Preclinical Evaluation of a Bispecific Low-Molecular Heterodimer Targeting Both PSMA and GRPR for Improved PET Imaging and Therapy of Prostate Cancer

Matthias Eder,1* Martin Schäfer,1 Ulrike Bauder-Wüst,1 Uwe Haberkorn,2 Michael Eisenhut,1 and Klaus Kopka1

1Division Radiopharmaceutical Chemistry, German Cancer Research Center (DKFZ), Heidelberg, Germany
2Department of Nuclear Medicine, University of Heidelberg, Heidelberg, Germany

BACKGROUND. It has recently been reported that metastases of prostate cancer usually show highly heterogeneous or partly lost prostate-specific membrane antigen (PSMA) expression. In order to image and treat both PSMA positive and negative tissues PSMA targeting probes need to be extended by a further specificity. Since prostate cancer cells usually express both PSMA and gastrin-releasing peptide receptor (GRPR) a bispecific low-molecular heterodimeric molecule, addressing both targets at the same time, may significantly improve prostate cancer imaging and therapy.

METHODS. The nonapeptide BZH3 representing the GRPR binding part was combined with the urea-based PSMA inhibitor Glu-urea-Lys(Ahx)-HBED-CC. The syntheses of the compounds were performed according to standard Fmoc-solid phase peptide synthesis. The binding properties were analyzed by competitive cell binding and internalization experiments. The in vivo targeting properties were investigated by means of biodistribution studies.

RESULTS. Cell binding experiments revealed high binding affinities to both GRPR and PSMA expressing cell lines. The heterodimer bound with IC50-values essentially matching the IC50 values of the respective monomers (25.0 ± 5.4 nM for PSMA and 9.0 ± 1.8 nM for GRPR, respectively). In vivo, the heterodimer showed dual targeting of PSMA (5.4%ID/g for PSMA-positive tumors) and GRPR receptors (3.3% ID/g for GRPR-positive tumors) while exhibiting fast pharmacokinetic properties. The clearance from background was comparable to the monomeric PSMA-targeting reference.

CONCLUSIONS. The heterodimeric molecule is a promising agent for PET imaging of primary and recurrent prostate cancer covering two receptor entities which might lead to an improved diagnostic sensitivity and therapeutic efficiency.

KEY WORDS: prostate cancer; tumor heterogeneity; heterodimeric radiopharmaceuticals; bispecific PET imaging
metastatic, and hormone-refractory carcinomas making it to an ideal target for therapy and imaging of prostate cancer [5–7]. Since there are many treatment options available, it is often challenging in terms of finding the optimal clinical strategy. Non-invasive imaging of upregulated PSMA levels is able to rapidly provide important information on the stage of the disease and the location of metastatic lesions. As a consequence, PET/CT imaging using radiolabeled PSMA inhibitors might have high impact on patient management and clinical decision making for patients with biochemical recurrence after initial therapy or during therapeutic interventions.

However, the diagnostic significance of those examinations is often reduced or difficult to interpret as tumors usually present with a high grade of heterogeneity [8]. Even in the same patient tumor lesions might differ in terms of their receptor expression levels, kind of receptors expressed or their grade of malignancy and resistance after therapy. It has recently been reported that there are lesions with highly heterogeneous or partly lost PSMA expression in patients which are generally PSMA positive whereas complete PSMA negative cases were found to be rare [9].

In the context of therapy or critical clinical decisions, it is important to image and treat with the highest possible sensitivity. Lack of PSMA expression would significantly reduce the image quality that might result in the failure to notice a lesion and subsequently in a false-negative finding. In case of recurrent prostate cancer, this could have an influence on further therapeutic strategies, such as optional salvage radiation therapy when distant metastases are missing. Any approach enhancing the sensitivity of PET imaging in the context of biochemical recurrence of prostate cancer has, therefore, the potential to increase the clinical significance of the diagnostic examination.

