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# Computational studies and synthesis of <sup>131</sup>I-iodine-labeled nocardiotide A analogs as a peptide-based theragnostic radiopharmaceutical ligand for cancer targeting SSTR2†

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Radiolabeled peptides belong to a highly specific group of radiotracers used in oncology, particularly for diagnostics and cancer therapy. With the notable advantages of high binding affinity and selectivity to cancer cells, they have proven to be very useful in nuclear medicine. As a result, efforts have been focused on discovering new peptide sequences for radiopeptide preparation. Nocardiotide A, a cyclic hexapeptide comprising the amino acids cyclo-Trp-Ile-Trp-Leu-Val-Ala (cWIWLVA) isolated from *Nocardioopsis* sp., has shown significant cytotoxicity against cancer cells, rendering it a suitable candidate for the process. Therefore, the present study aimed to design a stable and effective radiopeptide by labeling nocardiotide A with iodine-131 (<sup>131</sup>I), ensuring that its affinity to SSTR2 is not compromised. *In silico* study showed that structural modification of nocardiotide A labeled with <sup>131</sup>I-iodine exhibited good affinity value, forming hydrogen bonds with key residues, such as Q.102 and T.194, which are essential in SSTR2. Based on the results, cyclic hexapeptides of cWIWLVA were selected for further synthesis, and its peptide product was confirmed by the presence of an ionic molecule peak *m/z* [M + Na]<sup>+</sup> 855.4332 (yield, 25.60%). *In vitro* tests conducted on cWIWLVA showed that cWIWLVA can bind to HeLa cancer cells. Radiopeptide synthesis was initiated with radiolabeling of cWIWLVA by <sup>131</sup>I using the chloramine-T method that showed a radiochemical yield of 93.37%. Non-radioactive iodine labeling reaction showed that iodination was successful, which detected the presence of di-iodinated peptide (I<sub>2</sub>-cWIWLVA) with *m/z* [M + Na]<sup>+</sup> 1107.1138. In summary, a radiopeptide derived from nocardiotide A showed great potential for further development as a diagnostic and therapeutic agent in cancer treatment.

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## 1 Introduction

Radiolabeled peptides, also known as radiopeptides, serve as a carrier to transport radioisotopes to the cancer site for its diagnosis and therapy.<sup>1</sup> They specifically target overexpressed peptide receptors in cancer tissues, exhibiting selectivity towards tumor-specific cells. This unique property, stemming from their high-affinity characteristics, enables the radiopeptide to be highly effective in early cancer detection and treatment initiation. With these advantages, the search and development of novel peptide compounds are on the rise.<sup>2</sup> One of the promising candidates is a radiopeptide designed to target the peptide

receptor of somatostatin receptor 2 (SSTR2),<sup>3,4</sup> offering enhanced visualization for cancer imaging and targeted therapy.<sup>5</sup>

Naturally occurring peptides with cytotoxic properties were predominantly developed as anticancer peptides (ACPs) due to their ability to traverse cell membranes and penetrate the cancer cell.<sup>6</sup> To enhance penetration, it is crucial to utilize peptides with low molecular weight (*M<sub>w</sub>* < 10 kDa). It is also required to resist proteolytic degradation through possible modification by cyclization, resulting in increased affinity.<sup>7</sup>

Nocardiotide A, a cyclic hexapeptide composed of cyclo-Trp-Ile-Trp-Leu-Val-Ala (cWIWLVA), is derived from marine sponges of *Callyspongia* sp. This compound exhibits significant cytotoxicity against human MM.1S multiple myeloma, human HeLa cervix carcinoma, and murine cells CT26 colon carcinoma with IC<sub>50</sub> 8, 11, and 12 μM mL<sup>-1</sup>, respectively.<sup>8</sup> The distinctive biological activity demonstrated by nocardiotide A makes it an attractive candidate for the development of an ACP. Furthermore, the total chemical synthesis of this compound has been achieved through a combination of solid-phase peptide synthesis (SPPS) and liquid-phase peptide synthesis (LPPS),

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resulting in a yield of 20%.<sup>9</sup> Given present evidence on its selective anticancer activity and the ease of synthesis, nocardiotide A holds a great potential as a lead compound that can be modified, synthesized, and developed into a radiopeptide for cancer detection and therapy.

