Journal of Nuclear Medicine, published on February 9, 2023 as doi:10.2967/jnumed.122.264944

PSMA-GCK01 - A Generator-Based ^{99m}Tc-/^{188}Re-Theranostic Ligand for the Prostate-Specific Membrane Antigen

Running title: (Pre)clinical evaluation of PSMA-GCK01

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Word Count: 4995

This work received financial support from Telix Pharmaceuticals Ltd.

ABSTRACT

Introduction: Prostate-specific membrane antigen (PSMA)-theranostic has been introduced with Gallium-68 and Lutetium-177, the currently most used radionuclides. However, Rhenium-188 is a well-known generator based therapeutic nuclide completing a theranostic tandem with Technetium-99m and may offer an interesting alternative to the current state of the art. In the present work, we aimed towards the development of a PSMA-targeted ^{99m}Tc-/^{188}Re-theranostic tandem.

Methods: The ligand HYNIC-iPSMA was chosen as lead structure. Its HYNIC chelator has limitations for ^{188}Re-labeling and was exchanged by MAS_3 to obtain PSMA-GCK01, as precursor for stable ^{99m}Tc- and ^{188}Re-labeling. ^{99m}Tc-PSMA-GCK01 was used for the *in-vitro* evaluation of the novel ligand and comparison with ^{99m}Tc-EDDA/HYNIC-iPSMA. Planar imaging using ^{99m}Tc-PSMA-GCK01 and organ biodistribution with ^{188}Re-PSMA-GCK01 were done using LNCaP-tumor bearing mice, respectively. Finally, the theranostic tandem was applied for imaging and therapy in three prostate cancer patients in compassionate care.

Results: An efficient radiolabeling of PSMA-GCK01 with both radionuclides was demonstrated. Cell-based assays with ^{99m}Tc-PSMA-GCK01 vs ^{99m}Tc-EDDA/HYNIC-iPSMA revealed comparable uptake characteristics. Planar imaging and organ distribution revealed a good tumor uptake of both, ^{99m}Tc- and ^{188}Re-PSMA-GCK01 at 1 h and 3 h p.i. with low uptake in non-target organs. In patients, similar distribution patterns were observed for ^{99m}Tc-PSMA-GCK01 and ^{188}Re-PSMA-GCK01 and also in comparison of Tc/Re-PSMA-GCK01 with ^{177}Lu-PSMA-617.

Conclusion: The novel ligand PSMA-GCK01 labels stable with ^{99m}Tc- and ^{188}Re - both are generator based radionuclides – and, thus, provides access to on-demand labeling at reasonable costs. The preclinical evaluation of the compounds revealed favorable characteristics of the PSMA-targeted theranostic tandem. This result was further confirmed by a successful translation into first-in-human application.

Key words: PSMA, Rhenium-188, Technetium-99m, Theranostic, SPECT

INTRODUCTION

The development of novel theranostics has dominated the recent activities in the field of oncological nuclear medicine. Most common theranostics are based on so-called matched pairs in which the diagnostic and therapeutic radiopharmaceuticals are labeled with radionuclides from different elements – sometimes even different molecules are used. The only precondition is that both (or all) radiopharmaceuticals within the matched pair show a very similar biodistribution (*1,2,3*).

A combination of nuclides using the same precursor that perfectly fulfills this demand is Technetium-99m with Rhenium-188 (and Rhenium-186) (*4*). Technetium-99m is still one of the most widely used radionuclides worldwide and rhenium (with its isotopes Re-186 and Re-188) is the only element resembling its *in-vivo* chemistry nearly perfectly. Moreover, both nuclides (Tc-99m and Re-188) are available from radionuclide generators disclosing potential application in areas without strong nuclear infrastructure. Finally, Rhenium-188 might help to surpass potential shortages in the supply of Lu-177, which may arise from lack of high-flux neutron facilities (*5*). This renders technetium/rhenium-based theranostic radiopharmaceuticals an attractive combination, in particular for smaller hospitals using "only" SPECT in their NM departments, as well as for application in developing countries (*6*). Another more current aspect underscores the need for SPECT based PSMA imaging and, therefore, Technetium-99m ligands: the patient selection for the recently approved ^{177}Lu-PSMA-617 (Pluvicto) affords confirmation of sufficient PSMA uptake in a preliminary diagnostic scan. For these mandatory diagnostic scans, current PET infrastructure may demonstrate to be the bottleneck - A challenge that might be met by suitable SPECT ligands including ligands primarily developed for diagnostic purpose (e.g., ^{99m}Tc-MIP-1404 (*7*) and ^{99m}Tc-EDDA/HYNIC-iPSMA (8)).

The aim of this work was to develop a ^{99m}Tc-/^{188}Re-theranostic tandem targeting the prostate-specific membrane antigen (PSMA). As lead structure the ligand HYNIC-iPSMA was chosen, which is already in advanced clinical stage in its Technetium labeled form ^{99m}Tc-EDDA/HYNIC-iPSMA (*8,9,10*). Technetium-99m is coordinated via the HYNIC-chelator in ^{99m}Tc-EDDA/HYNIC-iPSMA. However, it is rather unlikely that HYNIC is a suitable chelator for Rhenium-188 (*6*). Thus, we replaced the chelator in HYNIC-iPSMA by the more suitable MAS3 – a classical N_3S-chelator, which is suitable for ^{188}Re-coordination (Figure 1) (*11,12*). In the following, we will provide a summary on our research results thus far including first-in-man application under compassionate use.

MATERIALS AND METHODS

General

The precursor synthesis was accomplished by well-known standard methods and is described in the supplementary information (*13,14*). The $SnCl_2$ for Rhenium reduction was trace metal basis and acquired from Merck (Taufkirchen, Germany). The remaining chemicals for tracer synthesis were all Ph. Eur. or *ad injectabilia* grade and acquired from Merck (Taufkirchen, Germany) or BBraun (Melsungen, Germany) or. Waters Sep-Pak Light Alumina N cartridges, Sep-Pak Light QMA cartridges and Sep-Pak Light tC18 cartridges were purchased from Macherey und Nagel (Dueren, Germany). Dionex OnGuard II Ag cartridges were purchased from Thermo Fischer (Schwerte, Germany).

Technetium-99m was obtained from a TekCis generator (Curium, Berlin, Germany) in form of ^{99m}Tc-$NaTcO_4$ in 0.9 % saline according to the manufacturer instructions. Rhenium-188 was obtained in form of ^{188}Re-$NaReO_4$ from a ^{188}W/^{188}Re generator (OnkoBeta GmbH, Garching, Germany) by elution with 10 mL 0.9 % saline (BBraun, Melsungen, Germany). ^{99m}Tc-EDDA/HYNIC-iPSMA was produced from commercial kits according to the instructions. The reagent-kits were acquired from Telix Pharmaceuticals Ltd (Brussels, Belgium).

Chemistry and Radiochemistry

*^{99m}Tc-labeling of PSMA-GCK01.*Phosphate buffer for the labeling was prepared from 890 mg $Na_2PO_4 \cdot 2\ H_2O$ in 9.5 mL water for injection and 0.5 mL 2 m NaOH (pH = 11.5-12.0). For ^{99m}Tc-labeling, 500-800 µL pertechnetate solution (1.5-2.5 GBq / mL in 0.9 % saline) were mixed with 200 µL phosphate buffer, 100 µL tris(2-carboxyethyl)phosphin (TCEP; 28.9 mg / mL in phosphate buffer) 20 µL precursor solution (1 mg / mL). The resulting mixture (pH 8.0-8.5) was heated at 98 °C for 10 minutes. The mixture was diluted with approx. 1 mL of 0.9 % saline and passed through a C-18 cartridge (SepPak plus light tC18, preconditioned with 5 mL EtOH, followed by 10 mL water). The product was eluted with 1 mL 70 % ethanol and diluted with 9 mL PBS (prepared from 9 mL 0.9 % saline and 1 mL phosphatebuffer concentrate, BBraun *ad injectabilia*, both). Finally, the product was passed through a 0.22 µm sterile filter. Aliquots

of the reaction mixture were withdrawn directly after the reaction, after cartridge separation (before dilution with PBS) and of the final product formulation and analyzed by RP-HPLC.

^{188}Re-labeling of PSMA-GCK01. ^{188}Re was eluted from the ^{188}W/^{188}Re-generator using 10 mL 0.9 % NaCl. The eluate was postprocessed according to ref. (*15*). Briefly, potential tungsten breakthrough was retained on an SepPak Alumina(N) cartridge. The eluate was dechlorinated using a Dionex OnGuard II Ag cartridge and the perrhenate was concentrated using a SepPak QMA cartridge, preconditioned with 5 mL 1 m K_2CO_3, followed by 10 mL deionized water. The perrhenate was eluted from the QMA-cartridge using 1 mL of 0.9 % NaCl (BBraun).

