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Design, synthesis, and anti-tumor activities of novel Brevinin-1BYa peptidomimetics

Shili Xiong [a,d], Nan Wang [b,d], Chao Liu [b], Huaxing Shen [b], Zengqiang Qu [c], Lijun Zhu [c], Xiaosong Bai [a], Hong-gang Hu [b], Wei Cong [b,*], Liang Zhao [a,*]

[a] Luodian Clinical Drug Research Center, Institute for Translational Medicine Research, Shanghai University, Shanghai, China
[b] Institute of Translational Medicine, Shanghai University, Shanghai, China
[c] Department of Invasive Technology, Eastern Hepatobiliary Surgery Hospital, Shanghai, China

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ABSTRACT

Brevinin-1BYa is an amphibian skin-derived peptide that exhibits promising anti-microbial activity against gram-positive and -negative bacteria. However, the anti-tumor activity of Brevinin-1BYa remains unclear, and, more importantly, its therapeutic application is limited owing to its poor protease and reduction stability. In this study, a series of novel Brevinin-1BYa derivatives, including *O*-linked *N*-acetyl-glucosamine glyclopeptides and disulfide bond mimetics, were designed and synthesized. Additionally, their anti-tumor activity against human prostate cancer cell line C4-2B, human NSCLC cell line A549 (adenocarcinoma), and human hepatoma cells line HuH-7 was investigated. Among these, the thioether bridge substituted peptidomimetic Brevinin-1BYa-3 displayed improved reduction stability, more stable secondary structure, greater protease stability, and increased anti-tumor activity compared with the original peptide, rendering it a promising leading compound for drug development, particularly for applications against malignant tumors.

Amphibian skin has been revealed to be a valuable source for discovering novel drug candidates with various biological activities, including anti-bacterial, anti-viral, anti-fungal, and anti-tumor activities, among others.[1–4] Several compounds with unusual architecture and remarkable biological activity, especially a number of peptides including Aurein2.2,[5] Magainin II,[6] Ascaphin8,[7] Dermaseptin-S1,[8] Temporin A,[9] and Brevinin-1BYa,[10] have become the focus of recent research.[11]

Brevinin-1BYa is an anti-microbial peptide (AMP) that was originally isolated from the skin secretions of the North American foothill yellow-legged frog (*Rana boylii*).[12] It has been reported that Brevinin-1BYa performed with high potential against gram-positive and gram-negative bacteria, including methicillin-resistant Staphylococcus aureus (MRSA), as well as against a range of opportunistic yeast pathogens such as *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, and *Candida tropicalis*.[10] Brevinin-1BYa has been regarded as a promising candidate for anti-bacterial drug development, but few studies have focused on its anti-tumor activity. Considering the good anti-tumor activity of Brevinin-2R1, another AMP from the Brevinin family,[13] the anti-tumor activity of Brevinin-1BYa is explored herein. Meanwhile, it was also noticed that Brevinin-1BYa contains a cyclic disulfide-bridged loop at the C-terminal end of the peptide that was unstable *in vivo* because of a reducing circumstance, disulfide isomerases, and enzymatic cleavage and the degradation then leads to structural distortion and activity loss.[14,15] To overcome these difficulties, synthetic disulfide surrogates including thioether, amide, and triazole bridges with improved reduction stability and conformational rigidity have been introduced into the peptide backbone to obtain Brevinin-1BYa-1, Brevinin-1BYa-2, and Brevinin-1BYa-3 (Fig. 1).[16–19] In addition, it has been reported that glycosylation could improve the chemical properties of peptides, such as hydrophilicity, oral bioavailability, and conformation stability,[20] and thus two glycopeptides, Brevinin-1BYa-4 and Brevinin-1BYa-5, were designed and synthesized by acetylglucosamine glycosylation at serine and threonine residues, respectively (Fig. 1).

* Corresponding authors.
E-mail addresses: viviencong@shu.edu.cn (W. Cong), zhaoliangphar@163.com (L. Zhao).
[d] Shili Xiong and Nan Wang contribute equally to this work.