The choice of a radioisotope that can be easily incorporated into the substrate with minimal structural modification is crucial for the production process. In the previous computational study, nocardiotide A labeled by technetium-99m showed radiolabeling had an impact with decreasing the binding free energies.<sup>10</sup> Among the available options for radiolabeling, <sup>131</sup>I stands out as one of the most favorable. It possesses a relatively long half-life of 8.05 days as well as emits gamma radiation (82%, 0.364 MeV) and beta particles (86%, 0.607 MeV). This combination of characteristics makes <sup>131</sup>I an ideal candidate for the development of theragnostic agents, which can be utilized in diagnostic procedures, therapy, and monitoring the efficacy of therapy in nuclear imaging.<sup>11</sup> A variety of small-molecule polypeptides have radiolabeled by iodine-131.<sup>12–14</sup> In addition, the iodine radioisotope also presents as <sup>123</sup>I for diagnostic application with SPECT and <sup>124</sup>I for PET imaging.

The labelling process using <sup>131</sup>I involves its incorporation into tyrosine or histidine residues within the peptide. However, the original structure of nocardiotide A does not provide accessibility to these residues. To circumvent this limitation, a modification of nocardiotide A can be conducted by incorporating histidine and tyrosine into the original structure. Therefore, the present study aims to design an analog of nocardiotide A and synthesize the selected peptide sequence based on docking results.<sup>15–17</sup> Additionally, cytotoxicity was assessed and a preliminary study on radiolabelling of the newly developed peptide by <sup>131</sup>I for radiopeptide candidates was conducted.

## 2 Experimental

### 2.1 Docking studies

A complete 3D structural model of SSTR2 was obtained from RCSB with PDB ID 7XAT. In order to introduce a radiolabeling target into the original nocardiotide A, its analogs were designed by incorporating histidine and tyrosine. The molecular interaction assessment of the <sup>131</sup>I labeled nocardiotide A analogs with SSTR2 were conducted for both mono-iodinated [<sup>131</sup>I-peptide] and di-iodinated peptide [<sup>131</sup>I<sub>2</sub>-peptide] structures as shown in Fig. 1. The obtained structure from geometry optimization was subsequently utilized for molecular docking simulation, where the interactions study of selected analogs to the SSTR2 was performed by AutoDock Vina 1.1.6.<sup>18,19</sup> To determine hydrogen and hydrophobic bonds, MGLTools 1.5.6 and Discovery Studio 2020 were employed. Finally, molecular dynamics simulations were performed using AMBER18, running for 200 ns for each peptide and labeled iodine peptide with the best value of free binding energy in SSTR2.

### 2.2 Synthesis of the selected peptide

The SPPS method was employed to synthesize linear hexapeptides using 2-chlorotrityl chloride resin and the Fmoc

strategy. A mixture of dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) served as the solvent, while *N,N'*-diisopropylcarbodiimide (DIC) and oxyma were utilized as coupling reagents. The Fmoc group was removed with 20% piperidine solution in DMF, and the completion of the coupling protocol was monitored by the chloranil test. In the synthesis, all amino acids used were Fmoc-protected and all side-chain functionalities were protected with TFA labile groups. To release the linear peptides from the resin, a mixture of acetic acid (AcOH), trifluoroethanol (TFE), and dichloromethane (DCM) in a ratio of 2 : 2 : 6 was used as the cleavage solution for a duration of 2 hours. This resulted in linear hexapeptides with protected side chains characterized by TOF-ESI-MS (Waters Q-ToF MS Xevo). Following this, the protected precursor was used for the cyclization reaction without purification.