A typical ^{188}Re-labeling mixture consisted of 120 µL citrate solution (100 mg / mL), 80 µL GCK-01 precursor solution (1 mg/mL in MeCN/H_2O 50:50 v/v), 40 µL 30 % ascorbic acid solution (in water), 800 µL perrhenate in 0.9 % NaCl (postprocessed as described above, 6-12 GBq) and 48 µL $SnCl_2$ (50 mg/mL in 1 m HCl). The pH of the mixture was usually 2.0-3.5. The mixture was heated at 96 °C for 60 min. After cooling to ambient temperature, the mixture was neutralized to a pH 0f 7.5 using 0.5 M sodiumphosphate, heated for additional 5 min at 96 °C, diluted with 1 mL 0.9 % NaCl and passed through a SepPak plus light tC18 cartridge (preconditioned with 5 mL Ethanol and 10 mL water). The cartridge was washed with 2-3 mL 0.9 % NaCl and the product eluted with 1 mL 70 % EtOH. The solution containing the product was diluted 1:9 into PBS (prepared from 9 mL 0.9 % NaCl and 1 mL sodiumphosphate concentrate; BBraun *ad injectabilia*, both) containing 2 % sodium ascorbate solution. The (radiochemical) yield was determined by division of the isolated product activity by the starting activity. The radiochemical purity was determined by radio HPLC for the isolated product (after cartridge separation and formulation).

Preclinical evaluation

In vitro and toxicological evaluation of ^{99m}Tc-/^{188}Re-PSMA-GCK01. The evaluation has been conducted by well-known standard methods *(16,17)*. Detailed information is provided in the supplementary information.

Cellular uptake experiments were conducted in analogy to a previously described procedure (*16*). A detailed description is provided in the supplementary information.

In vivo and organ distribution experiments. All animal experiments were conducted in compliance with the current laws of the Federal Republic of Germany (Animal license number: 35-9185.81/G-127/(18). For *in vivo* planar imaging and organ distribution experiments, 8-week-old BALB/c *nu/nu* mice (male) were subcutaneously inoculated in the left shoulder 6 million LNCaP cells in 50 % Matrigel (Corning) in Opti-MEM I medium. The studies were performed when the tumor size reached approx. 1 cm^3 (8-12 weeks after inoculation). Mean body weight was 23 ± 2 g on the day of investigation.

In vivo planar imaging. For the in-vivo planar imaging, 100 µL of a formulation containing 5-10 MBq ^{99m}Tc-labelled compound in PBS (approx. 0.1 µg precursor, 1 nM, 5-10 MBq/nM) was injected into the tail vein of a LNCaP tumor bearing mouse (n = 1). The animal was anesthetized with Isoflurane (Abbvie, Wiesbaden, Germany), placed on the Gamma IMAGER – S/C (Paris, France) in prone position to perform planar imaging (using Gamma Acquisition und GammaVision+ software). The scan was started directly after administration of the activity and the mouse was scanned for 10 minutes. The scan was repeated after 30, 90, 180 minutes and 24 hours. An activity standard (approx. 1 MBq of the respective tracer) was prepared in a closed HPLC-sample flask and placed next to the animal during all timepoints of the measurement.

Ex vivo organ distribution with ^{188}Re-PSMA-GCK01. For *ex-vivo* biodistribution, LNCaP tumor bearing mice were injected with 100 µL of a PBS-formulation containing approx. 1 MBq of the respective ^{188}Re-PSMA-GCK01 (approx. 0.1 µg precursor, 1 µg precursor / mL), each. The animals were sacrificed by CO_2-asphyxiation at 1 h p.i. and 3 h p.i., respectively. Organs of interest were dissected, blotted dry, weighted and the radioactivity was determined on a gamma counter (Packard Cobra II, GMI, Minnesota, USA) and calculated as % ID/g.

Clinical imaging and therapy

After written informed consent had been obtained, three patients with metastatic castration-resistant prostate cancer received PSMA-RLT and the related companion diagnostic under compassionate care regulations. Prospective

clinical trial registration is not required for compassionate care that is performed under individual medical indication. The ethical committee of the University Hospital Heidelberg approved the retrospective evaluation (permission S-732/18). The patients were Gleason score 9 (N=2) or 10 (N=1), all were metastatic to lymph-nodes and bone but without visceral lesions, all had previously received standard androgen-deprivation therapy, abiraterone/prednisolone and docetaxel, N=2 had additionally received enzalutamide and N=1 apalutamide, N=2 had additionally received cabazitaxel, all were BRCA1/2-wildtype and naïve to PARP-inhibitors, no one was a promising candidate for $^{223}RaCl_2$ (low uptake in bone-scan or bulky lymph-nodes). One patient had previously received Ipilimumab/Nivolumab and 2 cycles of ^{177}Lu-PSMA-617, the other two patients were ^{177}Lu-PSMA-naïve.

To demonstrate target positive disease a diagnostic scan was performed approx. 1 week in advance of therapy using 600 MBq ^{99m}Tc-PSMA-GCK01 (molar activity: 75-125 MBq/nmol) and images were acquired 2-4 h p.i. with an Low-Energy-High-Resolution collimator at 140 keV +/- 10% photopeak (E.Cam, Siemens, 18 cm/min). At the day-1 of therapy 3.7 GBq ^{188}Re-PSMA-GCK01 (molar activity 56-112 MBq/nmol) were administered, followed by serial planar scans (20 min – 48 h as clinical available) centered at the 155 keV (+/-10%) photopeak, however with a High-Energy collimator due to down-scatter of up to 2.12 MeV Bremsstrahlung (E.Cam, Siemens, 18 cm/min). After 1-2 d (approx. 2-3 physical half-lifes of ^{188}Re), when septum-penetration of Bremsstrahlung became negligible, 3.7 GBq ^{177}Lu-PSMA-617 was injected and serial images were acquired with a Medium-Energy collimator; to avoid cross talk with the primary 155 keV photons of Re-188 only the upper photopeak of Lu-177 at 210 keV (+/-10%) was used. Dual photo-peak imaging within such a short time interval enables an intra-individual comparison of two therapeutic ligands, while relevant treatment-related effects have not yet to be considered. The timeline of imaging time-points is illustrated with Figure 2. However, under these circumstances no scatter-subtraction techniques could be applied to obtain sufficient quantitative data.

RESULTS

Chemistry and Radiochemistry

Precursor. The identity of the precursor was confirmed by HPLC-MS m/z = 977.386 (calc. m/z ($[M+H^+]^+$) = 977.392) and m/z = 999.367 (calc. m/z ($[M+Na^+]^+$) = 999.374). The purity was analyzed by HPLC and was larger than 95 %. The only detectable impurity was the oxidized disulfide. Details are provided in supplementary Figures 1-3.

^{99m}Tc-PSMA-GCK01. The radiosynthesis of ^{99m}Tc-PSMA-GCK01 reliably delivered the product in radiochemical yields of 81±3 % and purities above 97,8±0,7 % after cartridge separation (n = 8). Molar activity was in the range of 75-125 MBq/nmol. Residual TCEP was removed quantitatively by cartridge separation. No signs of degradations were observed over a period of 7h. Details are provided in supplementary Figures 4 and 5.

^{188}Re-PSMA-GCK01. After Rhenium-188 postprocessing approx. 79±6 % of the total activity was retrieved in 1 mL of saline without any detectable tungsten-188 breakthrough (n = 9). Subsequent labeling yielded ^{188}Re-PSMA-GCK01 in radiochemical yields of 78±3 % and with a radiochemical purity of more than 96±3 % (n = 6). Molar activity was in the range of 56-112 MBq/nmol. Only minor signs of degradation were observed after 3 h (approx. 3 % degradation of RCP). Details are provided in supplementary Figure 6.

Preclinical evaluation

In vitro evaluation. The binding characteristics of PSMA-GCK01 and HYNIC-iPSMA were evaluated with the respective ^{99m}Tc-labeled tracers; the results are summarized in Table 1. ^{99m}Tc-PSMA-GCK01 showed a plasma protein binding of 98 %. The RCP of the free fraction showed no signs of degradation over 4h (by repetitive HPLC measurements, see supplementary Figure 7).

In vivo and organ distribution experiments. The images acquired by *in vivo* planar imaging are shown in Figure 3. ROI analysis and standardization on the internal standard provided a rough estimation of the observed uptake values of ^{99m}Tc-PSMA-GCK01.

The results of the organ distribution of ^{188}Re-PSMA-GCK01 are depicted in Figure 4: The tumor uptake of the ligand is approx. 5 % ID/g at 1 h p.i. rising to approx. 11 % ID/g at 3 h p.i.. Besides, the ligand showed an uptake of 70 % ID/g (1 h p.i.) and 91 % ID/g (3 h p.i.) in kidneys and 11 % ID/g (1 h p.i.) and approx. 4 % ID/g (3 h p.i.) in the spleen. Moreover, renal excretion is reflected by urine "uptake" of 36 % ID/g (1 h p.i.) and 71 %ID/g (3h p.i.), respectively. All further organs showed only minor tracer uptake. Detailed results are provided in the supplementary information.

Toxicological investigation. No test article-related mortality was observed. No difference in organ weights, or macroscopic observations were made at terminal or recovery sacrifice.

