, [<0.01 mCi]) of ^{89}Zr-7E11 in 1% bovine serum albumin (BSA) were added to each tube (*n*=3; final volume: 550 µL) and the samples incubated on an orbital mixer for 60 min. at room temperature. Cells were then pelleted by centrifugation (600G for 2 min.), resuspended and washed twice with ice-cold PBS before removing the supernatant and counting the ^{89}Zr-radioactivity associated with the cell pellet. The count data were background corrected and compared with the total number of counts in control samples. Immunoreactive fractions were determined by linear regression analysis of a plot of (total/bound) activity versus (1/[normalized number of cells]), and calculated as 1/y-intercept. (Suppl. Fig. 4).

Biodistribution studies

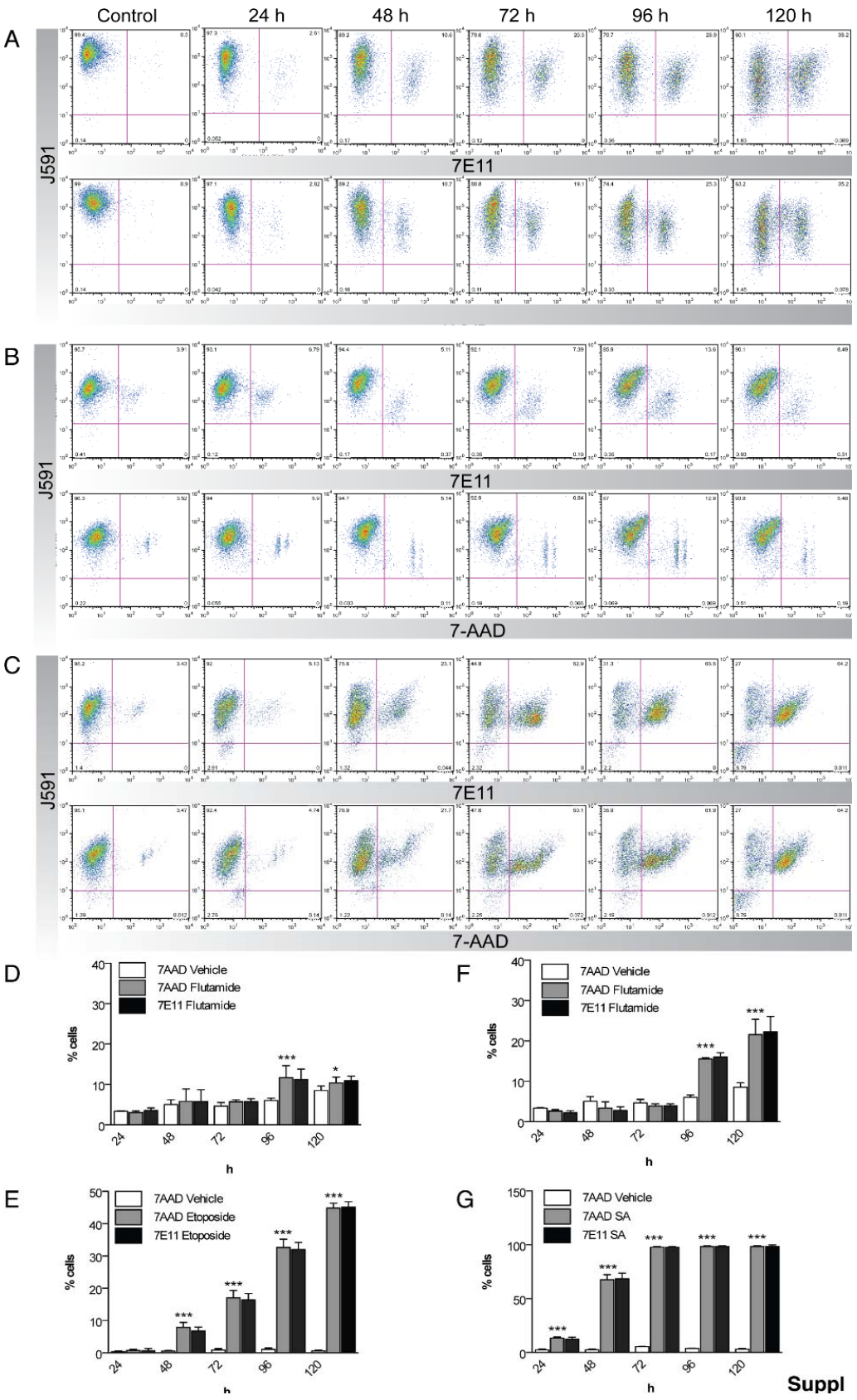
In vivo biodistribution studies were conducted (*n* = 5 per group) to evaluate the uptake of ^{89}Zr-7E11 in mice bearing LNCaP xenograft (50–200 mm^3). Mice were randomized before the study and received ^{89}Zr-7E11 (0.55–0.74 MBq, [15–20 µCi], 3–4 µg of mAb, in 200 µL sterile saline) *via* retro-orbital injection. The mass of ^{89}Zr-7E11 formulation injected into each animal was measured and used to determine the total number of counts (counts per minute, [c.p.m.]) by comparison to a standard syringe of known activity and mass. Count data were background and decay-corrected and the percentage injected dose per gram (%ID/g) for each tissue sample was calculated by normalization to the total amount of activity injected in each mouse.

