Published in final edited form as:

Bioconj Chem. 2016 November 16; 27(11): 2658–2668. doi:10.1021/acs.bioconjchem.6b00418.

Investigation of the Biological Impact of Charge Distribution on a NTR1-Targeted Peptide

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Abstract

The neurotensin receptor 1 (NTR1) has been shown to be a promising target, due to its increased level of expression relative to normal tissue, for pancreatic and colon cancers. This has prompted the development of a variety of NTR1-targeted radiopharmaceuticals, based on the neurotensin (NT) peptide, for diagnostic and radiotherapeutic applications. A major obstacle for the clinical translation of NTR1-targeted radiotherapeutics would likely be nephrotoxicity due to the high levels of kidney retention. It is well-known that for many peptide based agents renal uptake is influenced by the overall molecular charge. Herein, we investigated the effect of charge distribution on receptor binding and kidney retention. Using the [(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13) targeting vector, three peptides (¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6), with the Lys moved closer (K6) or further away (K2) from the pharmacophore, were synthesized. *In vitro* competitive binding, internalization/efflux and confocal microscopy studies were conducted using the NTR1-positive HT-29, human colon cancer cell line. The ¹⁷⁷natLu-K6 demonstrated the highest binding affinity (21.8 $\pm$ 1.2 nM) and the highest level of internalization (4.06% $\pm$ 0.20% of the total added amount). *In vivo* biodistribution, autoradiography and metabolic studies of ¹⁷⁷Lu-radiolabeled K2, K4 and K6 were examined using CF-1 mice. ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 gave the highest levels of *in vivo* uptake in NTR1-positive tissues, whereas ¹⁷⁷Lu-K2 yielded nearly two-fold higher renal uptake relative to the other radioconjugates. In conclusion, the position of the Lys (positively charged amino acid) influences the receptor binding, internalization, *in vivo* NTR1-targeting efficacy and kidney retention profile of the radioconjugates. In addition, we have found that hydrophobicity of the radioconjugates

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Supporting Information. Graphs describing the biodistribution results of ¹⁷⁷Lu-K2, K4 and K6 with 0.5% BSA as well as ¹⁷⁷Lu-N1 are given in the Supporting Information. In addition, an example chromatogram of the purification of ¹⁷⁷natLu-K6 is presented.

The authors have no potential conflict of interest relevant to this article.

and/or generated radiometabolites likely play a role in the unique biodistribution profiles of these agents.

Introduction

The neurotensin receptor 1 (NTR1) is a G-protein coupled receptor that has been linked to proliferative action in a number of cancers, including colon and pancreatic cancer^{1, 2}. In addition, or as a consequence of this proliferative pathway, NTR1 has also been shown to be overexpressed on the cellular surface of these cancers. These findings have resulted in significant interest in developing NTR1-targeted radiopharmaceuticals for diagnostic and therapeutic purposes^{3–12}. To date, NTR1-targeted agents have been largely based on neurotensin (NT), a thirteen amino acid peptide that has demonstrated nanomolar binding affinity to the receptor. Researchers have principally focused on targeting vectors based on the last six (NT(8-13)) or eight (NT(6-13)) amino acids of the NT sequence. Of the two targeting vectors, the NT(6-13) peptide demonstrates a marginally higher binding affinity to the NTR1¹³. One major obstacle that hampered the development of NTR1-targeted radiopharmaceuticals early on was the rapid proteolytic degradation of the NT in the serum^{14, 15}. This situation is aggravated by the inability of *in vitro* serum studies to adequately predict *in vivo* stability which led to significant underperformance of the NTR1-targeted agents in preclinical and clinical studies^{6, 16}. The *in vivo* instability of these agents has led to the development of more stable, synthetic derivatives of the NT pharmacophore, which demonstrate significantly better *in vivo* performance^{7, 10, 11, 17}.

The development of receptor-targeted, radiotherapeutic agents based on small peptide targeting vectors has been an active area of research over the last three decades^{18–20}. However, the translation of these radiotherapeutic peptides, including NTR1-targeted agents, into the clinic has been, in many cases, hampered by high renal uptake which can result in dose-limiting toxicities^{21, 22}. A variety of reports have investigated both the mechanism and structure-activity relationships corresponding to the renal uptake and retention of radiolabeled peptides^{21, 23, 24}. From these studies, it has been recognized that the molecular charge is a significant factor in the renal uptake of radiolabeled peptides, including NTR1-targeted agents which typically contain two to three positively-charged amino acids (Arg and Lys)^{23, 25, 26}. This high renal uptake has been a major hurdle for the potential development and translation of radiotherapeutic, NTR1-targeted agents.

Our laboratory has recently begun exploring the development of NTR1-targeted agents using a stabilized [(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13) targeting vector (Lys-Pro-(N- α -Me)Arg-Arg-Pro-Dmt-Tle-Leu)³. Although this pharmacophore exhibits excellent NTR1-positive tumor uptake, it has a total charge of +2 and exhibits significant renal uptake, reducing its potential for radiotherapeutic applications. While the molecular charge/renal uptake relationship for NTR1-agents has been established, we are interested in determining if charge distribution impacts the receptor avidity and renal uptake and retention of these agents. Herein, we investigate the biological impact of the translation of the Lys⁶ relative to the rest of the pharmacophore. Using a series of NTR1-targeted agents, Figure 1, we explore

the structure-activity relationship of these agents using *in vitro* and *in vivo* models of the HT-29 human colon adenocarcinoma cell line.