The cyclization of peptide was performed in the solution phase using 1.14 equivalents of PyBOP under a diluted concentration of 2.0 mM DMF in the presence of basic DIPEA (1.65 eq.) and NaCl in H<sub>2</sub>O (5 eq.). The reaction mixture was stirred for 24 hours at room temperature and monitored by thin-layer chromatography using iso-propanol : *n*-hexane (8 : 2) as the eluent. Subsequently, it was extracted using ethyl acetate and saturated NaCl solution. The ethyl acetate phase was collected and evaporated to remove the solvent. The crude cyclic product from the extraction was subjected to deprotection by 95% TFA in water.<sup>20</sup> The peptide synthesis procedures are shown in S.1,† with the resulting product being purified, characterized by TOF-ESI-MS and RP-HPLC (Agilent 1260). Analytical RP-HPLC was performed with C18 column (ZORBAX Eclipse XBD C18 4,6 × 150 mm) at a flow rate of 1.0 mL min<sup>-1</sup> (with wave length detection of 254 nm) with 100% H<sub>2</sub>O (solvent A) and 100% acetonitrile (solvent B). The analytical HPLC starts from initial settings 100% A for 10 min and is followed by a linear gradient of 100% A and 0% B to 20% A and 80% B over 40 min. The gradient remained at this position for 25 min before switching back to initial settings of 100% A for another 5 min.

### 2.3 In vitro study

To evaluate the cytotoxic activity of the selected peptide, an MTT test was performed on HeLa cancer cells, with synthesized cWIWLVA serving as the lead compound for comparison. The cells were cultured in RPMI medium supplemented with FBS and penicillin–streptomycin, and then incubated in a 5% CO<sub>2</sub> incubator at 37 °C. After 48 h, the cultures were transferred into 96 well plates with a certain density per well and incubated for an additional 48 h. They were treated with peptides at various concentration range and incubated for another 48 h. Following the treatment period, 100 μL of culture medium containing MTT at a concentration of 5 mg mL<sup>-1</sup> was added to each well, and the plates were incubated for 3 hours at 37 °C. The purple formazan crystals that formed were dissolved by adding 10% sodium dodecyl sulphate (SDS) stopper reagent and left in the dark overnight. Furthermore, the quantity of formazan, which was presumed to be directly proportional to the number of viable cells, was measured using an ELISA reader by calculating



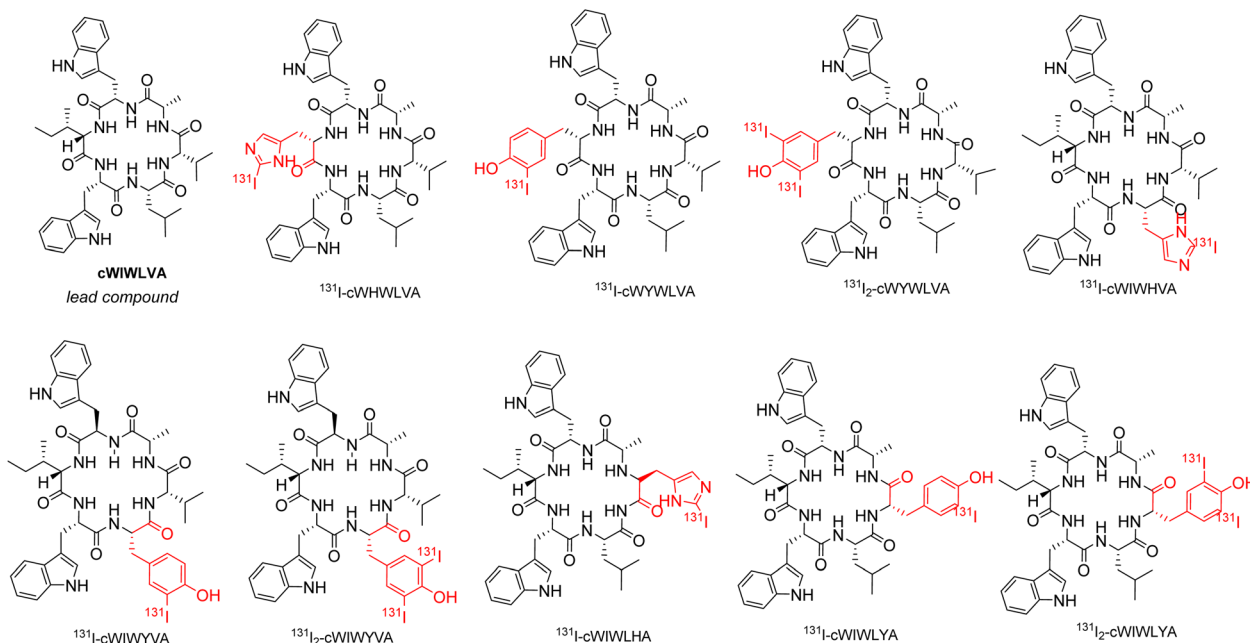


Fig. 1 Chemical structure of nocardiotide-A (cWIWLVA) and its analogs labeled by  $^{131}\text{I}$ .