Small-animal immuno-PET imaging

PET imaging experiments were conducted on a microPET Focus 120 scanner (Concorde Microsystems). Mice (n = 5) were administered ^{89}Zr-7E11 formulations (8.8–11.1 MBq, [280–300 μCi], 62–67 μg of mAb, in 200 μL sterile saline for injection) *via* tail-vein injection. Approximately 5 min. prior to recording PET images, mice were anesthetized by inhalation of 1% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and placed on the scanner bed. PET images were recorded at various time-points between 24–120 h post-injection. List-mode data were acquired for between 10 and 30 min. using a γ-ray energy window of 350–750 keV, and a coincidence timing window of 6 ns. For all static images, a minimum of 20 million coincident events were recorded. Data were sorted into 2-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128×128×63 (0.72×0.72×1.3 mm) matrix. The reconstructed spatial resolution for ^{89}Zr was 1.9 mm full-width half maximum (FWHM) at the center of the field-of-view (FOV). The image data were normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection but no attenuation, scatter, or partial-volume averaging correction was applied. An empirically determined system calibration factor (in units of [mCi/mL]/[cps/voxel]) for mice was used to convert voxel count rates to activity concentrations. The resulting image data were then normalized to the administered activity to parameterize images in terms of %ID/g. Manually drawn 3-dimensional volumes-of-interest (VOIs) were used to determined the maximum and mean %ID/g (decay corrected to the time of injection) in various tissues. Images were analyzed by using ASIPro VM™ software (Concorde Microsystems).

