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Review

Buforins: Histone H2A-derived antimicrobial peptides from toad stomach

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ABSTRACT

Antimicrobial peptides (AMPs) constitute an important component of the innate immune system in a variety of organisms. Buforin I is a 39-amino acid AMP that was first isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans*. Buforin II is a 21-amino acid peptide that is derived from buforin I and displays an even more potent antimicrobial activity than its parent AMP. Both peptides share complete sequence identity with the N-terminal region of histone H2A that interacts directly with nucleic acids. Buforin I is generated from histone H2A by pepsin-directed proteolysis in the cytoplasm of gastric gland cells. After secretion into the gastric lumen, buforin I remains adhered to the mucous biofilm that lines the stomach, thus providing a protective antimicrobial coat. Buforins, which house a helix-hinge-helix domain, kill a microorganism by entering the cell without membrane permeabilization and thus binding to nucleic acids. The proline hinge is crucial for the cell penetrating activity of buforins. Buforins also are known to possess anti-endotoxin and anticancer activities, thus making these peptides attractive reagents for pharmaceutical applications. This review describes the role of buforins in innate host defense; future research paradigms; and use of these agents as human therapeutics.

Contents

1. Introduction

Amphibians, like other vertebrates, are constantly exposed to multiple harmful microbes that can easily penetrate the mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts. Therefore, the capacity to overcome microbial infections is essential for vertebrate survival [1,2]. Most mucosal surfaces do not contain abundant phagocytic cells under normal conditions. Thus, surface epithelial cells are of critical importance in mediating the host's innate immune response, which is the first line of defense against microbial maladies [3,4]. In response to microbial infection, a variety of host-defense compounds are secreted from specialized glands on the dorsal surface and into the gut of the amphibian. These include amines, alkaloids, and cationic antimicrobial peptides (AMPs) [5–7].

AMPs are derived through the proteolysis of precursor proteins/peptides that are encoded by the host genome and synthesized on ribosomes. These defense peptides are short (10–50 amino acids) and contain an overall positive charge (in general, +2 to +9) and a substantial proportion (>30%) of hydrophobic residues [8–10]. These properties permit AMPs to fold into amphipathic α-helix and/or β-sheet structures upon contact with negatively charged microbial membranes. These structures can then insert themselves into the membranes of infectious particles and create pores. Energy and ionic gradients are subsequently lost, and cell lysis occurs within minutes [11–14]. Furthermore, biological studies of AMPs have demonstrated that, in addition to killing microorganisms by membrane insertion, some of these peptides function in regulating cell proliferation, extracellular matrix production, and cellular immune responses [15–17].

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Table 1
Amino acid sequences of buforins

Peptide	Amino acid sequence
Buforin I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY
Buforin II	TRSSRAGLQFPVGRVHRLLRK
Buforin IIb	RAGLQFPVGRLLRRLLRRLLR
Histonin	RAGLQFPVGKLLKKLLKRLKR

Thus, AMPs provide a powerful defense system that can both protect the mucosal surfaces from infection and signal host cells to change their behavior in response to external injury.

Buforin I is a 39-amino acid AMP that was first isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans* [18]. Derived from buforin I, buforin II is a 21-amino acid AMP that displays a more potent antimicrobial activity than does its parent peptide (Table 1). The buforins share complete sequence identity with the N-terminal region of histone H2A, which specifies the protein's DNA binding activity. Historically, histone function has been studied mainly in connection with DNA stabilization and regulation of gene expression. However, a growing collection of evidence suggests that histones are involved in a multitude of biological functions [19]. For example, buforin I highlights the role of histones in innate immunity. And buforin II has attracted the attention of researchers because of its unique mechanism of antimicrobial action. Indeed, buforins kill a microorganism by translocating into the cell, without membrane permeabilization, and thus binding to nucleic acids [20]. This review focuses on the current status of buforins in terms of their structures; roles in innate host defense; mechanisms of antimicrobial action; and other biological activities. The potential exploitation of buforins as therapeutics is also discussed.

