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HV-BBI—A novel amphibian skin Bowman–Birk-like trypsin inhibitor

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ABSTRACT

Here we describe the isolation of a novel C-terminally amidated octadecapeptide—SVIGCWTKS IPPRPCFVK-amide—that contains a disulphide loop between Cys^5 and Cys^{15} that is consistent with a Bowman–Birk type protease inhibitor, from the skin secretion of the Chinese Bamboo odorous frog, *Huia versabilis*. Named HV-BBI, the peptide is encoded by a single precursor of 62 amino acid residues whose primary structure was deduced from cloned skin cDNA. The precursor exhibits the typical organization of that encoding an amphibian skin peptide with a highly-conserved signal peptide, an intervening acidic amino acid residue-rich domain and a single HV-BBI-encoding domain located towards the C-terminus. A synthetic replicate of HV-BBI, with the wild-type K (Lys-8) residue in the presumed P1 position, was found to be a potent inhibitor of trypsin with a K_i just slightly less than 19 nM. Substitution at this site with R (Arg) resulted in a significant reduction in potency (K_i 57 nM), whereas replacement of K with F (Phe) resulted in the complete abolition of trypsin inhibitory activity. Thus, HV-BBI is a potent inhibitor of trypsin and the lysyl (K) residue that occupies the P1 position appears to be optimal for potency of action against this protease.

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Amphibian skin is a multifunctional organ that is morphologically and biochemically adapted to subserve both general physiological and more specific survival roles, such as in chemical defence against predators [3,20]. The complex cocktails of bioactive molecules that constitute defensive secretion arise from the highly-specialized dermal granular or poison glands that are particularly well-developed in many anurans. These cocktails contain, among other minor components, proteins, biogenic amines, alkaloids and a plethora of bioactive peptides, and as a consequence, extracts of amphibian skin have been used for centuries in folk medicine due to their possession of a wide spectrum of potent pharmacological actions [3,9,12,20].

A multitude of peptides have been discovered in many species of anuran amphibian, with peptides from some five hundred species of frogs and toads from six continents, having been isolated, over the past few decades [3,9,12,20]. Some interesting families of bioactive peptides have been studied in great detail. These include the bradykinins and related peptides, the tachykinins, the bombesins and related peptides, and the skin opiates [3,20]. Perhaps no family, or rather cluster of families, has received as much attention as those that possess antimicrobial properties [12,28]. However, many novel peptides remain to be discovered and with the recent advances in analytical and mass spectrometric technologies, many hitherto considered unique peptides have been found to be widely-distributed in many structurally-related forms across different species.

One such prototype peptide is peptide leucine–arginine (pLR), originally isolated from skin secretion of the North American leopard frog, *Lithobates* (formerly *Rana*) *pipiens*, due to its potent stimulation of histamine release from mast cells and subsequently found inhibitory effect on bone marrow stem cell granulopoiesis [26]. Thereafter, a group of analogous peptides, named ranacyclins, were isolated from several species of European ranids by nature of their antimicrobial activity. Peptide tyrosine–arginine (pYR), an analogue with an identical bioactivity profile to pLR, was isolated from the skin secretion of the North American gopher frog, *Lithobates capito* (formerly *Rana sevosa*) [16] and several additional variants from other North American ranids [2]. Due to the presence of a Bowman–Birk-like protease inhibitor motif [10,24–25] contained within the single disulphide-bridged loop that characterizes this group of inhibitors, it was deemed likely that these amphibian peptides could constitute a new set of trypsin inhibitors [25]. Thus, the cumulative data on the bioactivity of this peptide family from discovery of its prototype [10,16,22,24–26], would suggest a wide spectrum of action on multiple targets.

In this study, we report the identification, structural characterization, and cloning of skin biosynthetic precursor cDNA of a novel member of the family from the skin secretion of the Bamboo Leaf Odorous frog, *Huia versabilis*, which we have named HV-BBI (*Huia versabilis* Bowman–Birk inhibitor). This peptide exhibits several amino acid substitutions within the presumed inhibitory loop when compared to the prototype peptide, pLR [26]. In addition, we have performed an initial structure/activity study on the P1 position of the inhibitory site by comparing the trypsin inhibitory potency of synthetic wild-type with two, site-substituted analogues.

