

Proof of Therapeutic Efficacy of a Novel ¹⁷⁷Lu-Labeled Neurotensin Receptor 1-Antagonist in a Colon Carcinoma Xenograft Model

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Running title

Therapeutic effect of a NTR1-radiotracer

ABSTRACT

Purpose: Increased expression of neurotensin receptor 1 (NTR1) has been shown in a large number of tumor entities like pancreatic or colon carcinoma. Hence, this receptor is a promising target for diagnostic imaging and radioligand therapy (RLT). Based on favorable biodistribution data of the NTR1-targeting agent ^{111}In -3BP-227, we investigated the therapeutic effect of its ^{177}Lu -labeled analog on the tumor growth of NTR1-positive HT29 colon carcinoma xenografts.

Methods: 3BP-227 was labeled with ^{177}Lu . To assess its biodistribution properties, single photon emission computer tomography (SPECT) and computed tomography (CT) scans of HT29-xenografted nude mice injected with ^{177}Lu -3BP-227 were acquired and ex vivo tissue activity was determined. To evaluate therapeutic efficacy, two groups of mice received the radiopharmaceutical either in a median dose of 165 MBq (129-232 MBq, $n = 10$) or 110 MBq (82-116 MBq, $n = 10$), whereas control mice were injected with vehicle ($n = 10$). Tumor sizes and body weights were monitored for up to 49 days. Renal function and histological morphology were evaluated.

Results: Whole-body SPECT/CT images allowed clear tumor visualization with low background activity and high tumor-to-kidney and -liver ratios. Ex vivo biodistribution data confirmed high and persistent uptake of ^{177}Lu -3BP-227 in HT29 tumors (19.0 ± 3.6 vs. 2.7 ± 1.6 %ID/g at 3 and 69 h p.i., respectively). The application of ^{177}Lu -3BP-227 resulted in a distinct delay of tumor growth. Median tumor doubling time for controls was 5.5 (2.8; 7.0) days, compared to 17.5 (5.5; 22.5) days for the 110 MBq and 41.0 (27.5; 55.0) days for the 165 MBq group. Compared to controls, median relative tumor volume at day 23 p.i. was reduced by 55% ($P = 0.034$) in the 110 MBq and by 88% ($P < 0.01$) in the 165 MBq group. Renal histology and clinical chemistry results did not differ between radiotherapy groups and controls, suggesting absence of therapy-induced acute renal damage.

Conclusion: These data demonstrate that the novel NTR1-targeting, theranostic agent 3BP-227 is an effective and promising candidate for RLT with a favorable preliminary safety profile and high potential for clinical translation.

Key Words:

NTR1-radiotracer, xenograft, SPECT/CT, theranostic, oncology

INTRODUCTION

The successful diagnosis and therapy of somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors with ^{177}Lu and ^{90}Y -labeled somatostatin analogs (1,2) demonstrates the high potential of neuropeptide receptor-based imaging and RLT. Since the range of somatostatin receptor-positive tumor entities is limited, other neuropeptide receptor systems such as the neurotensinergic system have been evaluated as targets for RLT. Neurotensin receptors (NTR's) mediate the effects of the neuropeptide hormone neurotensin, which acts as neuromodulator and peripherally as para- and endocrine factor in the gastrointestinal system (3). Physiologically, NTR's are mainly expressed in the central nervous as well as intestinal and hepatic system (4). Out of the three known NTR subtypes, NTR1 is the primary mediator of neurotensin signaling due to its subnanomolar affinity to its natural ligand.

In colon carcinoma, breast cancer, and pancreatic adenocarcinoma, NTR1 expression has been associated with neoplastic progression (5–8). Furthermore, autoradiographic studies have shown that NTR1 is expressed in pancreatic adenocarcinoma in high densities of 10^3 - 10^4 dpm/mg in primary tumors (9) and metastases (8). Ewing's sarcoma, small cell lung cancer and medullary thyroid carcinoma express similar densities, although a few individual tumors express receptor densities below 10^3 dpm/mg (10). These receptor densities are in a similar range as the somatostatin receptor subtype 2 (sst2) densities found in neuroendocrine tumors when tested autoradiographically using an sst2 agonist (11). Therefore, NTR1-targeting RLT may become a similarly effective therapy for NTR1-expressing tumor entities as somatostatin analog-based RLT for neuroendocrine tumors is today.

Using an sst2 antagonist, an approximately ten-fold higher number of binding sites has been demonstrated in various tumor entities compared to an sst2 agonist (12). Several other studies have also revealed higher numbers of binding sites and increased tumor uptake for radiolabeled somatostatin and gastrin-releasing peptide receptor antagonists compared to agonists (13–15), which can be explained by the recognition of more conformational receptor states by the antagonist compared to the agonist (16). Hypothesizing that antagonist-based NTR1-targeting radiotracers might exhibit better tumor-to-normal tissue uptake ratios than previously reported agonistic agents (17,18), Osterkamp et al. decided to modify the known NTR1 antagonist SR142948A (19,20) in order to allow attachment of the chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (21). This strategy allows the formation of highly stable complexes with metal radionuclides such as ^{111}In , ^{68}Ga , ^{90}Y and ^{177}Lu , and therefore enables use of

the novel antagonistic derivatives as theranostic agents for a wide range of clinical applications like SPECT or PET and RLT. We reported recently the favorable biodistribution profiles of the ^{111}In -labeled NTR1 antagonists 3BP-227 and 3BP-228, exhibiting high tumor and low normal tissue uptake, with 3BP-227 yielding a more persistent tumor uptake (22). The aim of this follow-up study was to evaluate the antitumoral effect of ^{177}Lu -labeled 3BP-227 in the standard xenograft model in NTR1-targeted RLT, subcutaneous HT29 colon carcinoma tumors, and ultimately to gauge its potential for clinical translation.

MATERIALS AND METHODS

Radiolabeling

3BP-227 (35-40 μg) in buffer (0.4 M acetate, 0.325 M gentisic acid, pH 5) was incubated with 2.0-3.1 GBq $^{177}\text{LuCl}_3$ (in 0.04 M HCl, Isotope Technologies Garching GmbH). The mixture was heated at 95°C for 30 minutes. After cooling, the product was analyzed by thin layer and high performance liquid chromatography as previously described (22), and formulated in 0.9% NaCl for intravenous administration.

Cell Culture and Tumor Model

NTR1-positive HT29 human colorectal carcinoma cells (European Collection of Authenticated Cell Cultures and Cell Lines Services) (23) were grown in McCoy's 5A modified medium (Biochrom) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (10000 U/ml) and streptomycin (10 mg/ml) at 37°C in 5% CO_2 .