References

1. Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J. & Bunn, P.A., Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *Journal of Immunological Methods* **72**, 77-89 (1984).
2. Lindmo, T. & Bunn, P.A., Jr. Determination of the true immunoreactive fraction of monoclonal antibodies after radiolabeling. *Methods in Enzymology* **121**, 678-691 (1986).
3. Holland, J.P., *et al.* ^{89}Zr-DFO-J591 for immunoPET imaging of prostate-specific membrane antigen (PSMA) expression in vivo. *J Nucl Med* **51**, 1293-1300 (2010).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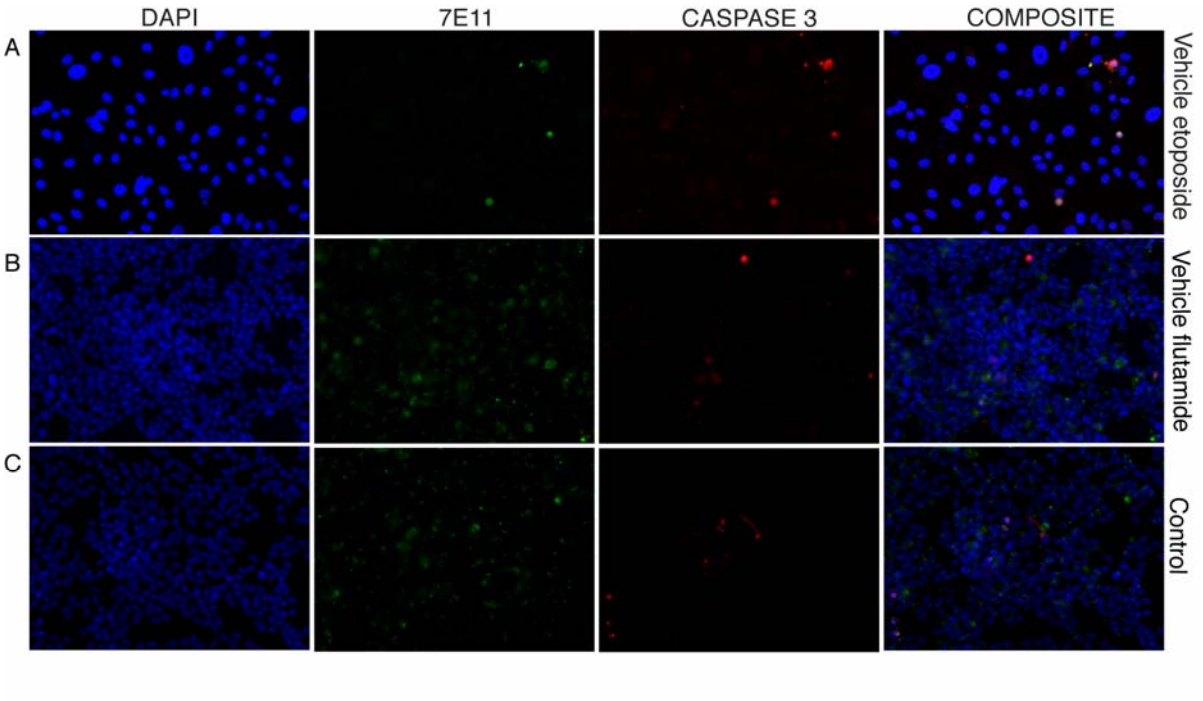
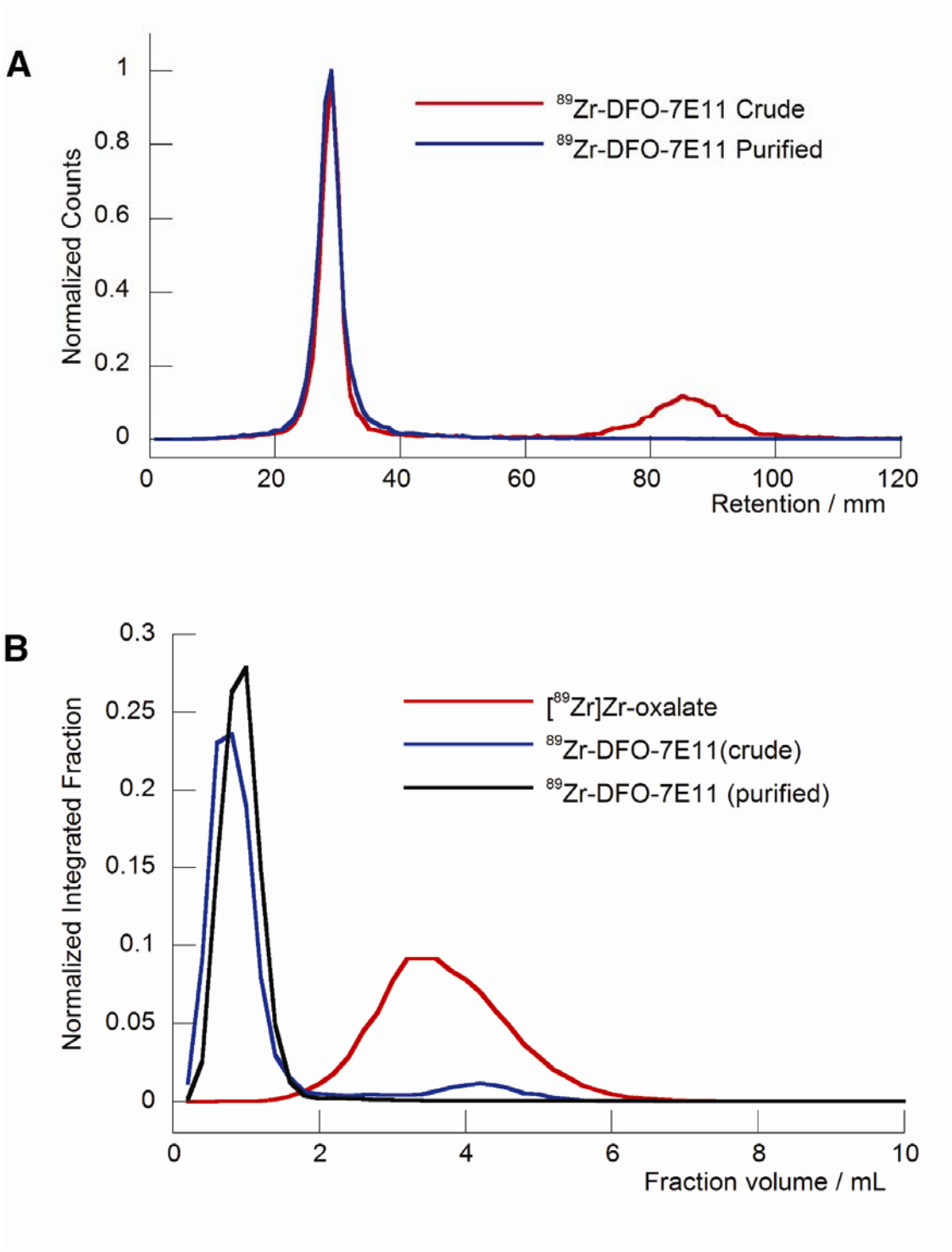
Suppl. Fig. 1

