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Synthesis and Cancer Cell Targeting of a Boron-Modified Heat-Stable Enterotoxin Analog for Boron Neutron Capture Therapy (BNCT)

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Abstract

Heat-stable enterotoxin (STa) is a peptide toxin that induces acute diarrhea by binding to guanylyl cyclase C (GC-C) in intestinal epithelial cells. Interestingly, GC-C is highly expressed not only in intestinal cells but also in certain colorectal cancer cells, such as T84 and Caco-2 cells. This unique expression pattern provides STa as an effective candidate for therapeutic applications in cancer suppression or as a probe for detecting cancer cells. Recently, we developed attenuated forms of several STa analogs, including STa topological isomers, and evaluated their efficacy in detecting GC-C on Caco-2 cells, which enables the use of STa in human applications. Therefore, in this study, we investigated the potential application of a ^{10}B-labeled STa derivative, [Mpr^{5},D-Lys^{16}(BSH)]-STp(5–17) topological isomer, in boron neutron capture therapy (BNCT), for establishing a novel therapeutic strategy for colorectal cancer. The ^{10}B-labeled STa peptide clearly exhibited Caco-2 cell killing activity upon neutron irradiation in a concentration-dependent manner, indicating that STa is an effective candidate drug for BNCT. To our knowledge, this is the first report of using STa in boron neutron capture therapy (BNCT).

Keywords: BNCT; BSH; Caco-2; heat-stable enterotoxin; guanylin

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1. Introduction

Heat-stable enterotoxin (STa) is a peptide toxin produced by enterotoxigenic *Escherichia coli* that causes acute diarrhea, particularly in infants, and is divided into two types, STh and STp [1–3], as shown in Figure 1A. STa also possesses high homology to the intrinsic peptide hormones, guanylin and uroguanylin [4,5], as shown in Figure 1A. The toxic activity of STa is mediated by a 13-amino acid core toxic region that is stabilized by three intramolecular disulfide bonds, which are critical for its receptor binding [6–8]. STa exerts its effects by binding to guanylyl cyclase C (GC-C), which is a receptor protein for guanylin and uroguanylin, in intestinal epithelial cells, leading to elevated intracellular cGMP levels [9–11]. The increased cGMP levels stimulate protein kinase C, resulting in the phosphorylation of the cystic fibrosis-related chloride channel (CFTR). This cascade ultimately manifests as severe diarrhea resulting from water secretion through CFTR [12].

Thus, this cascade is induced by the stimulation of the receptor, GC-C. Interestingly, GC-C is abundantly expressed in certain colonic cancer cells, such as T84 and Caco-2 cells [13,14]. Recently, this selective expression has made STa an attractive candidate as a therapeutic peptide for cancer suppression or as a probe for detecting cancer cells [15,16].