Experiments were approved by the local animal welfare committee and performed according to national regulations. Mice were kept in an individually ventilated cage system under standard conditions with food and water ad libitum. HT29 cells ($2\text{-}3 \times 10^6$ cells) were inoculated subcutaneously in the flank of 6-8 wk old athymic nu-nu female mice (Nude-*Foxn1^{nu}*, Harlan Laboratories).

SPECT/ CT Imaging

SPECT/CT whole-body imaging was performed at 3, 20, 45, 69 and 93 h after injection of ^{177}Lu -3BP-227 in two mice (159.7 and 130.7 MBq, respectively). Image acquisition was carried out under isoflurane anesthesia (1%-2% isoflurane in 2:1 $\text{O}_2\text{:N}_2\text{O}$ volume ratio) with a four head NanoSPECT/CT™ scanner (Mediso Ltd.). CT and SPECT were co-registered. Whole-body CT scans were acquired at pixel sizes of 192 μm and reconstructed with the manufacturer's software (InVivoScope 1.43) at isotropic voxel sizes of 200 μm . Whole-body SPECT scans were acquired using a multi-pinhole collimator set (SCIVIS),

each collimator with 9 pinholes (1.4-mm pinhole diameter, ≤ 1.0 mm nominal resolution). Photopeaks were set to the default values of the NanoSPECT for ^{177}Lu (57, 113 and 208 keV, each $\pm 10\%$). Acquisition time was 60 minutes. SPECT scans were reconstructed using the manufacturer's software (HiSPECT; SCIVIS) at isotropic voxel output sizes of 300 to 600 μm , depending on count rates.

Ex Vivo Biodistribution Study

Mice were injected with 4.0 ± 0.7 MBq ^{177}Lu -3BP-227 and sacrificed under isoflurane anesthesia 3, 20 or 69 h later ($n = 5$ per time point). Tissues of interest and urine were removed and weighed, and their activity content was determined by well counter measurement. Tissue counts and injected dose for individual mice were decay-corrected to the time of euthanasia. Tissue uptake is expressed as the percentage injected dose per gram of tissue (%ID/g).

Radioligand Therapy

Radiopharmaceutical or vehicle injection was performed seven days after tumor cell inoculation (day 0). At this time tumors have reached a mean volume of 90 mm^3 . Mice were divided into three groups of $n = 10$, receiving either a median dose of 110 MBq (82-116 MBq) or 165 MBq (129-232 MBq) of ^{177}Lu -3BP-227, or vehicle (0.9% NaCl) via a lateral tail vein. One mouse in the 165 MBq group had to be excluded from analysis due to a failed injection. Tumor measurements by digital caliper, determinations of body weight and assessments of clinical status were performed thrice weekly beginning at the day of active agent or vehicle injection (day 0). Tumor volume was calculated according to the formula $V = (\pi/6)LW^2$ (L = largest, W = shortest diameter of the tumor). As the average tumor sizes in the three groups were not equal on day 0, individual tumor measurements were normalized to their initial value at day 0 and are presented as relative tumor volume ($V_{\text{day p.i.}}/V_0$). Endpoint criteria were defined as relative body weight (body weight at day p.i. divided by weight on day 0) < 0.85 , tumor volume > 1500 mm^3 or largest diameter ≥ 15 mm, exulceration or invasive growth of the tumor and clinical abnormalities like apathy, labored respiration, impaired movement or abnormal posture. The study was terminated after a maximum observation time of 49 days.

Kidney Function and Morphology

In order to investigate potential induction of renal damage by ^{177}Lu -3BP-227, kidney sections were histologically examined. For this purpose, kidneys of 3 mice of the 165 MBq, 110 MBq and control group

were collected after they reached endpoint criteria or the end of the investigation period. Organs were fixed in 4% paraformaldehyde and embedded in paraffin. Dewaxed sections were either stained with periodic acid-Schiff reagent, hematoxylin-eosin or Masson Trichrom staining. Specimens were evaluated by a trained nephropathologist. Furthermore, blood samples were taken via heart puncture directly after euthanasia. Serum concentrations of creatinine and urea were measured utilizing standard techniques of clinical chemistry. Neutrophil gelatinase-associated lipocalin and cystatin C concentrations were determined by enzyme-linked immunosorbent assay (Mouse NGAL ELISA Kit, BioPorto Diagnostics A/S; Mouse Cystatin C ELISA Kit, Boster Biological Technology Co., Inc.) following manufacturers' instructions.

Data Analysis

OsiriX™ MD (open source version 1.3, 64-bit) was used to analyze SPECT/CT data. Regions of interest for kidneys and tumors were drawn manually on transaxial CT slices. Volumes of interest were compiled of 2D-regions of interest delineating the entire extent of each examined tissue and transferred to corresponding SPECT slices for quantification of counts in the volumes of interest.

Dosimetric calculations for selected tissues were performed using the sphere model of OLINDA/EXM software (version 1.1) (24) and mono-exponential fitting of kinetic data. The tumor mass was assumed to be constant using the barycenter of the mass over time function. The resulting absorbed doses have to be considered rough estimates due to the limited number of time points and the imperfect tumor's spherical shape.

SPSS Statistics (IBM, 83-bit version 22.0.0.0) was used for statistical analysis and data visualization. Statistical analysis of differences of medians of relative tumor volume, tumor doubling times, relative body weight and kidney parameters was performed applying Kruskal-Wallis-test followed by Mann-Whitney-U-test for multiple comparisons or Mann-Whitney-U-test for analyzing differences between two groups. For evaluation of the results of the RLT study, a Kaplan-Meier-analysis and log rank tests were performed. The end point was defined as reaching a relative tumor volume of ≥ 2 . Differences of *P* values less than 0.05 were considered statistically significant.

RESULTS

Radiolabeling

¹⁷⁷Lu-radiolabeling yields of 3BP-227 were $\geq 95\%$ based on thin layer and high performance liquid chromatography. Specific activities were 56.5 MBq/nmol for the imaging and 59.9 MBq/nmol for the biodistribution study, 59.3 MBq/nmol for the 110 MBq group and 87.6 MBq/nmol for the 165 MBq group in the therapy setting.

