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Identification, Screening, and Application of Natural Peptides from Toad

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Mini Review

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ABSTRACT

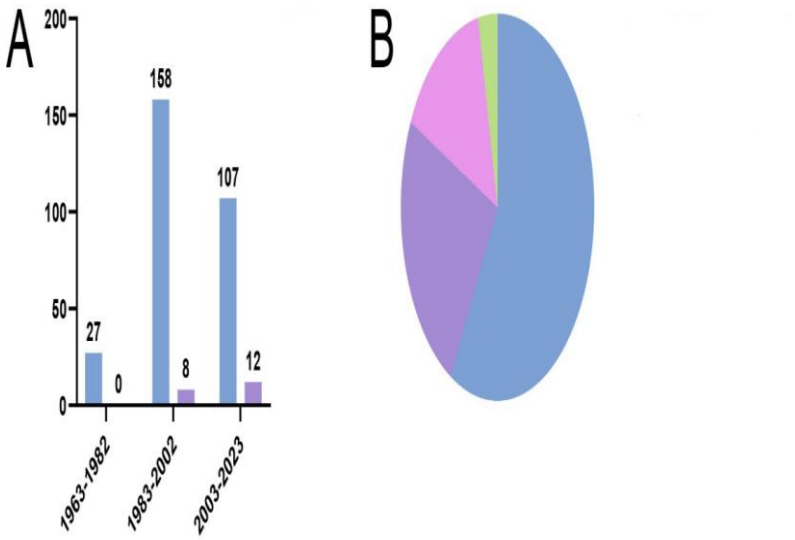
Natural peptide drugs have good targeting properties and lower toxicity compared to small molecule drugs. A large number of animal-derived peptides have been gradually discovered over the past 60 years. These natural peptides, with potential biological activity, can be used to treat human diseases. In addition, some natural peptides possess species specificity, which can be used for quality control of peptide drugs, thereby improving the consistency and clinical safety of medications. In this mini review, we present the current strategies for identifying and screening natural peptides from toad, as well as their applications in quality control, species-based identification, and the discovery of new peptide leads.

Keywords: Toad; Peptidomics; Natural peptides; Peptide leads

INTRODUCTION

Peptides (molecular weight <10 kDa) have the characteristics of strong targeting compared to small molecular compounds. Animal-derived natural peptides with better targeting properties and lower toxicity promote them potentially valuable for pharmaceutical development. Several natural peptides from microorganisms, plants, and animals have been gradually developed as drugs, such as cyclosporin and hirudin [1,2]. Among these active peptides, many originate from amphibians, especially toads and frogs [3]. Over the past 60 years, there have been increasing researches on peptide compounds derived from toads and frogs. The active natural peptides mainly exhibited antimicrobial, antitumor, and antiviral activities (Figures 1A and 1B).

Figure 1. (A) The literature reports on toad and frog peptides in the past 60 years. (B) Functional distribution of active peptides derived from toads and frogs. **Note**: ( ) Toad; ( ) Frog; ( ) 58.06% Antibacterial; ( ) 24.19% Antitumor; ( ) 14.52% antiviral; ( ) 3.23% Others.



In our recent researches, by systemically analysis toad skin, toad venom, and cinobufacini preparations, we discovered some natural peptides derived from the *Bufo gargarizans* showed species specificity [4]. Combining transcriptomics and peptidomics analysis, we have identified numerous characteristic natural peptides, which have been further used for quality control, species identification, and activity screening (Table 1).

Table 1. Information of natural peptides from Toad venom, Cinobufacini capsules and Cinobufacini injections.