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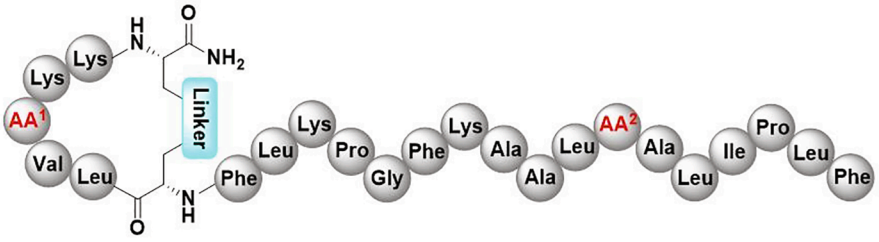
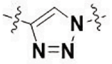
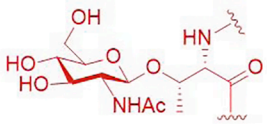


	Linker	AA¹	AA²
Brevinin-1BYa		L-Thr	L-Ser
Brevinin-1BYa-1		L-Thr	L-Ser
Brevinin-1BYa-2		L-Thr	L-Ser
Brevinin-1BYa-3		L-Thr	L-Ser
Brevinin-1BYa-4		L-Thr(β-D-GlcNAc)	L-Ser
Brevinin-1BYa-5		L-Thr	L-Ser(β-D-GlcNAc)

L-Thr(β-D-GlcNAc)



L-Ser(β-D-GlcNAc)