SPECT/CT Imaging

¹⁷⁷Lu-3BP-227-SPECT/CT imaging of HT29-xenografted nude mice visualized the tumor clearly. At 3 h p.i. distinct background activity was still present, representing circulating radiotracer as well as urorenal and intestinal accretion (Fig. 1). By 20, 45, 69 and 93 h p.i. tumor uptake clearly exceeded uptake in kidneys and liver. The tumor-to-kidney activity ratio of the animal depicted in Figure 1 continuously increased from 1.19 at 3 h p.i. to 5.66 at 93 h p.i. (3.26 at 20 h p.i., 3.89 at 45 h p.i., 4.65 at 69 h p.i.). Tumor-to-liver activity ratio was 1.35 at 3 h p.i., rising to a constantly high level in the following acquisitions (3.20 at 20 h p.i.; 3.21 at 45 h p.i.; 3.31 at 69 h p.i.; 3.76 at 93 h p.i.). These data indicate a slower washout from tumor than from renal and hepatic tissue. Tumor uptake was persistent – at 93 h p.i., still 34% of the decay-corrected activity determined at the first acquisition was still present in the tumor. The second mouse in the imaging study showed a very similar distribution profile (Supplemental Figure 1).

Ex vivo Biodistribution Study

The ex vivo biodistribution data for ¹⁷⁷Lu-3BP-227 confirmed a high and persistent tumor accumulation (Fig. 2; Supplemental Table 1). HT29 uptake at 3 h p.i. was 19.0 ± 3.6 %ID/g, 8.6 ± 4.0 %ID/g at 20 h p.i. and 2.7 ± 1.6 %ID/g at 69 h p.i.. A mean absorbed radiation dose of 830 mGy/MBq ¹⁷⁷Lu was estimated for the tumor. Low and over time decreasing activity was measured in kidneys. Tumor-to-kidney ratios rose from 5.2 ± 2.5 at 3 h p.i. to 6.3 ± 4.3 at 20 h and 15.9 ± 19.3 at 69 h after injection, respectively, indicating a faster washout of these critical organs than from target tissue. At each time point after injection, tumor uptake exceeded uptake in all other examined tissues considerably. In our dosimetry estimation this translated into low extrapolated human body and organ doses (Supplemental Table 2).

Radioligand Therapy

Two groups of HT29-grafted nude mice were injected with either a median dose of 110 MBq (82-116 MBq) or 165 MBq (129-232 MBq) of ¹⁷⁷Lu-3BP-227, corresponding to an average injected mass of

approximately 1.9 nmol in both groups. Tumor volumes of control animals having received 0.9% NaCl vehicle injections instead of ^{177}Lu -3BP-227 increased rapidly with a median doubling time of 5.5 (2.8; 7.0) days (Fig. 3). Treatment with ^{177}Lu -3BP-227 induced a marked slowing of tumor growth. In the 110 MBq group the median doubling time was 17.5 (5.5; 22.5) days ($P = 0.022$ vs. control) and in the 165 MBq group it was prolonged to 41.0 (27.5; 55.0) d ($P < 0.001$ vs. control; $P = 0.015$ vs. 110 MBq group). At day 23 p.i., most animals in the control group reached tumor volumes that necessitated euthanasia. At this time, median relative tumor volume in the 110 MBq group was reduced by 55% ($P = 0.034$) and by 88% in the 165 MBq group ($P < 0.01$) compared to controls. Differences in relative tumor volume between the 110 MBq group and controls were statistically significant from day 7 p.i. ($P < 0.05$). Between the 165 MBq group and controls, differences were statistically significant from day 4 p.i. ($P < 0.01$). Application of 110 MBq of ^{111}Lu -3BP-227 stabilized tumor size up to a median relative tumor volume level of approximately 2 until day 20 p.i., whereupon tumor growth proceeded similar to controls. The observation of animals receiving 110 MBq was terminated at day 38 p.i. when most of the mice reached endpoint criteria. In comparison to the 110 MBq group, median relative tumor volume in the 165 MBq group was 89% lower at this time ($P = 0.009$). The effect of 165 MBq of ^{177}Lu -3BP-227 was more persistent with a median relative tumor volume of only 2.4 by day 49 p.i.. Absolute tumor volume data are shown in supplemental figure 2.

Kaplan-Meier analysis (Fig. 3) was performed in order to demonstrate tumor growth behavior of individual mice. Four out of nine mice in the 165 MBq group and one animal of ten in the 110 MBq group did not reach the end point, set at reaching twice the initial tumor volume. Log rank tests showed a significantly increased tumor doubling time after application of ^{177}Lu -3BP-227 in comparison with controls (110 MBq vs. control $P = 0.006$, 165 MBq vs. control $P < 0.001$). Applying 165 MBq of the compound had a significantly stronger effect than 110 MBq ($P = 0.013$).

Evaluation of Acute Toxicity

Comparing the relative body weight of the three groups, 165 MBq and 110 MBq groups showed in relation to the controls at day 3-5 p.i. an intermittent weight loss of 7.2% ($P = 0.001$) and 4.7% ($P < 0.001$), respectively. At day 7 p.i. the intervention groups recovered their initial body weight. Hereafter, progression of body mass of the 165 MBq group and the control converged, with no statistically significant differences, whereas body weights of the 110 MBq group initially stayed stable but then increased

continuously, remaining however on a overall lower level than the other two groups (Supplemental Figure 3).

None of the examined functional kidney parameters revealed a significant or relevant change for both 165 and 110 MBq groups versus controls (Fig. 4). No pathologic abnormalities, such as tubular damage, thrombotic microangiopathy, interstitial nephritis or glomerulonephritis, were detectable. No histological differences between kidneys of ^{177}Lu -3BP-227- and vehicle-injected mice were visible.

DISCUSSION

This study presents the first evaluation of the therapeutic effect of an NTR1-antagonistic radiopharmaceutical. 3BP-227 is equipped with a DOTA chelator, allowing incorporation of a range of diagnostic and therapeutic metal radionuclides (e.g., ^{68}Ga for PET, ^{111}In for SPECT, ^{90}Y and ^{177}Lu for RLT). This enables use of 3BP-227 as a theranostic agent, i.e., 3BP-227 labeled with a diagnostic radionuclide could be used to assess NTR1 receptor expression and suitability for 3BP-227-based RLT in an individualized precision medicine approach. This is important as NTR1 expression in different tumor types can vary substantially (9,8,10,25) and patient selection is warranted for a successful treatment.