RESULTS

Synthesis and radiolabeling

Three NT analogs (Figure 1) with linking groups consisting of one lysine (Lys) and four D-leucine ((D)Leu) in the paradigm DOTA-X-[(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(7-13), where X= Lys-(D)Leu-(D)Leu-(D)Leu-(D)Leu, K2; (D)Leu-(D)Leu-Lys-(D)Leu-(D)Leu, K4; and (D)Leu-(D)Leu-(D)Leu-(D)Leu-Lys, K6, were synthesized by SPPS. The three analogs, K2, K4 and K6, were named based on the position of Lys in the peptide sequence. RP-HPLC retention time, mass spectrometric identification and yields of the unlabeled conjugates and ^{nat}Lu-conjugates are listed in Table 1. The isolated yields of the conjugates were relatively poor, likely due to the sterics resulting from conjugation to the secondary amines of the prolines and (N- α -Me)Arg³. The purified conjugates were subsequently radiolabeled with ¹⁷⁷/^{nat}LuCl₃ (t_{1/2} = 6.73 d) by co-incubation in an ammonium acetate buffer (pH = 5.5) for 45 min at 90 °C. The radiochemical yields for ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4, and ¹⁷⁷Lu-K6 were 77.5%, 79.14% and 75.88%, respectively.

In addition to the radioconjugates, a Cy5 fluorescent analog of K6 (Cy5-K6) was synthesized in order to perform cell trafficking studies. Cy5-K6 was prepared directly from SPPS, Figure 2, with an overall isolated yield of 2.5%.

Distribution coefficient

To evaluate the impact of the Lys position on the water solubility of the radioconjugates, octanol-PBS distribution coefficients at pH 7.4 were measured. The log D_{oct/water} values for ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4, and ¹⁷⁷Lu-K6 were -2.53 ± 0.02 , -2.59 ± 0.02 and -2.63 ± 0.01 , ($P < 0.05$), respectively. While, as expected, the distribution coefficients were similar, the relative hydrophilicities were ¹⁷⁷Lu-K6 > ¹⁷⁷Lu-K4 > ¹⁷⁷Lu-K2, indicating that movement of the Lys closer to pharmacophore increases hydrophilicity, albeit slightly.

In vitro competitive binding studies

The NTR1 binding affinity of the unlabeled conjugates and ^{nat}Lu-labeled conjugates was investigated by competitive binding studies using HT-29 cells. The IC₅₀ values are given in Table 1. For the unlabeled conjugates, the IC₅₀ value for K2 was the highest ($P < 0.001$) signifying the poorest binding analog investigated. In contrast, K6 had the lowest IC₅₀ value ($P < 0.001$) indicating the highest affinity to the NTR1. For the ^{nat}Lu-labeled K2, K4 and K6, the same trend was found (i.e., binding affinity of ^{nat}Lu-K6 > ^{nat}Lu-K4 > ^{nat}Lu-K2). This reveals that the proximity of the Lys to the pharmacophore plays a role in increasing the binding strength of the conjugate to the NTR1.

In vitro internalization and efflux studies

The effect of the position of the positive charged amino acid (Lys) on the rates of internalization (Figure 3) and efflux (Figure 4) of the radioconjugates was investigated in HT-29 cells. At time points 15, 30, 60 and 120 min, the cellular uptake (internalized and

surface-bound radioactivity) of the radioconjugates was represented as a percentage of total activity added. ^{177}Lu -K6 exhibited a statistically higher level of uptake ($P < 0.0001$) relative to ^{177}Lu -K2 and ^{177}Lu -K4 at each time point from 15 min to 120 min. The internalization of ^{177}Lu -K2 and ^{177}Lu -K4 was not significantly different over the initial 60 min time period. However, by 120 min post-incubation, a statistically significant increase was observed for ^{177}Lu -K4. At the 120 min post-incubation time point, the overall uptake for the radioconjugates were ^{177}Lu -K6 ($4.06 \pm 0.20\%$) $>$ ^{177}Lu -K4 ($2.64 \pm 0.04\%$) $>$ ^{177}Lu -K2 ($1.91 \pm 0.09\%$).

Efflux studies were conducted by incubating the radioconjugates with HT-29 cells for 2 h, washing the cells with media to remove extracellular radioactivity and monitoring the release of radioactivity from the cells into the extracellular environment. The effluxed fractions of ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6 were calculated at the 0.5, 1, 2, 4 and 24 h time points. By the 1 h time point, the effluxed fraction for ^{177}Lu -K6 ($15.74 \pm 1.13\%$) was substantially lower than ^{177}Lu -K2 and ^{177}Lu -K4 ($24.78 \pm 0.43\%$ and $25.15 \pm 0.93\%$ respectively, $P < 0.0001$). Statistically lower levels of efflux were observed for ^{177}Lu -K6 relative to the other radioconjugates for the remaining time points. By the 24 h time point, ^{177}Lu -K6 had effluxed only 20% of the internalized radioactivity relative to 28% and 31% for ^{177}Lu -K4 and ^{177}Lu -K2, correspondingly.