Since prostate cancer cells usually express both PSMA and GRPR a heterodimeric molecule, combining both specificities, may improve the sensitivity of PSMA-targeted prostate cancer imaging. While 95% of all prostate cancers express PSMA, GRPR is overexpressed in 84% of all human prostate cancers [10]. GRPR expression can be found in the majority of lymph node metastases and in 52.9% of bone lesions [11]. Compared to benign prostate tissue, GRPR expression is higher in malignant lesions [12]. Moreover, as the expression of GRPR was reported to be enhanced in early stage prostate cancers and lower in high grade disease [13] the expression profile of GRPR seems to be contrary to PSMA which is higher in later and poorly differentiated stages of the disease. Thus, the combination of these two specificities for the diagnosis and therapy of prostate cancer might be of high clinical impact. The accuracy of imaging prostate cancer and its disseminated metastases might profit from the here presented bispecific PSMA-/GRPR-targeted radiotracer.

The aim of this work is the preclinical proof of concept and evaluation of the $^{68}$Ga-labeled heterodimer Glu-urea-Lys-HBED-CC-BZH3. BZH3 represents a high affinity Bombesin analog showing uptake in GRPR-expressing tumors [14]. Peptidomimetic urea-based PSMA inhibitors reveal high specific uptake in prostate tumors [15] and as they represent small hydrophilic molecules they might be well suited for the combination with a peptide without affecting its targeting and pharmacokinetic properties.

**MATERIALS AND METHODS**

The chemicals were of analytical grade and were used without further purification. Analysis of the synthesized molecules was performed using reversed-phase high performance liquid chromatography (RP-HPLC; Chromolith RP-18e, 100 mm × 4.6 mm; Merck, Darmstadt, Germany) with a linear A–B gradient (0% B to 100% B in 6 min) at a flow rate of 4 ml/min (analysis) or 6 ml/min (purification). Solvent A consisted of 0.1% aqueous TFA and solvent B was 0.1% TFA in CH$_3$CN. The HPLC system (L6200 A; Merck-Hitachi, Darmstadt, Germany) was equipped with a UV and a gamma detector (Bioscan; Washington, USA). UV absorbance was measured at 214 and 254 nm, respectively. Mass spectrometry was performed with a MALDI-MS Daltonics Microflex system (Bruker Daltonics, Bremen, Germany). $^{68}$Ga (half-life 68 min; $\beta^+$ 89%; $E_{\beta^+}$ max 1.9 MeV) was obtained from a $^{68}$Ge/$^{68}$Ga generator based on pyrogallol resin support [16].

**Synthesis of BZH3 and Glu-Urea-Lys(Ahx)**

The BZH3 Peptide was synthesized using standard Fmoc-peptide chemistry on a Rink Amide 4-methylbenzhydrylamine resin (Merck, Darmstadt) [14]. Subsequent cleavage from the resin with trifluoroacetic acid/triisopropylsilane/water (95/2.5/2.5) followed by RP-HPLC purification resulted in the peptidic building block. The peptide was isolated via RP-HPLC. Glu-urea-Lys(Ahx) was synthesized as previously described [17].

**Synthesis of the HBED-CC Conjugated Heterodimeric Molecule**

The monoactive tetrafluorophenyl (TFP) ester of $N, N'$-bis(2-hydroxy-5-(carboxyethyl)benzyl) ethylenedi-
amine-\(N,N\)-diacetic acid (HBED-CC) and the bis-activated ester (HBED-CC)TFP\(_2\) were synthesized as previously described [18,19]. The purified protected BZH3 or Glu-urea-Lys(Ahx) were reacted with 1.2 equivalents of HBED-CC-TFP-ester in presence of two equivalents of DIPEA in \(N, N\)-dimethylformamide (DMF) to form the monovalent and monospecific reference compounds. After HPLC purification the protecting groups were removed by trifluoroacetic acid treatment at room temperature for 1 hr resulting in the products BZH3-HBED-CC and Glu-urea-Lys(Ahx)-HBED-CC. Mass spectrometry confirmed the identity of the compounds (Table I).

The heterodimeric conjugate Glu-urea-Lys(Ahx)-HBED-CC-BZH3 was synthesized by incubating the bis-activated ester with one equivalent of BZH3. After 4 hr at RT, an excess of 10 equivalents of bis(\textit{tert}-butyl) protected Glu-urea-Lys(Ahx) was added to the reaction mixture and incubated for 16 hr at RT. The formed molecule (Scheme 1) was purified by semi-preparative HPLC. The remaining protecting groups were removed by trifluoroacetic acid treatment at room temperature during two hours. Mass spectrometry confirmed the identity of the compound (Table I) which was obtained in 18.5% overall chemical yield.