2. Synthesis of buforin I and its role in innate host defense

Compared with other amphibian AMPs, such as magainin 2, buforin I shows much stronger antimicrobial activities *in vitro* against a broad spectrum of microorganisms. In addition to its powerful antimicrobial activity, a second striking feature of buforin I is that it shares complete sequence identity with the N-terminal region of histone H2A [21]. In the eukaryotic nucleus, DNA is packaged as chromatin, which consists of DNA bound to histones and other nonhistone proteins. Histones are basic proteins that form a spool around which nuclear DNA is wrapped, and they constitute the major protein component of chromatin. Histones are known to function in chromatin structure formation, nuclear targeting, and the regulation of gene expression. However, it also has been reported that histones, especially histone H2A, display weak antimicrobial activity [22–24]. However, the use of histone H2A as an antimicrobial agent by eukaryotic cells might be problematic, because histone H2A is a large protein that contains numerous functional domains that carry out its myriad functions.

In contrast, by virtue of its amphiphaticity and composition of positively charged amino acids, buforin I houses a domain that resembles the conserved AMP structural motif more closely than does the corresponding domain in histone H2A. The observation that histone H2A serves as a precursor of an AMP has given rise to a series of intriguing experiments designed to decipher the mode of histone H2A processing in the toad stomach as part of the innate host defense. Biochemical and immunohistochemical analyses have revealed that, in the gastric mucosal cell, histone H2A is synthesized in excess of the amount required for DNA packaging and accumulates within cytoplasmic secretory granules. Furthermore, our group has shown that a fraction of the newly synthesized histone H2A pool is acetylated and thus targeted for translocation to the nucleus, whereas the remaining unacetylated histone H2A, upon secretion into the gastric lumen, is processed by pepsin C isozymes to yield buforin I [25].

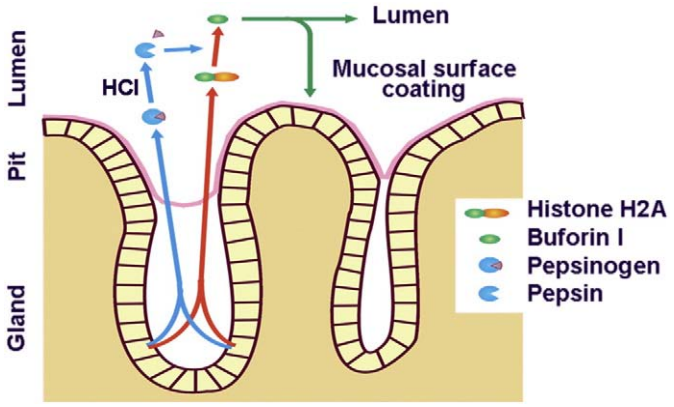

Fig. 1. Postulated mechanism of buforin I production in toad stomach. In the gastric mucosal cell, histone H2A is synthesized in excess of the amount required for DNA packaging and thus accumulates within cytoplasmic secretory granules. Upon secretion into the gastric lumen, histone H2A is processed by pepsin to the potent antimicrobial peptide buforin I, which remains adhered to the mucous biofilm that lines the stomach surface; this provides the stomach with a protective antimicrobial coat. HCl also is secreted by the gastric mucosal cell and participates in the conversion of inactive pepsinogen to active pepsin.

Buforin I production is known to be closely synchronized with the secretion of hydrochloric acid and pepsinogen by gastric mucosal cells; this gastric acid catalyzes the conversion of inactive pepsinogen to the proteolytically active pepsin (Fig. 1).

As is the case with other anurans, the toad swallows its prey intact and stores this microbe-laden food in the stomach for some period of time before digestions begins; thus the secretion of buforin I into the gastric lumen serves to inhibit bacterial growth. Moreover, after secretion, buforin I remains adhered to the mucous biofilm that coats the surface of the stomach (Fig. 1) and thus provides this organ with a protective antimicrobial coat that works in conjunction with mucosal immune cells to combat microbial infections. It also has been suggested that AMPs on the mucosal surfaces of vertebrates aid in wound repair [26–28].