the absorbance at 595 nm.<sup>21,22</sup> The  $\text{IC}_{50}$  values of the selected peptide were estimated and compared to those of the lead compound using Prism GraphPad 8.4.3 software.

#### 2.4 Radiolabeling of peptide

The process began by dissolving 3 mg of the peptide in a small amount of DMSO, then  $\text{H}_2\text{O}$  was added to up 1.5 mL. The reagents including chloramine-T ( $8 \text{ mg mL}^{-1}$ ) and sodium metabisulfite ( $7 \text{ mg mL}^{-1}$ ) were individually dissolved in 1 mL of  $0.05 \text{ mol L}^{-1}$  PBS (pH 7.4). Radioisotope  $^{131}\text{I}$  (3 mCi) was added to the 450  $\mu\text{L}$  peptide solution followed by 100  $\mu\text{L}$  chloramine-T. The mixture was vortexed at room temperature for 2 minutes and 100  $\mu\text{L}$  sodium metabisulfite was then added to stop the reaction. The radiolabelling efficiency was assessed by thin layer chromatography (Whatmann 3MM) using 0.9% saline as the mobile phase. The stability of radiolabeled peptide at room temperature was then evaluated for several days, while its stability in human plasma was observed for 72 hours. Radiochemical yield was estimated by TLC scanner (TLC 204, Comacer) and  $\gamma$ -counter (The Capintec™ CAPRAC-t Wipe-Test Counter).

Subsequently, an additional synthesis of the non-radioactive iodine labeling reaction was conducted, and its successful iodination was confirmed by TOF-ESI-MS. The experiment was performed using the same method that was started by replacing  $^{131}\text{I}$  with 0.6 mg KI dissolved in 1 mL of  $0.05 \text{ mol L}^{-1}$  PBS (E. Merck). The experiment was conducted in a small glass vial, with a molar ratio of peptide : KI : chloramine-T set at 1 : 1 : 1.

### 3 Results and discussion

In this study, analogs of nocardiotide A were designed as a radiopeptide labelled with  $^{131}\text{I}$ , specifically targeting SSTR2. Their binding affinity is essential in cancer imaging as it directly

affects the quality of visualizing cancer cells within the body.<sup>6,23</sup> Therefore, interactions between the designed analogs labelled with  $^{131}\text{I}$  and SSTR2 were explored to determine the effect of geometry changes upon the radiolabelling process.

To develop a radiopeptide based on  $^{131}\text{I}$ , it is necessary to incorporate either histidine or tyrosine into the peptide structure. Consequently, modification to the original structure of nocardiotide A is required.

According to a previous synthetic study conducted by Muhajir *et al.*, the site between alanine and tryptophan at the C terminus and N-terminus, respectively, was selected for the cyclization of the linear precursor, rendering the two residues irreplaceable. Previous studies highlighted the significant role of tryptophan as amino acid residues in the effects of ACP on cancer cells, particularly in terms of their toxicity. Considering this, the modification of nocardiotide A analogs conducted, allowing for the presence of two tryptophan in the peptide analogs' structure.<sup>24</sup> To achieve this, amino acids histidine and tyrosine were incorporated individually to replace valine, leucine, and isoleucine residues in the original structure. As a result, 6 nocardiotide A analogs, namely cWHWLVA, cWIWHVA, cWIWLHA, cWIWLYA, cWIWYVA, and cWYWLVA were designed and subjected to a docking study. The addition of  $^{131}\text{I}$  to tyrosine and histidine by radioiodination through direct labeling resulted in the chemical structure shown in Fig. 1.

