**SUPPLEMENTARY METHODS**

**Generation of MEDI3726 and Isotype Control ADC**

The antibody components of MEDI3726 and the isotype control antibody-drug conjugate (ADC) were recombinantly produced in transiently transfected CHO cells (Evitria, Zurich, Switzerland) and purified on Protein A before conjugation. To achieve a DAR of 2, the following conjugation steps were followed. A 50 mM solution of Tris (2-carboxyethyl) phosphine hydrochloride in PBS pH 7.4 was added (50 molar equivalent/antibody, 13.8 micromoles, 277 L) to a 21.0 mL solution of antibody (41.5 mg, 277 nmol) in reduction buffer containing PBS and 1 mM EDTA and a final antibody concentration of 2 mg/mL. The reduction mixture was agitated at 65 rpm at room temperature in an orbital shaker for 24 h or until full reduction was observed by ultrahigh-pressure liquid chromatography). The reduced antibody was buffer-exchanged by spin filter centrifugation into a conjugation buffer containing PBS pH 7.4 and 1 mM EDTA to remove all the excess reducing agent, then sterile-filtered. Tesirine (22) was added as a dimethyl sulfoxide (DMSO) solution (10 molar equivalent/antibody, 1.38 micromoles, in 2.1 mL DMSO) to 18.5 mL of the reduced antibody solution (20.75 mg, 138 nmol) for a 10% (v/v) final DMSO concentration at a final antibody concentration of 1.0 mg/mL. The solution was mixed for approximately 1.5 h at room temperature. The conjugation was quenched by addition of N-acetyl cysteine (6 micromoles, 60 L at 100 mM), and the ADC was purified by spin filtration with a 15 mL Amicon Ultracell 50 kDa molecular weight cut-off spin filter.

**Drug-to-antibody Ratio Determination by Reduced Reversed Phase Liquid Chromatography**

Antibody or ADC (approximately 35 μg in 35 μL) was reduced by the addition of 10 μL borate buffer (100 mM, pH 8.4) and 5 μL dithiothreitol (0.5 M in water), and heated at 37°C for 15 min. The sample was diluted with 1 volume of acetonitrile:water:formic acid (49%:49%:2% v/v), and injected onto a Widepore 3.6μ XB-C18 150 x 2.1 mm (P/N 00F-4482-AN) column (Phenomenex Aeris) at 80°C, in an ultraperformance liquid chromatography (UPLC) system (Shimadzu Nexera) with a flow rate of 1 ml/min equilibrated in 75% Buffer A (trifluoroacetic acid, 0.1% v/v), 25% buffer B (acetonitrile:water:trifluoroacetic acid 90%:10%:0.1% v/v). Bound material was eluted by using a gradient from 25% to 55% Buffer B over 10 min. Peaks of UV absorption at 214 nm were integrated. The peaks for native antibody light chain (L0), native antibody heavy chain (H0), and each of these chains with added drug-linkers (labelled L1 for light chain with one drug and H1, H2, H3 for heavy chain with 1, 2 or 3 attached drug linkers) were identified for each ADC or antibody. The UV chromatogram at 330 nm was used for identification of fragments containing drug linkers (i.e., L1, H1, H2 and H3).

A pyrrolobenzodiazepine (PBD)/protein molar ratio was calculated for both light chains and heavy chains:

$$\frac{Drug}{Protein}ratio on light chain=\frac{\%Area at 214 nm for L1}{\%Area at 214 nm for L0 and L1}$$

$$\frac{Drug}{Protein}ratio on heavy chain =\frac{\sum\_{n = 0}^{3}n×\left(\%area at 214 for Hn\right)}{\sum\_{n = 0}^{3}\%area at 214 for Hn}$$

Final DAR was calculated as:

$$DAR=2 × \left(\frac{Drug}{Protein}ratio on light chain+\frac{Drug}{Protein}ratio on heavy chain\right)$$

DAR measurement was carried out at 214 nm, because it minimized the interference from drug-linker absorbance.

**Size-exclusion Chromatography Determination of Purity**

Antibody or ADC (approximately 20 μg in 20 μL) was placed into an insert high-pressure liquid chromatography vial, and 10 μL was injected into a Waters Acquity UPLC BEH200 SEC 1.7 μm 4.6 x 150 mm ( P/N 186005225) column running isocratically with 0.3 mL/min PBS containing 10% isopropanol for 10 min; or a Phenomenex Yarra 3μm SEC-3000 4.6x300 mm (P/N 00H-4513-E0) column running isocratically with 0.35 mL/min PBS containing 10% isopropanol for 16 min. Peaks of UV absorption at 280 nm and 214 nm were integrated.