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Materials and methods

Specimen biodata and secretion harvesting. Chinese bamboo leaf odorous frogs, *H. versabilis* ($n = 3$, 10–12 cm snout-to-vent length), were captured in the Five Fingers Peak Nature Reserve, Hainan Island, People's Republic of China. All frogs were adults and skin secretion was obtained from the dorsal skin using gentle transdermal electrical stimulation as previously described [31]. The stimulated secretions were washed from the skin using de-ionized water, snap frozen in liquid nitrogen, lyophilized and the lyophilizates stored at −20 °C prior to analysis.

Chromatographic fractionation. Five milligrams of lyophilized skin secretion were dissolved in 0.5 ml of 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to reverse-phase HPLC fractionation using a Thermoquest gradient system fitted with an analytical column (Phenomenex C-5; 0.46 cm × 25 cm). This was eluted with a linear gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected and the effluent absorbance was continuously monitored at λ214 nm. Samples (100 μl) were removed from each fraction in triplicate, lyophilized and stored at –20 °C prior to trypsin inhibition assay.

Trypsin inhibition assay. Trypsin (10 μl of a 0.1 μM stock solution in 1 mM HCl) was added to the wells of a micro-titre plate containing substrate (Phe-Pro-Arg-NHMec, obtained from Sigma/Aldrich, Poole, Dorset, UK) (50 μM) and either reconstituted chromatographic fraction (33%), in the first instance or, subsequently, synthetic peptide replicates (0.1–100 μM) in 10 mM phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl (final volume 210 μl). Each determination was carried out in triplicate. The rate of hydrolysis of substrate was monitored continuously, at 37 °C, by measuring the rate of increase of fluorescence, due to production of 7-amino-4-methylcoumarin (NH_2Mec) at 460 nm (excitation 360 nm) in a CYTOFLUOR® multi-well plate reader Series 4000 spectrofluorimeter.

Chymotrypsin inhibition assay. Inhibitory activity assays on wild-type HV-BBI and the various P1-varients against chymotrypsin were performed exactly as detailed in Trypsin inhibition assay, except that the fluorogenic substrate utilised was Succinyl-Ala-Ala-Pro-Phe-NHMec (obtained from Bachem, UK).

Synthesis of HV-BBI and P1 site-substituted analogues. Wild-type HV-BBI (Lys^8), and the P1 site-substituted analogues, (Arg^8)-HV-BBI and (Phe^8)-HV-BBI were synthesized by standard solid-phase Fmoc chemistry, using a Protein Technologies PS3™ automated peptide synthesizer. Following cleavage from the resin, deprotection and oxidative disulphide bond formation, peptides were purified by reverse-phase HPLC and both molecular masses and MS/MS fragmentation profiles were employed to confirm purity and identity.

Identification of HV-BBI precursor cDNA from a skin secretion-derived library. A 5 mg sample of lyophilized skin secretion was dissolved in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5′- and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length HV-BBI nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as previously described [5–6]. Briefly, the 3′-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and a sense primer (S: 5′-GTIATHGGIT GYTGGACIAA-3′) that was complementary to the amino acid sequence, -VIGCWTK-, of HV-BBI. The 3′-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from these 3′-RACE products were used to design a specific antisense primer (AS: 5′-CCAAATTAGATGACTT CCAATTCAAGT-3′) to a conserved site within the 3′-non-translated region of OVTI cDNAs. 5′-RACE was carried out using this specific primer in conjunction with the NUP RACE primer and resultant products were purified, cloned and sequenced.

