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Supplementary Materials for

Engineering a Prostate-Specific Membrane Antigen–Activated Tumor Endothelial Cell Prodrug for Cancer Therapy

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The PDF file includes:

Supplementary Methods

Determination of plasma and tissue levels of 12ADTβAsp-GluγGluγGluγGlu (G202) prodrug:

Calibration standards are first prepared by adding 5μL of the respective 10x calibration standard and 5uL of the Ser-12ADT Internal Standard (IS) (1μM final) to plasma from control mice. IS (1μM) is similarly added to plasma samples from G202 treated animals. Plasma sample are deproteinated with the addition of 150uL acetonitrile containing 0.1% Formic acid (CH3CN/0.1% FA) and after mixing, samples were centrifuged at 15,000RPM x 5 minutes. Resulting supernatant was analyzed by LC/MS analysis. Tissues were homogenized using a hand held glass homogenizer (Kimble Kontes size 22) in a wet ice bath. The tissues were homogenized in a volume of 2x Complete Protease inhibitor [Roche Diagnostics (Indianapolis, IN)] buffer equal to 3 to 9 times the weight (g) of tissue being processed. Samples were then deproteinated with (CH3CN/0.1% FA) and processed as above.

Calibration standards and samples were analyzed by liquid chromatography coupled to a quadripole mass spectrometer (LC/MS/MS) [Applied Biosystems ABI 3000 triple quadrapole MS]. A multistep gradient elution HPLC method was developed to separate the G202 prodrug, 12ADTβAsp analog and 12ADT. Ser-12ADT was added as an internal standard. The mobile phase is composed of 5% Acetonitrile/water/0.1% Formic Acid (Solv A) and 100% Acetonitrile/0.1% Formic Acid (SolvB) and initial conditions are 60% Solv A/ 40% SolvB at a flow rate of 0.25mL/min. The injection volume is 10uL and the separation is carried out in a 100 x 2 millimeter, 2.5 micron reversed phase column (Phenomenex Luna part number 00D-4446-B0; Torrance, CA) At 1 minute following injection a linear gradient raises the mobile phase composition to 100% Solv B over 7 minutes with a return to initial conditions at 9

minutes. Within this gradient the analytes – listed with their respective ion transition pairs - elute at the following retention times; 12ADT (778/216.4) = 4.75 min, G202 (1409.8/216) = 5.19 min, 12ADTβAsp (893.5/216.4) = 6.28min and the internal standard Ser-12ADT (865.5/216) = 6.57min. Although all analytes of interest were detected from each mouse sample at one time using the same acquisition method, a separate calibration curve for each compound was prepared. For all other tissue samples, individual calibration curves were prepared using that respective tissue matrix and processed as above. Each analyte was discriminated based on the individual extracted ion chromatogram and the areas of G202, 12ADTβAsp and 12ADT were converted into a ratio with the IS. The lowest levels of detection are 10 pmoles for G202 and 1 pmole for 12ADT and 12ADTβAsp.

Crystal structure determination:

Procedures for solubilization and crystallization of the 12ADTβAsp complex of rabbit SERCA1a, as well as for crystal stabilization and mounting, were essentially as described previously for other thapsigargin derivatives (Winther 2010). Data were collected at the PXI beamline (X06SA) of the Swiss Light Source at the Paul Scherrer Institute in Villigen. Diffraction data were processed and scaled in the space group $P4_12_12$ at 50 – 2.9 Å resolution using XDS (Kabsch 1993), and the structure determined and refined using PHENIX (Adams 2002) yielding a final R_{work} of 21.7% and an R_{free} of 25.4% (suppl. Table 2). A phospholipid head group was located at a position observed earlier (Winther 2010) and interacts with the free aminoacid of the 12ADTβAsp, near the binding pocket for cyclopiazonic acid (Laursen 2009).