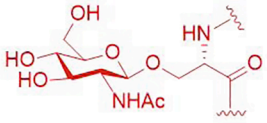
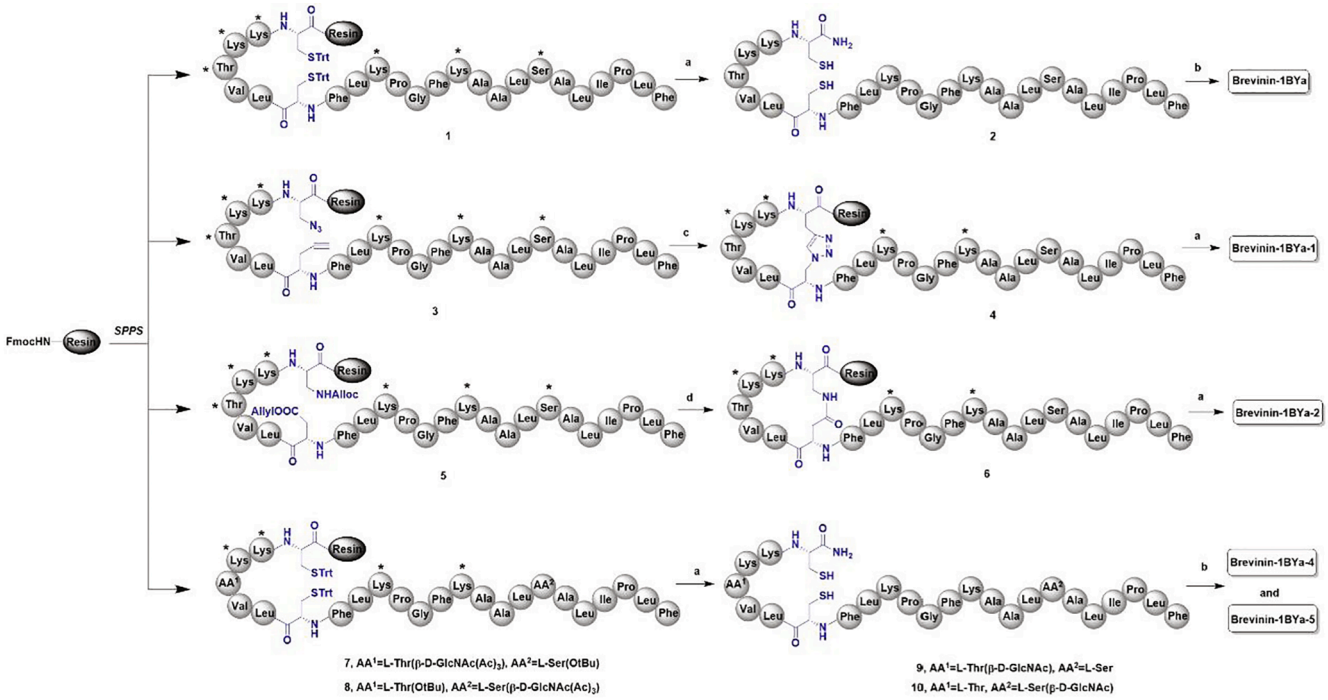
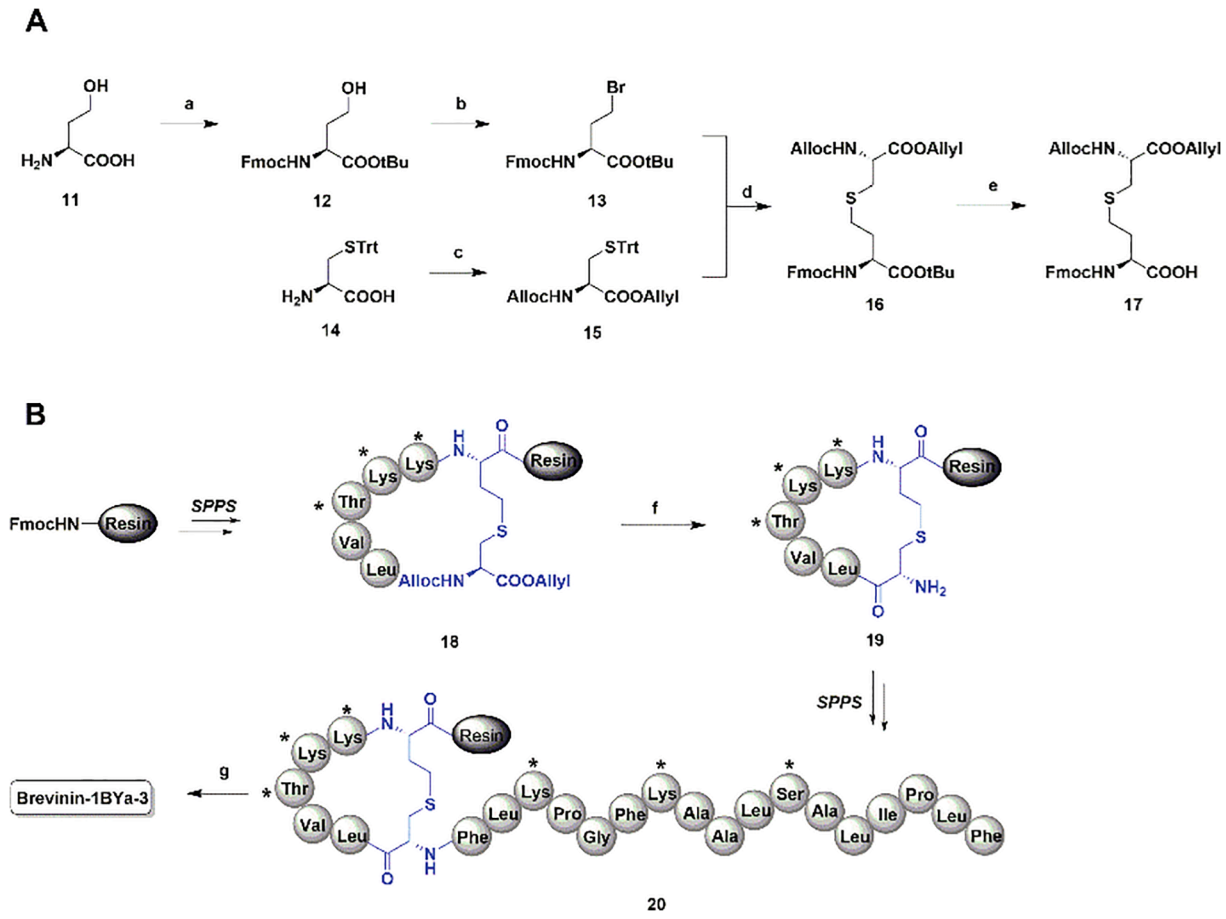


Fig. 1. Structures of Brevinin-1BYa and derivatives.