Results

Isolation and structural characterization of HV-BBI

An inhibitor of trypsin activity was identified in fraction #62 following reverse-phase HPLC fractionation of *H. versabilis* skin secretion (Fig. 1A). MALDI-TOF analysis of this fraction, using a Perseptive Biosystems Voyager DE system in linear mode, indicated a single peptide with an m/z of 2013.95 and a high degree of purity (Fig. 1B). The primary structure of the peptide was established, using an Applied Biosystems 491 Procise microsequencer, as: SVIGCWTKSIPPRPCFVK. This structure is compared with other pLR/ranacyclin family members in Table 1. The peptide was deemed to be C-terminally amidated by nature of its consistent decrement of a single mass unit when compared to the computed mass of the peptide incorporating a free acid at the C-terminus. A synthetic replicate of this peptide, named HV-BBI, was tested for inhibitory activity against trypsin. The peptide was found to behave as a competitive reversible inhibitor of trypsin; 'progress curves' for the hydrolysis of the fluorogenic substrate in the presence of competing concentrations of peptide are shown in Fig. 2A. Initial rates (v_i) for product formation were estimated from these progress curves and these were used to generate "Morrison plots" (v_i against [I]; see side panel in Fig. 2A) from which a K_i value of 18.8 ± 1.8 nM was determined for the inhibition of trypsin [23].

Effect of P1 site substitution on inhibitory activity

Substitution of Lys-8, the predicted residue occupying the P1 position within the inhibitory loop, with Arg (R) resulted in 3-fold decrease in inhibitor effectiveness ($K_i = 54.2 + 5.6$ nM). In addition, both the Lys-P_1 and Arg-P_1 variants exhibited no inhibitory activity against chymotrypsin even when tested at a concentration of 100 μM. When Lys-8 was replaced by Phe (F) it eliminated trypsin inhibitory activity completely (this peptide was without effect even at a concentration of 100 μM, data not shown). In contrast, this Phe-variant exhibited modest inhibitory activity towards chymotrypsin as evidenced by the substrate hydrolysis progress curves shown in Fig. 2B. The resultant 'Morrison plots' (side panel in Fig. 2B) derived from these progress curves yielded a K_i value of approximately 389 nM for this peptide against chymotrypsin. The kinetic data for each peptide are summarised in Table 2.

Molecular cloning of the HV-BBI biosynthetic precursor

The HV-BBI biosynthetic precursor-encoding cDNA was successfully cloned from the skin secretion-derived library using the RACE protocol described above. An open-reading frame for (HV-BBI) consisted of 62 amino acid residues (Fig. 3). The N-terminal Ser (S) residue of the mature HV-BBI is flanked by a classical processing site (-KR-) and the C-terminal asparagine residue is flanked C-terminally by a glycine residue that serves as an amide donor (Fig. 4). The nucleotide sequence of HV-BBI has been deposited in EMBL Nucleotide Sequence Database under Accession codes AM850139.