Based on its advantageous physical properties for RLT especially of smaller tumors (half-life of 6.7 days, maximum energy of 0.5 MeV corresponding to tissue penetration depth of 2 mm), we chose the β -emitter ^{177}Lu as therapeutic radionuclide for this study. Consistent with our previous report on the theranostic potential of 3BP-227 (22), the biodistribution profile of ^{177}Lu -labeled 3BP-227 in NTR1-positive HT29 tumor-bearing mice confirmed high tumor accumulation and low uptake in normal tissues, leading to favorable tumor-to-kidney activity ratios (e.g., 3.2 at 20 h p.i.). Persistent uptake of radiopharmaceuticals in normal tissues can compromise their therapeutic applicability due to potential radiotoxicity. In particular, the accumulation of peptide radiopharmaceuticals in the kidneys has been described and may cause dose-limiting renal toxicity (26). The observed high tumor-to-kidney uptake ratios for ^{177}Lu -3BP-227 suggest that therapeutically effective doses of the radiopharmaceutical are unlikely to cause intolerable radiation damage to the kidneys.

Interestingly, tumor uptake of ^{177}Lu -3B-227 appeared to be significantly higher than of its ^{111}In -labeled analog (22) (e.g., $P = 0.02$ at 20 and 24 h p.i., respectively). This observation cannot be attributed to differences in affinity: the half maximal inhibitory concentration of both compounds has been

determined to be similar in an in vitro functional calcium assay (5.35 nM for ^{nat}In- vs. 5.95 nM for ^{nat}Lu-3BP-227) as well as in a radioligand binding assay (0.76 nM for ^{nat}In- vs. 0.59 nM for ^{nat}Lu-3BP-227) (21). More likely the discrepancy in ¹⁷⁷Lu- versus ¹¹¹In-3BP-227 uptake in tumors as well as other tissues is due to variation in experimental parameters such as batch-to-batch variability of xenografts and administered mass dose, and a head-to-head comparison would be necessary to determine if there are any true differences in the biodistribution of 3BP-227 labeled with different radiometals.

Administration of a single therapeutic dose of ¹⁷⁷Lu-3BP-227 to HT29-xenografted mice led to statistically significant and persistent tumor growth inhibition with a distinct dose-dependent response. A more than three-fold increase of tumor doubling time corresponding to a tumor growth delay of approximately 2 weeks was observed for the 110 MBq group compared to controls. The antitumor effect was even more pronounced in the 165 MBq group with a more than seven-fold increase in tumor doubling time and a tumor growth delay of more than 5 weeks. So far, such a high degree of tumor growth inhibition has not been achieved with NTR1-targeted radiotherapeutics. Comparable studies investigating the antitumoral potential of NTR1-agonistic radioligands were performed using peptidic neurotensin analogs based on the biologically active sequence 8-13. García-Garayoa et al. examined the metabolically stabilized neurotensin derivative (NHis)Ac-Arg-(N-CH₃)-Arg-Pro-Dmt-Tle-Leu (NT-XIX), the ^{99m}Tc-labeled version of which displayed a promising biodistribution pattern for theranostic applications with tumor-to-kidney ratios of 1.9-2.7 between 1.5-24 h p.i. (17). Labeled with Rhenium-188, a significant inhibition of HT29 tumor growth by this peptide was shown in the first week after administration of the first fraction. In subsequent weeks, however, tumor growth did not differ significantly between therapy and control groups, resulting in only marginally increased tumor doubling times under ¹⁸⁸Re-NT-XIX treatment. Maschauer et al. investigated the therapeutic efficacy of the ¹⁷⁷Lu-labeled, metabolically stabilized neurotensin derivative NLys-Lys-Pro-Tyr-Tle-Leu (¹⁷⁷Lu-NT127) (18). Compared to controls, a significant reduction of HT29 tumor volume of 50% was shown 4 wk after the application of a single dose of 50 MBq. However, relatively high tracer uptake in excretory organs was observed, leading to tumor-to-kidney ratios as low as 0.03-0.04 between 1-24 h p.i.. This unspecific accumulation led to significant radiation-induced damage to liver and kidney tissues, hindering clinical translation of ¹⁷⁷Lu-NT127-RLT.

In contrast to previously reported neurotensin derivatives, the medium-sized NTR1-antagonist ¹⁷⁷Lu-3BP-227 elicits a promising therapeutic effect with a favorable biodistribution profile. In line with the

observed biodistribution pattern showing low accumulation and absorbed radiation doses in excretory organs, there was no evidence of radiation-induced acute side effects to the kidneys after administration of ^{177}Lu -3BP-227. Our dosimetry estimates show that even when administering a rather high cumulative patient dose of 50 GBq ^{177}Lu -3BP-227, the absorbed radiation dose for the kidneys should be only around 6 Gy, which is far below the postulated 23 Gy threshold for increased probability of developing severe late kidney damage. While we have observed cross-reactivity of 3BP-227 with murine and human NTR1 (unpublished data), under- or overestimation of renal damage in patients through extrapolation from the xenograft model is still possible due to potential species differences in NTR1 kidney expression and/or radiotracer pharmacokinetics. The observed reduction in body weight gain was only transient and not directly dose-dependent. Taken together, these results suggest that ^{177}Lu -3BP-227, while having a statistically significant inhibitory effect on tumor growth, induces no overt signs of acute nephrotoxicity at the doses studied. However, radiation nephropathy has been observed to occur late (> 6 months) after RLT (27,28). Long-term toxicity as well as comprehensive dosimetry studies are needed to evaluate if measures for kidney protection such as dose fractionation and/or co-administration of renoprotective agents are necessary in conjunction with ^{177}Lu -3BP-227 therapy. Furthermore, considering the relatively high initial blood pool activity of ^{177}Lu -3BP-227, its haematotoxic safety profile needs to be assessed in future studies.

CONCLUSION

This study demonstrates the therapeutic potential of ^{177}Lu -labeled 3BP-227. ^{177}Lu -3BP-227 inhibited HT29 tumor growth to a high degree and in a dose-dependent manner without significant acute toxic effects on normal tissues. The favorable properties for both patient stratification and RLT applications underline the high potential of 3BP-227 as a theranostic agent for clinical translation. Further preclinical evaluations, for example evaluation in other tumor models as well as multi-dose, detailed dosimetry and toxicity studies, are currently ongoing and paving the way towards clinical studies of the therapeutic efficacy of ^{177}Lu -3BP-227 in cancer patients.