**Surface Plasmon Resonance Determination of Binding Kinetics for MEDI3726**

The equilibrium dissociation constant (KD) for the binding of MEDI3726 to human and cynomolgus monkey (Macaca fascicularis) (EHH56646.1) soluble recombinant ectomains of PSMA (shPSMA, scPSMA respectively) was measured using a BIAcore T200 (BIAcore, Inc.) instrument. ScPSMA and shPSMA proteins were immobilized to Biacore CM5 (GE Healthcare Life Sciences) sensor chips at 1000RU following the manufacturer’s instructions. A reference flow cell surface was also prepared by using the same immobilization protocol, but omitting the protein. Serially diluted MEDI3726 (concentrations ranging from 1.25 g/mL to 20 g/mL) or isotype antibody (concentrations ranging from 2.5 g/mL to 100 g/mL) was sequentially injected over the sPSMA surfaces and reference cell surfaces, connected in series at a flow rate of 30 L/min for 2 min, and allowed to dissociate in HBS-EP buffer (0.01M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20; GE Healthcare Life Sciences) at the same flow rate, for 60 min. The Biacore “multi-cycle kinetics” method was used. Data were analyzed by using the BIA evaluation software (GE Healthcare Life Sciences). Kinetic data were fitted to a 1:1 (Langmuir) model.

**Soluble PSMA Generation and Purification**

shPSMA (M99487.1), scPSMA, and soluble recombinant ectodomain of rat PSMA (NM\_057185.2, srPSMA), each of which was fused to a six-histidine tag peptide at the C-terminus, were expressed in transiently transfected CHO cells by Evitria, AG (Switzerland). All purification runs were performed at 4°C on the ÄKTA pure platform. srPSMA fused to a six-histidine tag at the C-terminus was purified on a nickel immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare), following the manufacturer’s instructions of washing in 20 mM phosphate, 0.5 M NaCl, and 20 mM imidazole pH 7.4 and eluting in 20 mM phosphate, 0.5 M NaCl, and 500 mM imidazole pH 7.4.

shPSMA and scPSMA antigens were purified on a J591 affinity column, because the IMAC purification was not adequate for those antigens. The J591 affinity column was generated by coupling 400 mg of J591 antibody in carbonate buffer pH 8.6 to 15 g CNBr-activated Sepharose resin to make 45 ml of affinity resin coupled at 8.9 mg of antibody per milliliter of resin. Remaining activated groups were quenched with ethanolamine. A mock run (1 mL/min) of binding, wash, and pH 11.5 and pH 3.0 elution buffers was performed on the column to remove residual uncoupled antibody. CHO cell culture supernatant was then applied to the column at 1 mL/min, which was washed with PBS/0.5 M NaCl (pH 7.4) and eluted with 100 mM glycine pH 11.5. Eluate was neutralized with 1 M Tris pH 7.0 and dialyzed three times with PBS in a ratio of 1:20. Purified proteins were analyzed by SDS PAGE gel electrophoresis and SEC.

**Soluble PSMA binding assay**

Human sPSMA (6 μg/mL in PBS) was dispensed (50 μL/well) in 96 well ELISA microplates and incubated overnight (4°C). Plates were blocked with 200 μl 3% BSA/PBS for 1 hour at 37°C. In a separate, non-binding plate, serial 3x dilutions of test samples, from 1 μg/mL, were made in PBS/Tween (0.05% (v/v)) /BSA. The sPSMA-coated plate was washed 3x (PBS/Tween 20 (0.05% (v/v))), samples transferred (50 μl/well) from the non-binding plate, and incubated at 37°C for 1 hour. The plate was washed 3x as before, an anti-human Fc conjugate (Goat anti-Human IgG – HRP (Fc specific), diluted 1:5000 in PBS/Tween/BSA dispensed (50 μL/well) and incubated at 37°C for 1 hour. The plate was washed 3x with PBS/Tween, then 2x with PBS. 1-Step Turbo TMB-ELISA Substrate (Thermo Scientific 34022) was dispensed (100 μL/well), incubated at ambient temperature for 30 min, then 0.6 M HCl (100 μL/well) added to stop reaction, and the optical density of each well was measured at 450 nm. Data were fitted without constraint, to a “sigmoidal, 4PL, X is log10 (concentration)” model (GraphPad Prism 6) to obtain the EC50.