Table S1. Tumor endothelial cell staining scores for individual tumor types

		Tumor Endothelial Cell PSMA Staining Score			
Tumor Type	n=	3+	2+	1+	0
Hepatocellular Primary	36	16	12	5	3
Hepatocellular Metastatic	6	0	5	0	1
Cholangiocarcinoma	4	0	1	1	2
Liver Total	**46**	**16**	**18**	**6**	**6**
Mesothelioma	36	0	2	7	27
Ovarian	34	4	9	14	7
Renal Ca Clear Cell	38	11	17	9	1
Renal Ca Non Clear Cell	8	2	2	2	2
Renal Cancer Total	**46**	**13**	**19**	**11**	**3**
Breast Ca Primary	34	0	13	8	13
Breast Ca Metastatic	10	0	2	7	1
Breast Cancer Total	**44**	**0**	**15**	**15**	**14**
Melanoma	44	5	12	8	19
Bladder TCC	94	3	6	32	53
Adenocarcinoma	21	0	1	3	17
Squamous	14	0	3	1	10
Signet Ring/Mucinous	5	0	0	0	5
Adenosquamous	2	0	2	0	0
Leiomyosarcoma	2	0	0	0	2
Bladder Cancer Total	**138**	**3**	**12**	**36**	**87**
Normal Tissue Type					
Normal Liver	9	0	0	0	9
Normal Kidney	9	0	0	0	9
Normal Breast	7	0	0	0	7
Normal Bladder	10	0	0	0	10

Table S2. Crystal structure data collection and refinement statistics.

Data collection	
Beamline	X06SA (SLS PX1)
Space group	$P4_12_12$
Wavelength (Å)	1.000 Å
Resolution range (Å)	50-2.95 (2.95 – 2.90)
Unit cell parameters	$a = b = 71.0$ Å, $c = 586.8$ Å
	$\alpha = \beta = \gamma = 90°$
No. of unique reflections	35,117 (1747)
Redundancy	12.9 (10.4)
Completeness (%)	99.8 (98.0)
R_{meas} (%)	10.1 (>100)
$\langle I/\sigma(I) \rangle$	17.6 (2.0)
Wilson B-factor	76.8
Complex per ASU (Z)	1
Solvent content (%)	63
Model refinement	
R_{work} (%)	21.7
R_{free} (%)	25.4
No. amino acid residues	994
No. of atoms, total	7777
R.m.s. deviations bond length (Å)	0.005
R.m.s. deviations bond angles (°)	0.745
Ramachandran analysis	
Favoured (%)	97.0
Outliers (%)	0.3

Table S3. Effect of G202 on laboratory values in cynomolgus monkeys

[a]Lab Test	Days	0 mg/kg	10 mg/kg
Hemoglobin	1	13.7	13.1
(Hgb)	5	11.7	12.5
	12	12.5	9.6
	29	13.5	12.1
White Blood Cells	1	11.6	10.16
(WBC)	5	13.6	7.23
	12	11.24	11.35
	29	13.4	12.7
Absolute Neutrophil	1	3.51	3.66
Count	5	5.71	4.78
(ANC)	12	4.24	6.1
	29	3.12	4.52
Serum Creatinine	1	0.94	0.94
	5	0.84	1.84
	12	0.96	1
	29	1.08	0.9
Aspartyl Transferase	1	40	22.8
(AST)	5	47.4	42.4
	12	31.8	24
	29	36.6	23
Total Bilirubin	1	0.48	0.36
	5	0.66	0.74
	12	0.38	0.3
	29	0.38	0.25
Lactate Dehydrogenase	1	483	254
(LDH)	5	907	498
	12	588	500
	29	615	269

[a]Change in laboratory values in 6 cynomolgus monkeys (3 male and 3 female) after a single 3 day course of vehicle or 10 mg/kg G202