DISCLOSURE

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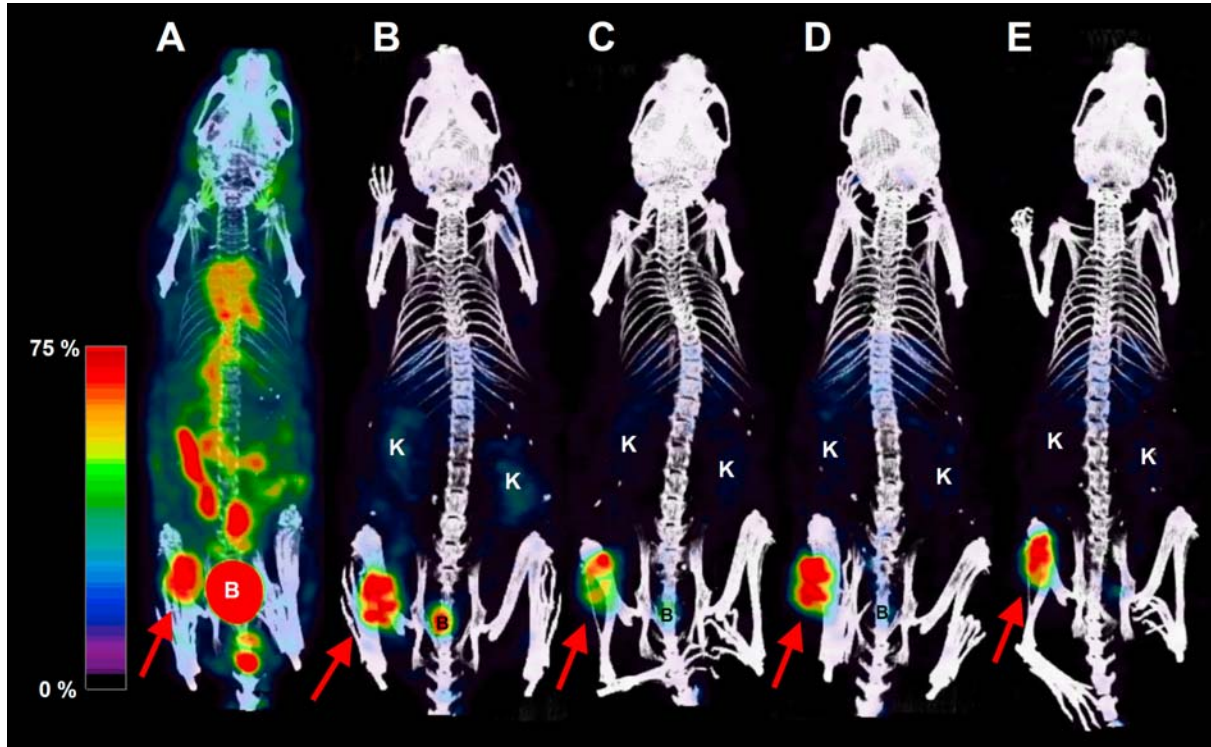


FIGURE 1. Co-registered whole-body SPECT/CT scans of a single female nude mouse grafted with HT29 tumor cells acquired 3 (A), 20 (B), 45 (C), 69 (D), and 93 (E) hours p.i. of 130.7 MBq of ^{177}Lu -3BP-227. Arrows show HT29 tumors. SPECT data were scaled to 0-75% of maximum count value in the tumor of the respective dataset. B = Urinary bladder, K = Kidney.

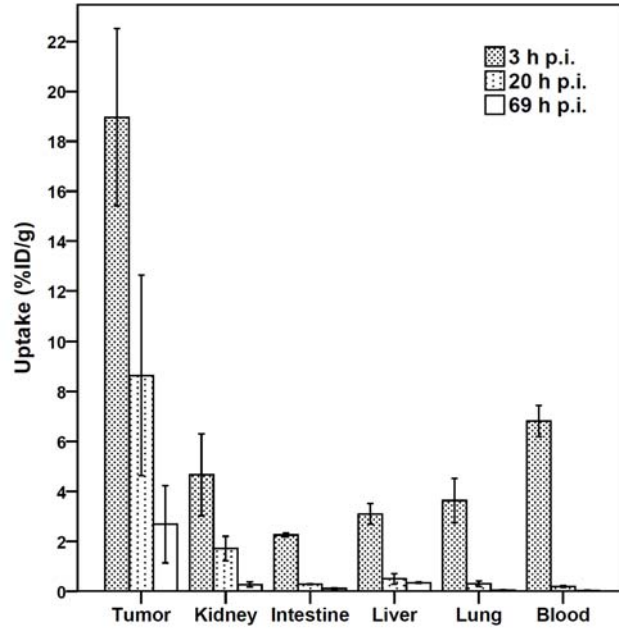


FIGURE 2. Uptake of ^{177}Lu -3BP-227 in selected tissues 3, 20 and 69 h after injection (mean %ID/g \pm SD).

n = 5 mice per time point.