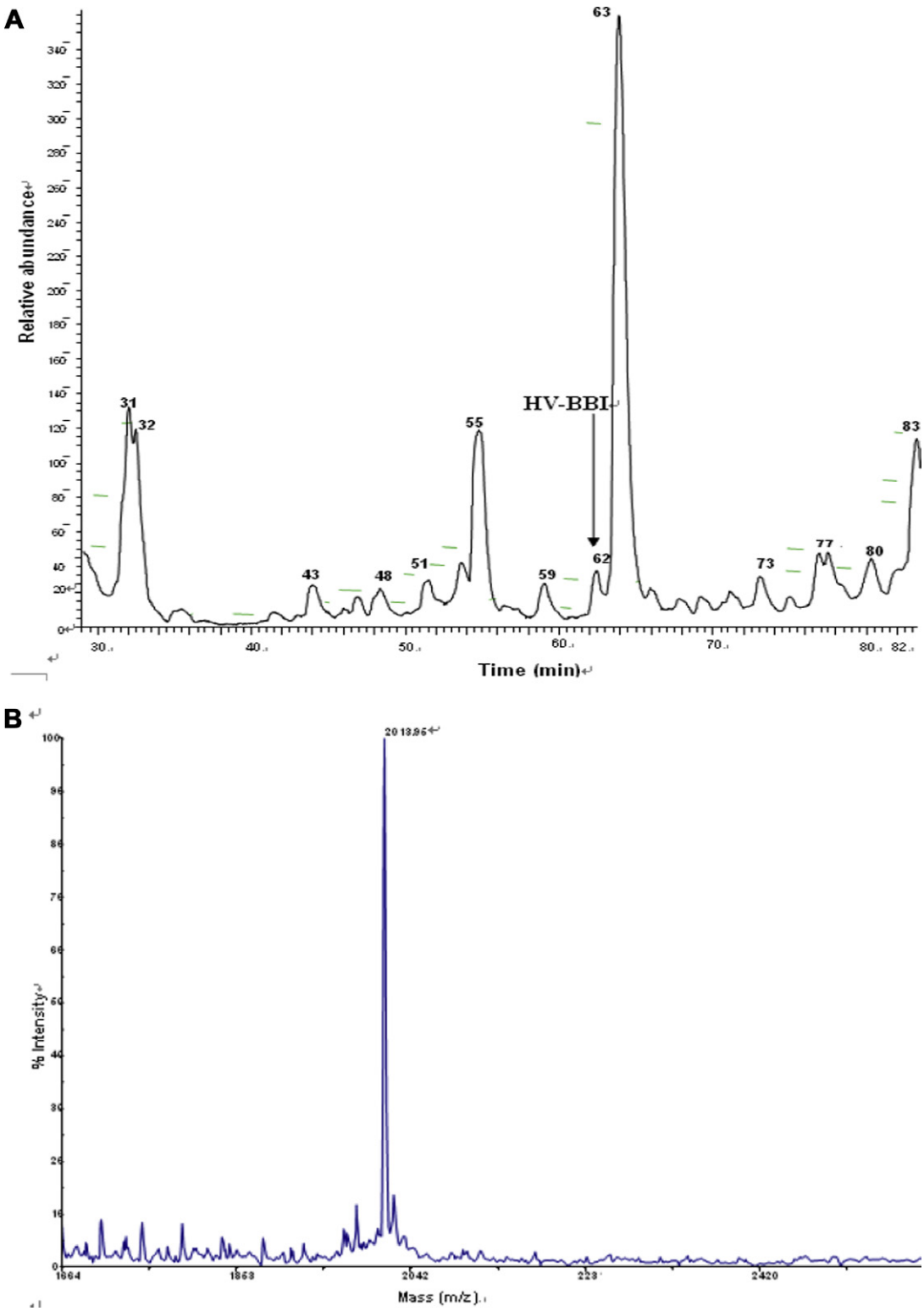

Fig. 1. (A) Region of reverse-phase HPLC chromatogram of *Huia versabilis* skin secretion indicating the absorbance peaks corresponding to HV-BBI. (B) MALDI-TOF mass spectrograms of HV-BBI in sequential reverse-phase HPLC fractions.

Discussion

Proteases and their inhibitors are ubiquitous molecules within biological systems indicative of their regulation of a plethora of fundamental functions. Proteases are generally classified according to the key catalytic amino acid residue within their active sites (serine, cysteine, threonine, aspartic acid) or the requirement for a metal cofactor essential for catalytic activity (metalloproteases). Likewise protease inhibitors that are proteinaceous in nature, are often classified according to the presence of a defined structural

Table 1
Alignment of the primary structure of HV-BBI with other pLR/ranacyclins from amphibian skin

HV-BBI	SVIGCWTKSIPPRPCFVK
Ranacyclin-T	GALRGCWTKSYPPKPCK
Ranacyclin-E	SAPRGCWTKSYPPKPCK
pLR	LVRGCWTKSYPPKPCFVR
pYR	YLKGCWTKSYPPKPCFSR
	****** **

Fully conserved residues indicated by asterisks.

motif (Kunitz, Kasal, Bowman–Birk) [19] or to reflect the actual class of protease that they inhibit (cystatins, serpins, and tissue inhibitors of metalloproteases (TIMPS)) [4,13,18]. Serine proteases are one of the most widely studied grouping of proteins and are involved in key regulatory processes such as peptide hormone precursor processing and release, blood coagulation and complement fixation, and additionally, for their intimate involvement in the pathogenesis of numerous diseases, including cancer, pulmonary emphysema and inflammation [17,19]. The inhibitors of this class of protease function by binding to their cognate enzyme in a substrate-like manner and forming a stable complex [19,27].