Buforin I is the first histone-derived AMP whose function as an innate immune effector has been studied in detail. Since then, the role of histones in innate immunity has been widely appreciated [29–38], and the proteolytic processing of histone H2A to yield an AMP also has been reported to occur in vertebrates other than the toad. Parasin I, a 19-amino acid peptide, was isolated from the skin mucus of catfish [39] and shown to be generated from histone H2A by cathepsin D-directed proteolysis. The inactive proenzyme procathepsin D is secreted to the mucosal surface and then processed to the active

Table 2
Antimicrobial activities of buforins

Microorganism	Minimal inhibitory concentration[a] (μg/mL)			
	Buforin I	Buforin II	Buforin IIb	Histonin
Gram-positive bacteria				
Bacillus subtilis	4	2	1	2
Staphylococcus aureus	4	4	1	2
Streptococcus mutans	8	2	0.5	2
Gram-negative bacteria				
Escherichia coli	8	4	1	2
Pseudomonas putida	4	2	2	2
Salmonella typhimurium	4	1	1	
Fungi				
Candida albicans	4	1	2	2
Cryptococcus neoformans	4	1	1	2
Saccharomyces cerevisiae	4	1	4	2

[a] Data taken from Refs. [18], [45], and [86].

mature enzyme (cathepsin D) by a matrix metalloprotease 2 that is induced in response to epidermal injury [40,41].

3. Structure of buforin II and its mechanism of antimicrobial action

Buforin II is a 21-residue peptide that is produced from buforin I by treatment with the endoproteinase Lys-C [18]. Buforin II contains residues Thr^{16} to Lys^{36} of buforin I (Table 1) and exhibits antimicrobial activity that is twice as potent as that of its parent peptide (Table 2). Most AMP structure–activity studies of buforins have been performed with buforin II as a model. The structure of buforin II was determined using NMR spectroscopy and restrained molecular dynamics [42]. Buforin II adopts a helix-hinge-helix structure in 50% trifluoroenthanol (TFE); the N-terminal extended α-helix includes residues Arg^5 to Phe^{10}, and the C-terminal α-helix includes residues Val^{12} to Lys^{21}. The helices are separated by a proline residue situated at amino acid position 11 (Fig. 2).

Although buforin II bears a structure similar to those of other amphiphatic α-helical AMPs, buforin II's mechanism of antimicrobial action appears to differ from those of AMPs that function by membrane permeabilization. Confocal fluorescence microscopic analysis and gel-retardation experiments have revealed that buforin II kills bacteria without cell lysis and has a strong affinity for DNA and RNA *in vitro* [20]. Kobayashi et al. investigated the interaction of buforin II with phospholipid membranes and compared these results with those of similar experiments with magainin 2 [43]. These researchers used equipotent tryptophan-substituted peptides to fluorometrically monitor peptide–lipid interactions. Control circular dichroism studies showed that, like magainin 2, buforin II binds selectively to liposomes composed of acidic phospholipids. However, the fluorometric experiments revealed that, in contrast to magainin 2, buforin II translocates across the liposome membranes efficiently without inducing significant membrane permeabilization or lipid flip-flop. Furthermore, the Pro^{11} residue, which induces a kink in buforin II α-helix, is the key structural feature required for the buforin II's unique cell penetrating property.

A subsequent study revealed that buforin II crosses lipid bilayers in a manner similar to that of magainin 2—via the transient formation of a peptide–lipid supramolecular complex (toroidal) pore. However, the presence of Pro^{11} distorts the helical structure of buforin II, concentrating five basic amino acid residues in a limited amphipathic region (Arg^5-Lys^{21}); this structure destabilizes the pore by enhanced electrostatic repulsion and enables efficient translocation of buforin II into the microbial cell [44]. The importance of the Pro^{11} residue also was demonstrated by a systematic structure–activity relationship study [45]. In this study, antimicrobial potencies, secondary structures, and mechanisms of bacterial killing action were assessed for a series of structurally altered synthetic buforin II analogs. The results revealed that the proline hinge (Pro^{11}) is a key structural factor for the cell-penetrating property of buforin II, while the cell-penetrating efficiency, which depends on α-helical content, is a critical factor for determining antimicrobial potency. Indeed, these experiments showed that only a single amino acid substitution at the Pro^{11} position changes buforin II into a membrane-active magainin-like peptide. Conversely, insertion of a proline-hinge region (Arg^5-Gly^{11}) into the amino-terminal helix of magainin 2 switches this AMP from a membrane-permeabilizing peptide to a cell-penetrating one.