Response of PSMA positive cells to different treatments. Flow cytometry scatterplot of PC-3/PSMA+ treated with (**A)** 150 μm etoposide, LNCaP cells treated with **(B)** radiation therapy (20 Gy) and **(C)** flutamide (100 μM) at different time points (controls correspond to the 24 h time point). Cells are stained for J591-APC, 7E11-AF488 and 7AAD. J591 binds to the external epitope of PSMA, and therefore the corresponding staining does not change over treatment. 7AAD (lower panels) is used as an established marker of dying/dead cells and is compared to 7E11 staining (upper panels). Higher percentages of 7AAD and 7E11 stained cells are observed over time after the treatment compared to control. In all experiments, 7E11 and 7AAD staining correlated with each other. Data are reported in terms of percentage of dying/dead cells after treatment with (**D)** flutamide (10 μM) **(E)** flutamide (50 μM) on LNCAP cells and (**F)** etoposide (300 μM) and **(G)** sodium azide (0.2%) on PC-3/PSMA+ cells. Treatment was evaluated by comparing 7AAD staining of the cells treated with the only vehicle or control at the corresponding time point (*, $0.01<P<0.05$; **, $0.001<P<0.01$; ***, $P<0.001$).



Suppl. Fig. 2

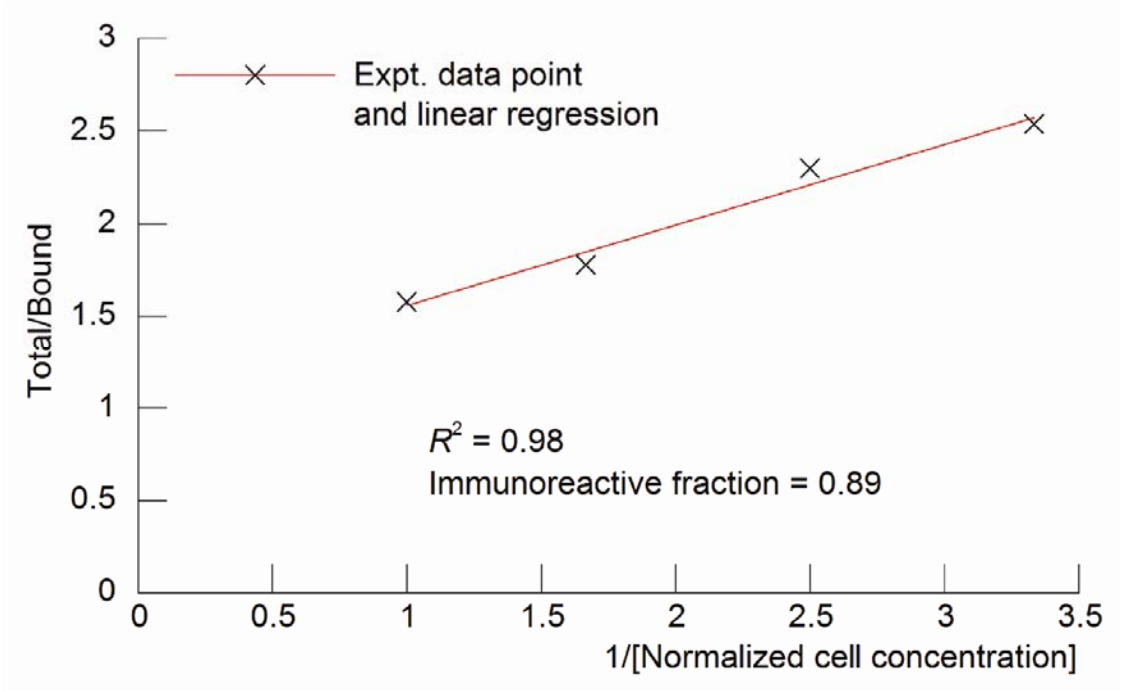
Immunofluorescence of control stained cells. Immunofluorescence assay of prostate cancer cells stained with DAPI, 7E11 and Caspase-3 obtained at 96 h after seeding. (**A)** PC-3/PSMA+ receiving etoposide vehicle (DMSO). (**B)** LNCaP cells receiving flutamide vehicle (ethanol) and (**C)** non-irradiated LNCaP cells. Magnification: 20 x.