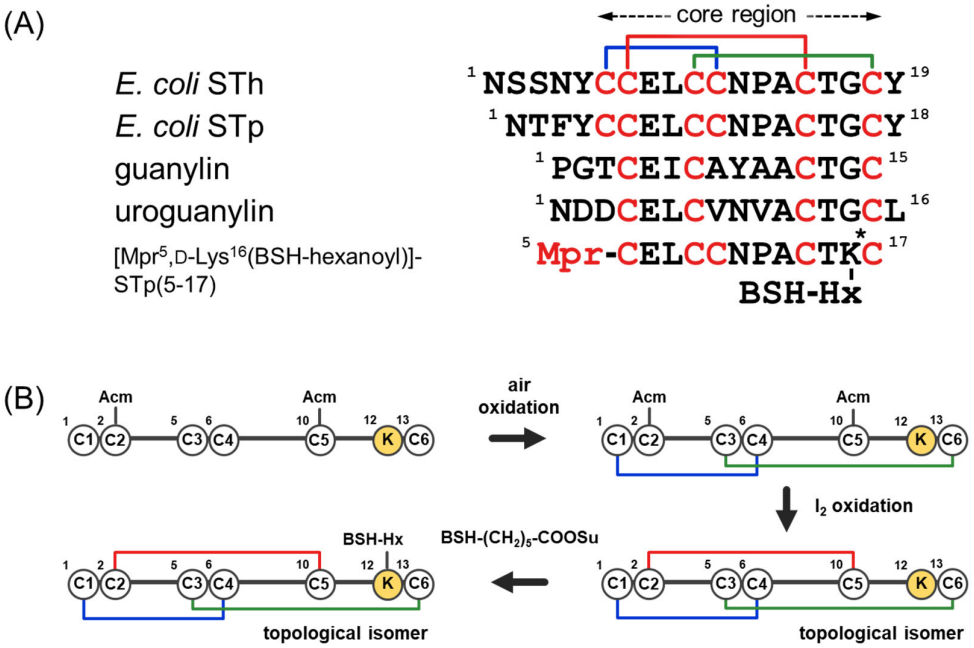


Figure 1. Sequence alignment of heat-stable enterotoxins and intrinsic peptide hormones, guanylin and uroguanylin (**A**) and scheme for synthesis of the [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer (**B**): (**A**) Thick lines indicate disulfide bonds and the region essential for biological activity is highlighted with a dashed line. BSH-Hx, K*, and Mpr represent BSH-hexanoyl, D-Lys, and mercaptopropionyl, respectively. (**B**) Air oxidation of the STa peptide predominantly forms native pairings between two disulfide bonds [7,8]. Iodine oxidation provides topological isomers of STa analogs [7,8]. C1 to C6 represent Mpr^5, Cys^6, Cys^9, Cys^{10}, Cys^{14}, and Cys^{17}, respectively. Blue, red, and green lines indicate disulfide bonds between C1–C4, C2–C5, and C3–C6, respectively.

Recent advances in chemical synthesis in our laboratory have allowed us to develop attenuated STa analogs with regioselective disulfide bond formation, maintaining their structural integrity at the GC-C binding site while reducing toxicity [17]. These innovations have paved the way for vaccine development and targeted therapies [17]. In this study, we aimed to leverage our recently synthesized attenuated STa derivatives for selective targeting of colonic cancer cells that overexpress GC-C.

Boron neutron capture therapy (BNCT) has attracted attention as a next-generation radiation therapy capable of targeting cancer at the cellular level [18,19]. In this therapeutic approach, boron-containing compounds such as boronophenylalanine (BPA) and sodium borocaptate (BSH) are commonly used. Among the key challenges in BNCT is achieving efficient and selective accumulation of boron compounds in target cells. To address this, various antibodies and peptides have been explored for boron delivery [20,21]. In this study, we aimed to enhance the tumor-targeting ability of BSH by conjugating it with streptavidin (ST), thereby facilitating efficient delivery of BSH to cancer cells for potential application in BNCT.

For this purpose, we modified the attenuated STa analog, [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer, by introducing a boron-containing moiety (BSH) to serve as a boron-10 (^{10}B) neutron capture nucleus [18]. Subsequently, we evaluated its potential application in boron neutron capture therapy (BNCT) [19]. By introducing BSH, we sought to selectively induce cell death in GC-C-expressing cells via neutron irradia-

tion. Herein, we describe the chemical synthesis of the attenuated STa derivative [17], [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer, and assess its efficacy in inducing radiation-mediated cell death in Caco-2 cells.

2. Materials and Methods

2.1. Materials

All chemicals and solvents were of reagent grade unless otherwise described. 6-Bromohexanoic acid and di(*N*-succinimidyl)carbonate were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. *S*-cyanoethylthio-dodecaborate containing only ^{10}B (Stella Pharma Co., Osaka, Japan) was prepared, as previously described [18]. The [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer was also prepared, as previously reported [17]. The scheme of the synthesis of the BSH derivatives is presented in Supplementary Figure S1.

2.2. Synthesis of [BSH-(CH$_2$)$_5$-COO]$^{3-}$·3[N(CH$_3$)$_4$]$^+$

6-Bromohexanoic acid (392 mg, 2.01 mmol) was added to a solution of *S*-cyanoethylthio-dodecaborate (100% ^{10}B, 668 mg, 1.83 mmol) in CH_3CN (20 mL), and the mixture was refluxed for 24 h. The mixture was concentrated in vacuo, and the resulting residue was subsequently suspended in acetone (100 mL). After filtration, the solution was mixed with 25% (*w*/*v*) N(CH$_3$)$_4$OH in methanol (1.54 mL, 3.65 mmol). The precipitate was recrystallized from H_2O to obtain [BSH-(CH$_2$)$_5$-COO]$^{3-}$·3[N(CH$_3$)$_4$]$^+$ (900 mg, 99%) as colorless crystals.