Confocal studies of the Cy5-K6

Using HT-29 cells, confocal microscopy images of the Cy5-K6 (pink) were taken at 0.5, 2 and 24 h, Figure 5. Additionally, early endosome (Rab5a, green), late endosome/lysosome (Rab7a, red) and acidic compartments (LysoTrackerTM, blue) were stained and their colocalization with Cy5-K6 quantified, Table 2. At 30 min post-incubation, the Cy5-K6 was shown primarily at the surface of the HT-29 cells with a small fraction of the Cy5-K6 entering the cell and co-localizing with the early endosome marker (green). By 2 h post-incubation, Cy5-K6 had distributed into vesicles that contained all three markers with low to moderate correlation. The Cy5-K6 demonstrated strong correlation/retention in vesicles that were both Rab7a- and LysoTrackerTM-positive by the 24 h post-incubation time point.

In vivo biodistribution studies

We investigated the *in vivo* biodistribution of ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6 at the 1, 4 and 24 h time points (Figure 6, 7 & 8). All three compounds, ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6, were effectively cleared from the blood after 4 h post-injection (0.04 ± 0.02 %ID/g, 0.06 ± 0.08 %ID/g and 0.03 ± 0.03 %ID/g, respectively). At all time points investigated, ^{177}Lu -K2 and ^{177}Lu -K6 exhibited substantially higher levels of liver uptake and retention, relative to ^{177}Lu -K4. At 1 h, the liver uptake values for ^{177}Lu -K2 and ^{177}Lu -K6 were 5.64 ± 0.47 %ID/g and 6.65 ± 0.71 %ID/g, correspondingly. By the 24 h time point, the liver uptake for ^{177}Lu -K2 and ^{177}Lu -K6 had dropped modestly to 4.73 ± 0.75 %ID/g and 5.67 ± 0.57 %ID/g, respectively. Additionally, ^{177}Lu -K6 demonstrated significantly higher lung uptake (5.08 ± 1.31 %ID/g) at 1 h post-injection relative to the other conjugates ($< 1.11 \pm 0.15$ %ID/g). The substantial level of uptake in the liver and lung prompted us to investigate if the biodistribution of the radioconjugates was hindered by aggregate formation. BSA has been shown to inhibit the aggregation of peptides/proteins in

solution^{27, 28}. The three radioconjugates were mixed with saline containing 0.5% BSA, to impede any potential aggregation of the radioconjugates. With this in hand, the 1 h biodistribution experiments were repeated (data presented in the supporting information, Figure S1) and yielded no substantial changes in organ uptake and overall distribution profiles.

The kidney uptake of ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 were statistically lower than that of ¹⁷⁷Lu-K2 at all time points. At 1 h, the kidney uptake of ¹⁷⁷Lu-K2 was 127.51 ± 13.14 %ID/g, two fold higher than that of ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 (64.8 ± 10.81 %ID/g and 68.98 ± 6.22 %ID/g, respectively). By 24 h post-injection, the kidney retention of ¹⁷⁷Lu-K2 had decreased substantially to 77.75 ± 10.15 %ID/g, but still significantly higher than ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 (48.75 ± 9.59 %ID/g and 43.96 ± 6.92 %ID/g, correspondingly).

To evaluate the *in vivo* targeting capabilities of the NTR1-targeted agents, the uptake of the radioconjugates in the small and large intestines (NTR1-positive tissues) were evaluated. At the initial 1 h time point, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 demonstrated a statistically higher uptake ($P < 0.001$) in the small intestines (4.25 ± 0.75 %ID/g and 4.46 ± 0.58 %ID/g, correspondingly) relative to ¹⁷⁷Lu-K2 (2.72 ± 0.48 %ID/g). By 24 h post-injection, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 retained 67.1 and 57.0 % of the 1 h uptake, while ¹⁷⁷Lu-K2 preserved 66.2 % of the initial uptake. Similar trends of uptake and retention for the radioconjugates were observed in the large intestines data. A blocking study was performed to demonstrate the NTR1-mediated nature of the intestinal uptake. Using the ¹⁷⁷Lu-K6 as our model radioconjugate, the blocking study at 1 h demonstrated a substantial reduction of 82.1% and 62.6 % in small and large intestinal uptake, respectively. Important to note, substantial reduction in uptake due to NTR1-blocking was not observed in the kidneys or liver, suggesting this uptake is not NTR1-mediated. However, interestingly, the lung uptake of ¹⁷⁷Lu-K6 was shown to be substantially lower upon NTR1-blocking implying it was NTR1-mediated.

Autoradiography of the kidney

The distribution of radioconjugates/radiometabolites in the kidneys at 10 and 60 min post-injection was investigated using autoradiography, depicted in Figure 9. The autoradiographs of the ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 show that even in the early time points, the radioactivity is largely localized in the cortex of the kidney. While the position of the Lys group does impact the extent of kidney retention, based on the biodistribution studies, these autoradiographic images demonstrate that it does not influence residual localization in the kidney.

Metabolic studies in mice

To evaluate the metabolism of the radioconjugates *in vivo*, metabolic analyses were performed on the blood and urine at 10 and 60 min, respectively, Figure 10. For the blood metabolism studies, the recovery efficiencies of the radioactive components from the samples were ~70 %. At 10 min post-injection, there were substantially higher levels of radioactivity observed in the blood for ¹⁷⁷Lu-K2 and ¹⁷⁷Lu-K4 (AUC: 22.56 and 14.99, respectively) compared to ¹⁷⁷Lu-K6 (AUC: 2.61). However, the relative percent of intact

radioconjugate were similar, ranging from 70.6 – 74.4 %. Three primary metabolites were observed in the blood for ^{177}Lu -K2 and ^{177}Lu -K6, while five were found for ^{177}Lu -K4. The radiometabolites isolated from the blood had shorter retention time relative to the intact radioconjugate suggesting an increased hydrophilicity. Urine analysis at the 1 h time point demonstrated that 43.2, 49.8 and 34.0 % of the signal corresponded to the intact ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6, respectively. Three to four major radiometabolites were observed for each radioconjugate. Correlative identification based on retention times of the radiometabolites in the blood and urine was somewhat obfuscated due the different experimental conditions (e.g., sample matrices, analyte concentrations, etc.) leading to shifts in retention times. However, the major radiometabolite peaks for both the blood and urine samples had similar retention times and, for the most part, shift patterns suggesting that many of the radiometabolites were likely chemically identical.