Peptide sequence	Mass	m/z	z	Accession	Category
NFTGDSIPC(+57.02)R	1165.5186	583.768	2	CL.4590	Toad venom
GVTIAQGGVLPNIQ	1365.7616	683.8903	2	Histone H2A	Cinobufacini capsules
PEPAKSAPAPK	1091.5974	364.8741	3	Histone H2B	Cinobufacini capsules
T(+42.01)SQYARSLGGGQ	1265.6	633.8099	2	Keratin	Cinobufacini capsules
AP(+15.99)GQPQLQISGQ	1238.6255	620.3234	2	RNA polymerase	Cinobufacini capsules
GIADALGKAYH	1114.577	558.2979	2	Hemoglobin	Cinobufacini capsules
A(+42.01)SLQLINVN	1012.5553	507.2861	2	Glutamyl transferase	Cinobufacini capsules
A(+42.01)DKTELIQKAK	1285.7241	643.8701	2	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	Cinobufacini capsules
AVFPSIVGRPR	1197.6891	400.2411	3	beta-actin	Cinobufacini injections
PEPAKSAPAPK	1091.5974	364.8734	3	Histone H2B type 1	Cinobufacini injections
GIADALGKAYH	1114.577	558.2979	2	Hemoglobin subunit beta-2	Cinobufacini injections
GVTIAQGGVLPNIQ	1365.7616	683.8903	2	H2A histone family member J	Cinobufacini injections

Modifications: Carbamidomathylation (+57.02), acetylation of the N-terminus (+42.01), oxidation (+15.99).

By utilizing cell affinity screening techniques, we have also discovered natural peptide leads with excellent anti-tumor activity. Here, we discuss the identification, screening methods, and applications of natural peptides from toad.

LITERATURE REVIEW

Identification and analysis of natural peptides

Peptidomics: The common peptide identification methods include high-performance liquid chromatography and infrared spectroscopy. For identification of complex peptide structures, nuclear magnetic resonance or Mass Spectrometry (MS) were applied [5,6]. In recent years, with rapid development in mass spectrometry, peptidomics based on MS has become more mature. Compared with other methods, this method has high sensitivity and high throughput, and can be used to identify post-translational modification sites of natural peptides [7]. We utilized peptidomics to identify the natural peptides in cinobufacini injections and capsules by searching against a self-built transcriptome database, and obtained 230 and 716 natural peptides, respectively [8]. Additionally, we identified a total of 939 unique peptides from toad venom, which possessed excellent anti-tumor properties [9]. The identification of these natural lead peptides lays a solid foundation for the subsequent development of innovative peptide drugs.

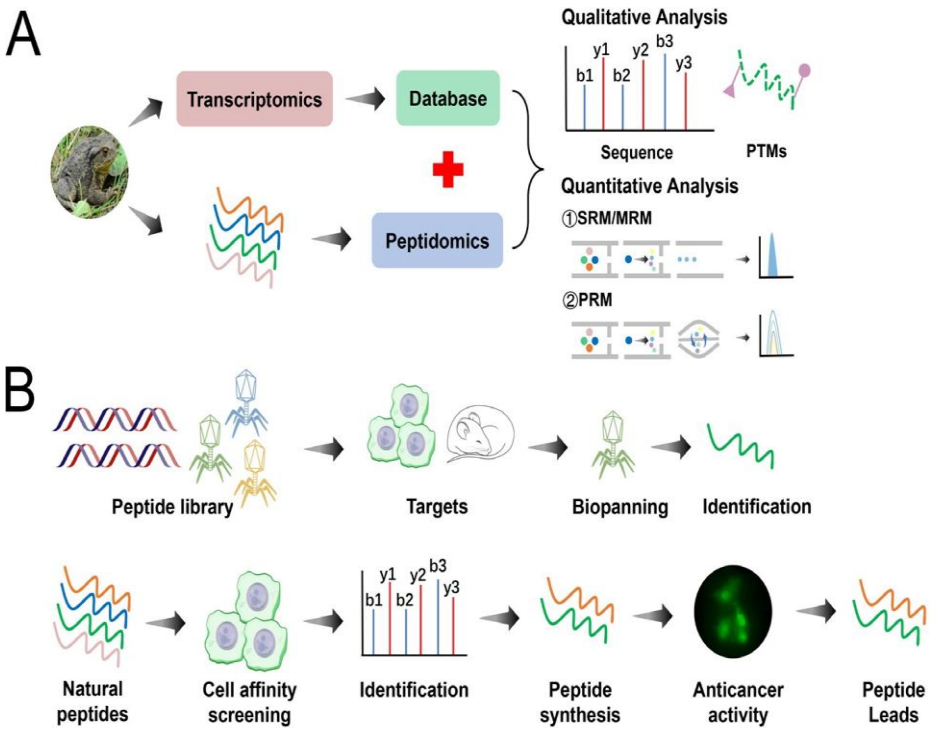
Targeted quantification of peptides: Peptide quantification techniques based on mass spectrometry have a matured methodology, progressing from the earliest Selected Reaction Detection (SRM) to Multiple Reaction Detection Mode (MRM), and later to Parallel Reaction detection Mode (PRM) [10]. SRM and MRM require setting the ion pair information of the target peptide segment. They offer good stability and a linear range in analysis, making them as the gold standard for targeted quantification. However, these two modes are susceptible to interference by complex matrices and are limited by the setting ion pairs. In contrast, the PRM detection mode benefits from the extremely high sensitivity of high-resolution MS and can detect the target peptide segment without selecting the product ion [11]. In our study, we established a quantitative method for