Clinical imaging and therapy

In the three compassionate care patients, no acute adverse events were observed neither following injection of ^{99m}Tc- nor ^{188}Re-PSMA-GCK01. Visually, the gross biodistribution of diagnostic ^{99m}Tc-PSMA-GCK01 was similar to all other low-molecular-weight scintigraphic PSMA-ligands that have been developed previously (Figure 5a), with combined renal and hepatointestinal clearance, non-target accumulation in salivary glands and low perfusion dependent background in the remainder organs (*8,9,18,19*). Considering the limitations that the mono-energetic 140 keV pure gamma-emitting ^{99m}Tc-PSMA-GCK01 was imaged with a LEHR collimator but ^{188}Re-PSMA-GCK01 had to be measured with a HE collimator to cope with the high level of scatter and bremsstrahlung (up to 2.12 MeV) in relation to the only 15% co-emission probability of 155 keV photons, the tagged radionuclide had no obvious influence regarding the biodistribution at 2-4 h p.i., respectively (Figure 5). Already 20 min p.i., the intensity of tumor targeting exceeds the intra-vascular blood-pool and delineation of the bladder demonstrates moderate clearance kinetics. Late images beyond 20 h p.i. demonstrate prolonged trapping in tumor lesions, additional hepatobiliary clearance into the intestine and low residual uptake in other organs (Figure 5b), thus in some degree PSMA-GCK01 is following a similar tumor-accumulation and excretion kinetics comparable with other Glu-Urea-based PSMA-ligands such as MIP1095, PSMA-617 or PSMA-I&T (*20,21,22*).

Using the distinct photopeaks of Re-188 at 155 keV, 48 h later followed by Lu-177 imaged at 210 keV an intra-individual comparison between ^{188}Re-PSMA-GCK01 and ^{177}Lu-PSMA617 within a 2 day interval is demonstrated in Figure 6. While tumor-targeting is almost equal at 20-24 and 44-48 h p.i., the PSMA-GCK01 demonstrates initially a higher liver-to-kidney uptake ratio which translates into better delineation of the intestine at 48 h p.i., which might imply a slightly shift from renal to hepato-intestinal clearance for PSMA-GCK01 compared to PSMA-617.

DISCUSSION

The focus of our investigation was the development of a PSMA-ligand suitable for ^{99m}Tc- and ^{188}Re-labeling based on the HYNIC-iPSMA lead structure. Since the suitability of HYNIC for ^{188}Re-labeling has been discussed controversially (*6*), we replaced the HYNIC unit with a similar-in-size spacer, linked to mercaptoacetyltriserine sequence as favorable chelator for radiolabeling with both Tc-99m and Re-188 (Figure 1). The precursor synthesis was obtained in high purity of more than 95 %. The only detectable “impurity” was the oxidized disulfide derivate (supplemental Figures 2 and 3), which is reduced to the desired precursor under the reductive labeling conditions.

The technetium labeling using TCEP as reducing agent delivered the product ^{99m}Tc-PSMA-GCK01 in high and reproducible yields and purities. In general, the radiochemical purity would allow for direct application of the product mixture containing the tracer. However, for clinical formulation we applied a cartridge separation of the tracer to get rid of any residual TCEP, which was confirmed by HPLC analysis (supplemental Figure 5). The reducing agent may impose a potential limitation: For broader application a kit should be developed using the more commonly applied $SnCl_2$ as reducing agent.

In case of the ^{188}Re-labeling the desired ^{188}Re-PSMA-GKC01 was produced in yields of approx. 75% (isolated after synthesis and purification) using relatively harsh conditions (low pH). The results are in good agreement with previous reports on such reactions (*23*). However, under these conditions the product is formed in two stereoisomers (supplemental Figure 6). We eventually converted the undesired isomer by terminal elevation of the pH to 7.0-7.5 and a short additional heating period. In case of the ^{99m}Tc-labeling the pH was sufficiently high to suppress formation

of the isomer. Thus, and since no isomer formation was observed in the plasma stability of ^{99m}Tc-PSMA-GCK01 (see below), we do not consider the potential isomer formation as drawback.

For the preclinical evaluation we first compared our ligand ^{99m}Tc-PSMA-GCK01 with ^{99m}Tc-EDDA/HYNIC-iPSMA in a cell based assay: Both compounds showed a comparable and specific uptake in LNCaP cells. Further displacement experiment with ^{99m}Tc-PSMA-GCK01 also revealed a comparable Ki of 26 (vs. 38 for ^{99m}Tc-EDDA/HYNIC-iPSMA). The ligand showed a relatively high plasma-protein binding of 98%. However, it is well-known for other PSMA ligands that high plasma-protein binding does not necessarily impose a problem for clinical application, recently even dedicated albumin-binding motifs have been suggested as an improvement when PSMA-ligands should be labeled with long physical half-life nuclides (*24,25*). More importantly, the free fraction in plasma did not show any signs of decomposition over a period of 4 hours. Unfortunately, we were not able to confirm this for the theranostic Rhenium tandem ^{188}Re-PSMA-GCK01 due to insufficient count rate (only 15,5 % probability of 155 keV emissions) in the respective HPLC samples. Hence, we decided to evaluate our ligands in a suitable animal model. In planar imaging with ^{99m}Tc-PSMA-GCK01, the ligand presents promising tumor uptake and retention in the LNCaP xenotransplant and moderate renal clearance according to semiquantitative ROI analysis. Quantitative data was obtained by further organ distribution experiments with the Rhenium analogue ^{188}Re-PSMA-GCK01: As expected, the results reflected the planar imaging quite well. Already at 1 h p.i. we observed intense tumor uptake reaching approx. 11 %ID/g at 3 h p.i.. In mice only renal clearance but minimal uptake in the liver was observed; However, it was already reported for PSMA-617 that renal vs. hepato-intestinal clearance is not comparable between animal-studies and human application (*14,26*). In sum, preclinically we achieved our goal to develop a promising PSMA-ligand for labeling with both Technetium and Rhenium for theranostic application. A potential limitation of the current preclinical study is the missing of a late timepoint (e.g., 24 or 48 h) in the organ distribution and a histopathological evaluation of eventual radiation-induced kidney toxicity of ^{188}Re-PSMA-GCK01 in mice. However, a dedicated clinical dosimetry study is already in preparation (including an initial extrapolation from ^{99m}Tc-PSMA-GCK01 to ^{188}Re-PSMA-GCK01), which is probably more predictive than the mice-to-men extrapolation that are otherwise needed for non-radioactive therapies. Another potential limitation is the missing of ^{99m}Tc-PSMA-GCK01 organ distribution data. We considered this data facultative since analogy of ^{99m}Tc-/^{188}Re-radiopharmaceuticals is widely accepted (see e.g., Ref 4) and consequently we preferred to reduced our demand for laboratory animals.

Toxicological investigation of PSMA-GCK01 according to current OECD guideline was ordered by a third-party preclinical research organization and revealed no toxicological effect up to 2 mg/kg in mice. Based on this data we conclude that GCK01 has a good safety profile and application of up to 2 µg/kg GCK01 in human will likely be well tolerated.

During compassionate use the promising tumor-targeting and acceptable fast clearance kinetics was confirmed in human beings. Dual photopeak-imaging enabled intra-individual comparison with the current standard-of-reference compound ^{177}Lu-PSMA-617. Our preliminary investigation suggests that ^{188}Re-PSMA-GCK01 and ^{177}Lu-PSMA-617 share the combined renal and hepato-intestinal clearance route and a relatively similar biodistribution between 2 h p.i. and-20 hi. However, by using a double-isotope imaging protocol, a quantitatively reliable treatment dosimetry could not be approximated, yet. Nevertheless, the novel ligands offer some promising benefits, which we would summarize as follows:

- Availability of a completely generator based theranostic tandem to the currently available ligands will help to facilitate PSMA-RLT, in particular in countries / regions with a less developed nuclear infrastructure. Moreover, it may help to reduce cost of PSMA-RLT.

- $^{188(/186)}$Re-PSMA-RLT might amend ^{177}Lu-PSMA-RLT in a “mixed nuclide therapy” with potential benefits in offering additional beta-emission energy profile (β_{mean}(Re-188) = 765 keV, β_{mean}(Re-186) = 347 keV, β_{mean}(Re-Lu-177) = 133 keV) improving therapy of bulk lesions (*27,28*).

- Shorter half-life and lower energy of gamma emission (Re-188: 16.9 h, 155 (15 %); Re-186: 3.7 d, 137 keV (9%); Lu-177: 6.7 d, 113 keV (6%) & 208 (10 %)) may disclose therapy in an outpatient setting eventually helping to circumvent the expected bottleneck in bed capacity of currently existing nuclear medicine departments after ^{177}Lu-PSMA-617 (half-life 6.7 d) approval (*29*).

In summary, we consider the novel PSMA-ligands ^{99m}Tc-/^{188}Re-PSMA-GCK01 a versatile and promising supplement in the currently available PSMA-ligand landscape. In particular, the broad availability of different therapeutic nuclides may lead to interesting synergetic effects, which cannot be predicted by now. A phase I/II clinical

trial with the novel ligands including dosimetry study is currently in preparation. The additional potential which may arise from the ligand ^{186}Re-GCK01 is still to be disclosed.

CONCLUSION

PSMA-GCK01 is characterized by robust labeling with both radionuclides of the theranostic ^{99m}Tc-PSMA-GCK01 / ^{188}Re-PSMA-GCK01 tandem, hence, they can be produced in high radiochemical yields using standard methodologies, respectively. Preliminary experiences in patients with metastatic castration-resistant prostate cancer were promising and thus further investigation of ^{99m}Tc/^{188}Re-PSMA-GCK01 in a prospective phase-1 trial was already initiated.