Discussion

To date, NTR1-targeted radiopharmaceuticals have been largely based on two targeting vector constructs: NT(6-13) and NT(8-13)^{3–5, 7, 8, 10, 17}. While the NT(8-13) contains all of the necessary interactions to ensure low nanomolar binding affinity to the NTR1, the NT(6-13) fragment includes a terminal Lys⁶ which has been used by a variety of investigators as a convenient functionalization site for the incorporation of chelation systems (e.g. DTPA and DOTA)^{5, 17, 29}. Recently, our laboratory reported a DOTA incorporated [(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13) analog, based on work from Gruaz-Guyon and co-workers⁵, in which the Lys⁶ remained unfunctionalized (charged)³. This analog exhibited significantly higher uptake in NTR1-positive tissues and kidneys compared to reported analogs that contain a functionalized (non-charged) Lys⁶^{5, 17}. In order to better understand the role Lys⁶ plays on tissue uptake, we investigated the impact of positional translation of the Lys, relative to the pharmacophore, on the biological performance of a series of NTR1-targeted peptides, ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6, Figure 1.

Evaluation of the receptor binding affinity of the NTR1-targeted analogs revealed that the position of the lysine has a modest impact on receptor binding. Binding affinities were found to decrease correlatively as the lysine is translated away from the main pharmacophore suggesting that Lys⁶ has a role in stabilizing the receptor-ligand complex. Based on what is known concerning NTR1-ligand interactions³⁰, we posit that the lysine is able to form weak charge-charge interactions with the electronegative rim of the binding pocket. The internalization efficacies of the NTR1-targeted agents were also found to correlatively decrease as the lysine moved toward the N-terminus of the peptide. This signifies that the ^{177}Lu -K6, with Lys⁶, is more efficient at inducing the internalization of the receptor, possibly due to enhanced stabilization/residence time of the NTR1-ligand complex. Efflux studies revealed that both ^{177}Lu -K2 and ^{177}Lu -K4 exhibited significantly higher levels of externalization relative to ^{177}Lu -K6. However, it is unclear to us how the structure of the NTR1-targeted agents resulted in this observation.

The cellular trafficking of fluorescently labeled NTR1-targeted agents has been previously reported by Vandenbulcke, Falciani and co-workers^{31, 32}. From our multicolor confocal microscopy studies, the initial uptake (i.e., 30 minute time point) of the Cy5-K6

demonstrated that internalized fluorescence was taken up, as expected, into the early endosomal compartments. Interestingly, the rate of Cy5-K6 uptake was much slower in HT-29 cells than what was observed by Vandenbulcke and co-workers using NTR1-transfected COS-7 cells³¹. This may be due to a number of factors including inherent differences in the two cell line phenotypes and levels of NTR1 expression. At 2h post-incubation, the Cy5-K6 demonstrated moderate levels of signal overlap ($0.36 \pm 0.11 - 0.56 \pm 0.18$) with the three compartmental markers. However, by 24 h of continuous exposure to Cy5-K6, strong levels of overlap, 0.75 ± 0.13 and 0.76 ± 0.15 , was observed for the Rab7a-positive (red) and LysotrackerTM-positive (blue) vesicles, correspondingly. Both Rab7a and LysotrackerTM are markers for late endosomes/lysosomes. Given the 24 h time point, it seems likely that most of the Cy5-K6 has matriculated to its terminal location in the lysosomes.

Using biodistribution studies in CF-1 mice, the *in vivo* targeting efficacy of the NTR1-targeted agents were evaluated. For our NTR1-positive tissue, we utilized the small and large intestines of the mouse, which are known to endogenously express NTR1³³. The small intestinal uptake for ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 were statistically higher than ¹⁷⁷Lu-K2 at all time points with $P < 0.01$. Similarly, uptake in the large intestines followed the same trend, but did not meet statistical significance. Blocking studies for ¹⁷⁷Lu-K6 confirmed the NTR1-mediated nature of the uptake in the intestinal tissues. Given the observed binding affinity trend of ¹⁷⁷Lu-K6 > ¹⁷⁷Lu-K4 > ¹⁷⁷Lu-K2, the uptake in the intestines suggests other factors than just binding affinity play a role in determining the *in vivo* NTR1-targeting efficacy of these agents.