Scheme 1. Synthesis route of Brevinin-1BYa and Brevinin-1BYa-1, -2, -4 -5. Conditions and reagents: a) TFA/TIPS/phenol/water (88:5:5:2, *v/v/v/v*), 4 h, RT; b) Guanidine hydrochloride, sodium dihydrogen phosphate, DMSO, PBS buffer, 24 h, RT; c) 2,6-lutidine, DIPEA, sodium ascorbate, CuBr acetonitrile/DMF, 8 h, rt; d) PyAOP, HOAt, NMM, NMP, 12 h, RT. Resin-bound peptides were protected on side chains at asterisk sites. The following protecting groups for amino acid side chains were used: *tert*-butyl (tBu; for Thr and Ser) and *tert*-butyloxycarbonyl (Boc; for Lys).



Scheme 2. Synthesis route of diaminodiacid (A) and Brevinin-1BYa-3 (B). Conditions and reagents: a) i) Na_2CO_3, FmocOSu, water/1,4-dioxane, 12 h, RT; ii) *tert*-butyl trichloroacetimidate, DCM, 12 h, rt; b) CBr_4, PPh_3, DCM, 12 h, RT; c) i) Na_2CO_3, allyl chloroformate, water/acetonitrile, 12 h, RT; ii) Allyl bromide, $NaHCO_3$, DMF, 12 h, RT; d) i) TFA, TIPs, DCM, 1 h, RT; ii) TBAB, $NaHCO_3$, water/EA, 12 h, RT; e) TFA/DCM (1:1, *v/v*), 2 h, RT; f) i) $Pd(PPh_3)_4$, $PhSiH_3$, DMF/DCM, 3 h, RT; ii) PyAOP, HOAt, NMM, NMP, 12 h, RT; g) TFA/TIPS/phenol/water (88:5:5:2, *v/v/v/v*), 4 h, RT. Resin-bound peptides were protected on side chains at asterisk sites. The following protecting groups for amino acid side chains were used: *tert*-butyl (tBu; for Thr and Ser) and *tert*-butyloxycarbonyl (Boc; for Lys).

The prototype peptide Brevinin-1BYa and its derivative were synthesized through 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS, Scheme 1). First, Brevinin-1BYa was realized *via* a solid-phase-coupling/liquid-phase-cyclization strategy. The on-resin peptide 1 was synthesized through SPPS using 5-chloro-1-[bis(dimethylamino) methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU) and *N,N*-diisopropylethylamine (DIPEA) as coupling reagents, and it was cleaved from the solid support to obtain the free peptide 2. The following oxidative folding of 2 with 10% DMSO in buffer provided the disulfide peptide Brevinin-1BYa in an overall yield of 53.2%. Next, by incorporating Fmoc-β-azido alanine and Fmoc-L-propargly glycine into peptide backbone through standard SPPS, the protected on-resin peptide 3 was assembled. On-resin intermediate 4 was obtained through a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction subjected to the treatment of lutidine, DIPEA, sodium ascorbate, and CuBr. After cleavage from the solid support and global sidechain deprotection with B reagent, Brevinin-1BYa-1 was obtained in 56.4% yield. The synthetic route of Brevinin-1BYa-2 was similar to that of Brevinin-1BYa-1. Fmoc-Lys(Alloc)-OH and Fmoc-Asp (OAllyl)-OH were coupled into the peptide to provide on-resin intermediate 5. De-protection of Alloc/Allyl protective groups with $Pd(PPh_3)_4$/$PhSiH_3$ in DMF/DCM was conducted and, following macrocyclization promoted by (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP)/3H-[1,2,3]-triazolo[4,5-*b*]pyridin-3-ol (HOAt)/*N*-methylmorpholine (NMM)/*N*-methyl-2-pyrrolidone (NMP), precursor 6 was obtained. Final de-protection and cleavage provided amide-bridged derivative Brevinin-1BYa-2 with a yield of 52.5%.