DISCLOSURE

FLG, CK, UH and JC are holding a patent application on PSMA-GCK01. This project was supported by a research grant from Telix Pharmaceuticals Ltd.. FLG is advisor at ABX Radiopharmaceuticals, SOFIE Biosciences, Telix pharma and Alpha Fusion. The toxicological study at Agilex Biolabs was sponsored by Telix Pharmaceuticals Ltd..

ACKNOWLEDGMENTS

We thank the working group Haberkorn at Otto-Meyerhof-Zentrum Heidelberg – in particular Annette Altmann, Marlene Tesch and Vanessa Kohl– for conducting the preclinical investigations. We thank Dr. Alesia Ivashkevich and Dr. Michael Wheatcroft for their support and assistance of the toxicological study.

KEY POINTS

Question: Can we prepare a PSMA ligand suitable for ^{99m}Tc- and ^{188}Re-labeling based on HYNIC-iPSMA as lead structure?

Pertinent Findings: An exchange of the chelator moiety to MAS_3 yielded PSMA-GCK01, a ligand suitable for ^{99m}Tc- and ^{188}Re-labeling maintaining the good binding characteristics of HYNIC-iPSMA.

Implications for patient care: PSMA-GCK01 offers a suitable platform for the decentralized production of the theranostic tandem for prostate cancer patients. In particular, it provides the basis for diagnosis of PCa in smaller nuclear medical centers limited to SPECT and may help to circumvent potential bottlenecks in the Lutetium-177 supply chain.

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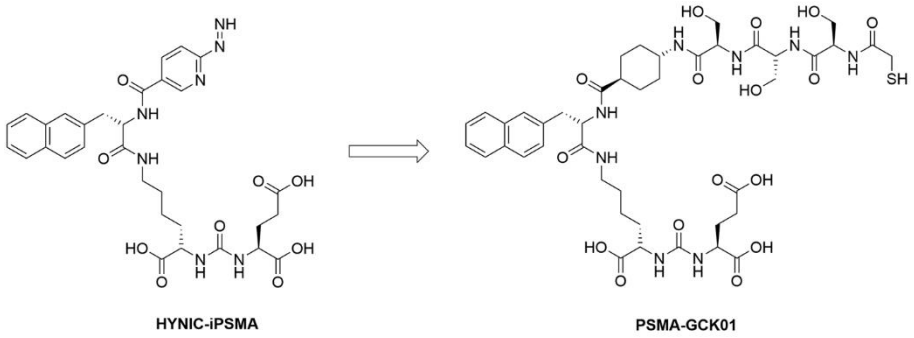
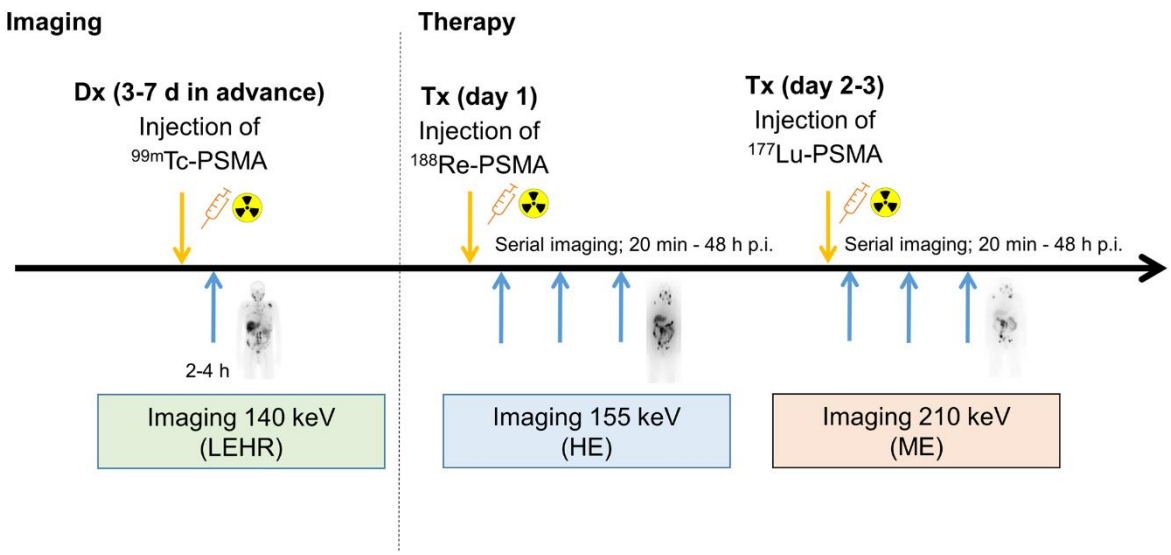


Figure 1: Structures of HYNIC-iPSMA and PSMA-GCK01



391

392 *Figure 2: Dual-photo-peak imaging for intra-individual comparison of Re-PSMA-GCK01 versus Lu-*
393 *PSMA617 PK*

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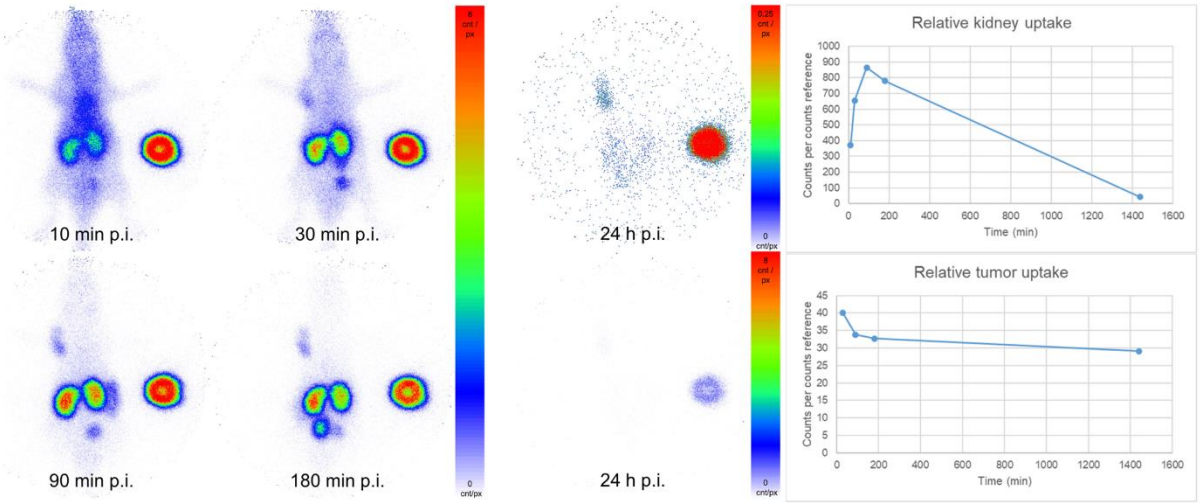

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396 *Figure 3: Planar imaging of ^{99m}Tc-PSMA-GCK01 in a LNCaP tumor bearing mouse (Red spot at the right*
397 *side is an internal standard).*

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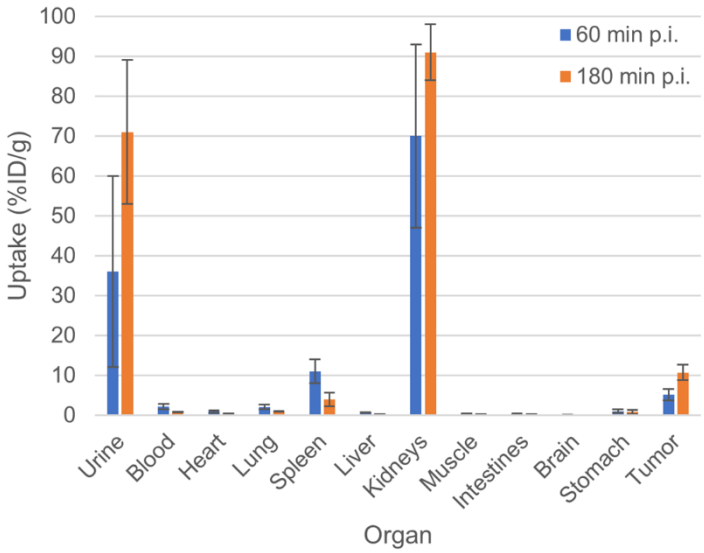
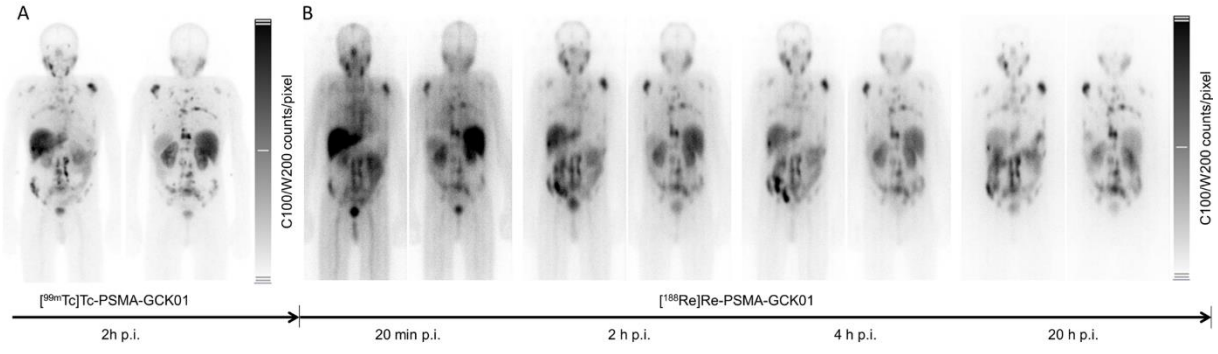