4 peptides in cinobufacini injection and toad skin using the MRM mode. This method can be applied to quality control, improving the consistency and safety of the drug. Additionally, we employed the PRM mode for targeted detection of natural peptides identified in cinobufacini capsules and toad venom.

Screening of natural active peptides

Currently, the screening methods for natural active peptides mainly include random screening strategy, phage display technology, and the cell affinity peptidomic screening method [12,13]. We have summarized these methods as applied to natural peptide screening in Supplementary Table 1. The cell affinity screening method we developed is used to screen anti-tumor active peptides and can identify Cell Binding Peptides (CBPs) at the cellular level. In our previous study, we applied this method to screen active peptides derived from toad venom on SMMC-7721 and Hela cells. Through peptidomics, we identified 76 potential cell-binding peptides. We found these peptides with biotin- labeled were able to internalize in tumor cells in cytoplasm, nucleus and other organelles by fluorescence microscopy analysis. This method offers high throughput screening for unknown natural active peptides and can also identify different post- translational modification sites, providing unique advantages over other methods (Figures 2A and 2B).

Figure 2. Workflow: (A) Identification and analysis of natural peptides. Natural peptides were extracted from toad, the sequence and Post-Translational Modifications (PTMs) of peptides were analysis by peptidomics with a self-built transcriptome database. The target peptides could analysis by selected reaction detection (SRM), Multiple Reaction Detection (MRM) or parallel reaction detection (PRM). (B) Screening natural active peptides by phage display and cell affinity screening.