During synthesis of the designed derivatives of Brevinin-1BYa, it was noticed that not all types of disulfide surrogates could be obtained through a post-chain-assembly cyclization strategy in which the disulfide bond or its substitutes were formed after the peptide backbone had already been completed, especially the thioether bridge-substituted Brevinin-1BYa-3. The residual containing halogen sidechain hardly tolerated the base condition required by Fmoc SPPS because of its nucleophilicity. Hence, our interest was focused on the diaminodiacid strategy based on pre-prepared diaminodiacids for synthesis of peptide mimics with thioether as disulfide surrogates. Since every step was achieved *via* peptide coupling reactions, the difficulty in generating non-peptidic structures on peptides could be avoided in this strategy. Briefly, installation of the *tert*-butyl (tBu) protective group followed by capping of the Fmoc group on L-Homoser-OH (**11**) provided Fmoc-Homoser-OtBu (**12**). Compound **12** was subjected to carbon tetrabromide and triphenylphosphine, which provided bromine-substituted homoserine Fmoc-Homoser(Br)-OtBu (**13**). L-Cys(Trt)-OH was further protected with allyloxycarbonyl (Alloc) and allyl groups to obtain Alloc-Cys(Trt)-OAllyl (**15**). After removal of trityl protection, Alloc-Cys(Trt)-OAllyl (**15**) was reacted with compound **13** to obtain protected diaminodiacid 16 in a high chemical yield. Finally, the diaminodiacid building block 17 was prepared from diaminodiacid 16 by removing the *tert*-butyl protection group using trifluoroacetic acid (Scheme 2A). Next, to obtain on-resin peptide 18, the key intermediate 17 was successfully attached to resin through standard Fmoc SPPS using 2-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) and 3*H*-[1,2,3]-triazolo[4,5-*b*]pyridin-3-ol (HOAt) as the coupling reagent. After allyl and Alloc group de-protection via $[Pd(PPh_3)_4]$/$PhSiH_3$,

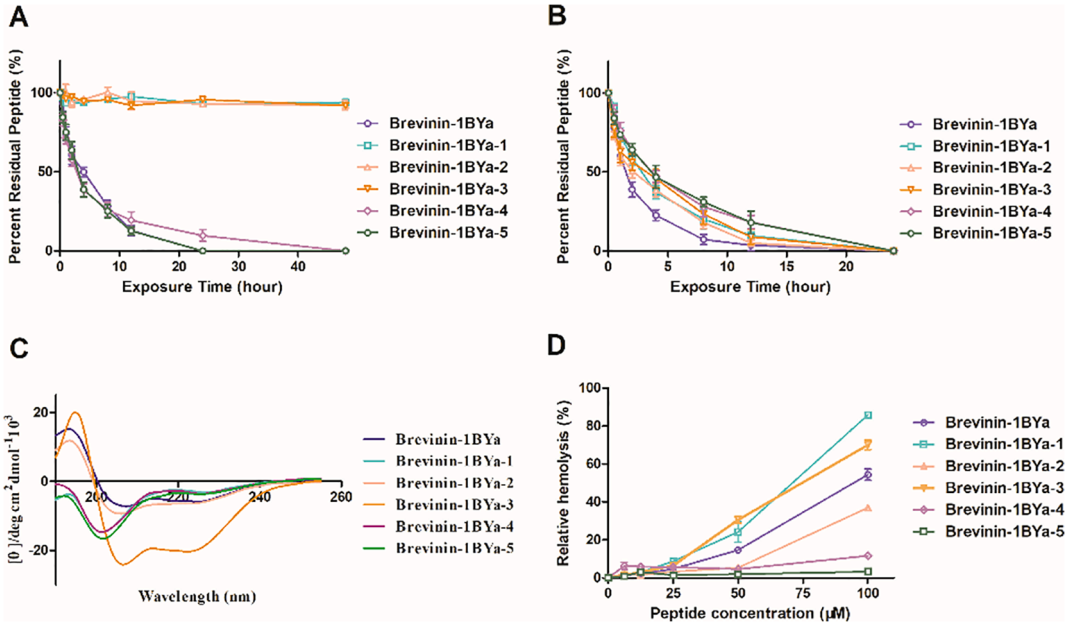


Fig. 2. A) Reduction stability of Brevinin-1BYa vs. its derivatives under DTT treatment. B) Protease stability of Brevinin-1BYa vs. its derivatives under α-chymotrypsin treatment. C) CD spectra of Brevinin-1BYa vs. its derivatives in TFE aqueous solution. D) Hemolytic activity of Brevinin-1BYa vs. its derivatives with fresh rabbit blood. Data points are displayed as the mean ± standard error of mean (SEM) of duplicate independent experiments. Percentage of residual peptide was monitored *via* HPLC.