399

400 *Figure 4: organ distribution of approx. 1 MBq ^{188}Re-PSMA-GCK01 in LNCaP tumor bearing mice (n = 3 per*
401 *timepoint). Exact values are provided in supplementary Table 1.*

402



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404 *Figure 5: ^{188}Re-GCK01 PK (sequential imaging)*

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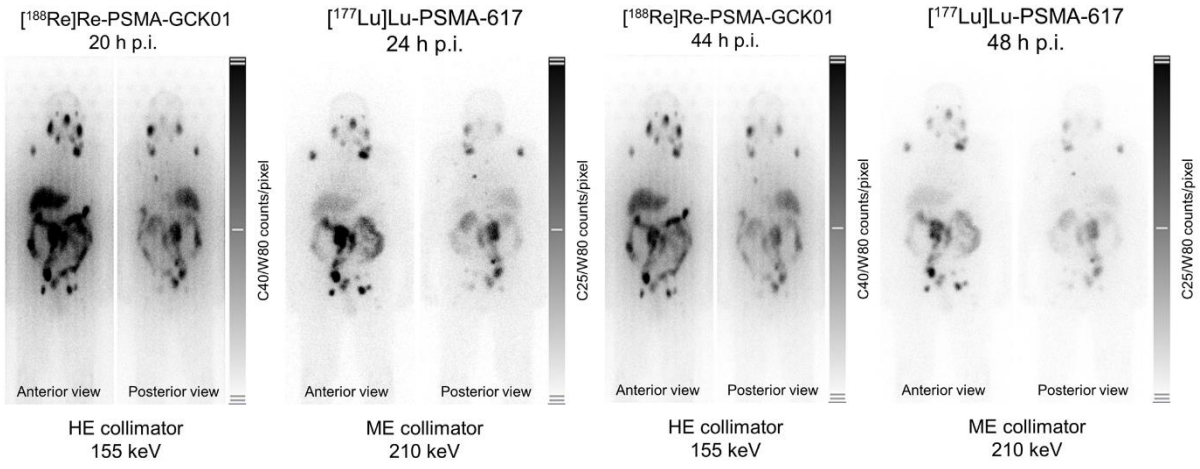

406

407 *Figure 6: Intra-individual comparison of 3.7 GBq Lu-PSMA-617 and 3.7 GBq Re-PSMA-GCK01 at 20-24 and*
408 *44-48h p.i., respectively.*

409

410 *Table 1: Binding characteristics of ^{99m}Tc-EDDA/HYNIC-iPSMA and ^{99m}Tc-PSMA-GCK01*

Substance	Uptake [%AD/10^6cells]	Unspecific uptake [%AD/10^6cells]	Specific uptake [%AD/10^6cells]	Ki [nm]
^{99m}Tc-EDDA/HYNIC-iPSMA (reference compound)	20.3±0.3	1.64±0.07	18.7±0.3	38
^{99m}Tc-PSMA-GCK01	19.6±4.8	1.2±0.6	18.4±4.2	26

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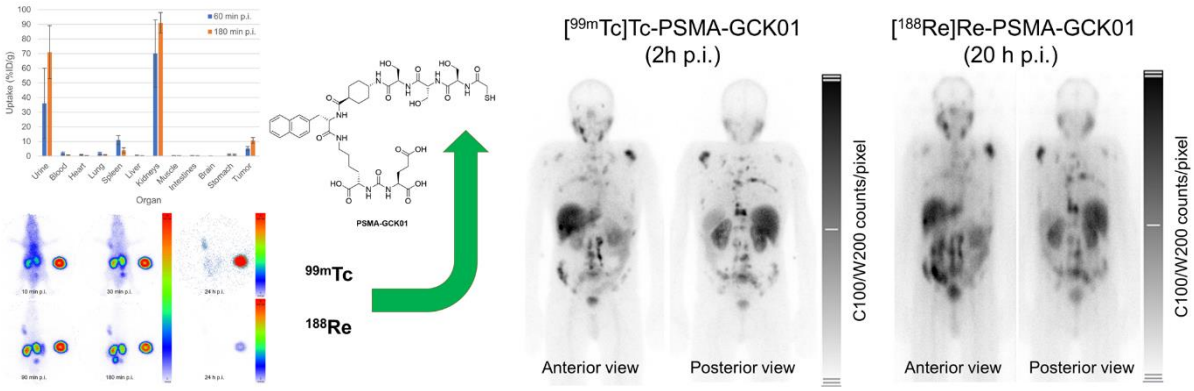

413

414 Graphical Abstract

PSMA-GCK01 - A Generator-Based ^{99m}Tc-/^{188}Re-Theranostic Ligand for the Prostate-Specific Membrane Antigen

Supplementary information

Contents

S1: Preparative Methods & Cell Culture

Preparation of the Precursor

The synthesis of the pharmacophore was accomplished by a well-known procedure: The isocyanate of the glutamyl moiety was generated *in situ* by adding a mixture of 3 mmol of bis(tert-butyl) L-glutamate hydrochloride and 1.5 mL of N-ethyldiisopropylamine (DIPEA) in 200 mL of dry CH_2Cl_2 to a solution of 1 mmol triphosgene in 10 mL of dry CH_2Cl_2 at 0°C over 4 h. After agitation of the reaction mixture for 1 h at 25°C, 0.5 mmol of the resin-immobilized (2-chloro-tritylresin) ε-allyloxycarbonyl protected lysine in 4 mL DCM was added and reacted for 16 h with gentle agitation. Subsequently the resin was filtered off and dried.

For the synthesis of the precursor, an aliquot of the resin carrying approx. 50 µmol pharmacophore was used. For deprotection, the resin was swollen in CH_2Cl_2 (Dichloromethane, DCM) and reacted with a mixture of 10 mg $(PPh_3)_4Pd^0$ and 60 mg dimethylaminoborane in 3 mL DCM for 15-30 minutes. Subsequently the resin was washed with DCM, 5% aminoethanol in DCM (5 min shaking), Methanol, Dimethylformamide (DMF) (3-5 times each).

The linker was build-up by means of standard solid phase peptide synthesis (SPPS) using fluorenylmethoxycarbonyl (Fmoc) as protective group. Each coupling was conducted with 3 equivalents of the respective Fmoc protected aminoacid, 2.96 equivalents of HATU and 8-12 equivalents of diisopropylamine (DIPEA) for 30 minutes at room temperature under agitation in DMF. Removal of the fmoc group was conducted by reaction with 20 % piperidine in DMF for 5 minutes at room temperature (3 times each). Between the individual steps, the resin was washed with DMF (5 times each).

The chelator consists of 3 D-Serines (tert-Butyl protected OH) and a termial mercaptoacetyl group and was build using the same procedure as described for the linker. The coupling of the mercaptoacetylgroup was conducted using acetyl protected 2-mercaptoacetic acid (same procedure as for the linker/chelator). For removal of the terminal acetyl-protective group, the solvent was changed to acetonitrile (MeCN) and the deprotection was conducted with 35 µl hydrazinehydrate in 2 mL MeCN for 10-20 minutes at room temperature under agitation. Then the resin was washed with MeCN, DMF and DCM (5 times each). Finally, the compounds were cleaved from the resin using TFA containing 2.5 % water and 2.5 % triisopropylsilane (TIS) (2-3 mL) for 30-45 min at room temperature. The cleavage cocktail was filtered, diluted with 20 mL DCM and the solvents removed under reduced pressure. The crude product was purified by preparative HPLC. Identity of the compounds was confirmed by HPLC-MS (Figure S1) and the purity observed by HPLC (Figure S2).

Preparation of [^{99m}Tc]Tc-PSMA-GCK01 for in vitro analysis

Phosphate buffer was prepared from 890 mg $Na_2PO_4 \cdot 2\ H_2O$ in 9.5 mL water for injection and 0.5 mL 2 m NaOH. After dissolution of the salts, the buffer was sterile filtered, the pH was determined using pH stripes (pH = 11.5-12.0). The labeling mixture consisted of 1 µl precursor solution (1 mg / mL in MeCH/H_2O 20:50; approx. 1 nmol), 20 µl phosphate buffer, 10 µl tris-carboxyphenylphosphin (TCEP; 28.7 mg / mL in phosphate buffer; 0.1 molar solution) and 4-10 µL pertechnetate in saline (0.9 % NaCl; generator eluate) containing an activity of approx. 7.5 MBq. The mixture was filled to 100 µl with saline (0.9 % NaCl; 59-65

µL). The pH of the reaction mixture was 8.0-8.5 (tendency towards 8.5). The mixture was heated at 98 °C for 10 minutes. After cooling to room temperature, the mixture was diluted to 1 mL by addition of saline (0.9 % NaCl, ligand concentration approx. 1 µm). An aliquot was analyzed by HPLC to determine the radiochemical yield (RCY) (Figure S4). Aliquots of the product mixture containing the ^{99m}Tc-labeled ligand were further diluted to a concentration of approx. 100 nM (precursor) in PBS with and without a 10000 fold excess of 2-(Phosphonomethyl)-pentandioic acid, 2-Phosphonomethyl pentanedioic acid (2-PMPA) as competitor.

