Detailed synthesis of the linker part and following conjugation of the chelator

The resin-bound and tert-butyl-protected binding motif (6) was dried *in vacuo* and split into three portions. The following coupling of the linker moiety was performed according to standard Fmoc protocol. Relative to the resin (3 x 0.1 mmol), 4 equiv. of Fmoc-L-2-Nal-OH were activated with 3.92 equiv. of HBTU (*O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate) and 4 equiv. of DIPEA in DMF. After 2 min, the solution was added to the resin-immobilised Glutamate-urea-Lysine and shaken for 1 hour. The Fmoc-protecting group was cleaved with a mixture of DMF and piperidine (1:1). The product (7) was reacted with 4 equiv. of *trans*-4-(Fmoc-aminomethyl)cyclohexanecarboxylic acid (*N*-Fmoc-tranexamic acid) activated with 3.92 equiv. of HBTU and 4 equiv. of DIPEA. After deprotection of the Fmoc group with a mixture of DMF and piperidine (1:1), the conjugation of the chelator to (**8**) was performed using 3.95 equiv. HBTU and 4 equiv. of DOTA-tris(tBu)ester. The final product (PSMA-617) was cleaved from the resin and deprotected with trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (95:2.5:2.5).

Analytical methods

For analytical (100 mm × 4.6 mm) and semipreparative (100 mm × 10 mm) RP-HPLC Chromolith RP-18e columns were used and eluted with a linear

gradient from 0 to 100% acetonitrile in water (0.1% TFA) over 6 min at a flow rate of 4 mL/min or 6 mL/min, respectively. The UV detector was adjusted for 214 and 254 nm, respectively.

MALDI-MS was carried out with a Daltonics Microflex system. A full-scan single mass spectrum was gained by scanning from m/z = 400 to 2000.

Determination of radiochemical stability, distribution coefficient and protein binding

For the radiochemical stability, 5 nmol of PSMA-617 was labeled with 70 MBq of [^{68}Ga]Ga^{3+} eluate, 50 MBq of [^{67}Ga]$GaCl_3$ or 20 MBq of [^{177}Lu]$LuCl_3$, respectively. After applying the samples the TLC plate was placed in pre-equilibrated TLC chambers with 0.1 M sodium citrate. R_f ([^{67}Ga/^{68}Ga/^{177}Lu]Ga/$LuCl_3$) = 0.0; R_f ([^{67}Ga/^{68}Ga/^{177}Lu]Ga/Lu-PSMA-617) = 0.9. The respective TLC plate was cut in the middle and detected in a gamma counter (Packard Cobra II, GMI).

In order to analyze the lipophilicity, the distribution coefficient between n-octanol and HEPES (0.1 M; pH 7.4) (logD) was determined. [^{68}Ga]Ga-PSMA-617 was added in two-phase system under shaking, and samples were collected from both phases and analysed with a gamma counter.

Binding to serum proteins was analyzed on a Superdex^{TM} 75 5/150 GL column (GE Healthcare) after 1 hour incubation at 37 °C. The runs were

performed using phosphate-buffered saline (PBS; pH 7.4) as the mobile phase at a flow rate of 0.4 mL/min. The UV absorbance was measured at 280 nm.

Binding affinity assay procedure

The cell-based assay was performed using 10^5 LNCaP cells/well. The radioligand (Glu-urea-Lys-(Ahx))$_2$-[^{68}Ga]Ga(HBED-CC) (*19*) was added as 0.75 nM solution to 12 different concentrations of non-labeled, ^{nat}Ga- or ^{nat}Lu-labeled PSMA-617 (from 0 to 5000 nM). After 1 hour incubation at 37 °C, the cells were washed with ice-cold PBS and the cell-bound radioactivity was measured in a gamma counter.

Internalization assay procedure

Briefly, 10^5 LNCaP cells were incubated for 45 min with ^{68}Ga- or ^{177}Lu-labeled PSMA-617 (32 nM) at 4 °C and 37 °C, respectively. 2-PMPA was used for confirmation of specific cellular uptake. The surface-bound radioactivity was removed with glycine · HCl (50 mM; pH 2.8) and the internalized fraction using 0.3 M NaOH. The collected fractions were measured in a gamma counter and calculated as %IA/10^6 cells.

µPET imaging

For µPET imaging, the mice were anaesthetized (2% sevoflurane, Abbott) and placed into the small animal PET scanner (Inveon PET, Siemens). After a 20 min transmission scan, mice were injected with ^{68}Ga-labeled PSMA-617 and a 50 min dynamic scan and a static scan from 100 to 120 min p.i. was performed. Images were reconstructed iteratively using the space alternating generalized expectation maximization method (SAGE, 16 subsets, 4 iterations) applying median root prior correction and were converted to standardized uptake value (SUV) images. Quantitation was done using a ROI technique and expressed as SUVmean.