Amphibian skin secretions, long known for containing complex cocktails of biologically-active peptides, have yielded protease inhibitors representing several different classes. Kunitz-type protease inhibitors have been isolated and structurally-characterized from the skin secretion of the North American crawfish frog, *Rana areolata* [1], and the Madagascan tomato frog, *Dyscophus guineti* [11]. Additionally, *R. areolata*, in common with four species of bombinid toad investigated (*Bombina bombina*, *variegata, orientalis* and *maxima*) [7], contained a novel inhibitor of trypsin/thrombin in defensive skin secretions that shared a common motif with an inhibitor from the parasitic nematode, *Ascaris suum* [15]. More recently, a Kasal-type inhibitor of post-proline cleaving enzyme was found in the skin secretions of the South American phyllomedusid

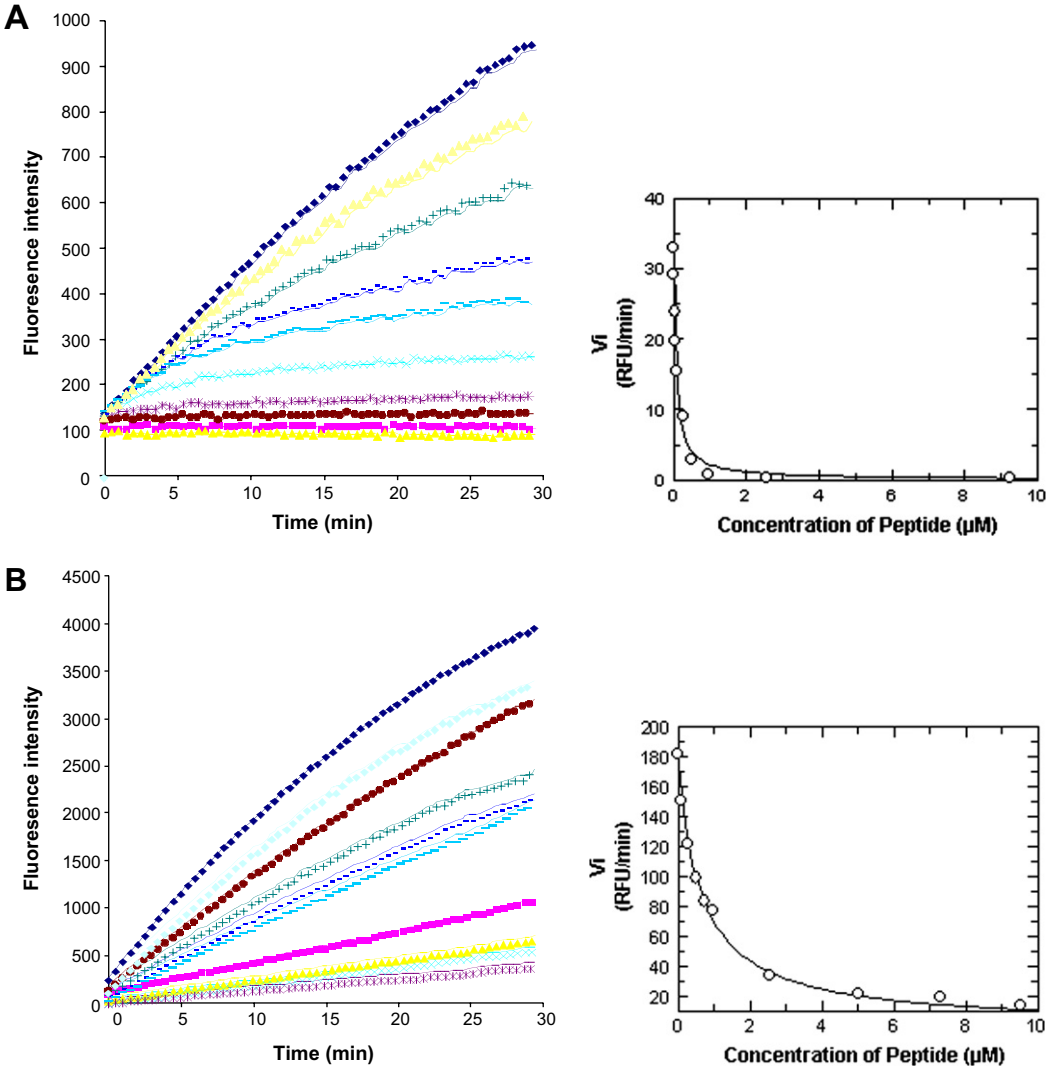

Fig. 2. (A) Progress curves for the trypsin-catalysed hydrolysis of Phe-Pro-Arg-NHMec in the presence of varying concentrations of peptide SVIGCWTKSIPPRPCFVK-amide. Assays were conducted at 37 °C, in 10 mM phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl, employing peptide concentrations of 0 (◆); 0.007 μM (▲); 0.026 μM (+); 0.05 μM (–); 0.7 μM (–); 0.25 μM (×); 0.5 μM (×); 0.9 μM (●); 2.5 μM (■); and 9.5 μM (▼). Side panel is corresponding Morrison plot of initial rate (v_i) for substrate hydrolysis at each of the indicated concentrations of peptide. (B) Progress curves for the chymotrypsin-catalysed hydrolysis of succinyl·Ala-Ala-Pro-Phe-NHMec in the presence of varying concentrations of peptide SVIGCWTFSIPPRPCFVK-amide. Assays were conducted at 37 °C, in 10 mM phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl, employing peptide