Cellular uptake and competitive binding

LNCaP (lymph node carcinoma of the prostate; CRL-1740; DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) cells were seeded in 6-Well plates in RPMI medium containing 20 % FBS (Pan Biotech, Aidenbach, Germany) and grown to a confluency of 70-80 % in an incubator at 37°C with humidified air equilibrated with 5% CO_2 (approx. 2 days).

Prior to the uptake experiment, the medium was removed and the cells were incubated with 1 mL of a 1 nm ligand solution prepared by 1:99 dilution in RPMI medium of the 100 nM ligand formulations with and without competitor for *in vitro* analysis (see supporting information). After 1 h the medium containing the ^{99m}Tc-ligand was removed, the cells were washed twice with 1 mL PBS and lysed twice using lysis buffer (0.3 M NaOH containing 0.2 % SDS; 700 µL each). The cellular uptake was determined from the activity in the lysed fraction. The unspecific uptake was determined from the cellular uptake of the cells incubated with the ligand in presence of the competitor. Each experiment was conducted as triplicate.

For determination of Ki, competitive binding experiments were conducted. Four six-well plates were prepared as described above. After removal of the medium the cells were incubated with 1 mL of a 1 nm ligand solution prepared by 1:99 dilution of the 100 nM ligand formulations for *in vitro* analysis (without competitor; see supporting information) in RPMI medium containing different concentrations of competitor (2-PMPA, 10E-4/-5/-6/-7/-8/-9 mol/L). Further processing was conducted as described above.

Plasma protein binding and stability

Blood was drawn from 3 volunteers and the plasma was separated by centrifugation at 2000 RPM. 400 µL Plasma were incubated at 37 °C with 100 µL ^{99m}Tc-PSMA-GCK01 (clinical protocol / approx. 150 MBq/mL).

For Plasma protein binding, 100 µL of the test solution were removed after 1 h of incubation, diluted with 0.9 % saline and an ultra-filtration was performed with 500 µL of the dilution using Amicon Ultracel 3k centrifugal filters (Merck Millipore Ltd, Corck, Ireland) in an Eppendorf Centrifuge was performed at 12k RPM. The filter was subsequently washed with 400 µL saline and the unified filtrates, as well as the filter unit were measured on a Gamma counter.

For plasma stability, 400 µL MeCN were added to 100 µL test solution for protein precipitation (after 1, 2 and 4 h of incubation). The mixture was centrifuged at 13k RPM for 5 minutes and 100 µL of the solution were pipetted into another Eppendorf vessel. The protein precipitation was completed by addition of another 100 µL MeCN and subsequent centrifugation at 13k RPM for 5 minutes. Then, the plasma free fraction was analyzed using Gamma-HPLC.

Toxicological investigation of PSMA-GCK01

A toxicological investigation of PSMA-GCK01 was conducted under full GLP by an external facility (Agilex Biolabs, Thebarton, Australia) under study number TLX-003. Briefly, animals (Healthy CD-2 mice, 6-8 Weeks old, 30-40 g) were divided into three groups and received daily repetitive dose 0.0 mg/kg (vehicle), 0.2 mg/kg (low dose) and, 2.0 mg/kg (high dose) PSMA-GCK01. The animals were either sacrificed one day (Main) or 14 days (Recovery) after receiving the final dose of PSMA-GCK01 and investigated by adequate pathological procedures. The design of this study was adapted from and is in accordance with OECD guideline for testing of chemicals (No. 407).

S2: Analytical Methods

a) Identity of the precursor (HPLC-MS)

HPLC-MS (gradient A: 0 % A (0min) 100 % A (20 min) linear gradient, 0.2 mL/min; A + B = 100 %; solvent A: MeCN + 0.1 % trifluoroacetic acid, solvent B: water + 0.1 % trifluoroacetic acid; Column: Hypersil Gold aQ 200X2.1 mm, 1.9 µm particle size).

Analysis was conducted on an Agilent Infinity 1200 System with binary pump (Bin Pump SL), autosampler (HIP ALS SC), column compartment (Col Comp), and an UV detector (VWD SC+). The system was connected to a Thermo Fisher Exactive ESI MS system. The system was controlled by Thermo Xcalibur Version 3.0.63. Confirmation of identity is assessed by comparing the mass/charge values in the resulting integrated electropherograms (Figure S1).

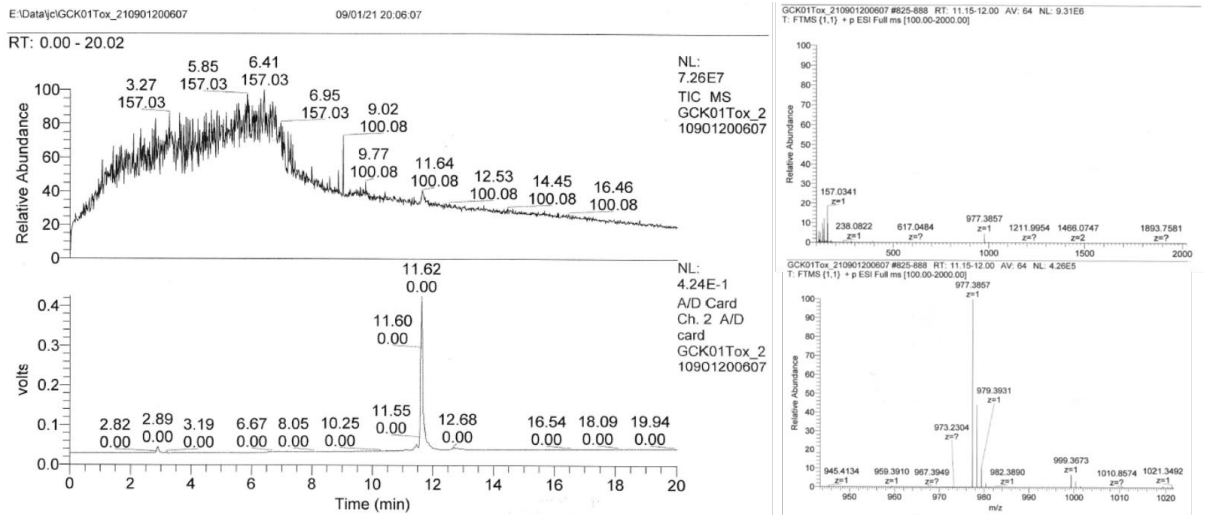

Figure S1: HPLC-MS after separation of PSMA-GCK01 precursor; Identity is confirmed by the peak at m/z = 977.386 (calc. m/z ($[M+H^+]^+$) = 977.392) and m/z = 999.367 (calc. m/z ($[M+Na^+]^+$) = 999.374)

b) *Purity of the precursor*

HPLC-gradient: 0 % A (0min) 80 % A (10 min) linear gradient, 2 mL/min; A + B = 100 %; solvent A: MeCN + 0.1 % trifluoroacetic acid, solvent B: water + 0.1 % trifluoroacetic acid; Column: Chromolith Performance C18e 100X3 mm). UV detection was carried out at 226 nm wavelength. Analysis was conducted on an Agilent Infinity 1100 System with binary pump (G1312A Bin Pump), an autosampler (G1313A ALS), and an UV detector (G1314A VWD). The system was controlled using ChemStation for LC systems Rev. D.01.03.

On the system described above the retention time of PSMA-GCK01 is 3.8±0.2min and the retention time of the corresponding disulfide 4.6±0.2 min. A sample chromatogram with visible amounts of the disulfide is shown in Figure S2. Identification of the disulfide was conducted after intentional disulfide formation by HPLC-MS (Figure S3).