^{1}H and ^{13}C NMR spectra were measured on a JNM-ECZR (500 MHz, JEOL Ltd., Tokyo, Japan). LR ESI-MS measurements were performed on an expression CMS® (Advion Interchim Scientific® (Ithaca, NY, USA). ^{1}H-NMR (500 MHz, DMSO-D$_6$): δ 3.03–3.18 (48H), 2.14–2.22 (2H), 1.69–1.77 (2H), 0.56–1.41 (19H). ^{13}C-NMR (126 MHz, DMSO-D$_6$): δ 175.5, 54.3, 32.6, 32.6, 29.9, 26.8. MS (neg. ESI, *m*/*z*): 323 Da [M + 2Na]$^-$ (theoretical: 323 Da).

2.3. Synthesis of [BSH-(CH$_2$)$_5$-COOSu]$^{2-}$·2[N(CH$_3$)$_4$]$^+$

Di(*N*-succinimidyl)carbonate (118 mg, 0.462 mmol) was added to a solution of [BSH-(CH$_2$)$_5$-COO]$^{3-}$·3[N(CH$_3$)$_4$]$^+$ (210 mg, 0.420 mmol) in CH_3CN (10 mL), and the mixture was stirred for 4 h at room temperature [18]. The mixture was concentrated in vacuo, and the resulting residue was re-dissolved in CH_3CN (2 mL). The solution was added to $CHCl_3$ (50 mL), and the resulting solid was collected. The precipitate was recrystallized from H_2O to obtain [BSH-(CH$_2$)$_5$-COOSu]$^{2-}$·2[N(CH$_3$)$_4$]$^+$ (142 mg, 64.5%) as colorless crystals.

^{1}H-NMR (500 MHz, DMSO-D$_6$): δ 3.04–3.13 (32H), 2.75–2.83 (4H), 2.56–2.65 (2H), 2.18–2.25 (2H), 1.50–1.60 (2H), 0.58–1.46 (15H). ^{13}C-NMR (126 MHz, DMSO-D$_6$): δ 170.3, 169.0, 54.4, 32.0, 31.7, 30.3, 28.1, 25.4, 24.3. MS (neg. ESI, *m*/*z*): 398 Da [M+Na]$^-$ (theoretical: 398 Da).

2.4. Synthesis of BSH-(CH$_2$)$_5$-CO- Conjugated [Mpr^5,D-Lys^{16}]-STp(5–17) Topological Isomer

[BSH-(CH$_2$)$_5$-COOSu]$^{2-}$·2[N(CH$_3$)$_4$]$^+$ (110 nmol) was dissolved in CH_3CN (10 µL) and mixed with [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer (100 nmol) [17] in 1% $NaHCO_3$ solution (90 µL, pH 9). The reaction mixture was allowed to stand for 24 h at room temperature and subjected to RP-HPLC. The purified peptide, [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17)·2[N(CH$_3$)$_4$]$^+$ topological isomer was identified by MALDI-TOF MS and amino acid analyses, as described previously [17,22,23]. The molecular mass of the STa peptide was determined by means of a MALDI-7090 (SHIMADZU Co., Kyoto, Japan) in the positive ion mode. Mass spectrometric analyses of the peptides were carried out using 3,5-dimethoxy-4-hydroxycinnamic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) as a matrix.

The target peptide provides 1776.5 Da (theoretical, 1776.5 Da) for the mass value, $[M+Na]^+$, of [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17)·2[$N(CH_3)_4]^+$ by mass spectrometric analysis. Notably, no significant disulfide exchange reactions were observed under the reaction conditions, including isomerization between the topological isomer and the native type peptide of [Mpr^5,D-Lys^{16}]-STp(5–17).