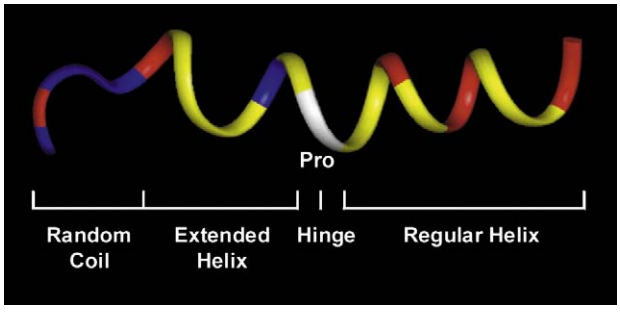

Fig. 2. Ribbon-model representation of the backbone structure of buforin II in 50% TFE. The N-terminal random coil, the extended helix, the hinge, and the C-terminal regular helix form an overall amphipathic structure. The amino acid residues are colored as follows: positively charged residues, red; other hydrophilic residues; blue; proline, white; other hydrophobic residues, yellow (Reproduced from [45]. Copyright (2000) National Academy of Sciences, U. S. A.).

Because buforin II was shown to bind nucleic acids *in vitro*, it has been hypothesized that buforin II kills a microorganism by interacting with its nucleic acids after translocation across the cell membrane [20]. Although the proposed mechanism is quite intriguing, many questions remain to be answered. The connection between nucleic acid binding and antimicrobial activity has not been demonstrated directly, and it is unclear whether buforin II and nucleic acids interact in a specific manner or whether they only bind to each other because of their opposite net charges. Uyterhoeven et al. recently characterized the nucleic acid binding properties of buforin II using molecular modeling and a fluorescent intercalator displacement assay [46]. These researchers observed that, in addition to non-specific electrostatic attractions between a cationic peptide and nucleic acids, specific side chains (R^2 and R^{20}) of buforin II form interactions with DNA that are stronger than the nonspecific electrostatic ones. Moreover, disruption of the buforin II–DNA interactions by substituting basic residues of buforin II with alanine generally decreases the antimicrobial activity of buforin II. This observation supports the assertion that buforin II kills bacteria through its interaction with nucleic acids, although it does not preclude buforin II from having other as yet unidentified intracellular targets. Indeed, pyrrhocoricin and other members of the proline-rich AMP family, such as drosocin and apidaecin, appear to achieve their antimicrobial activity by binding to the bacterial heat shock protein DnaK [47], preventing chaperone-assisted host protein folding and inhibiting the strongly related ATPase activity of DnaK [48].

4. Other biological activities of buforins

Although buforin I and II have been shown primarily to possess a broad spectrum of antimicrobial activity against many pathogens and drug-resistant microbes [18,45,49–56], recent studies suggest that these AMPs have other biological effects as well, such as the inhibition of botulinum neurotoxins [57] and of tissue factor-initiated coagulation [58]. In fact, several AMPs display anti-endotoxin activity that is stimulated by binding of the peptide to bacterial lipopolysaccharide (LPS) and lipoteichoic acid, which results in the prevention of the sepsis and septic shock associated with the presence of pathogenic Gram-negative and Gram-positive bacteria [9,59,60]. Similarly, buforin II is able to prevent lethal endotoxemia in the rat model of peritonitis [61–63]. In this model, administration of a mono-dose of buforin II lowers intra-abdominal bacterial concentration and mortality. Furthermore, the concentrations of LPS and the LPS-induced, sepsis-mediating host cytokine TNF-α also are reduced dramatically in the blood of septic rats treated with buforin II. In these rats, intra-abdominal sepsis was induced via cecal ligation and single puncture.