The comparison of ¹⁷⁷Lu-K6 with our previously published analog ¹⁷⁷Lu-N1, which used the same [(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13) pharmacophore, yielded interesting disparities³. The *in vitro* binding and internalization characteristics of ¹⁷⁷natLu-N1 were similar to ¹⁷⁷Lu-K6. However, the uptake of ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6, in the small intestines (4.25 ± 0.75 and 4.46 ± 0.58 %ID/g, correspondingly) at 1h were significantly higher than observed for ¹⁷⁷Lu-N1 (3.31 ± 0.41 %ID/g; biodistribution profile shown in Figure S2 in the supporting information). This increased uptake is likely associated with the decreased hydrophilicity of this series of agents relative to ¹⁷⁷Lu-N1 ($\log D_{\text{oct/water}} = -3.15 \pm 0.05$) leading to enhanced circulation times. This agrees well with the blood retention profile of ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6, which are approximately three fold higher at 1 h p.i. than the more hydrophilic ¹⁷⁷Lu-N1. Despite being more hydrophobic, ¹⁷⁷Lu-K2 yielded poorer intestinal uptake than ¹⁷⁷Lu-N1 due to the comparably lower NTR1 binding affinity.

All of the NTR1-targeted agents demonstrated significantly ($P < 0.0001$) higher kidney uptake (5–10 fold) than observed with the previously reported analog, ¹⁷⁷Lu-N1 (13.05 ± 0.18 %ID/g at 1 h). This uptake in the kidneys is not NTR1-mediated as demonstrated by our blocking study. It is well-known that radiolabeled peptides are taken up by the kidney through active (e.g., megalin and cubulin) and passive (e.g., pinocytosis) means^{34, 35}. Additionally, the overall charge of the radioconjugate has been shown to influence the extent of renal uptake. Since the overall charge (+2) of ¹⁷⁷Lu-N1 and this series of NTR1-targeted agents are identical, we attribute this substantial increase in renal uptake to the increased hydrophobicities of ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6.

As demonstrated by autoradiography studies, the retention of the radioactivity in the kidney occurred in the cortex, which is consistent with literature reports for neurotensin and other small receptor-targeted peptides³⁶. ¹⁷⁷Lu-K2 demonstrated approximately two-fold higher kidney uptake/retention relative to ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6. Based on the distribution coefficients, the differences in the hydrophobicities of the radioconjugates were negligible. Additionally, no substantial differences in the metabolism of ¹⁷⁷Lu-K2 relative to the other radioconjugates were noted. Currently, it is unclear why the structure of ¹⁷⁷Lu-K2 yields such a significant increase in kidney uptake. We hypothesize that this dissimilarity is due to unexpected higher renal uptake by one of the uptake mechanisms for ¹⁷⁷Lu-K2 and/or one of its resulting radiometabolites.

For both ¹⁷⁷Lu-K2 and ¹⁷⁷Lu-K6, unexpected retention was observed in the liver and/or lung. For the DTPA and DOTA containing NTR1-targeted agents reported to date, significant uptake in these tissues has not been observed^{3-5, 16, 17}. Since these peptides are significantly more hydrophobic than previously reported analogs, initial thoughts were that aggregation of the peptides might be contributing to the uptake in these non-target tissues. Yet, co-formulation with BSA did not substantially alter the biodistribution profiles of the three radioconjugates. Interestingly, some reported ^{99m}Tc-NTR1-targeted agents have been shown to have high liver and lung uptake^{11, 37}. Distribution coefficients were not reported for these analogs. However, based on their structures, we conjecture that hydrophobicity may be a contributing factor.

Conclusion

In summary, we investigated the impact of Lys charge distribution on the biological performance of a series of NTR1-targeted peptides. It was found that localization of the Lys closer to the pharmacophore lead to modest, but significant, increases in NTR1 affinity and rates of NTR1-mediated internalization. *In vivo* NTR1-targeting was best for radioconjugates (¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6) with the Lys functionality closest to the targeting vector. The charge distribution of the Lys had an unexpected and substantial impact on uptake in the liver, lungs and kidneys. Additionally, the comparison of these findings with the literature suggests that hydrophobicity plays a modest role in NTR1-mediated tissue uptake, but was particularly impactful for non-specific uptake by non-target organs.

Materials

Acetonitrile, formic acid, *N,N*-diisopropylethylamine (DIEA), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N,N'*-dicyclohexylcarbodiimide (DCC), methyl-*t*-butyl ether, *N*-methylpyrrolidone (NMP), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), LysoTracker™ Blue DND-22, CellLight® early endosome-green fluorescent protein (GFP) and CellLight® late endosome-red fluorescent protein (RFP) were purchased from Fisher Scientific (Fair Lawn, NJ). Fluorenylmethyloxycarbonyl (Fmoc)-protected natural amino acids, Fmoc-(D)leu-OH, Fmoc-Leu-Wang resin (100–200 mesh), and (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) were purchased from NovaBiochem (Hoherbrunn, Germany). Fmoc-N-Me-Arg(Pbf)-OH was produced by ChemPep, Inc. (Wellington, FL). Fmoc-2,6-

dimethyl-L-tyrosine (Dmt) was from Ontario Chemicals, Inc (Guelph, ON, Canada). Fmoc-Tle-OH was purchased from CreoSalus (Louisville, KY). Lutetium-177 chloride ($^{177}\text{LuCl}_3$) was obtained from Perkin Elmer (Waltham, MA) with a specific activity of 32.3 Ci/mg. Naturally abundant lutetium chloride ($^{nat}\text{LuCl}_3$), triisopropylsilane and 3,6-dioxa-1,8-octanedithiol were from Sigma-Aldrich (St Louis, MO). McCoy's 5A medium (1X; Iwakata & Grace Mod.) with L-glutamine was obtained from Mediatech, Inc. (Manassas, VA). TrypLE™ Express was purchased from Invitrogen (Grand Island, NY). Penicillin-Streptomycin solution and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from HyClone Laboratories, Inc (Logan, UT). Heparin was purchased from Elkins-Sinn, Inc (Cherry Hill, NJ). Fetal Bovine Serum (FBS) was purchased from Gibco™ by Life Technologies Corporation (Grand Island, NY). BD Cytotfix™ Fixation buffer was purchased from BD Biosciences (San Jose, CA). The O.C.T compound for tissue embedding was from Sakura Finetek USA, Inc. (Torrance, CA).