2.5. GC-C Binding Activity of BSH-$(CH_2)_5$-CO- Conjugated [Mpr^5,D-Lys^{16}]-STp(5–17) Topological Isomer

The GC-C binding assay of the STp topological isomers of [Mpr^5,D-Lys^{16}]-STp(5–17) and [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17)·2[$N(CH_3)_4]^+$ was performed, as described previously [17]. Briefly, Caco-2 cells on a 10 cm dish were harvested with PBS (5 mL) and centrifuged at 1000× *g* for 10 min at 4 °C. The cells were suspended in DMEM (5 mL) and then divided into small portions (182 µL). The aliquots were again centrifuged at 1000× *g* for 10 min at 4 °C and resuspended/mixed with the FTC-STp peptide (10^{-5} M) solution in DMEM (200 µL) containing several concentrations of STp analogs at room temperature for 30 min. The reaction mixtures were centrifuged at 1000× *g* for 10 min at 4 °C, resuspended with DMEM (500 µL) containing 10% FBS, transferred to a 24-well plate, incubated for 1 h at 37 °C in a 5% CO_2 incubator, and observed by fluorescence microscopy using a FITC filter set. The fluorescein densities of the cells were analyzed using ImageJ software ver. 1.54p. Binding assays were conducted in duplicate.

2.6. Irradiation of Caco-2 Cells Bound to the [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) Topological Isomer

Caco-2 cells were harvested in DMEM containing 10% FBS in two 6 cm dishes (70% confluence) and centrifuged at 1000× *g* for 5 min. After washing cells with PBS, they were aliquoted in 12 Treff tubes (Toho K.K., Tokyo, Japan). Several concentrations of [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer in DMEM (100 µL) containing 10% FBS were added to Caco-2 cells (approximately 1 × 10^5 cells) in DMEM (100 µL) containing 10% FBS in the Treff rubes for 2 h prior to irradiation. Cells were irradiated for 1 h in UTR-KINKI (Kindai University, Osaka, Japan), which was operated at 1 W output [24]. The neutron dose rate was estimated to be 0.2 Gy/hour because neutrons and gamma rays are emitted at a ratio of 1:1. In clinical applications of the BNCT method, a neutron dose of 2 Gy is typically used [19,25]. However, in this experiment, a dose of 0.2 Gy was used to minimize cellular damage. The irradiated cells were transferred to a 24-well plate containing DMEM containing 10% FBS (300 µL) and incubated for 24 h in a 5% CO_2 incubator. The attached cells were counted as described previously [17]. Notably, Caco-2 cells detached for the adhesion assay were incubated in the presence or absence of the [Mpr^5, D-Lys^{16}(BSH-hexanoyl)]-STp(5–17)·2[$N(CH_3)_4]^+$ peptide (1 × 10^{-5} M) by the method reported previously [26], and no difference in reattached cell numbers was observed, indicating that the peptide did not exert cytotoxic effects under these conditions.

2.7. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The HPLC apparatus was comprised of a HITACHI ELITE LaChrom system (L2130) equipped with a Hitachi L-3000 detector and a D-2500 chromato-integrator (Hitachi High-Tech Corporation, Tokyo, Japan). Peptides were purified by RP-HPLC using a Cosmosil 5C_{18}-AR-II column (4.6 × 150 mm, Nacalai tesque Inc., Kyoto, Japan), as previously described [17,26]. The peptides were separated by a linear gradient of CH_3CN in 0.05% TFA, increasing at a rate of 1%/min from solvent A (0.05% TFA/H_2O) to solvent B (0.05% TFA/CH_3CN) at a flow rate of 1 mL/min.