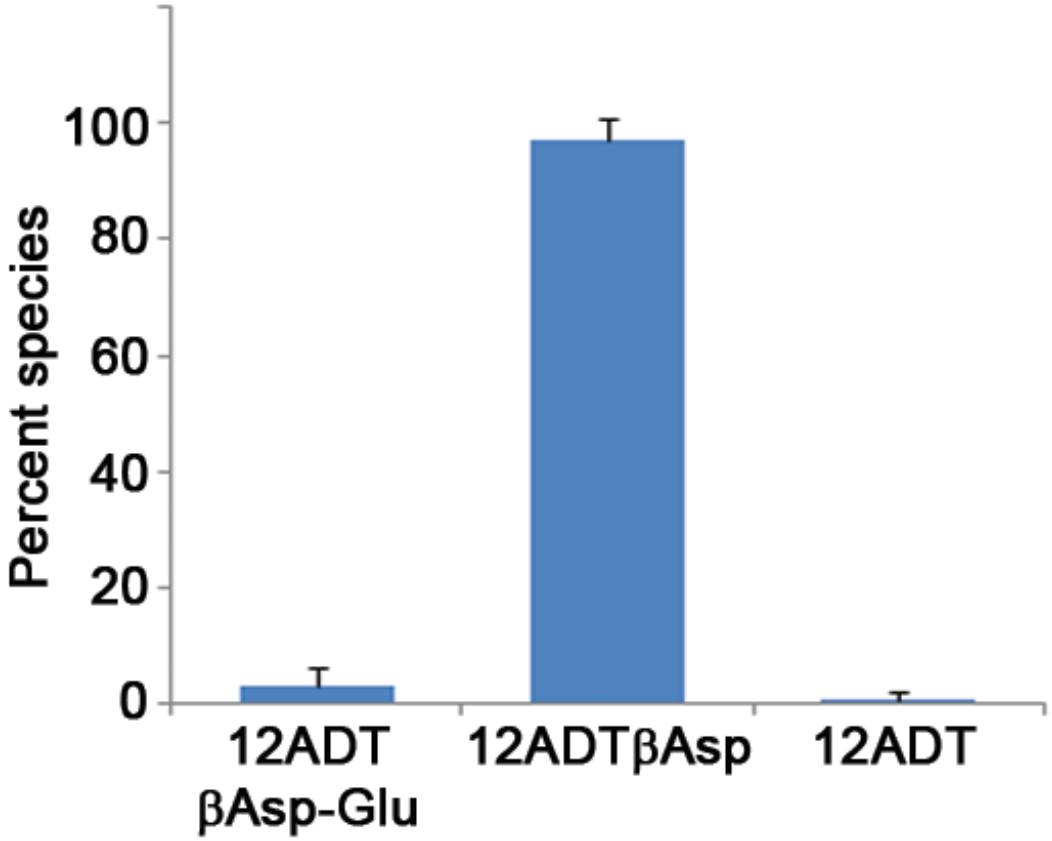

Fig. S1. PSMA hydrolysis of the 12ADTβAsp-Glu dipeptide. Fc-PSMA (10 μM) was incubated with 10 μM dipeptide and hydrolysis evaluated by HPLC analysis as described in methods. Percent of each species after 24 hrs determined from area of each individual peak divided by total peak area of all three species.