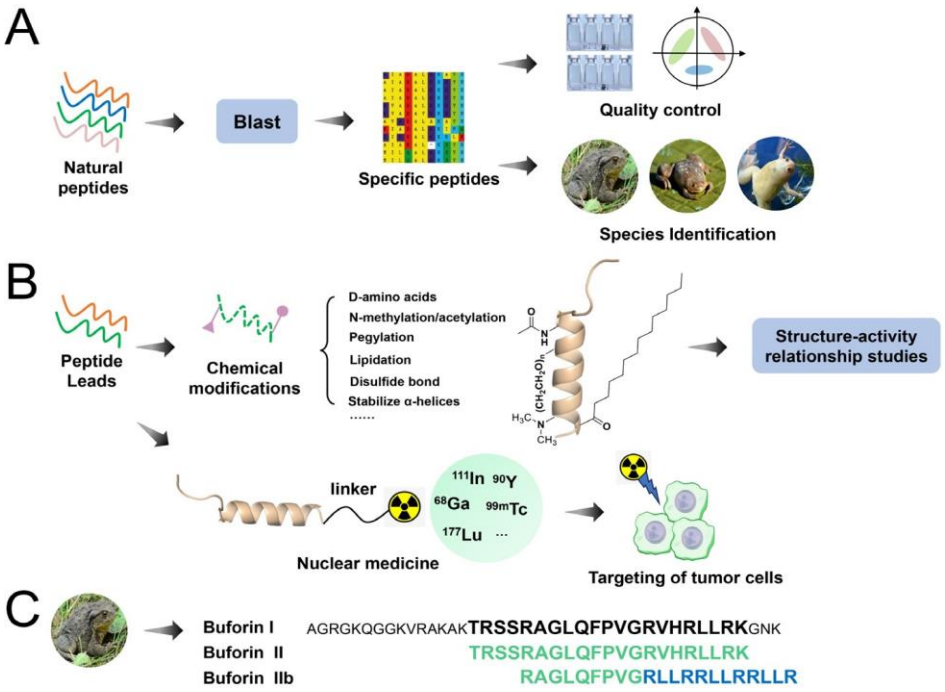
DISCUSSION

Application of natural peptides

Quality control of drugs: In the past, the quality control of drugs mainly focused on small molecules, while neglecting the peptide components in Chinese medicine. This oversight may result in poor batch-to-batch consistency of drugs. We established a quantitative method for 4 characteristic peptides in cinobufacini injection by employing targeted quantitative techniques. We applied this method to quality control in different batches of the drug and found certain variations among batches. Analyzing the causes of these differences will contribute to improving the consistency of cinobufacini injection and enhancing its clinical safety. We compared the homologous sequences of natural peptides identified in cinobufacini capsules and cinobufacini injection, found that some peptides were exclusively derived from *Bufo gargarizans*, indicating species specificity (Table 1). These peptides can be utilized for species identification in peptide drugs, which is beneficial for enhancing drug quality.

Peptide leads: Natural active peptide can possess improved activity and safety through further structural modifications for lead optimization, including D-amino acids, N-methylation, acetylation, pegylation, lipidation, disulfide bond and stabilization of α-helices. Additionally, natural peptides with good stability and targeting can be coupled with nuclides to form nuclear medicine, which can specifically target tumor tissues for treatment [14]. Buforin I, which was a typical example of a natural peptide, discovered in 1996 by Park from the stomach of *Bufo gargarizans*. It was further lysed by protease Lys-C to obtain buforin II, which exhibited stronger antibacterial activity [15]. By introducing the α-helix sequence (3 × RLLR) at the C-terminal of buforin II, the antitumor activity peptide buforin IIb was obtained [16,17] (Figures 3A-3C).

Figure 3. Application of natural peptides. (A) Quality control of drugs by using specific peptides. Species identification by using species specific peptides. (B) Chemical modifications of peptide leads, including D-amino acids, N-methylation, acetylation, pegylation, lipidation, disulfide bond and stabilization of α-helices. The activity of peptides will be further studied by structure-activity relationship. Also, the peptide with good targeting property could couple with the radionuclides to target tumor cells. (C) A case of optimization of natural peptides, from buforin I to buforin II and buforin IIb.



The structure of peptides is closely related to their function, including the presence of an α-helix structure, hydrophobicity, and net charge [18]. Peptides with membrane- penetrating capability may also exhibit an α-helix structure. The presence of a hinge structure increases the conformational flexibility of peptides [19]. Additionally, positively charged and high hydrophobicity peptides can penetrate the hydrophobic regions of tumor cell membranes [20-23]. The identification of natural peptides has greatly benefited from the rapid development of mass spectrometry. However, detecting low-abundance peptides remains significantly challenging. In the future, the detection of low-abundance peptides could be achieved through targeted Data-Independent Acquisition (DIA) combined with PRM, such as the recently developed dia-PASEF and prm-PASEF methods. Furthermore, attention should be paid to PTMs of natural peptides as they may impact peptide activities, including phosphorylation, methylation, hydroxylation, acylation, among others.

CONCLUSION

The discovery of natural peptides holds great significance in drug quality control and the development of new drugs. The discovery and application of natural peptides, particularly those from toads, hold immense promise in the development of safer and more effective pharmaceuticals, improved quality control, and the exploration of novel therapeutic leads. Continued advancements in analytical techniques and understanding of peptide structure-function relationships will continue to drive progress in this field. Natural peptides exhibit distinct advantages, including strong targeting properties and lower toxicity compared to small molecule drugs. Over the past 60 years, research has uncovered a wealth of natural peptides with potential biological activities, with many originating from amphibians like toads. This includes the use of D-amino acids, N-methylation, acetylation, pegylation, and other modifications. Additionally, natural peptides with good stability and targeting can be used in nuclear medicine for specific targeting of tumor tissues. Techniques based on mass spectrometry, such as Selected Reaction Detection (SRM), Multiple Reaction Detection Mode (MRM), and parallel reaction detection mode (PRM), enable the quantification of specific peptides.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflicts of interest.

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