3. Results and Discussion

3.1. Development of the STa Topological Isomer for BNCT: Design and Synthesis of BSH-$(CH_2)_5$-CO- Conjugated [Mpr^5,D-Lys^{16}]-STp(5–17) Topological Isomer

Recent advancements in the regioselective formation of multiple disulfide bonds and topological regulation of a target STa peptide have enabled the successful development of attenuated STa derivatives for applications in STa-based vaccines and GC-C-targeted probes [18]. Using this synthetic approach, we aimed to develop boronated STa peptides for potential use in BNCT. For this purpose, the topological isomer of [Mpr^5,D-Lys^{16}]-STp(5–17) was selected due to its high detection efficiency and reduced toxic activity, which is approximately one-tenth that of the native STa peptide, as previously described [7,8,17]. This topological isomer adopts a left-handed spiral structure, which has been structurally elucidated by NMR spectroscopy [27], and retains sufficient GC-C binding ability despite its attenuated toxicity. Therefore, the topological isomer was utilized as a probe targeting GC-C-expressing Caco-2 cells [17]. This approach leverages the isomer's weak toxicity originating from the -Asn-Pro-Ala- sequence, the GC-C binding site, and demonstrates its potential for precise targeting with minimized side effects, such as cross reactions against intrinsic peptide hormones, guanylin, and uroguanylin.

In BNCT, the selective accumulation of ^{10}B at high concentrations in tumor cells is critical. Sodium borocaptate (BSH) is commonly used as a boron compound in BNCT; however, its passive accumulation in tumor cells limits its efficacy [19]. To overcome this limitation, STa analogs encapsulating BSH were synthesized as a boron delivery system. These analogs were designed to target GC-C-rich cells, such as Caco-2 cells, enhancing the selective delivery of ^{10}B. Therefore, BSH was introduced to the side chain of D-Lys^{16} residues in a STa analog, yielding [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer.

For our purpose, the topological isomer of [Mpr^5,D-Lys^{16}]-STp(5–17) was prepared according to previously reported methods [17] and conjugated to BSH, as shown in Figure 1B. To avoid the steric hindrance of the BSH group for receptor binding, a hexanoyl group was introduced to the BSH group as a spacer because the D-Lys residue served as a platform located in close proximity to the GC-C binding site (-Asn-Pro-Ala-). Indeed, even the native type of [Mpr^5, Lys^{16}(FTC)], in which the chromophore, FTC, is directly attached to the Lys^{16} residue without a spacer, showed a drastically reduced GC-C binding ability, as previously described [17]. Therefore, we employed the BSH-hexanoyl group for the conjugation. Thus, the [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer (Figure 2A) was treated with BSH-$(CH_2)_5$-COOSu (Figure 2B) to synthesize the target peptide, [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer. The conjugation reaction proceeded well, indicating that the steric hindrance of the D-Lys residue was not significant, as expected from the NMR analysis [17]. Indeed, the target peptide was yielded quantitatively, as shown in Figure 2C, and was confirmed by MALD-TOF MS and amino acid analysis.

3.2. GC-C Binding Activity of [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) Topological Isomer

The GC-C binding activity of the synthesized peptide was evaluated using previously established methods [17]. Competitive inhibition assays were performed with the native type [Mpr^5,D-Lys^{16}(FTC)]-STp(5–17) peptide (10^{-6} M) using 293T cells expressing recombinant GC-C, as described previously [17]. The [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer showed approximately 10^{-7} M for the IC_{50} value in the assay, as shown in Figure 3A. Thus, the topological isomer exhibited competitive inhibition ability, confirming its suitability as a GC-C-targeted probe. The [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer exhibited GC-C binding activity nearly identical to that of the original [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer (IC_{50}: approximately 10^{-7} M), as shown in

Figure 3A. This result clearly indicates that the conjugation at the D-Lys^{16} residue in STa does not significantly affect GC-C binding, and the hexanoyl group functions as a good spacer for BSH conjugation.