Several AMPs also display anticancer activity [64–69]. One such peptide, melittin, specifically kills cells in culture that express high concentrations of the *ras* oncogene product [70]. The AMPs cecropin and magainin also kill neoplastic cells at concentrations lower than those required to lyse normal host cells, such as peripheral blood lymphocytes [71,72]. Unlike several other cationic peptides, buforin II does not exhibit cytotoxic activity against normal eukaryotic cells. For example, buforin II is nearly nonhemolytic with respect to human erythrocytes, even at a concentration greater than 200 times the amount required to inhibit bacterial growth [the so-called minimal

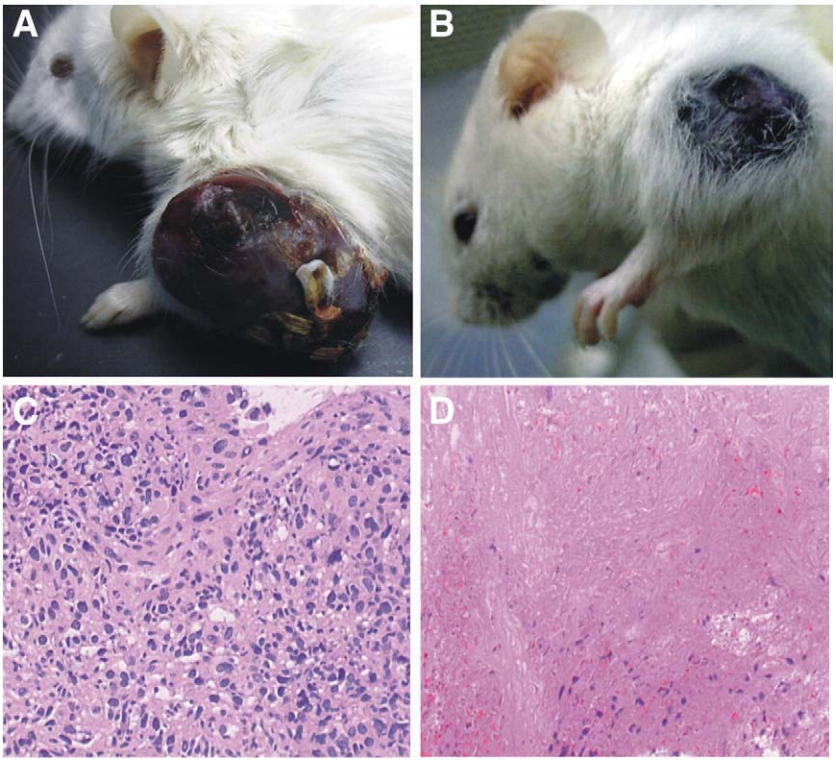

Fig. 3. Antitumor activity of buforin IIb. Spontaneous tumors produced in p53-deficient mice were treated with either phosphate-buffered saline (control) (A) or 20 mg/kg of buforin IIb (B) every 3 days for 2 weeks. Corresponding tumor sections were stained with hematoxylin and eosin (C and D). The cancerous cells are stained dark purple.

inhibitory concentration (MIC)] [43]. Also, when its cytotoxic activity is measured against human fibroblastic TM12 cells, buforin II has no effect on viability at a concentration of 100 μM [73]. However, a recent study showed that buforin II and buforin IIb–a synthetic analog of buforin II that contains a proline hinge between the two α-helices and a model α-helical sequence at the C-terminus (3×RLLR)–have selective cytolytic activity against 62 cancer cell lines with IC_{50} values (concentration of peptide at 50% cytotoxicity) in the range of 6 to 24 μg/mL [74]. The remarkable selectivity of buforin IIb for cancer cells results largely from the inability of the peptide to penetrate normal cell membranes. Cationic AMPs display selective toxicity toward bacteria, because their membrane surfaces contain an abundance of negatively charged phospholipids and polyanionic LPS [8]. Similarly, the outer surfaces of the plasma membranes of cancer cells contain high concentrations of negatively charged gangliosides [75,76]. In contrast, the impenetrable surface of normal mammalian cell membranes is composed mainly of neutral zwitterionic phospholipids and sterols [77]. Buforin IIb selectively targets cancer cells through interaction with the cell-surface gangliosides. Buforin IIb then traverses cancer cell membranes without damaging them and induces mitochondria-dependent apoptosis [74]. Buforin IIb also displays powerful cytotoxic activity when injected into solid tumors in p53-deficient mice (Fig. 3). These results suggest that buforin IIb may constitute a novel therapeutic agent for the treatment of cancers.