Molecular docking was conducted to analyze and identify the important residues responsible for the receptor–ligand interaction between the radiopeptide derived from nocardiotide A and SSTR2 (PDB ID 7XAT) within the proposed binding site of SSTR2 which defines as D89, F92, Q102, A104, M119, D122, N125, Q126, R190, C193, T194, Y205, I209, T212, G216, F265, W269, F272, Y273, N276, K291, V298, T301, Y302, N304 and S305.



Table 1 Docking of nocardiotide A and its analogs, labeled with iodine-131 in the active site of SSTR2

No.	Peptide	$\Delta G$ kcal mol <sup>-1</sup>	Hydrogen bond	Hydrophobic interaction
1	cWHWLVA	-9.7	Q102, L290, V103, K291	R184, S192, F294, V298
	<sup>131</sup> I-cWHWLVA	-9.6	T194	L99, I177, I195, F208, I209, F275, S279, F294, L290, V298
2	cWIWHVA	-10.5	Q102, T194, L290	F294, V298
	<sup>131</sup> I-cWIWHVA	-10.4	Q102, L290	V103, F294, V298
3	cWIWLHA	-10.1	Q102, T194	V298, L99, F294, L290
	<sup>131</sup> I-cWIWLHA	-9.6	Q102, T194	L99, R190, L290, F294, V298
4	cWIWLYA	-10.1	Q102, N186, L290	L99, R184, R190, T194, F294, V298
	<sup>131</sup> I-cWIWLYA	-9.9	N196	L99, I195, N196, L290, F294, V298
	<sup>131</sup> I <sub>2</sub> -cWIWLYA	-9.7	I195, N196	L99, F208, L290, F294, V298
5	cWIWYVA	-10.2	Q102	L290, K291, F294, V298
	<sup>131</sup> I-cWIWYVA	-10.3	Q102, L290	K291, F294, V298
	<sup>131</sup> I <sub>2</sub> -cWIWYVA	10.1	Q102	V103, L290, F294, V298
6	cWYWLVA	-9.9	T194, F275	F272, L290, F294
	<sup>131</sup> I-cWYWLVA	-9.9	T194, I195, F275	F208, F294
	<sup>131</sup> I <sub>2</sub> -cWYWLVA	-9.9	R184, T194, F275	I195, F208, F294

Table 1 provides a summary of the SSTR2-peptide interactions observed in the docking studies. The results of a molecular docking showed that both peptides and their labelled peptides were successfully accommodated in the binding site. These results presented the depiction of hydrogen bonds and hydrophobic interactions in the complex system, which played a crucial role in protein-ligand interactions and were related to binding affinity.<sup>25</sup> Hydrogen bonds identified in the designed and labeled peptides were following the previous *in silico* study. They corresponded to critical residues in the active site of SSTR2, which included Q.102 and T.194,<sup>26</sup> and this led to the high binding energy of the designed radiopeptide. The binding (affinity) energies ranged from -10.3 to -11.7 kcal mol<sup>-1</sup>, indicating minor structural alteration from the original substrate in iodine radiolabelling.

Although all designed radiopeptides showed different interactions with changed binding residue in the binding pocket of SSTR2, the computational study revealed that the designed radiopeptide showed no significant change in the affinity value for SSTR2 compared to the original peptide. Thus, for synthesis purposes, analogs cWIWLHA and cWIWLYA were selected as target peptides with the consideration of small residue leucine and alanine between histidine or tyrosine, which facilitated the process. Meanwhile, other analogs such as cWYWLVA, WHWLVA, cWIWHVA, and cWIWYVA contain two adjacent tryptophan residues, as well as two adjacent tryptophan and valine residues. They were initially considered challenging to synthesize due to steric hindrance.

The visualization of the computational study selected peptide cWIWLYA is shown below. Fig. 2 shows a superimposed image of cWIWLYA with its radiolabelled iodine. Fig. 3 shows the interactions of cWIWLYA labeled by <sup>131</sup>I in the active site of SSTR2, highlighting the significant amino acids that contributed to the ligand-protein binding.

The result of the molecular dynamic study showed that each peptide and labeled peptide were stable in the binding pocket for 200 ns, without significant conformational changes as seen on Fig. 4.