Table 1
Degradation half-life, helicity, HC_{50} and anti-tumor activity of Brevinin-1BYa and derivatives.

Peptide	DTT-$t_{1/2}$ (h)	α-chymo-trypsin-$t_{1/2}$ (h)	Helicity (%)	HC_{50} (μM)	IC_{50} (μM)		
					C4-2B	A549	Huh-7
Brevinin-1BYa	3.35	1.62	22.57	94.41	13.96	9.240	14.86
Brevinin-1BYa-1	>12	2.93	–	70.43	12.63	21.68	11.33
Brevinin-1BYa-2	>12	1.81	26.34	>100	16.77	17.10	16.59
Brevinin-1BYa-3	>12	2.51	83.65	70.43	9.233	11.15	10.83
Brevinin-1BYa-4	3.32	4.33	–	>100	20.51	20.93	23.37
Brevinin-1BYa-5	3.31	4.32	–	>100	18.72	32.65	39.60

macrocyclization was successfully accomplished with PyAOP, NMM, and HOAt as lactamization reagents to achieve cyclic peptide 19. Elongation with subsequent amino acids gave on-resin peptide 20. Finally, one-step global de-protection and cleavage with the TFA cocktail delivered Brevinin-1BYa-3 in a yield of 55.6% (Scheme 2B).

The synthesis of glycopeptide Brevinin-1BYa-4 and Brevinin-1BYa-5 was based on the key glycoamino-acid building block Fmoc-Ser(GlcNAc(Ac_3))-OH, which was readily incorporated into the peptide backbone through standard Fmoc SPPS to obtain on-resin peptides 7 and 8. The following on-resin de-protection of the acetyl protection group utilizing hydrazine hydrate provided the on-resin intermediates 9 and 10. After acidic cleavage, global de-protection, and oxidative folding, Brevinin-1BYa-4 and Brevinin-1BYa-5 were obtained in yields of 54.1% and 54.3%, respectively (Scheme 1).

To investigate the influence of disulfide substitutions on the redox stability of Brevinin-1BYa, a reduction stability experiment was carried out using dithiothreitol (DTT) as a reducing agent.[21] It was suggested that natural disulfide bridge-containing peptides, including Brevinin-1BYa, Brevinin-1BYa-4, and Brevinin-1BYa-5, were completely reduced in 48 h in aqueous DTT solution, while peptidomimetics Brevinin-1BYa-1, Brevinin-1BYa-2, and Brevinin-1BYa-3 remained intact after 48 h of treatment (Fig. 2A and Table 1). Furthermore, the protease stability experiment was investigated using α-chymotrypsin, which is a protease that preferentially cleaves large, hydrophobic amino acids such as tryptophan, phenylalanine, and leucine.[22] It was found that after 24 h of protease exposure, only slight improvement of protease stability was observed between Brevinin-1BYa and its disulfide bond mimetics, indicating that the disulfide mimics could significantly improve the reduction stability of peptides rather than the protease stability. However, it was also noticed that glycopeptides Brevinin-1BYa-4 and Brevinin-1BYa-5 performed 1.67- and 1.66-fold longer enzyme degradation half-lives, respectively, compared with the prototype peptide (Fig. 2B and Table 1), indicating the significant roles of glycosylation moieties for peptide protease stability as previously reported.[23] Next, the secondary structure of Brevinin-1BYa derivatives was measured by circular dichroism (CD, Fig. 2C and Table 1). CD analysis indicated that prototype Brevinin-1BYa performs an α-helix structure with a helicity of 22.57% which was consistent with previous report.[12] Brevinin-1BYa-1, -4 and -5 lost their α-helix structure and showed random conformations. Brevinin-1BYa-2, whose amide bond substitution was similar in size to disulfide bonds, maintained the secondary structure of the prototype drug. More importantly, it is worth noting that a huge improvement of helicity was observed for Brevinin-1BYa-3 (83.65%, 2.70-fold improvements compared to Brevinin-1BYa) whose thioether substitution was only one atom difference with disulfide bond, indicating that large steric disulfide bond replacements or inappropriate glycosylation sites were dramatically detrimental to their secondary structure maintaining.