### Table I. Analytical and Binding Data of the Investigated Conjugates

| Compound                                      | m/z calculated as [M+H]\(^+\) | m/z\(^a\) | Specificity | IC\(_{50}\) (nM)\(^b\) |
|-----------------------------------------------|-------------------------------|------------|-------------|------------------------|
| Glu-urea-Lys(Ahx)-HBED-CC                    | 947.4                         | 947.7      | PSMA        | 10.8 ± 1.1             |
| BZH3-HBED-CC                                 | 1800.0                        | 1800.8     | GRPR        | 8.9 ± 2.4              |
| Glu-urea-Lys(Ahx)-HBED-CC-BZH3               | 2213.5                        | 2214.2     | PSMA        | 25.0 ± 5.4             |
|                                              |                               |            | GRPR        | 9.0 ± 1.8              |

\(^a\)Mass spectrometry data of the free ligands ([M+H]\(^+\)).  
\(^b\)Affinity-related IC\(_{50}\) determined on LNCaP (PSMA\(^+\)) or PC-3 (GRPR\(^+\)) cells; representative binding curves are shown in the Supporting Information.

### 68Ga-Labeling

The conjugates (0.1–1 nmol in 0.1 M HEPES buffer, pH 7.5, 100 μl) were added to a mixture of 10 μl HEPES solution (2.1 M) and 40 μl \[^{68}\text{Ga}\]Ga\(^{3+}\) eluate (25–60 MBq). The pH of the labeling solution was adjusted to 4.2 using 30% NaOH. The reaction mixture

![Scheme 1. Synthesis of the bispecific heterodimeric molecule Glu-urea-Lys(Ahx)-HBED-CC-BZH3.](image-url)
was incubated at 80°C for 2 min. The radiochemical yield (RCY) was determined via analytical RP-HPLC.

**Cell Culture**

For binding studies and in vivo experiments LNCaP cells (metastatic lesion of human prostatic adenocarcinoma, ATCC CRL-1740), AR42J cells (rat pancreatic exocrine cell line which can be purchased from the European Collection of Cell Cultures), and PC-3 cells (bone metastasis of a grade IV prostatic adenocarcinoma, ATCC CRL-1435) were cultured in RPMI medium supplemented with 10% fetal calf serum and Glutamax (PAA, Austria). During cell culture, cells were grown at 37°C in an incubator with humidified air, equilibrated with 5% CO₂. The cells were harvested using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; 0.25% trypsin, 0.02% EDTA, all from PAA, Austria) and washed with PBS.

**Cell Binding and Internalization**

The competitive cell binding assay and internalization experiments were performed as described previously [17]. Briefly, the respective cells (10⁵ per well) were incubated with the radioligand (⁶⁸Ga-labeled [Glu-urea-Lys(Ahx)]₂-HBED-CC (19) or ¹²⁵I-Tyr⁴-BBN (Perkin Elmer), respectively) in the presence of 12 different concentrations of analyte (0–5,000 nM, 100 µL/well). After incubation, washing was carried out using a multiscreen vacuum manifold (Millipore, Billerica, MA). Cell-bound radioactivity was measured using a gamma counter (Packard Cobra II, GMI, Minnesota, USA). The 50% inhibitory concentration (IC₅₀) was calculated by fitting the data using a nonlinear regression algorithm (GraphPad Software). Experiments were performed three times.

To determine the specific cell uptake and internalization, 10⁵ cells were seeded in poly-L-lysine coated 24-well cell culture plates 24 hr before incubation. After washing, the cells were incubated with 25 nM of the radiolabeled compounds for 45 min at 37 and at 4°C, respectively. Specific cellular uptake was measured after washing four times with 1 ml of ice-cold PBS. Cells were subsequently incubated twice with 0.5 ml glycine-HCl in PBS (50 mM, pH 2.8) for 5 min to remove the surface-bound fraction. The cells were washed with 1 ml of ice-cold PBS and lysed using 0.3 N NaOH (0.5 ml). The surface-bound and the internalized fractions were measured in a gamma counter. The cell uptake was calculated as per cent of the initially added radioactivity bound to 10⁶ cells (% ID/10⁶ cells).