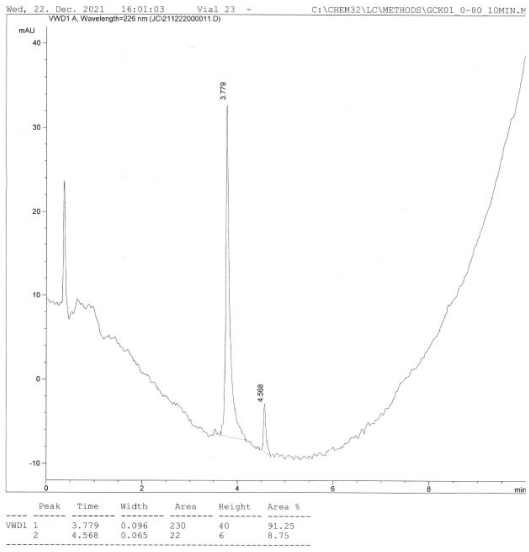

Figure S2: HPL-Chromatogram of PSMA-GCK01 in solution after some time for oxidation (disulfide formation); Identity of the disulfide was confirmed bx HPLC-MS

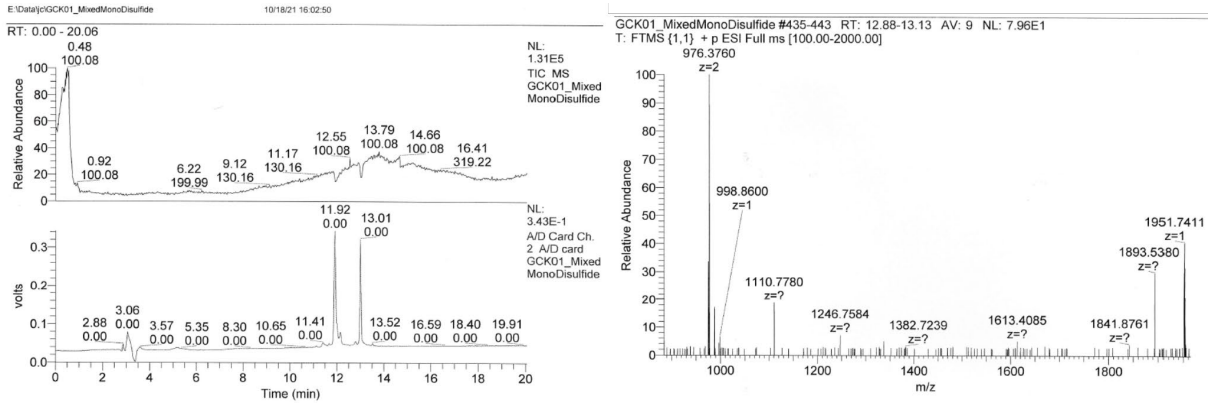

Figure S3: HPLC-MS after separation of PSMA-GCK01 precursor and intentional oxidation period (disulfide formation): Peak at 13.01 is the disulfide – The identity is confirmed by the peak at m/z = 1951.741 (calc. m/z ($[M+H^+]^+$) = 1951.761) and m/z = 976.376 (calc. m/z ($[M+2H^+]^{2+}$) = 976.384.

c) *Radiochemical purity*

HPLC-gradient: 0 % A (0min) 80 % A (5 min) linear gradient, 2 mL/min; A + B = 100 %; solvent A: MeCN + 0.1 % trifluoroacetic acid, solvent B: water + 0.1 % trifluoroacetic acid; Column: Chromolith Performance C18e 100X3 mm). UV detection was carried out at 226 nm wavelength. Analysis was conducted on an Agilent Infinity 1100 System with binary pump (G1312A Bin Pump), an autosampler (G1313A ALS), an UV detector (G1314A VWD) and a gamma-probe (Raytest, Straubenhardt, Germany). The system was controlled using ChemStation for LC systems Rev. D.01.03.

On the system described above the retention time of PSMA-GCK01 is 2.30 min, [^{99m}Tc]Tc-PSMA-GCK01 is 2.33 min and the Retention times of [^{188}Re]Re-PSMA-GCK01 are 2.12 min (undesired isomer) und 2.17 min. Sample chromatograms are shown in Figure S4.

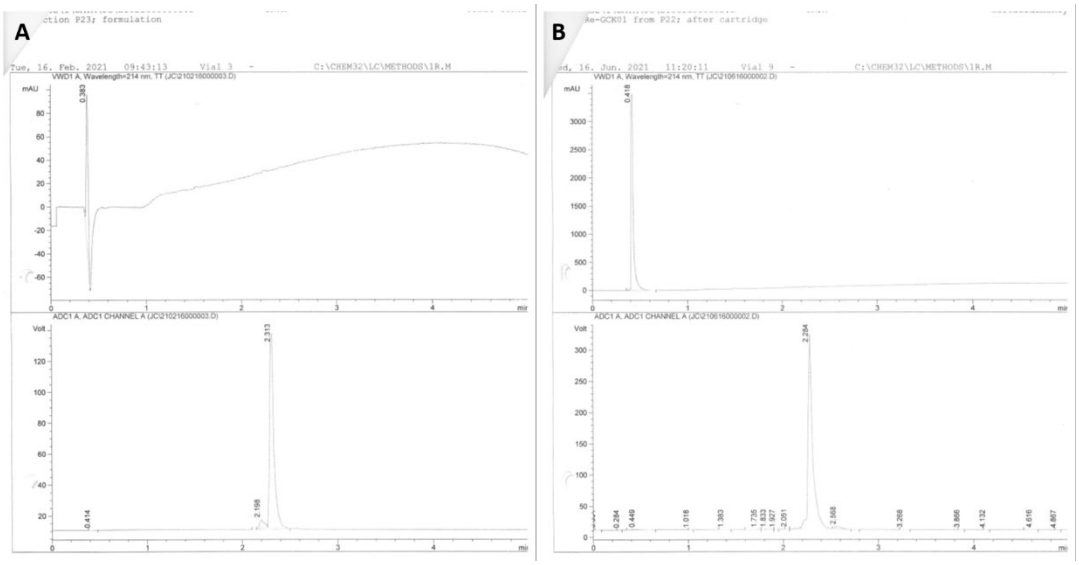

Figure S4: Typical radiochromatograms of (A) [^{99m}Tc]Tc-PSMA-GCK01 and (B) [^{188}Re]Re-PSMA-GCK01 (after formulation, each).

d) *Purity of the [^{99m}Tc]Tc-PSMA-GCK01 formulation*

Several radiochromatograms of [^{99m}Tc]Tc-PSMA-GCK01 are shown in Figure S5: First, in (A) the chromatogram directly after the conjugation reaction is shown. The presence of TCEP is causing a pronounced peak at a retention time of 0.47 minutes (UV-trace). After cartridge separation (B) (before PBS dilution, which would reduce sensitivity by a factor of 10) the TCEP peak is below LOD, demonstration quantitative separation by the cartridge purification process.

Second, example chromatograms for the shelf-life are shown in the lower part of Figure S5: In comparison to the formulation at 0h shelf-life (C), no signs of degradation were observed after approx. 7 h at room temperature (D) indicating more than 6 h shelf-life.

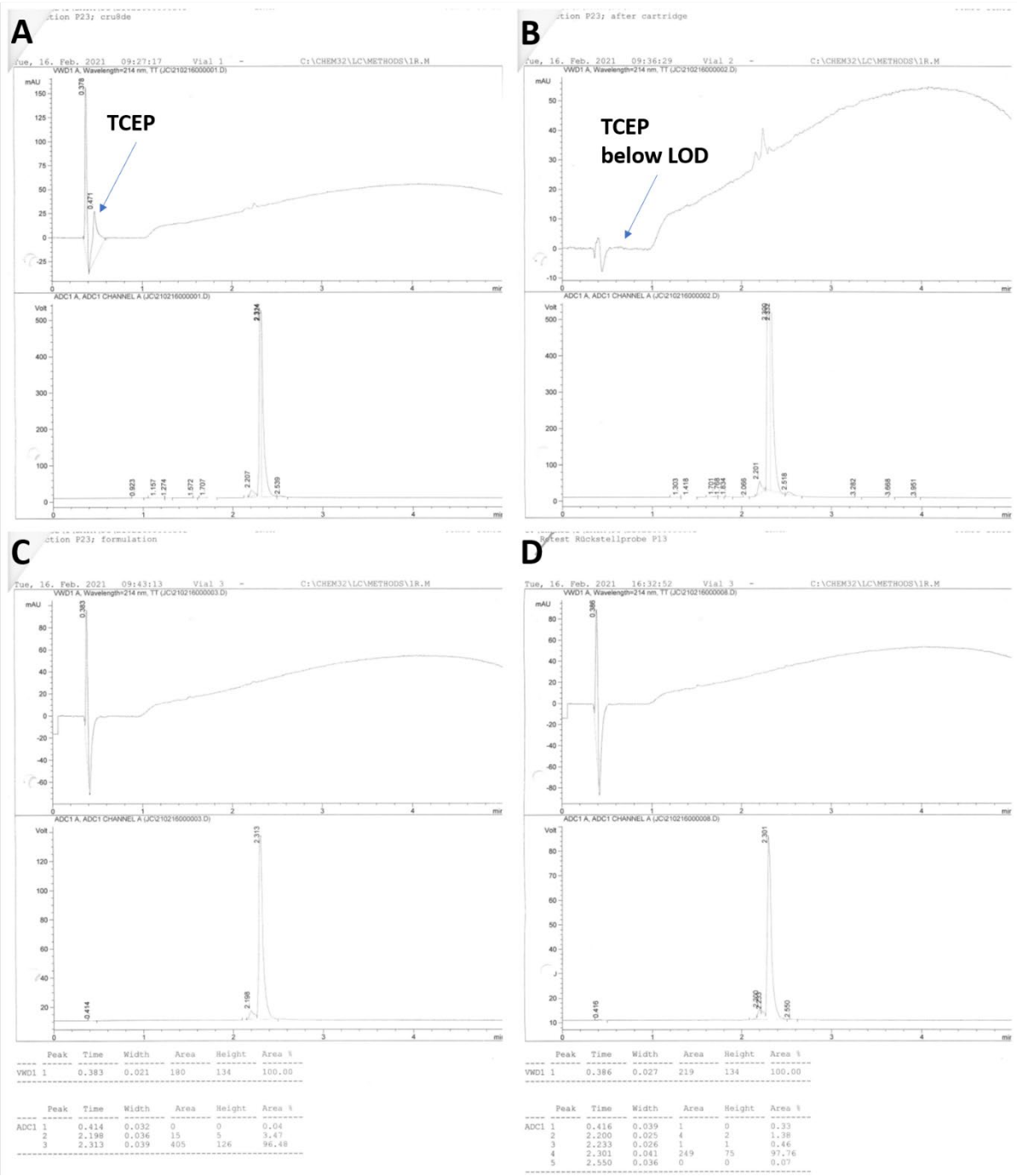

Figure S5: Radiochromatograms from [^{99m}Tc]Tc-PSMA-GCK01 before (A) and after (B) cartridge separation, formulation (C) and formulation after 7 h at room temperature (D).