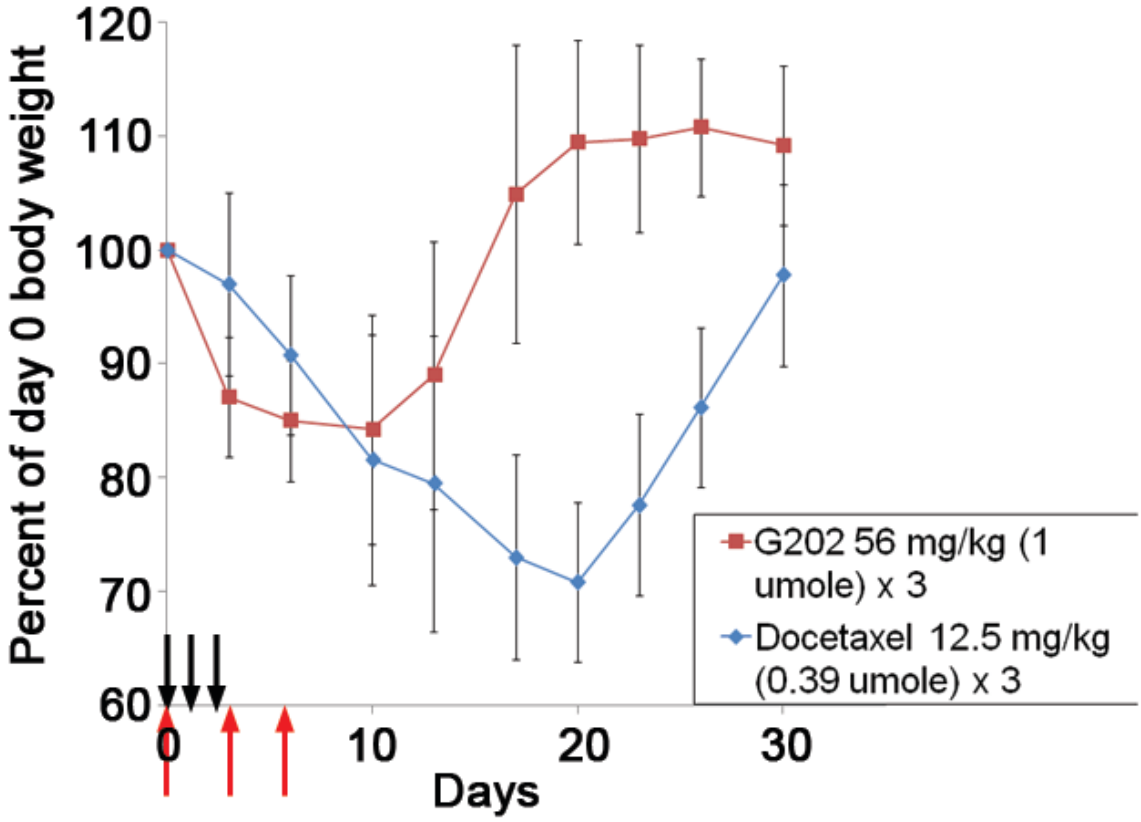

Fig. S2. Effect of G202 or docetaxel on body weight of LNCaP human prostate cancer xenograft-bearing mice. Animals were treated at the maximally tolerated in vivo dose of either G202 [56 mg/kg (1 μmole) x 3 doses) (n=9) or docetaxel ([12.5 mg/kg (0.39 μmole) x 3 doses] (n=8). Percent change of body weight compared to Day 0 pre-treatment weight for each individual animal was determined and average percent change for the entire group is plotted over a 30-day observation period. Black arrows indicate G202 treatment (days 0, 1, 2) and red arrows indicate docetaxel treatment (days 0, 3, 6). * indicates $P<0.05$ by student's t-test for less weight loss due to G202 treatment compared to docetaxel.

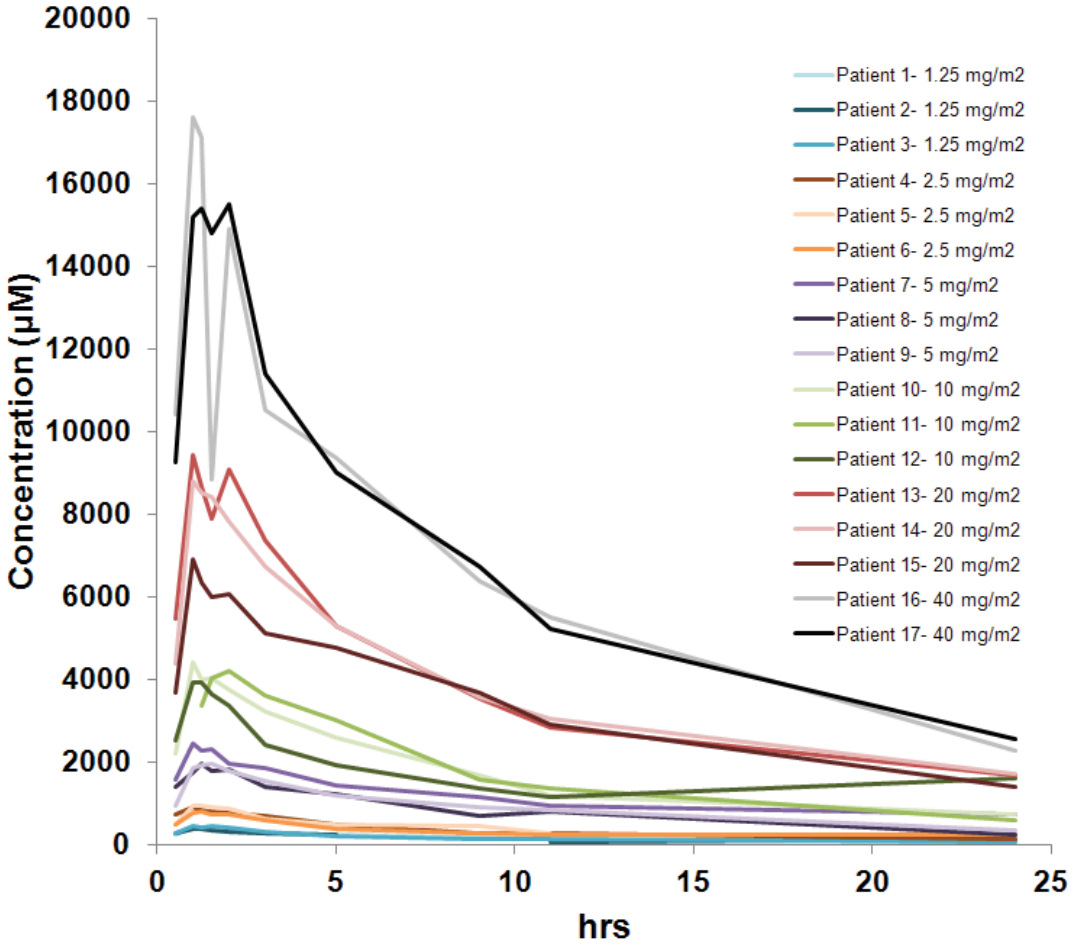

Fig. S3. Serum concentrations of G202 over a 24-hour period in 18 individual patients in 6 cohorts receiving G202 at doses ranging from 1.2 -40 mg/m^2 in phase 1 trial.