**Biodistribution and μPET**

Seven- to 8-week-old female (AR42J) or male (LNCaP) BALB/c nu/nu mice (Charles River Laboratories) were subcutaneously inoculated into the right trunk with 5 × 10⁶ cells of either AR42J or LNCaP (in 50% Matrigel) (Becton Dickinson, Heidelberg, Germany). The tumors were allowed to grow until approximately 1 cm³ in size. The radiolabeled compounds were injected into the tail vein (approx. 1 MBq per mouse; 0.06 nmol). At 1 hr after injection the animals were sacrificed. Organs of interest were dissected, blotted dry, and weighed. The radioactivity was measured using a gamma counter and calculated as %ID/g.

For the μPET studies, 5 MBq (ca. 100 pmol) of ⁶⁸Ga-labeled heterodimer were injected via a lateral tail vein into mice bearing AR42J or LNCaP tumor xenografts, respectively. Specific blocking was performed by coinjection of 0.5 µmol of the peptide BZH3 (GRPR-block) or 0.5 µmol of the PSMA inhibitor 2-PMPA (PSMA-block), respectively. The anesthetized animals (2% sevoflurane, Abbott, Wiesbaden, Germany) were placed in prone position into an Inveon small animal PET scanner (Siemens, Knoxville, Tenn, USA) to perform a 60 min-dynamic microPET scan starting at 1 min post injection.

**Statistical Aspects**

All experiments were performed at least in triplicate. Quantitative data were expressed as mean ± SD. If applicable, means were compared using Student’s t-test. P values < 0.05 were considered statistically significant.

**RESULTS**

**Synthesis and Radiolabeling**

The bispecific heterodimeric molecule Glu-urea-Lys(Ahx)-HBED-CC-BZH3 was synthesized by combining the peptidomimetic PSMA inhibitor Glu-urea-Lys and the GRPR binding peptide BZH3 via the bis-activated active ester HBED-CC-TFP2 (Scheme 1). As references, the corresponding monospecific HBED-CC conjugated counterparts Glu-urea-Lys(Ahx)-HBED-CC and BZH3-HBED-CC were synthesized according to standard Fmoc protocols. The yields of the peptidic monomers amounted to approximately 25%, whereas the yield of the heterodimeric compound was approximately 18.5% after HPLC purification.
The \(^{68}\)Ga complexation of all compounds resulted in high radiochemical yields of 99% after 2 min reaction time at 80°C. As a consequence, the radio-labeled compounds were subsequently used for in vitro and in vivo experiments without further purification.

**In Vitro Cell Binding Properties**

An in vitro competitive cell binding assay was performed in order to determine the binding potential expressed as IC\(_{50}\) values of Glu-urea-Lys(Ahx)-HBED-CC-BZH3 in comparison to the monospecific references Glu-urea-Lys(Ahx)-HBED-CC and BZH3-HBED-CC. The results are summarized in Table I. While the GRPR specificity of the heterodimeric molecule revealed a nearly identical binding affinity compared to BZH3-HBED-CC, the affinity of the PSMA specificity was reduced by a factor of 2.3 from 10.8 ± 1.1 to 25.0 ± 5.4 nM.

The functionality of both specificities was additionally investigated on the cellular basis by analyzing internalization and cell surface binding properties of the heterodimer (Fig. 1). \(^{68}\)Ga-labeled Glu-urea-Lys(Ahx)-HBED-CC-BZH3 was specifically internalized by LNCaP cells shown by competitive blocking with the PSMA inhibitor 2-PMPA (\(P < 0.001\)). While Bombesin (BBN) did not influence the cell surface or internalized fraction on PSMA\(^+\) and GRPR\(^+\) LNCaP cells (\(P = 0.53\) (cell surface binding), \(P = 0.49\) (internalized)), the signal on GRPR\(^+\) and PSMA\(^-\) PC-3 cells was significantly reduced (\(P = 0.004\) (cell surface binding), \(P = 0.008\) (internalized)) while the 2-PMPA block was not effective (\(P = 0.21\) (cell surface binding), \(P = 0.43\) (internalized)). These results indicate both PSMA and GRPR specific uptake and internalization of the heterodimer. In direct comparison with their respective \(^{68}\)Ga-labeled monomeric references Glu-urea-Lys(Ahx)-HBED-CC and BZH3-HBED-CC, the heterodimer did not show any statistically significant differences in terms of their specific cell surface binding and internalization properties on LNCaP and PC-3 cells, respectively (Fig. 2).