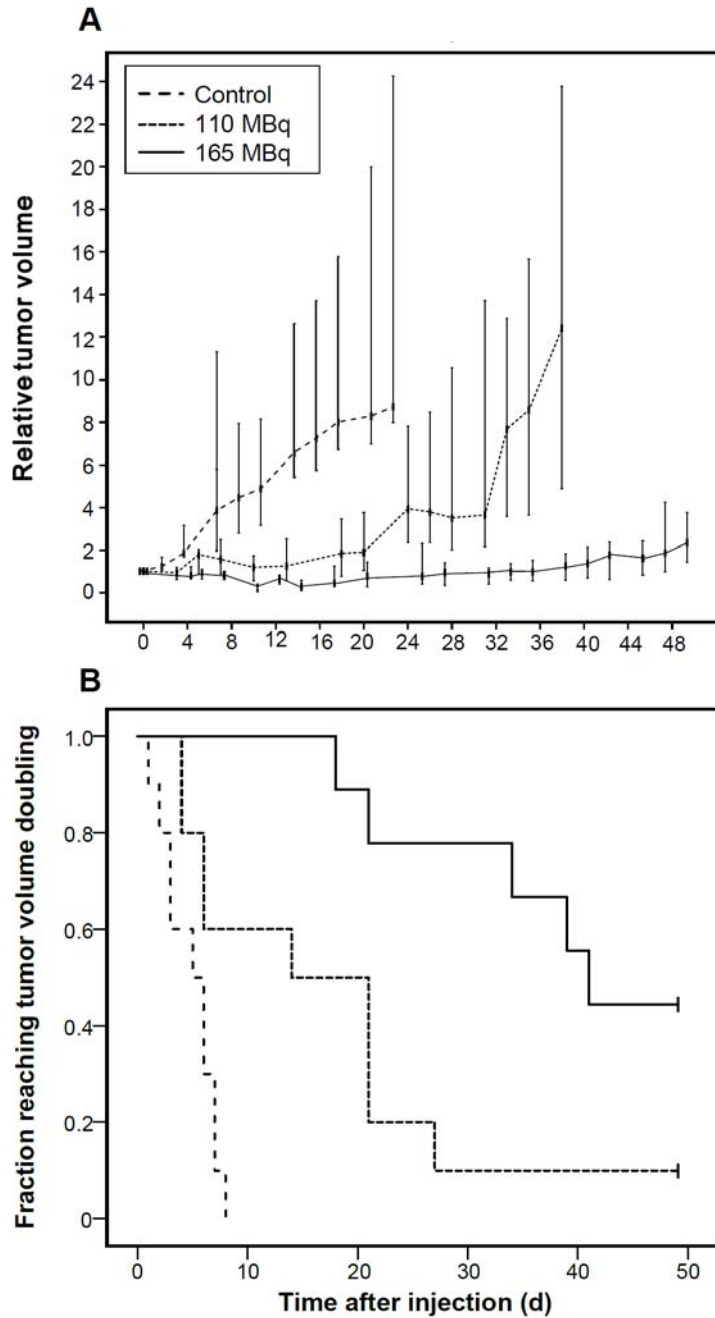


FIGURE 3. Therapeutic effect of ^{177}Lu -3BP-227 in female nude mice grafted with HT29 cells (A). Tumor volume data are normalized to the measurement at the day of ^{177}Lu -3BP-227 injection and represented as median and interquartile range. Kaplan-Meier plot (B) showing tumor progression of HT29 in the same study. The end point was set at reaching a relative tumor volume of ≥ 2 . Control: n = 10, 165 MBq group: n = 9, 110 MBq group: n = 10.

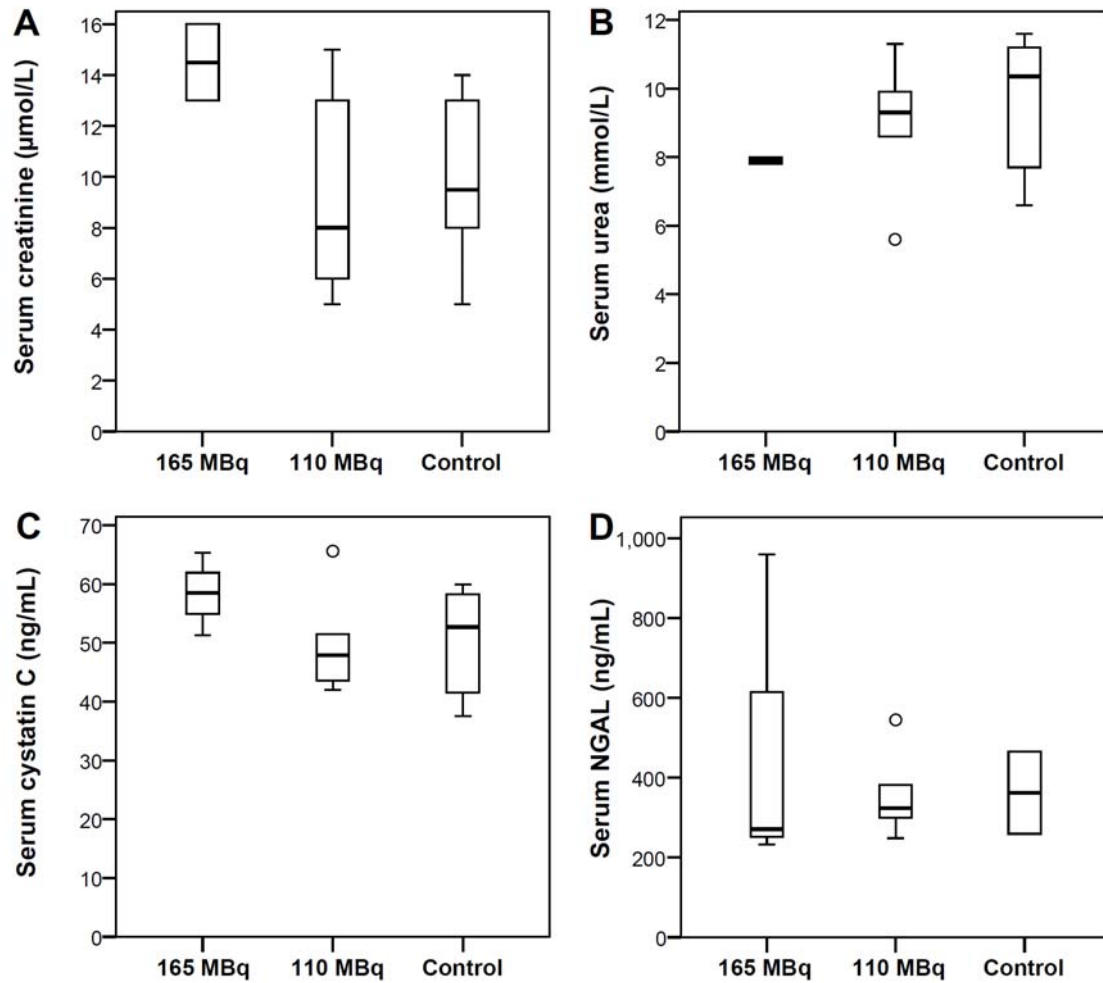
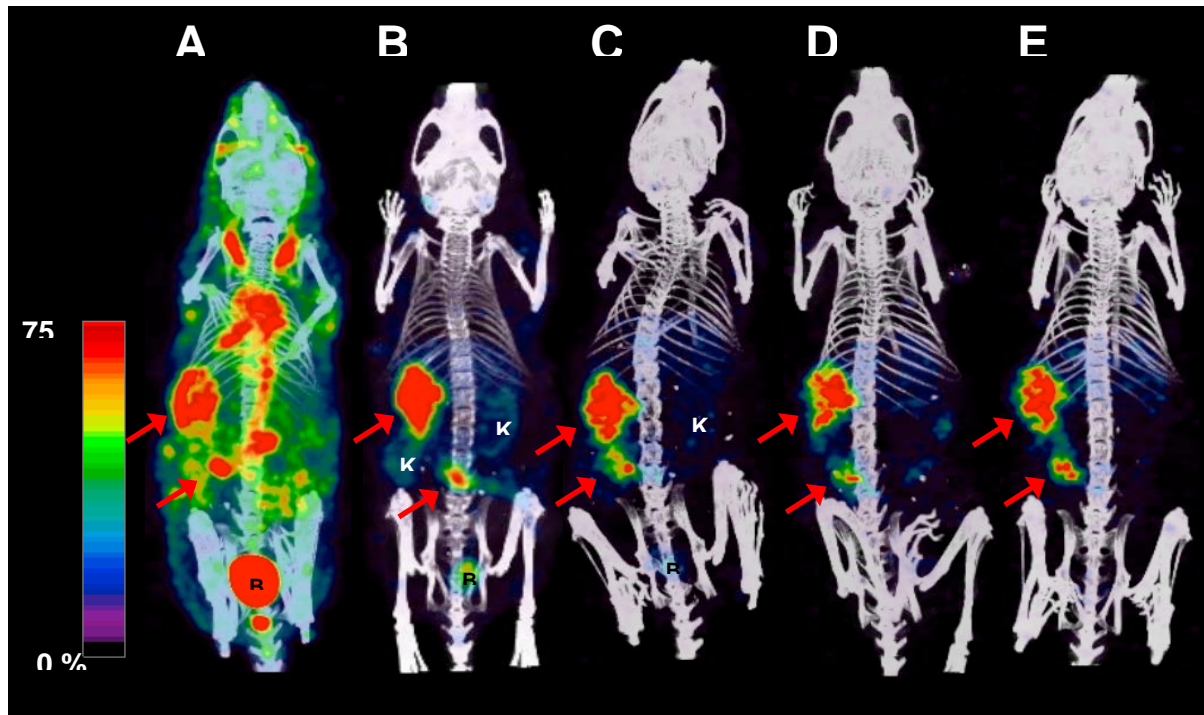
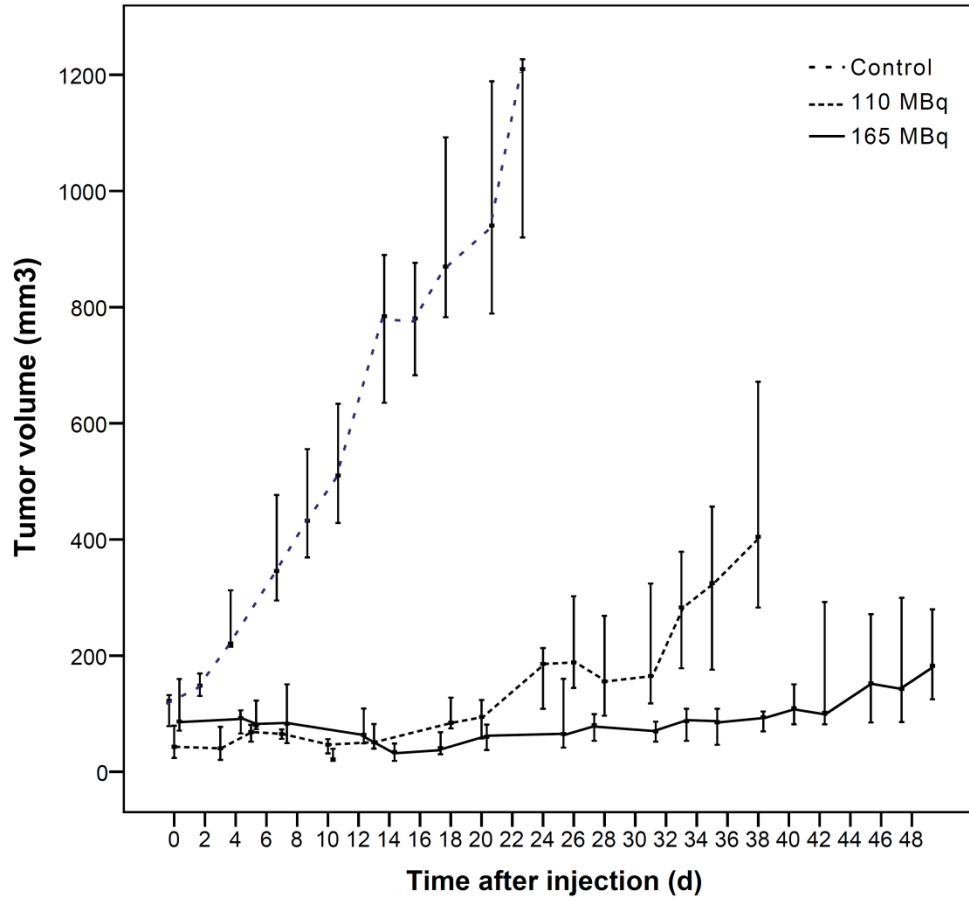