Suppl. Fig. 3

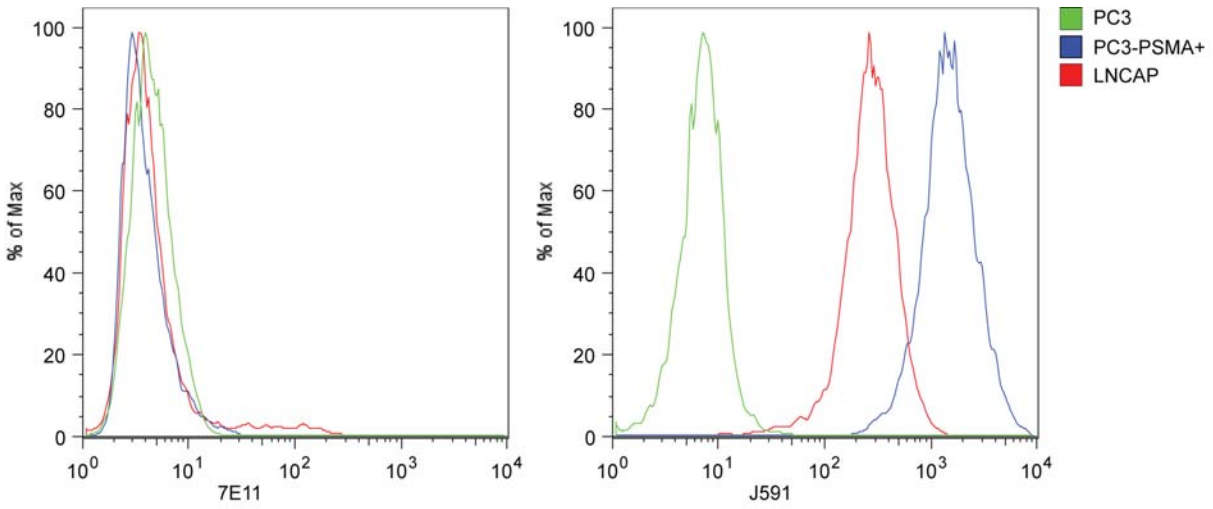
(A) Radio-ITLC. Typical radio-ITLC chromatograms of the crude (red) and purified (blue) ^{89}Zr-7E11. Eluant: DTPA(aq.), 50 mM, pH7. The ^{89}Zr-7E11 remains at the baseline ($R_f = 0.0$) and impurities run with the solvent front ($R_f = 1.0$). **(B) Size-exclusion chromatography.** Typical elution profiles observed by using PD-10 size-exclusion chromatography for the

purification of ^{89}Zr-7E11 from small-molecule (<30 kDa) ^{89}Zr-radiolabeled impurities and unreacted ^{89}Zr-oxalate (complexed as ^{89}Zr-DTPA). Species with molecular weights >30 kDa elute in the first 1.8 mL of solvent.



Suppl. Fig. 4

Cellular association of ^{89}Zr-7E11 with PC-3/PSMA+ (PSMA-positive) cells. The plot demonstrates that the ^{89}Zr-7E11 formulations are specific for expression of the PSMA antigen and have high immunoreactive fractions (0.89 ± 0.08). No weighting was applied to the data points. Data points are the mean of triplicate samples.



Suppl. Fig. 5

Flow cytometry histogram of PSMA expression in PC3 (wild-type), PC-3/PSMA+ (PSMA-positive), and LNCAP (PSMA-positive) cells.

Supplemental Video

Tridimensional Z-stack reconstruction of PC-3/PSMA+ cells labeled with 7E11 (green), activated Caspase-3 (red) and DAPI (blue) 96h after etoposide treatment.