Methods

Cell culture

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (U.S.) and cultured under vendor-recommended conditions. Cells were passaged twice weekly in McCoy's 5A medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

Mice Model

All animal experiments were conducted in accordance with the Principles of Animal Care outlined by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Eight-week-old CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed five per cage in a light- and temperature-controlled environment. Food and water were given ad libitum.

Solid-Phase Peptide Synthesis (SPPS)

Peptides were synthesized on an automated solid-phase Liberty microwave peptide synthesizer from CEM (Matthews, NC), employing traditional Fmoc chemistry. Briefly, the Fmoc-Leu-Wang resin (100 µmol of the resin-substituted peptide anchors) was deprotected by piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. Fmoc-protected amino acids (300 µmol) were activated with COMU and sequentially conjugated to the resin. The resulting peptide was orthogonally deprotected and cleaved from the resin by shaking in a cocktail consisting of triisopropylsilane (0.125 ml), water (0.125 ml), 3,6-dioxa-1,8-octanedithiol (0.125 ml), and trifluoroacetic acid (4.625 ml) for 3 hours. The cleaved peptide was subsequently precipitated and washed thrice using cold (0 °C) methyl-*tert*-butyl ether (40 ml×3). The crude peptides were dried under vacuum.

Synthesis of Cyanine5-K6 as fluorescent dye

Cyanine5-K6 was synthesized by conjugation of the Cyanine5 (Cy5) carboxylic acid to the NH₂-K6-Wang resin. Briefly, a mixture of Cy5-COOH (6 μ mol), NH₂-K6-Wang-resin (6 μ mol), COMU (30 μ mol) and DIEA (30 μ mol) was dissolved in DMF and shaken overnight at room temperature. After the reaction, the Cy5-K6 was cleaved from the Wang resin and purified by HPLC.

HPLC Purification and Analysis Methodology

HPLC/MS analyses were performed on a Waters (Milford, MA) e2695 system equipped with a Waters 2489 absorption detector and a Waters Q-ToF Micro electrospray ionization mass spectrometer. Sample purification for *in vitro* studies was performed on a Phenomenex (Torrance, CA) Jupiter 10 μ m Proteo 250 $\times$ 4.6 mm C12 column with a flow rate of 1.5 mL/min. For bulk sample purification, a Phenomenex Jupiter 10 μ m Proteo 250 $\times$ 10 mm C12 column was used with a flow rate of 5.0 mL/min. HPLC solvents consisted of H₂O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For unlabeled and ¹⁷⁷/_{nat}Lu-conjugates of K2, K4 and K6, an initial gradient of 85% A: 15% B linearly decreased to 70% A: 30% B over a 15-minute time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient 5% A: 95% B and re-equilibrated to the starting gradient.

Labeling with ^{nat}LuCl₃

Naturally abundant Lutetium (^{nat}Lu) was substituted for ¹⁷⁷Lu in the ES-MS and *in vitro* binding studies. The conjugate (0.10 mg, 0.06 μ mol) was dissolved in ammonium acetate buffer (0.5 M, 200 μ L, pH 5.5) and mixed with a solution of ^{nat}LuCl₃ (1.7mg, 6 μ mol). The solution was heated for 45 min at 90 °C. After cooling to room temperature, ^{nat}Lu-conjugates were then peak purified by RP-HPLC. All ^{nat}Lu-conjugates were 95% pure before mass spectrometric characterization and *in vitro* binding studies were performed.

Radiolabeling with ¹⁷⁷LuCl₃

The conjugate (50 μ g, 30 nmol) was dissolved in ammonium acetate buffer (0.5 M, 100 μ L, pH 5.5). ¹⁷⁷LuCl₃ (37 MBq, 1 mCi, 0.18 nmol) was added to the vial containing the conjugate, and the solution was heated for 45 min at 90 °C. To separate radiolabeled peptides from unlabeled peptides on the HPLC, 4–5 mg of CoCl₂ was added and incubated for 5 min at 90 °C to increase the hydrophobicities of the unlabeled conjugates [20]. After cooling to room temperature, evaluation and purification of the radiolabeled conjugates were performed on a Waters 1525 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (Poway, CA) Flow Count radiometric detector system. An example chromatogram demonstrating the separation of ¹⁷⁷/_{nat}Lu-K6 is given in Figure S3 in the supporting information. The collected radioconjugate was concentrated with an Empore (Eagan, MN) C18 high performance extraction disk followed by elution with ethanol/sterile saline solution (6:4, 400 μ L) to provide the radiolabeled conjugates in high purity. When required, a BSA-saline solution was added to the radioconjugate to give a solution containing 0.5% BSA.

Distribution coefficient

The distribution coefficient was determined (n = 6, 2 technical (tech) and 3 biological (bio) repeats) for each ^{177}Lu -labeled radioconjugate. In a 1.5 ml centrifuge tube, 0.5 mL of 1-octanol was added to 0.5 mL of phosphate-buffered saline (pH 7.4) containing the radiolabeled peptide (500,000 cpm). The solution was vigorously stirred for 2 min at room temperature and subsequently centrifuged ($8000 \times g$, 5 min) to yield two immiscible layers. Aliquots of 100 μL were taken from each layer and the radioactivity of each was quantified by an LTI (Elburn, IL) Multi-Wiper nuclear medicine gamma counter.