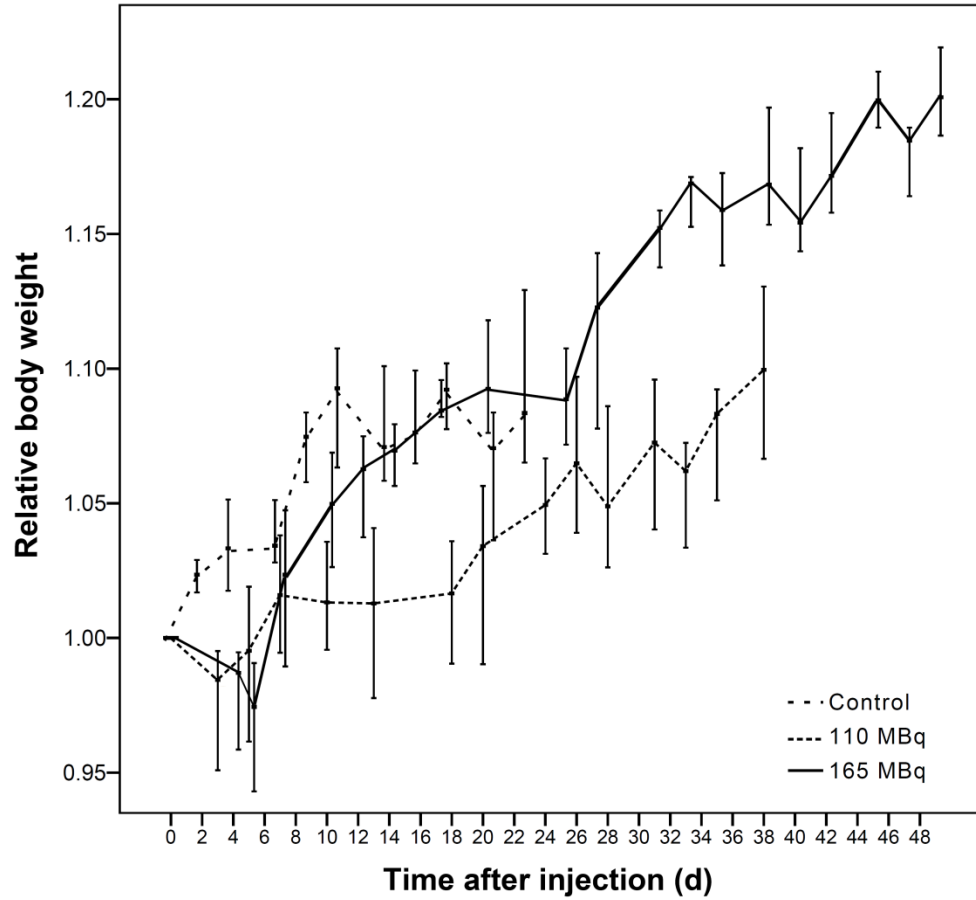
FIGURE 4. Serum concentrations of functional kidney biomarkers - creatinine (A), urea (B), cystatin C (C), Neutrophil gelatinase-associated lipocalin (NGAL) (D) - of female nude mice grafted with HT29 cells obtained after injection of a median dose of 165 MBq or 110 MBq of ^{177}Lu -3BP-227 or 0.9% NaCl as control. Sample sizes (165 MBq group/ 110 MBq group/ Control): A 2/5/6, B 2/5/6, C 3/5/6, D 3/5/2. Statistical analysis comparing biomarker concentrations was performed by Kruskal-Wallis-test with no significant differences detected.