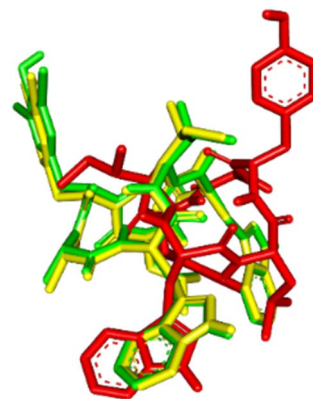


Fig. 2 Superimpose of cWIWLYA (red), <sup>131</sup>I-cWIWLYA (yellow) and <sup>131</sup>I<sub>2</sub>-cWIWLYA (blue) in the active sites of SSTR2.

All amino acids used in the synthesis of analogs cWIWLHA and cWIWLYA were Fmoc-protected, with the indole group, histidine side chain, and tyrosine side chain being protected by *tert* butyloxycarbonyl (Boc) group, trityl (Trt) group, and *tert*-butyl (*t*Bu), respectively. The procedure for synthesizing nocardiotide A analogs is based on its total synthesis by Muhajir *et al.*, where alanine and tyrosine were selected as C termini and N termini for cyclization due to a combination of small and large residues. The protected linear peptides cWIWLHA and cWIWLYA were prepared by attaching the first amino acid Fmoc-L-Ala-OH onto resin. This was followed by the attachment of the second amino acid Fmoc-L-His(Trt)-OH or Fmoc-L-Tyr (*t*Bu)-OH, which was then continued to the sixth amino acid Fmoc-L-Trp(Boc)OH (Fig. S.1).<sup>†</sup> Furthermore, the crude of protected linear WIWLHA and WIWLYA were characterized by ESI-MS showing confirmed molecular ion peaks at  $m/z$   $[M + H]^+$  1267.6594 (calculated  $m/z$  1267.6556) with a yield of 9.20% and  $[M + H]^+$  1107.629 (calculated  $m/z$  1107.6130) with a yield of 52.90% (Fig. S.2).<sup>†</sup>

During the synthesis of linear WIWLHA precursors, an issue arose with the yield of the synthesized product. It was suspected





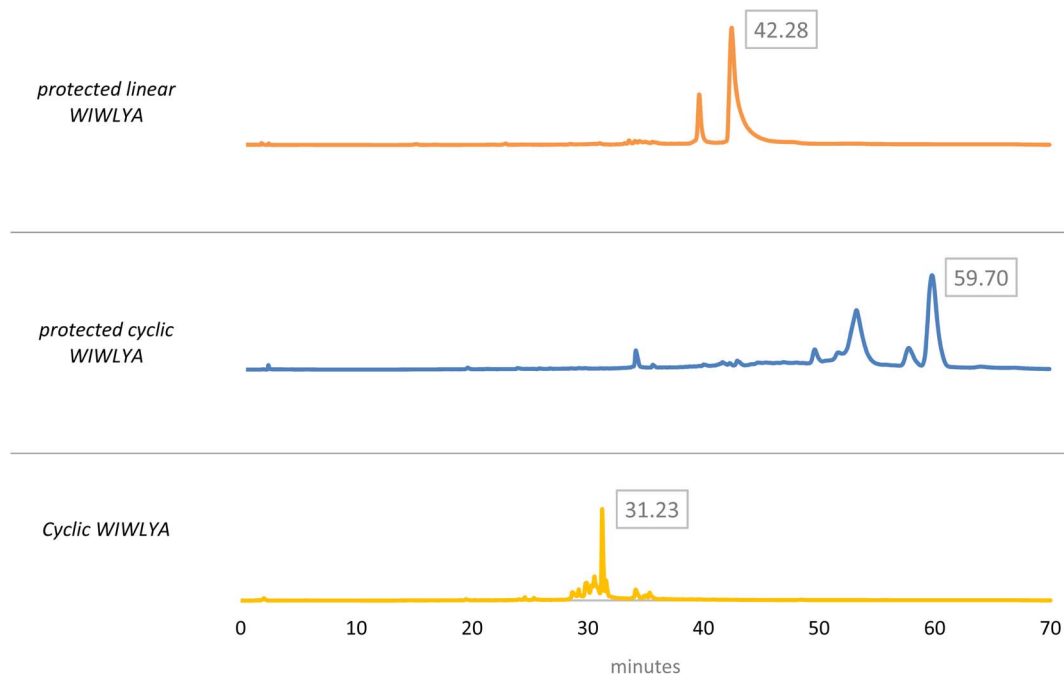


Fig. 5 Analytical HPLC chromatogram of the cWIWLYA cyclization synthesis process.