**Organ Distribution**

In order to demonstrate the functionality of both specificities in vivo, organ distribution studies with tumor bearing xenografts were performed. Figure 3 and Table II show that the tumor uptake of the \(^{68}\)Ga-labeled heterodimer Glu-urea-Lys(Ahx)-HBED-CC-BZH3 in PSMA positive LNCaP tumors (5.44 ± 1.54%ID/g) and in GRPR positive AR42J tumors (3.34 ± 1.04%ID/g) were clearly reduced either by co-injection of 0.5 \(\mu\)mol 2-PMPA (1.06 ± 0.24%ID/g; \(P = 0.008\)) or 0.5 \(\mu\)mol Bombesin (0.90 ± 0.13%ID/g; \(P = 0.016\)), respectively. Furthermore, the normal organ distribution profile indicates PSMA-mediated specific uptake in the kid-

![Fig. 1](image-url)
neys and the spleen on the one hand and GRPR-mediated specific uptake in the pancreas on the other hand. The uptake in the kidneys (110.46 ±8.80%ID/g) and the spleen (34.08 ±4.92%ID/g) was significantly blocked to 16.04 ±3.94%ID/g (P <0.001) and 2.75 ±1.61%ID/g (P <0.001) (2-PMPA block). GRPR-mediated uptake in the pancreas was reduced from 8.60 ±5.59%ID/g or 9.09 ±6.2%ID/g, respectively, to 1.23 ±0.14%ID/g (P =0.09 or P <0.001, respectively) by blocking with an excess of BBN (0.5 μmol). The clearance of the 68Ga-labeled heterodimer Glu-urea-Lys(Ahx)-HBED-CC-BZH3 from other important organs like heart, lung, liver, and intestine as well as from background (blood and muscle) occurred rapidly.

**DISCUSSION**

The diagnosis of recurrent prostate cancer is still challenging because patients who would benefit from...
Fig. 4. Whole-body coronal $\mu$PET imaging of athymic male nude mice bearing PSMA$^+/GRPR^-$ LNCaP and PSMA$^+/GRPR^+$ AR42J tumor xenografts, respectively. The $^{68}$Ga-labeled heterobispecific molecule Glu-urea-Lys(Ahx)-HBED-CC-BZH3 was evaluated by a dynamic $\mu$PET scan resulting in images at 40–60 min post injection and the corresponding time-activity curves of tumor and muscle (A–D). A: Glu-urea-Lys(Ahx)-$[^{68}$Ga$]$Ga-HBED-CC-BZH3 in LNCaP tumor xenografts; (B) Glu-urea-Lys(Ahx)-$[^{68}$Ga$]$Ga-HBED-CC-BZH3 in LNCaP tumor xenografts blocked by co-injection of 500 nmol 2-PMPA; (C) Glu-urea-Lys(Ahx)-$[^{68}$Ga$]$Ga-HBED-CC-BZH3 in AR42J tumor xenografts; (D) Glu-urea-Lys(Ahx)-$[^{68}$Ga$]$Ga-HBED-CC-BZH3 in AR42J tumor xenografts blocked by co-injection of 500 nmol Bombesin peptide. Approximately 5 MBq (~100 pmol)/mouse were injected. Time-activity curves were taken from the dynamic PET measurements and are expressed as SUV mean (standardized uptake values).

The Prostate
accurate imaging and therefore from salvage radiotherapy usually have low PSA values and very small metastases. At low PSA values Choline-PET/CT often fails to visualize recurrences leading to vague clinical decisions.

In comparison to Choline-PET/CT, PSMA-PET/CT was shown to be superior in terms of imaging metastasis of recurrent prostate cancer as the tumor uptake is often higher while the clearance from background occurs faster [1] resulting in high quality non-invasive visualization of even small lesions of recurrent prostate cancer. However, even this new imaging agent has limitations because tumors often show heterogeneous receptor expression. In case of prostate cancer, it was indeed reported that metastases partly show highly heterogeneous PSMA distribution or partly loss of PSMA expression [9]. This finding might explain the failure to image distant metastases.

Any effort which is able to enhance the imaging sensitivity of prostate cancer would improve the quality of the therapeutic strategy in the daily clinical routine of managing prostate cancer recurrences. A suitable strategy to enhance the sensitivity of an imaging agent is to address an additional specificity against another cell surface receptor which is also over-expressed on the respective tumor entity and to combine these two specificities in one and the same dual-targeting molecule.