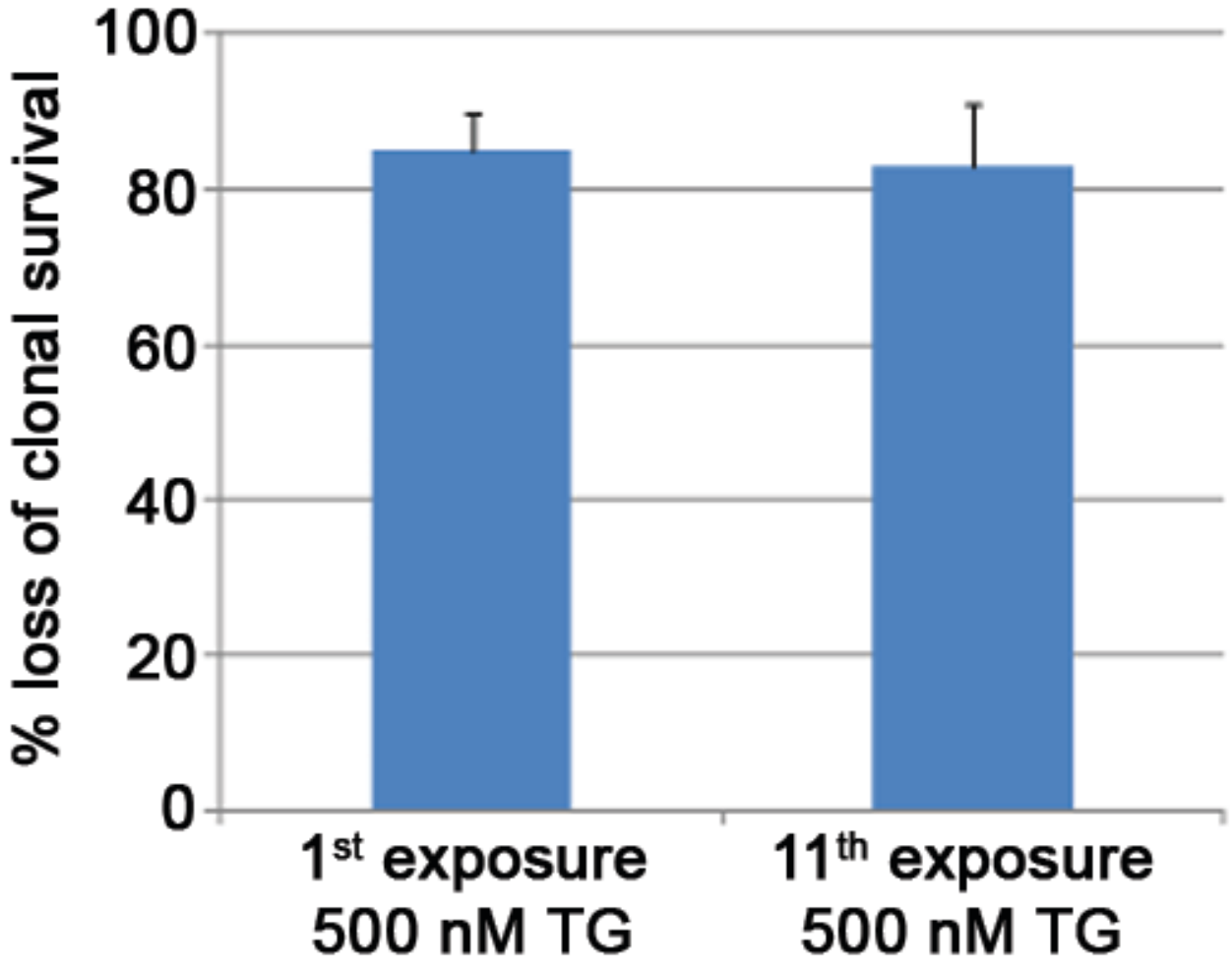

Fig. S4. Clonal survival of PC-3 human prostate cancer cells after repeated exposure to 500 nM TG. Cells were exposed to TG for 48 hrs, than plated to determine clonal survival. Replicate surviving clones were expanded and then reexposed to TG. This process was repeated x 11 cycles.