With Brevinin-1BYa and its analogs in hand, their anti-tumor activities were measured using the CCK-8 test with the human prostate cancer cell line C4-2B, the human NSCLC cell line A549 (adenocarcinoma), and the human hepatoma cell line HuH-7. The results are summarized in

Table 1. Among the disulfide mimics, Brevinin-1BYa-1 and Brevinin-1BYa-2 showed a slight loss of anti-tumor activities compared to prototype peptide, except for Brevinin-1BYa-3. The thioether bridge containing Brevinin-1BYa-3 showed the highest anti-tumor activities against the C4-2B and HuH-7 cell lines (IC_{50} = 9.233 and 10.83 μM, respectively), with values that are 1.50- and 1.38-fold greater, respectively, than those of Brevinin-1BYa. These results suggested that a spatially appropriate linker is indispensable for the anti-tumor activities of Brevinin-1BYa.[24] Meanwhile, excessive biological activity decreases were observed with the glycopeptides Brevinin-1BYa-4 and Brevinin-1BYa-5, suggesting that an inappropriate spatial location of the carbohydrate block might impart significant structural and conformational changes upon the peptide and perturb the interactions between the peptide and target protein. However, the carbohydrate block could indeed improve enzyme stability.[23] Further hemolytic activity (HC_{50}, Fig. 2D and Table 1) was evaluated by fresh rabbit blood hemoglobin releasing from erythrocyte suspensions. Data indicated that the HC_{50} of initial Brevinin-1BYa was 94.41 μM and that of the derivative peptides ranged from 65.19 μM to >100 μM. Among them, Brevinin-1BYa-4 and -5 displayed the lowest hemolytic activity. These results demonstrated that glycosylation moiety could dramatically ameliorate the hemolytic activity which was a significant characteristic of AMPs. All in all, both of the peptide derivatives showed no obvious hemolytic activity at the IC_{50}.

In summary, the anti-tumor activity of AMP Brevinin-1BYa was investigated, and a novel series of *O*-linked *N*-acetyl-glucosamine glycopeptides and disulfide bond mimetics of Brevinin-1BYa successfully were synthesized through either a post-chain assembly cyclization strategy or diaminodiacid-based strategy to improve the structural rigidity and redox stability of the prototype peptide. *In vitro* data suggested that the reduction stability of disulfide mimics Brevinin-1BYa-1, Brevinin-1BYa-2, and Brevinin-1BYa-3 was significantly improved, while all of the peptidomimetics exhibited slightly improved protease stability compared with the original peptide. For disulfide mimics, although thioether-bridged Brevinin-1BYa-3 showed a higher anti-tumor activity compared with the prototype peptide, a certain loss of anti-tumor activity was observed in Brevinin-1BYa-1 and Brevinin-1BYa-2. According to the previous report, the main mechanism of Brevinin family's anticancer action is membrane properties modification, especially membrane permeability which destroys the structure of the cell membrane, and then causes intracellular material leaking out and ultimate cell death, most likely the same as for pathogens. Together with the CD data, these results indicated that inappropriate non-peptidic bridges may possibly change the bridge length, which distorted the α-helix structure that was significant to Brevinin-1BYa's biological activities. Moreover, it was also noted that, even if the carbohydrate moiety could improve the protease stability and ameliorate the hemolytic activity of the prototype peptide, the inappropriate spatial location was dramatically detrimental to its secondary structure and biological activity, probably caused by the interruption of cell membrane binding. To further investigate these problems, studies of Brevinin-1BYa peptidomimetics containing different bridges, such as diselenium and olefin bond, are currently under way.[25–27] Meanwhile, Brevinin-1BYa glycosylated derivatives focusing on the different spatial locations and types of carbohydrate moiety will also be investigated and the results disclosed in due course.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127831.

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