5. Potential exploitation of buforins as human therapeutics

The emergence and rapid horizontal spread of antibiotic-resistant traits in bacteria of human and veterinary clinical significance have been a driving force in the search for new classes of antibiotics [78]. AMPs have been regarded as a potential solution to serious worldwide problems caused by infectious diseases [15]. The potential value of AMPs for clinical purposes includes their use as single antimicrobial agents, synergistic agents to existing antibiotics, immunostimulatory agents, and endotoxin-neutralizing agents [79]. Buforins display many of the desirable features of a novel antibiotic. They exhibit antimicrobial activity, with MICs as low as 0.25–4.0 μg/mL, against clinical isolates of Gram-positive and Gram-negative bacteria, and are unaffected by classical antibiotic-resistance mutations [49–53,55]. Moreover, buforins are not toxic with respect to mammalian cells, show synergy with classical antibiotics, neutralize endotoxins, and are active in animal models [49,54,61–63,73,74].

Despite the fact that buforins show great potential as novel antibiotics, a number of issues must be solved before these AMPs can be developed as human therapeutics. For example, short α-helical peptides, such as the buforins, are cleaved *in vivo* by endogenous mammalian proteases, severely reducing an AMP's therapeutic value. In particular, trypsin-like enzymes attack proteins at basic residues, which are an obligate feature of AMPs. In this regard, there are strategies for protecting peptides from proteases, including liposomal incorporation or chemical modification [80]. Recently, Meng and Kumar reported that incorporation of hexafluoroleucine at selected sites (Leu^{18} and Leu^{19}) of buforin II results in simultaneous enhancement of potency and increased resistance to protease degradation. These observations suggest that fluorination may be an important strategy for increasing the stability of buforin II [81].

Another impediment in the development of AMPs for therapeutic use is that it is difficult to produce these peptides in a cost-effective manner. Because they have relatively high molecular sizes compared to most other antibiotics, AMPs must be produced using recombinant techniques in order to keep the cost of production low [82]. Numerous biological expression systems have been introduced for the cost-effective production of AMPs [83]. Because of their natural destructive behavior toward microorganisms and relative sensitivity to proteolytic degradation, AMPs are often produced by fusing the peptides to a fusion partner protein in the heterologous hosts; this approach neutralizes the innate bacterial toxicity of AMPs and increases their expression levels. After purification, the recombinant fusion proteins are cleaved to release the AMPs.

For example, buforin II can be expressed in *Escherichia coli* by fusing the AMP to an anionic peptide that neutralizes the positive charges of buforin II; this modification shields host bacterial cells from the lethal effects of the AMP [84]. In this system, the fusion peptide is expressed in tandem repeats to increase the production yield. This multimeric expression is subsequently improved through stabilization of the long transcripts with a DEAD-box protein or by carrying out recombinant protein expression in an oxidizing environment using *trxB* mutant *E. coli* as the host cells [85]. In another study a truncated fragment of the *E. coli* PurF protein (F4) was used as a fusion partner for histonin, a synthetic analogue of buforin II Table 1 [86]. F4 reinforces the formation of inclusion bodies and, hence, prevents the host-lethal effects and proteolytic degradation of the expressed recombinant histonin. Using these systems, our group has been able to produce about 107 mg of buforin II and 167 mg of histonin from 1 L of *E. coli* culture.

For the separation of AMPs from its fusion partner, the recombinant fusion protein is cleaved with CNBr [84] or furin [86]. However, these chemical or enzymatic cleavage methods are never 100% efficient and make the purification of the AMPs complicated and expensive. Therefore, these techniques cannot yet be performed on an industrial scale. In this regard, an intein fusion approach offers an interesting opportunity for the production of AMPs, because intein can direct its own excision from an intein fusion protein in the presence of thiols [87,88]. Several groups have used the intein-mediated system to produce various AMPs [89–92]. However, the production yields of AMPs using the intein-mediated system were very low. Therefore, many groups, including us, are currently working on the improvement of the intein-mediated system or the development of a novel method to produce AMPs in *E. coli* as a natural form without using any chemical or enzymatic cleavage step. We believe that these efforts may lead to a cost-effective solution for the mass production of AMPs, so that AMPs may soon fulfill their promise as a solution of overcoming the serious worldwide problems caused by infectious diseases.

Acknowledgements

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