**Anti-PBD binding assay**

Streptavidin-coated MSD plates (Meso Scale Discovery, Rockville, MD cat no L15SA-1) were washed 3x (300 μL/ well) with wash buffer and blotted dry. Biotinylated anti-PBD antibody, 14B3-B7, (0.5 μg/mL in PBS) was dispensed (75 μL/ well) on the streptavidin-coated MSD plates. The plates were incubated overnight at 4°C, washed 3x with wash buffer (0.05% tween/ PBS), (300 μL/ well) and blotted dry. Blocking buffer (3% BSA in PBS) (150 μL/ well) was dispensed and incubated at room temperature for 1-2 hours. Test samples (100 μg/mL) in human or cynomolgus plasma or PBS, were diluted (5 μL of sample plus 89 μL of assay buffer (0.1%BSA/ 0.05% tween/ PBS) in a polypropylene offline plate (row A) and a 2.7x dilution series prepared in assay buffer. MSD plates were washed 3 x with wash buffer (300 μL / well) and blotted dry. The test sample dilution series was transferred (75 μL/ well) to the MSD plate and incubated at room temperature for 90 minutes, shaking at 500 rpm, then washed and blotted dry as before. “Detector” sulfo-tag-labelled anti-human-IgG1-Fc (batch B1015-0858) (75 μL/ well, 0.2 μg/mL) was dispensed and incubated for 1 hour at room temperature, shaking at about 500 rpm. MSD plates were washed and blotted dry as before. Read buffer T (MSD) (150 μL/ well, 2x) was dispensed and plates read (electrochemiluminescence) on an MSD machine. ECL data were analyzed and EC50 values were determined in GraphPad Prism using the ‘log(agonist) vs. response –Variable slope (4 parameters)’ algorithm, fitting either unconstrained or constrained at top and bottom values and Hill slope. These EC50 values were also expressed relative to the -80°C control sample.

**On-cell Binding Determination by Flow Cytometry**

LNCaP and CWR22Rv1 cells were dissociated by using TrypLE (Life Technologies) and suspended in their respective growth media. Cells were counted on a ViCell cell counter (Beckman Coulter) and brought to a concentration of 106 cells/mL. Cells were transferred in duplicate to a 96-well plate (Falcon) at 5 × 104 cells per well and centrifuged at 1,200 rpm at 4ºC. Pelleted cells were suspended in 180 μL of flow cytometry buffer (PBS pH 7.2, 2% FBS, on ice), and ADC was individually added to cells (20 μL of serial dilution, with a final concentration of 10 μg/mL to 0.0006 μg/mL). Antibodies and cells were incubated at 4ºC for 1 h, then washed with flow cytometry buffer and pelleted by centrifugation (twice at 1,200 rpm). After the final spin, cell pellets were resuspended in AlexaFluor 647-conjugated antihuman secondary antibody (150 μL, 8 μg/mL, in PBS pH 7.2, 2% FBS) and incubated at 4ºC for 1 h. Cells were washed with flow cytometry buffer and centrifuged (2 × 1,200 rpm), then resuspended in 135 μL of flow cytometry buffer. 4′,6-diamidino-2-phenylindol was added (15 μL from a 10X stock, 3 μM final concentration, Sigma Aldrich) to each cell suspension to act as a live or dead stain. Fluorescence data from the cells was collected by using a LSRII flow cytometer (Beckton Dickson), and data were analyzed by using FlowJo analysis software (Version 9, FlowJo, LLC). Binding curves were generated by using GraphPad Prism (Version 6, GraphPad Software, Inc.).

**MEDI3726 Internalization and Lysosomal Trafficking**

For microscopic resolution of the cells, the LNCaP or PC3 cells (50,000 cells/mL in growth medium, 100 μL per well) were plated on glass-bottom microplates (Sensoplate, Greiner BioOne, 655892) coated with poly-D-lysine (Sigma P1149: 1mg/mL in PBS). The plates were incubated at 37°C and 5% carbon dioxide for 2 days to allow the cells to adhere. Culture medium was aspirated from the microplates and replaced (100 μL/well) with MEDI3726 or isotype control ADC, (both 30 μg/mL in growth medium) or growth medium only. The microplates were incubated for 24 h to allow cellular internalization of the ADCs. Cells were then fixed at 4°C for 15 min with neutral buffered formalin (Sigma HT5014) (100 μL per well), then washed three times with Microscopy Wash Buffer (ProClin 150 [Supelco 49376] 0.04% (v/v) in PBS [Sigma P3813-10PAK]). Cells were permeabilized with 50 μL/well of Microscopy Permeabilization Buffer (Triton X-100 [Fluka BP151-100, 0.5% [vol/vol]), ProClin 150, 0.04% [vol/vol] in PBS) for 15 min at room temperature. After the permeabilized cells were washed, nonspecific binding sites were blocked for 1 h at 37°C with BlockAid (Life Technologies B10710). The BlockAid was aspirated from the microplates, which were then incubated for 1 h at 37°C with mouse anti-LAMP1 (CD107a) antibody (Becton Dickson 555798) (2.5 μg/mL) in BlockAid. Microplates were washed three times with Microscopy Wash Buffer and incubated for 1 h at 37°C with antihuman immunoglobulin G (IgG) Alexa Fluor 488 (Thermo Fisher A11013) and anti-mouse IgG Alexa Fluor 546 (Thermo Fisher A11003) (both diluted 1:200 in BlockAid). After three further washes in Microscopy Wash Buffer, the cells were counterstained for 15 min at room temperature with Hoechst 33342 (Thermo Fisher 62249, 10 μg/mL in D-PBS). All samples were imaged with a Zeiss Axio Observer Z1 microscope equipped with an αPlan Apochromat 63× NA 1.46 oil immersion objective (Zeiss 420780-9970-000) and Axiocam 506 mono CCD camera.