In vitro competitive binding studies

As described previously^{3, 38}, the half maximal inhibitory concentration (IC_{50}) for each conjugate was determined (n = 6, 2 tech and 3 bio repeats) for each ^{177}Lu -labeled radioconjugate using the HT-29 human colon cancer cell line. In these studies, ^{177}Lu -N1 (^{177}Lu -DOTA- β -Ala-[N- α -Me⁸,Dmt¹¹,Tle¹²]NT(6-13)) served as the competitive radioligand for comparing the relative effectiveness of the unlabeled and labeled conjugates. HT-29 cells ($\sim 1 \times 10^6$) were suspended in 100 μL of McCoy's 5A medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 37 °C for 45 min in the presence of ^{177}Lu -N1 (100,000 cpm, 100 μL) and various concentrations of the unlabeled conjugates and $^{\text{nat}}\text{Lu}$ -conjugates (100 μL). At the end of the incubation, the cells were centrifuged, aspirated and washed with media five times. The cell-associated radioactivity was measured using a gamma counter and the IC_{50} values determined by nonlinear regression using the one-binding site model of GraphPad Prism 5 (U.S.).

In vitro internalization and efflux studies

The *in vitro* internalization and efflux studies were performed as stated previously^{38, 39}. HT-29 cells ($\sim 1 \times 10^6$) were suspended in 100 μL of McCoy's 5A medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). Cells were incubated at 37°C with each ^{177}Lu -radioconjugate (100,000 cpm) for up to 2 h. At 15, 30, 60 and 120 min time points, cells were washed five times with media to remove the unbound peptide. Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The amount of radioactivity remaining in each cellular pellet was assigned as the internalized fraction. The cellular uptake of the radioconjugates were presented as a percentage of total activity added with groups representing internalized and surface-bound radioactivity.

For efflux studies, HT-29 cells ($\sim 1 \times 10^6$) were incubated in six-well plates overnight. On the day of the experiment, HT-29 cells were incubated for 2 h at 37°C in the presence of 100,000 cpm of each ^{177}Lu -radioconjugate. Cells were washed five times with medium (1 ml) to remove the unbound peptide followed by the addition of fresh medium. At 0, 0.5, 1, 2, 4 and 24 h, the medium for each time point was harvested for quantitative analysis of ligand efflux. Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The cells were then lysed using a 10 % aqueous SDS solution to quantify the remaining internalized fractions. The radioactivity in the effluxed, surface-bound and internalized fractions for each radioconjugate was determined using a gamma counter. The effluxed fraction is expressed as

a percentage of the total radioactivity in the well, which is the sum of the effluxed, surface-bound and fraction remaining in the cell.

Confocal microscopy images of the Cy5-K6

HT-29 cells were cultured on a Lab-Tek chambered #1.0 borosilicate coverglass disk (4 well) overnight at a concentration of 0.05 million cells per well. The CellLight™ early endosome-GFP and CellLight™ late endosome-RFP reagents were added to each well and incubated overnight in a humidified atmosphere with 5% CO₂ at 37 °C. After incubation, HT-29 cells were washed with fresh medium and incubated with LysoTracker Blue DND-22 for 2 h at a concentration of 1 nM. After again washing the cells, the Cy5-K6 was diluted in McCoy's 5A medium and incubated with HT-29 cells. At 0.5, 2 and 24 hour, the unbound Cy5-K6 was washed off. The formaldehyde fixation buffer was added to the cells and incubated for 10 min. The cells were washed with PBS and fluorescent images were obtained using an excitation wavelength of 373 nm (blue), 488 nm (green), 555 nm (red) and 650 nm (Cy5). Confocal microscopy images were taken on a Leica LSM 510 META Microscope equipped with an argon laser.

In vivo biodistribution studies

Biodistribution studies were carried out using healthy CF-1 mice. Each mouse (average weight, 25 g) received an intravenous bolus, via the tail vein, of ¹⁷⁷Lu-radiolabelled K2, K4 or K6 (370 kBq, 10 µCi) in 100 µL of saline. At 1, 4 and 24 hours post injection, the mice were sacrificed and the amounts of radioactivity in the tissues were counted with a NaI (TI) well detector from AlphaSpectra, Inc. (U.S.). The excised tissues were weighed and results were expressed as percentage of injected dose per gram of tissue (%ID/g). Blocking studies were carried out by co-injection with excess unlabeled K6 (250 µg).

Metabolic studies in mice

For metabolic evaluation assays, ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4 or ¹⁷⁷Lu-K6 (37 MBq, 1mCi) was injected in the tail vein of CF-1 mice. After 10 min, blood was drawn from the heart of the animals under anesthesia and collected with prechilled polypropylene tubes, containing heparin, and placed on ice. Blood samples were centrifuged at 2000 g/4°C for 10 mins, plasma was collected, mixed with chilled acetonitrile (1:1 v/v ratio), and centrifuged at 15,000 g/4°C for 10 min. Supernatants were concentrated to a smaller volume under a gentle N₂ gas, diluted with saline, and filtered through a Millex GV filter (0.22 µm). After 1 h, urine was collected, filtered with a Millex GV filter (0.22 µm). All samples were subsequently analyzed by RP-HPLC using the conditions previously stated.