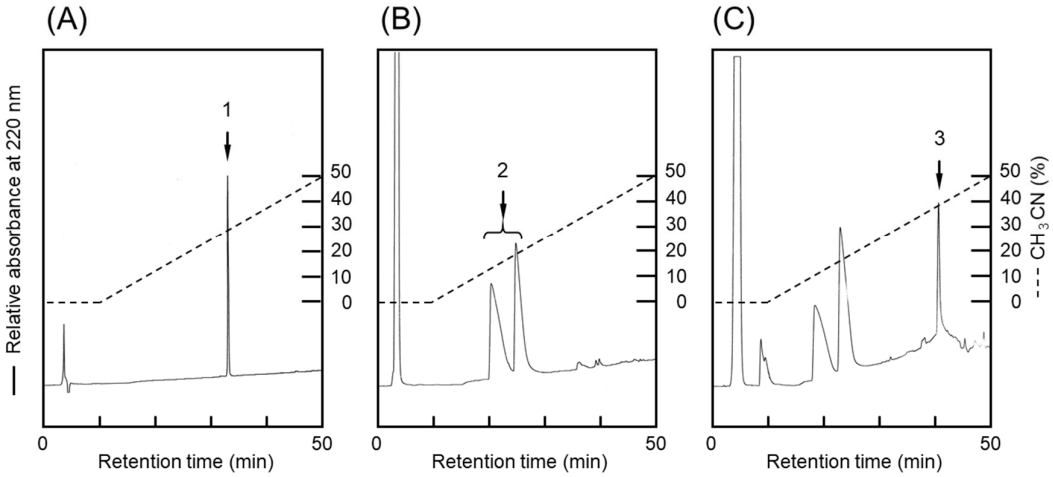


Figure 2. RP-HPLC profiles of the purified [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer (**A**), BSH-$(CH_2)_5$-COOSu (**B**), and the reaction mixtures (**C**). Peaks 1, 2, and 3 indicate the [Mpr^5,D-Lys^{16}]-STp(5–17) topological isomer, BSH-$(CH_2)_5$-COOSu, and [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer, respectively. The retention times of peaks 1, 2 (earlier peak), 2 (later peak), and 3 were 32.9, 20.3, 24.9, and 40.8 min, respectively.

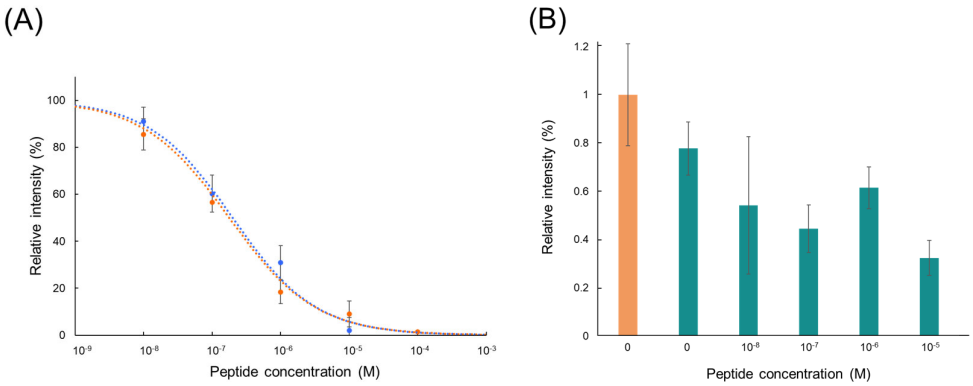


Figure 3. Competitive binding assay of STa analogs using G-C-expressing 293T cells (**A**) and recovery of STa-treated Caco-2 cells after neutron irradiation (**B**): (**A**) [Mpr^5,D-Lys^{16}(FTC)]-STp(5–17) (10^{-6} M) was used for the competitive binding assay. The orange and blue lines represent the data for the topological isomers of [Mpr^5,D-Lys^{16}]-STp(5–17) and [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17), respectively. The experiments were performed, as previously described [17]. (**B**) Orange and green bars represent data without and with neutron irradiation, respectively. Both experiments were performed in duplicate.

3.3. Neutron Irradiation of Caco-2 Cells

To assess the potential of the synthesized peptide in BNCT, neutron irradiation experiments were performed using GC-C-expressing human colonic Caco-2 cells. A slight difference in cell viability was observed between irradiated and non-irradiated conditions, as shown by the orange and green bars in Figure 3B. Due to gamma irradiation and physical factors during sample handling (such as temperature increase), a relatively large difference in cell viability was observed between irradiated and non-irradiated samples. Nonetheless, the differences observed among the irradiated samples were more pronounced and clearly reflected the effects of the respective treatments. A dose-dependent induction of cell death was observed in a concentration-dependent manner in the [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-