**Statistical analysis for the prostate cancer cell line xenograft studies**

The time-to-endpoint (TTE) for analysis was calculated for each mouse by using the following equation:

TTE (days) = (log10 [endpoint volume] – *b*)/*m*

where endpoint volume is expressed in mm3, *b* is the intercept, and *m* is the slope of the line obtained by linear regression of a log-transformed tumor growth data set. Prism (GraphPad) for Windows 6.07 was used for graphical presentations and statistical analysis of log-rank test. For all analyses, *P* < 0.05 was considered statistically significant.

**Statistical analysis for the LuCaP PDX studies**

Statistical assumptions were assessed prior to analysis of raw data or log10-transformed data (when assumptions were not met when applied to raw data). Data were analyzed by ANOVA followed by Dunnett’s pairwise testing for raw or log10 transformed data (where appropriate). If assumptions were not met (when applied to raw or log10 transformed data), a Kruskal-Wallis test followed by Dunn’s test for pairwise testing was applied to the raw data. Both Dunnett’s and Dunn’s pairwise testing adjusts for multiple comparisons. Adjusted *P* values were reported for Dunnett’s and Dunn’s pairwise testing. A *P* value of <0.05 was considered statistically significant. GraphPad Prism 7 was used for statistical analyses.

**Immunohistochemistry of Tumor Xenografts**

The PSMA immunohistochemistry (IHC) assay used monoclonal mouse anti-human PSMA clone 3E6 (Dako) and mouse IgG1 isotype control (R&D System) as primary antibodies at a dilution of 2 µg/mL. Xenograft tumors were fixed in buffered formalin and embedded in paraffin and sectioned at 5 µm. Deparaffinized and rehydrated slides were placed in citrate buffer pH 6.0 in a pressure-cooker for 5 min at 110°C. After the slides were cooled and rinsed in Tris-buffered saline plus 0.1% Tween 20 (TBST), the IHC assay was run on the Dako autostainer by using the Dako EnVision+ System-HRP (DAB) kit. Briefly, the peroxidase block was applied to slides with a solution of 3% hydrogen peroxide for 10 min followed by a TBST wash. Serum-free protein block was applied for 15 min before the slides were incubated with anti-PSMA or the mouse IgG1 isotype control antibody for 1 h. Following another TBST wash, the slides were incubated with Dako Envision+system-HRP Labeled Polymer anti-mouse secondary antibody for 30 min After the slides were rinsed in TBST, 3,3′-diaminobenzidine tetrahydrochloride was applied for 10 min, and the slides were rinsed and counterstained with Mayer’s hematoxylin. Staining was evaluated for the intensity of expression and the frequency of cell staining.

For the -H2AX IHC, LuCaP 73 xenograft-bearing animals were dosed once, and xenograft tumors were collected 48 h later for formalin-fixed paraffin-embedded generation. IHC detection of -H2AX was performed on deparaffinized 5 µm sections by using antibody rabbit anti-phospho-histone H2A.X (Ser139) (20E3) or rabbit monoclonal antibody IgG (both from Cell Signaling Technology), diluted 1:500 in Dako antibody diluent with Background Reducing Component (Cat# S3022) and Ventana-prediluted rabbit negative control antibody, as the primary antibodies. The IHC assay used the Ventana Discovery ULTRA Staining Module. Briefly, the deparaffinized sections were pretreated with Cell Conditioning 1 Mild solution for 40 min and incubated with Inhibitor for 8 min. Primary and negative control antibodies were applied and incubated for 20 min at 37°C, then incubated with the secondary antibody Multimer anti-Rabbit HRP OmniMap for 16 min. After the primary antibodies were detected with ChromoMap DAB, Ventana predilute Hematoxylin and Bluing Reagent was used for counterstaining.

**Toxicology Study of MEDI3726 in Cynomolgus Monkey**

In the GLP toxicology study, monkeys (5/sex/group) were administered IV injections of 0.15/0.3 mg/kg or 0.6 mg/kg MEDI3726 on Days 1 and 22. Animals were necropsied on Days 29 (main study; 3/sex/group) and 71 (recovery; 2/sex/group). Assessment of toxicity was based on clinical observations, body weight, clinical pathology (hematology, clinical chemistry, urine, and coagulation), body temperature, physical and neurological examinations, neurobehavioral evaluation, ophthalmic examination, cardiovascular assessment (electrocardiogram [ECG] and blood pressure), respiratory rate, organ weights, and anatomic pathology findings.