There are several studies showing the potential of heterodimers by synergistic tumor targeting [20–24]. However, these examples also indicate a major problem of heterodimeric molecules. If the pharmacokinetic properties are disadvantageously affected by heterodimerization, separate application of both monospecific compounds targeting different receptors would potentially have higher clinical impact. As an example, the somatostatin and integrin targeting heterodimer c(RGDiD)-[111In]DTPA-Tyr2-octreotate which was intended for simultaneous imaging and therapy purposes showed unfavorable pharmacokinetics in first preclinical studies which hampered further developments on this compound class [24,25].

Thus, besides the improvement of tumor uptake and sensitivity by synergistic targeting, heterodimers should at least exhibit similar or better pharmacokinetic properties than the clinically more attractive monospecific tracer. In our case, based on the currently available data of the radiolabeled small molecule inhibitors of PSMA it can be postulated that PSMA represents a key biological target of utmost clinical impact [1,4,26]. As a consequence, the pharmacokinetic properties need to be directly compared with the organ distribution profile obtained with the respective PSMA targeting monomer Glu-urea-Lys-(Ahx)-HBED-CC. The uptake of the herein presented heterodimer in important organs as well as the blood clearance are in good agreement with the organ distribution data previously published [17] indicating similar pharmacokinetic properties of the PSMA targeting monomer. The additional uptake of the heterodimer in comparison to the PSMA-targeted monomer in normal pancreas tissue should reveal negligible clinical problems, because metastases of prostate cancer in the pancreas are not expected to occur. Furthermore, as the tumor uptake of the heterodimer (5.44 ± 1.54%ID/g) is also comparable to the tumor accumulation of the monomeric Glu-urea-Lys-(Ahx)-HBED-CC (4.89 ± 1.34%ID/g [19]; P > 0.05) it can be concluded that the preclinical imaging quality of the new heterodimer is almost identical with the monomeric reference Glu-urea-Lys-(Ahx)-HBED-CC. This is also supported by the μPET imaging results of the presented study. Compared to former μPET images of the monomer Glu-urea-Lys-(Ahx)-HBED-CC [17,19] the heterodimer shows identical imaging contrasts in LNCaP tumor xenografts.

With regard to the GRPR specificity, the heterodimer shows specific GRPR-mediated tumor uptake (3.34 ± 1.04%ID/g; and 0.90 ± 0.13%ID/g blocked). The value is in line with in vivo data obtained from other heterodimeric molecules targeting GRPR expressing tumor xenografts (2.7%ID/g for 99mTc-HYNIC-RGD-BBN [27] and 4.0%ID/g for 64Cu-NO2A-RGD-BBN [28]). A slightly higher tumor uptake was found for the [66Ga]Ga-DOTA-labeled monomer BZH3 previously published by Schuhmacher et al. However, it was shown in this study that the tumor uptake of the monomer strongly depends on the absolute peptide amount injected [14]. Thus, the optimal in vivo concentration for the highest possible tumor uptake of the heterodimer has to be evaluated in a further dose study.

The specific blocking effect of the receptor-mediated uptake in healthy organs with proven physiological receptor expression (PSMA expression in murine kidneys and spleen and GRPR expression in murine pancreas) additionally indicates the remained functionality of both specificities. These findings were also supported by the in vitro evaluation of the heterodimer. Besides highly specific internalization and cell surface binding with nanomolar binding affinities to both specificities no significant deviations were observed in direct comparison with the respective monomers. Remarkably, the binding affinity of the heterodimer against PSMA presented with a reduction by a factor of 2.3 while the PSMA-specific cell uptake and internalization was not affected by the heterodimerization. Former studies [17,19,29] showed that mainly the internalization properties and to a lesser extent the binding affinity determined the in vivo tumor uptake which was indeed confirmed in this
study. Altogether the slight reduction of the PSMA binding affinity of the heterodimer neither influences the cell binding properties as shown by the internalization experiments nor its tumor targeting behavior as proven by the organ distribution studies.