Autoradiography of the kidney

The kidneys were collected from the metabolic studies, washed with deionized water, dried and immediately embedded in O.C.T compound on dry ice. Cryostat sections (10 µm) of tumor sample were exposed to the phosphor plate for 2 days. The phosphor plate was subsequently scanned by a Typhoon FLA 9500 variable mode imager (GE Lifesciences) at a 10 µm resolution.

Statistical analysis

Comparisons of groups for *in vitro* and *in vivo* studies were analyzed by the unpaired two-tailed Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank James R. Talaska and Janice Taylor at the Advanced Microscopy Core Facility at UNMC for assistance in collecting and interpreting the confocal microscopy images. The authors thank Chantey Morris at the UNMC Nanomaterials Characterization Core Facility (NIGMS 2P20 GM103480-08) for assistance using the Typhoon FLA 9500 variable mode imager. In addition, the authors would like to thank Dhruvkumar Soni and Sameer Alshehri for assistance with collecting data for the biodistribution studies. Lastly, the authors would like to gratefully acknowledge the National Institutes of Health (1 R01 CA179059 01A1), the Nebraska Department of Health and Human Services and the Nebraska Cancer and Smoking Disease Research Program (2017-21) for funding and support of this research.

Abbreviations

NTR1	neurotensin receptor 1
NT	neurotensin
FBS	Fetal bovine serum
BSA	Bovine serum albumin
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
SPPS	solid phase peptide synthesis

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Figure 1.

Structures of the ^{177}Lu -DOTA-X-[(N- α -Me)Arg⁸,Dmt¹¹,Tle¹²]NT(7-13) analogs (Lu-K2, Lu-K4 and Lu-K6) used in this study.

Figure 2.
Synthesis of the Cy5-K6 that was utilized in cell trafficking studies.

Figure 3.
The uptake of ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6 (internalization and surface bound) by HT-29 cells. Values are means $\pm$ SD ($n = 6$).

Figure 4.
The efflux of the internalized ¹⁷⁷Lu-K2, ¹⁷⁷Lu-K4 and ¹⁷⁷Lu-K6 by HT-29 cells. Values are means $\pm$ SD ($n = 6$).

Figure 5.
Confocal microscopy images of HT-29 cells with GFP-Rab5a, RFP-Rab7a, LysoTracker and Cy5-K6 at 30 min, 2 h and 24 h.

Figure 6.

Biodistribution studies at 1 h p.i. for ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6 in CF-1 mice. The blocking study was performed with co-administration of 250 μg of K6. The radioactivity uptake of tissues is expressed as a percentage of injected dose per gram of tissue (%ID/g). Values are means $\pm$ SD ($n = 5$).

Figure 7. Biodistribution studies at 4 h p.i. for ^{177}Lu -K2, ^{177}Lu -K4 and ^{177}Lu -K6 in CF-1 mice. The radioactivity uptake of tissues is expressed as a percentage of injected dose per gram of tissue (%ID/g). Values are means $\pm$ SD ($n = 5$).

Figure 8. Biodistribution studies at 24 h p.i. for $^{177}\text{Lu-K2}$, $^{177}\text{Lu-K4}$ and $^{177}\text{Lu-K6}$ in CF-1 mice. The radioactivity uptake of tissues is expressed as a percentage of injected dose per gram of tissue (%ID/g). Values are means $\pm$ SD ($n = 5$).

Figure 9.

Autoradiographic images of kidneys sections from CF-1 mice obtained at 10 and 60 min post-administration of $^{177}\text{Lu-K2}$, $^{177}\text{Lu-K4}$ and $^{177}\text{Lu-K6}$.

Figure 10.

(A) Metabolites of ^{177}Lu -K2 in the blood at 10 min (upper) and in the urine at 60 min (lower); (B) Metabolites of ^{177}Lu -K4 in the blood at 10 min (upper) and in the urine at 60 min (lower); (C) Metabolites of ^{177}Lu -K6 in the blood at 10 min (upper) and in the urine at 60 min (lower).

Table 1

Characterization of K2, K4 and K6

Analog	Mass Cal. ([M+2H] ²⁺)	Mass Obs. ([M+2H] ²⁺)	Retention Time (min)	Yields	IC ₅₀ ± SD (nM)
K2	962.21	962.49	8.93	5%	48.6 ± 1.2
K4	962.21	962.54	8.77	3%	31.0 ± 1.1
K6	962.21	962.04	8.00	4%	21.8 ± 1.2
^{nat} Lu-K2	1048.20	1048.51	8.82	69%	35.6 ± 1.1
^{nat} Lu-K4	1048.20	1048.56	7.42	76%	23.5 ± 1.1
^{nat} Lu-K6	1048.20	1048.55	7.47	71%	14.8 ± 1.1

Table 2

Colocalization of Cy5-K6

Overlap coefficient	30 min (n=4)	2 h (n=4)	24 h (n=6)
Rab7a+ and LysoTracker+	0.80 ± 0.12	0.75 ± 0.07	0.73 ± 0.09
Rab7a+ and Cy5-K6+	0	0.56 ± 0.18	0.75 ± 0.13
LysoTracker+ and Cy5-K6+	0	0.49 ± 0.14	0.76 ± 0.15
Rab5a+ and Cy5-K6+	0.51 ± 0.03	0.36 ± 0.11	0.62 ± 0.07