e) Purity of the [^{188}Re]Re-PSMA-GCK01 formulation

The ^{188}Re-labeling of MAS_3 derivates (including PSMA-GCK01) does lead to a mixture of diastereomers. This is shown in Figure S6 A and D. The product at 2.57 min is most likely an incompletely coordinated intermediate. However, by heating the mixture at elevated pH values

(above 7.0) the tracer can be converted to a single isomer, which was used for evaluation of [^{188}Re]Re-PSMA-GCK01. Since the RCY of [^{188}Re]Re-PSMA-GCK01 after this conversion step is higher than the RCP of both diastereomers at 2.92-3.32 min (data not shown), we rationalized that the third peak is incomplete coordination. It should be noted that this product is only partially converted to the desired [^{188}Re]Re-PSMA-GCK01, while a fraction dissociates to free Rhenium. Therefore, cartridge separation is conducted after the conversion step.

An example chromatogram for the shelf-life is shown in Figure S6 (C): Approx. 2.8 % degradation were observed in this case over a period of 3 h at room temperature.

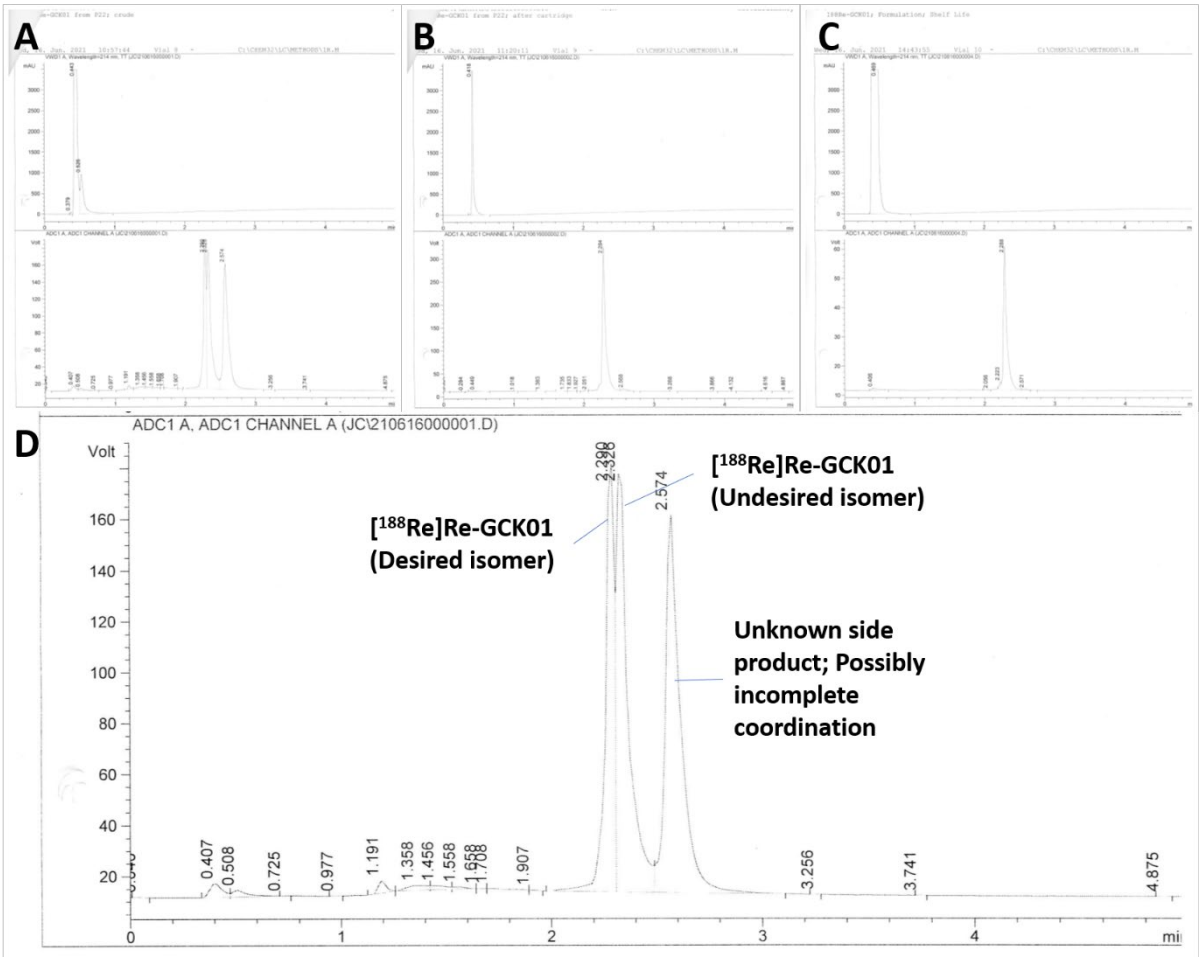

Figure S6: Radiochromatograms from [^{188}Re]Re-PSMA-GCK01 after ^{188}Re-labeling (A) and after cartridge separation (B), formulation after 3 h at room temperature (C) and zoom in chromatogram A (D)

f) Plasma Stability of [^{99m}Tc]Tc-PSMA-GCK01

Some results from the plasma-stability experiment with [^{99m}Tc]Tc-PSMA-GCK01 are shown in Figure S7: No signs of degradation were observed for the plasma free fraction at 60 min (A), 120 min (B) and 240 min (C) incubation time.

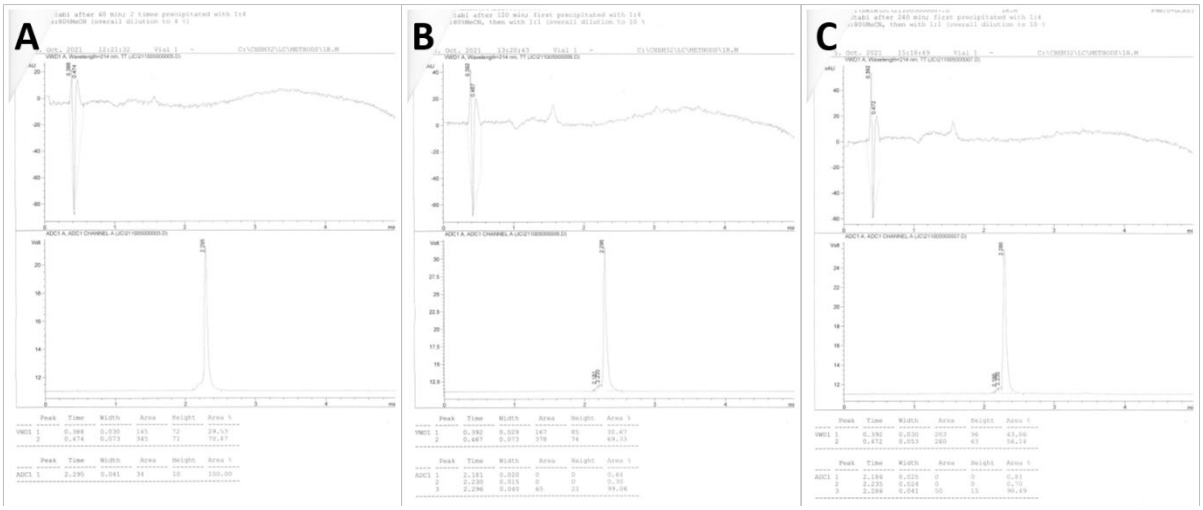

Figure S7: Radiochromatograms from the plasma stability experiment of [^{99m}Tc]Tc-PSMA-GCK01

S3: Detailed results of the organ distribution experiment

Table S1: Results of the ex-vivo biodistribution after injection of approx. 1 MBq of the respective [^{188}Re]Re-PSMA-GCK01

Organ	%ID/g 1 h p.i.	%ID/g 3 h p.i.
Urine	36±24	71±18
Blood	2.1±0.7	0.74±0.08
Heart	0.9±0.3	0.46±0.03
Lung	2.0±0.6	1.01±0.08
Spleen	11±3	3.9±1.7
Liver	0.68±0.05	0.28±0.01
Kidneys	70±23	91±7
Muscle	0.3±0.1	0.18±0.06
Intestines	0.36±0.06	0.24±0.04
Brain	0.04±0.02	0.030±0.004
Stomach	1.0±0.5	0.8±0.5
Tumor	5.1±1.4	10.7±1.9