CONCLUSIONS

Taken together, the herein presented $^{68}$Ga-labeled low-molecular heterodimer Glu-urea-Lys(Ahx)-HBED-CC-BZH3 showed bispecific targeting of both PSMA and GRPR receptors in vitro and in vivo. These findings will putatively result in the improved detection of PSMA- and GRPR-associated prostate cancer while keeping the pharmacokinetic and PSMA-targeting properties of the radiotracer constant. With regard to aforementioned clinical aspects, the herein presented heterodimer has the potential to improve prostate cancer imaging by enhancing the sensitivity of the recently introduced and highly promising PSMA-PET/CT method [1,2]. Since the expression of PSMA is often heterogeneous in PSMA positive patients a tracer additionally targeting the GRPR specificity might considerably improve the detection of recurrent prostate cancer at low PSA levels as well as the management of the therapy of the systemic disease.

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REFERENCES

1. Afshar-Oromieh A, Malcher A, Eder M, Eisenhut M, Linhart HG, Hadaschik BA, Holland-Letz T, Giesel FL, Kratochwil C, Huufe S, Haberkorn U, Zechmann CM. PET imaging with a $^{68}$Ga-labeled PSMA ligand for the diagnosis of prostate cancer: Biodistribution in humans and first evaluation of tumour lesions. Eur J Nucl Med Mol Imaging 2013;40(4):486–495.
2. Afshar-Oromieh A, Haberkorn U, Eder M, Eisenhut M, Zechmann CM. $^{68}$Ga-labeled PSMA ligand as superior PET tracer for the diagnosis of prostate cancer: Comparison with 18F-FECH. Eur J Nucl Med Mol Imaging 2012;39(6):1085–1086.
3. Hillier SM, Maresca KP, Lu G, Merkin RD, Marquis JC, Zimmerman CN, Eckelman WC, Joyal JL, Babich JW. 99mTc-labeled small-molecule inhibitors of prostate-specific membrane antigen for molecular imaging of prostate cancer. J Nucl Med 2013;54(8):1369–1376.
4. Barrett JA, Coleman RE, Goldsmith SJ, Vallabhajosula S, Petry NA, Cho S, Armor T, Stubbs JB, Maresca KP, Stabin MG, Joyal JL, Eckelman WC, Babich JW. First-in-man evaluation of 2 high-affinity PSMA-avid small molecules for imaging prostate cancer. J Nucl Med 2013;54(3):380–387.
5. Ferner S, Hofer MD, Kim R, Shah RB, Li H, Möller P, Hautmann RE, Geschwend JE, Kuefer R, Rubin MA. Prostate-specific membrane antigen expression as a predictor of prostate cancer progression. Hum Pathol 2007;38(5):696–701.
6. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant human tissues. Clin Cancer Res 1997;3(1):81–85.
7. Bostwick DG, Pacelli A, Blute M, Roche P, Murphy GP. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: A study of 184 cases. Cancer 1998;82(11):2256–2261.
8. Marusyk A, Polyak K. Tumor heterogeneity: Causes and consequences. Biochim Biophys Acta 2010;1805(1):105–117.
9. Mannweiler S, Amersdorfer P, Trajanoski S, Terrett JA, King D, Mehes G. Heterogeneity of prostate-specific membrane antigen (PSMA) expression in prostate carcinoma with distant metastasis. Pathol Oncol Res 2009;15(2):167–172.
10. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: Relation to neoplastic transformation. Cancer Res 1999;59(5):1152–1159.
11. Ananias HJ, van den Heuvel MC, Helfrich W, de Jong IJ. Expression of the gastrin-releasing peptide receptor, the prostate stem cell antigen and the prostate-specific membrane antigen in lymph node and bone metastases of prostate cancer. Prostate 2009;69(10):1101–1108.
12. Beer M, Montani M, Gerhardt J, Wild PJ, Hany TF, Hermans T, Muntener M, Kristiansen G. Profiling gastrin-releasing peptide receptor in prostate tissues: Clinical implications and molecular correlates. Prostate 2012;72(3):318–325.
13. Yu Z, Ananias HJ, Carlucci G, Hoving HD, Helfrich W, Dierckx RA, Wang F, de Jong IJ, Elsinga PH. An update of radiolabeled bombesin analogs for gastrin-releasing peptide receptor targeting. Curr Pharm Des 2013;19(18):3329–3341.
14. Schuhmacher J, Zhang H, Doll J, Macke HR, Matys R, Hauser H, Henze M, Haberkorn U, Eisenhut M. GRP receptor-targeted PET of a rat pancreas carcinoma xenograft in nude mice with a $^{68}$Ga-labeled bombesin(6-14) analog. J Nucl Med 2005;46(4):691–699.
15. Chen Y, Foss CA, Byun Y, Nimmagadda S, Pullambhatla M, Fox JJ, Castanares M, Lupold SE, Babich JW, Mease RC, Pomper MG. Radiolabeled heterogeneous prostate-specific membrane antigen (PSMA)-based ureas as imaging agents for prostate cancer. J Med Chem 2008;51(24):7933–7943.
16. Schuhmacher J, Maier-Borst W. A new Ge-68/Ga-68 radioisotope generator system for production of Ga-68 in dilute HCl. Int J Appl Radiat Isot 1981;32:31–36.
17. Eder M, Schär C, Bauder-Wust U, Hull WE, Wangler C, Mier W, Haberkorn U, Eisenhut M. (68)Ga-complex lipophilicity and the targeting property of a urea-based PSMA inhibitor for PET imaging. Bioconjug Chem 2012;23(4):688–697.
18. Eder M, Wangler B, Knackmuss S, Legall F, Little M, Haberkorn U, Mier W, Eisenhut M. Tetrafluorophenolate of HBED-CC: A versatile conjugation agent for (68)Ga-labeled small recombinant antibodies. Eur J Nucl Med Mol Imaging 2008;35(10):1876–1886.
19. Schär C, Bauder-Wust U, Leotta K, Zoller F, Mier W, Haberkorn U, Eisenhut M, Eder M. A dimerized urea-based inhibitor of the prostate-specific membrane antigen for 68Ga-PET imaging of prostate cancer. JNM/MRI Res 2012;2(1):23.
20. Liu Z, Yan Y, Liu S, Wang F, Chen X. (18)F, (64)Cu, and (68)Ga labeled RGD-bombesin heterodimeric peptides for PET imaging of breast cancer. Bioconjug Chem 2009;20(5):1016–1025.