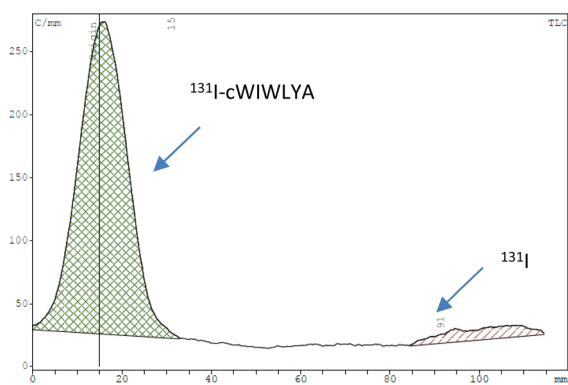


Fig. 6 Radiochromatogram profile of the cWIWLYA labeled iodine-131.

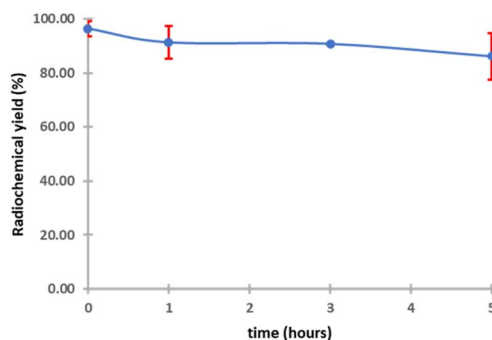


Fig. 8 Stability of  $^{131}\text{I}$ -cWIWLYA in plasma.

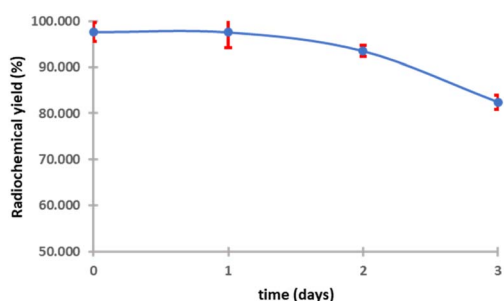


Fig. 7 Stability of  $^{131}\text{I}$ -cWIWLYA at room temperature.

labeling will give iodine addition to tyrosine residue at the primary site. The tyrosine moiety can be labeled twice, resulting in a mixture of mono and di-iodinated species. It should be noted

that the formation of di-iodinated tyrosine occurs at a faster rate than mono-iodinated.<sup>27</sup> The result of iodine-labeled cWIWLYA was characterized using ESI-MS, showing an  $m/z$   $[\text{M} + \text{Na}]^+$  1107.1138 (calculated  $m/z$  1107.2103) (Fig. S.9).<sup>†</sup> This confirmed that the product had 2 iodine atoms conjugated to the tyrosine group (di-iodinated). This result supported the notion that a nocardiotide A analog containing a tyrosine residue could be translated into a radioiodine-labeled peptide for the further development of a radiopeptide as a theragnostic agent.

## 4 Conclusions

The applied computational study showed that all the modified nocardiotide A and designed radiopeptides exhibited binding affinity to SSTR2. The peptide analog that has been successfully synthesized and easily labeled, makes it a prospective candidate for radiopeptide and theragnostic purpose and needs further studies.



## Author contributions

RJS: conducted computation, completed experiments, collected, and analyzed the data, and wrote the draft manuscript; RM: conducted supervision in peptide synthesis, analyzed data, and corrected the manuscript; FK: conducted supervision in computation, analyzed data, and corrected the manuscript; REK: supervised and corrected the manuscript; DHT: designed the method, conducted supervision, provided resources, and corrected the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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