SUPPLEMENTAL FIGURE 1. Co-registered whole-body SPECT/CT scans of a single female nude mouse grafted with HT29 tumor cells acquired 3 (A), 20 (B), 45 (C), 69 (D), and 93 (E) hours p.i. of 159.7 MBq of ^{177}Lu -3BP-227. Arrows show HT29 tumors that have grown irregularly as two closely located tumors and overlap the ipsilateral kidney. SPECT data were scaled to 0-75% of maximum count value in the tumor of the respective dataset. B = Urinary bladder, K = Kidney. Tumor-to-kidney activity ratios: 1.11 at 3 h p.i., 2.81 at 20 h p.i., 4.42 at 45 h p.i., 5.75 at 69 h p.i. and 7.84 at 93 h p.i. Tumor-to-liver activity ratios: 1.51 at 3 h p.i., 4.02 at 20 h p.i., 3.65 at 45 h p.i., 3.70 at 69 h p.i. and 3.62 at 93 h p.i..



SUPPLEMENTAL FIGURE 2. Absolute tumor volumes after injection of 165 MBq (n = 9) or 110 MBq (n = 10) of ¹⁷⁷Lu-3BP-227 or 0,9% NaCl as control (n = 10) in nude mice grafted with HT29 cells. Data are presented as median and interquartile range.



SUPPLEMENTAL FIGURE 3. Body weight development of nude mice grafted with HT29 cells after injection of 165 MBq (n = 9) or 110 MBq (n = 10) of ^{177}Lu -3BP-227 or 0,9% NaCl as control (n = 10). Body weight data are normalized to the measurement at the day of injection and represented as median and interquartile range.

SUPPLEMENTAL TABLE 1. Tissue uptake and tumor-to-normal tissue ratios of ¹⁷⁷Lu-3BP-227 in female nude mice grafted with NTR1-positive HT29 cells.

	3 h p.i. n = 5	20 h p.i. n = 5	69 h p.i. n = 5
Tumor	18.96 ± 3.55 [†]	8.64 ± 4.00 [‡]	2.68 ± 1.55
Heart	2.23 ± 0.29	0.16 ± 0.03	0.04 ± 0.02
Lungs	3.63 ± 0.89	0.30 ± 0.11	0.04 ± 0.01
Kidney	4.67 ± 1.64	1.71 ± 0.49	0.27 ± 0.11
Liver	3.10 ± 0.42	0.50 ± 0.21	0.34 ± 0.03
Spleen	1.96 ± 0.60	0.38 ± 0.06	0.07 ± 0.05
Stomach*	1.06 ± 0.80	0.18 ± 0.07	0.03 ± 0.01
Intestine*	2.26 ± 0.09	0.29 ± 0.02	0.11 ± 0.02
Adipose tissue	0.97 ± 0.48	0.15 ± 0.09	0.05 ± 0.03
Muscle	1.04 ± 0.13	0.18 ± 0.03	0.02 ± 0.02
Bone	1.03 ± 0.67	0.50 ± 0.13	0.26 ± 0.13
Brain	0.21 ± 0.12	0.03 ± 0.02	0.03 ± 0.03
Blood	6.82 ± 0.63	0.19 ± 0.03	0.03 ± 0.01
Urine	63.20 ± 29.06	3.58 ± 0.51	0.46 ± 0.07
Tumor/blood	2.78 ± 0.48	44.23 ± 13.45	85.04 ± 49.56
Tumor/liver	6.23 ± 1.44	32.65 ± 45.90	7.76 ± 4.56
Tumor/muscle	18.42 ± 4.07	49.63 ± 22.54	172.69 ± 206.60
Tumor/kidney	5.15 ± 2.47	6.33 ± 4.31	15.86 ± 19.28

Uptake is expressed as mean percentage of injected dose per gram of tissue (%ID/g) ± SD. *Organs with their content. [†]*P* = 0.0005 vs. ¹¹¹In-3B-227 3 h p.i. [‡]*P* = 0.022 vs. ¹¹¹In-3B-227 24 h p.i. (¹¹¹In-3BP-227 data: Schulz J, Rohracker M et al. Comparative Evaluation of the Biodistribution Profiles of a Series of Nonpeptidic Neurotensin Receptor-1 Antagonists Reveals a Promising Candidate for Theranostic Applications. *J. Nucl. Med.* 2016;57:1120-1123.)

SUPPLEMENTAL TABLE 2. Organ and effective doses (mSv/MBq) after application of ¹⁷⁷Lu-3BP-227.

Target Organ	Dose (mSv/MBq)
Brain	0.0002
Intestine	0.0106
Stomach Wall	0.0085
Heart Wall	0.0525
Kidneys	0.1250
Liver	0.0087
Lungs	0.0599
Muscle	0.0006
Red Marrow	0.0014
Osteogenic cells	0.0047
Spleen	0.0875
Effective Dose	0.0114