STp(5–17) topological isomer, as shown in Figure 3B. The effective dose (ED_{50}) of STa for inducing cell death in Caco-2 cells following radiation exposure was approximately 10^{-7} M to 10^{-6} M, indicating that the designed peptide can bind to GC-C and works well as a target probe for BNCT. Under the experimental conditions used in this study, ST did not exhibit cytotoxicity toward Caco-2 cells. Indeed, due to gamma irradiation and physical factors during sample handling (such as temperature increase), a relatively large difference in cell viability was observed between irradiated and non-irradiated samples. Nonetheless, the differences observed among the irradiated samples were more pronounced and clearly reflected the effects of the respective treatments. In Caco-2 cells, the internalization of GC-C via ST has been well studied, as ST is considered a potential drug delivery agent that targets cancer cells. Although ST itself does not enter cells, it can be internalized through binding to GC-C. However, under the conditions used in this study, only about 15% of the ST–GC-C complex was taken up by the cells after 1 h of incubation [28]. Although we have not yet measured the uptake of the complex of [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer and GC-C into the cells, it is reasonable to expect similar intracellular uptake for this peptide as well. Therefore, the result suggests that the observed cell death was mainly due to the radiation effect of BSH–ST remaining outside the cells, rather than internalization.

Although no toxicity data for STa in humans have been reported, toxicity testing using suckling mice (minimum effective dose = 1 ng/1.7 g body) [7,8] suggests that, based on body surface area (BSA) calculations, the equivalent dose for an adult human would be 2.88 μg (approximately 2 nmol). The toxicity of the [Mpr^5,D-Lys^{16}(BSH-hexanoyl)]-STp(5–17) topological isomer synthesized in this study was less than one-hundredth of that for the native type of the STa analog, which corresponds to the GC-C binding activity of the peptide hormones, such as guanylin and uroguanylin, as previously reported [29]. This indicates that its minimum effective dose for causing diarrhea is significantly higher (at least more than 288 μg), and therefore, the peptide is expected to exhibit minimal toxicity at the effective dose in BNCT. These results demonstrate the efficacy of the peptide in mediating STa-dependent cytotoxicity, thus validating its application in BNCT. Thus, effective results were achieved with only one-tenth of the standard 2 Gy radiation dose for BSH [16]. This approach minimizes damage to normal cells and demonstrates the ability of ST to selectively target and eliminate cancer cells.

4. Conclusions

To our knowledge, this study presents the first report of the use of STa for boron neutron capture therapy (BNCT). This study successfully demonstrated that the introduction of BSH into the STa peptide framework enables its application in BNCT. Our synthetic method with regioselective disulfide bond formation not only facilitates high-yield production of STa derivatives but also allows for diverse chemical modifications at the D-Lys^{16} position. Future studies focusing on further attenuation of STa toxicity and innovative chemical modifications are expected to yield more effective reagents for BNCT.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemistry7040111/s1. Supplementary Figure S1: Scheme for the preparation of *N*-succinimidyl BSH-hexanoate ester.

Author Contributions: Conceptualization, S.O., Y.H. (Yoshihide Hattori), N.S., M.G., S.K., H.I., T.T., S.S. and Y.H. (Yuji Hidaka); methodology, S.S. and Y.H. (Yuji Hidaka); validation, N.S., S.S. and Y.H. (Yuji Hidaka); formal analysis, S.O., Y.H. (Yoshihide Hattori), N.S., M.G., S.K., H.I., S.S. and Y.H. (Yuji Hidaka); investigation, S.O., N.S., T.T., S.S. and Y.H. (Yuji Hidaka); resources, Y.H. (Yuji Hidaka); data curation, N.S., S.S. and Y.H. (Yuji Hidaka); writing—original draft preparation, N.S. and Y.H.

(Yuji Hidaka); writing—review and editing, N.S., S.S. and Y.H. (Yuji Hidaka); visualization, N.S.; supervision, Y.H. (Yuji Hidaka); project administration, Y.H. (Yuji Hidaka); funding acquisition, N.S. and Y.H. (Yuji Hidaka). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article and Supplementary Materials.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

BNCT, boron neutron capture therapy; BSH, borocaptate; FTC, fluorescein thiocarbamoyl; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Mpr, mercaptopropionyl; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Tris/HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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