21. Liu Z, Yan Y, Chin FT, Wang F, Chen X. Dual integrin and gastrin-releasing peptide receptor targeted tumor imaging using 18F-labeled PEGylated RGD-bombesin heterodimer 18F-FB-PEG3-Glu-RGD-BBN. J Med Chem 2009;52(2):425–432.

22. Liu Z, Niu G, Wang F, Chen X. (68)Ga-labeled NOTA-RGD-BBN peptide for dual integrin and GRPR-targeted tumor imaging. Eur J Nucl Med Mol Imaging 2009;36(9):1483–1494.

23. Liu Z, Li ZB, Cao Q, Liu S, Wang F, Chen X. Small-animal PET of tumors with (64)Cu-labeled RGD-bombesin heterodimer. J Nucl Med 2009;50(7):1168–1177.

24. Bernard B, Capello A, van Hagen M, Breeman W, Srinivasan A, Schmidt M, Erion J, van Gameren A, Krenning E, de Jong M. Radiolabeled RGD-DTPA-Tyr3-octreotate for receptor-targeted radionuclide therapy. Cancer Biother Radiopharm 2004;19(2):173–180.

25. Capello A, Krenning EP, Bernard BF, Breeman WA, van Hagen MP, de Jong M. Increased cell death after therapy with an Arg-Gly-Asp-linked somatostatin analog. J Nucl Med 2004;45(10):1716–1720.

26. Eder M, Eisenhut M, Babich J, Haberkorn U. PSMA as a target for radiolabelled small molecules. Eur J Nucl Med Mol Imaging 2013;40(6):819–823.

27. Liu Z, Huang J, Dong C, Cui L, Jin X, Jia B, Zhu Z, Li F, Wang F. 99mTc-labeled RGD-BBN peptide for small-animal SPECT/CT of lung carcinoma. Mol Pharm 2012;9(5):1409–1417.

28. Jackson AB, Nanda PK, Rold TL, Sieckman GL, Szczodroski AF, Hoffman T, Chen X, Smith CJ. 64Cu-NO2A-RGD-Glu-6-Ahx-BBN(7-14)NH2: A heterodimeric targeting vector for positron emission tomography imaging of prostate cancer. Nucl Med Biol 2012;39(3):377–387.

29. Liu T, Toriyabe Y, Kazak M, Berkman CE. Pseudoirreversible inhibition of prostate-specific membrane antigen by phosphoramidate peptidomimetics. Biochemistry 2008;47(48):12658–12660.

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