concentrations of 0 (◆); 0.007 μM (▲); 0.026 μM (+); 0.05 μM (–); 0.7 μM (–); 0.25 μM (×); 0.5 μM (×); 0.9 μM (●); 2.5 μM (■); and 9.5 μM (▼). Side panel is corresponding Morrison plot of initial rate (v_i) for substrate hydrolysis at each of the indicated concentrations of peptide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 2
Inhibitor constants for 'wild-type' HV-BBI and P1-variants against trypsin and chymotrypsin

Peptide	K_i (nM) (trypsin)	K_i (nM) (chymotrypsin)
SVIGCWT**K**SIPPRPCFVK-amide	18.8 + 1.8	N.I.*
SVIGCWT**R**SIPPRPCFVK-amide	54.2 + 5.6	N.I.*
SVIGCWT**F**SIPPRPCFVK-amide	N.I.*	389.4 + 45.5

* No inhibition was observed using peptide concentrations up to and including 50 μM.
P1 position is highlighted.
K_i values are quoted as means + SEM for eight determinations.

frog, *Phyllomedusa sauvagei* [14]. Both Kunitz and Kasal-type trypsin inhibitors are found in the exocrine pancreatic secretions of mammals [19]. These amphibian skin protease inhibitors are thus representative of inhibitor classes found in the tissues and secretions of other animals so it is all the more surprising that pLR and its homologs, including the ranacyclins, found so far exclusively in the skin secretions of ranid frogs [22,26], should have counterparts only in the Bowman–Birk inhibitors (BBIs) of plants that are found particularly in the seeds of legumes, grasses and sunflowers [25]. A recent study on a pLR/ranacyclin family member, named ORB, from the oriental frog, *Odorrana grahami* [21],

```
                 M  F  T  L  K  K  S  L  L  L  L  F  F  L
1    TCCCAAAGAT GTTCACCTTG AAGAAATCCC TGTTACTTCT TTTCTTTCTT
     AGGGTTTCTA CAAGTGGAAC TTCTTTAGGG ACAATGAAGA AAAGAAAGAA

      G  T  I  S  L  S  L  C  E  Q  E  R  D  S  D  D  E
51   GGGACCATCT CCTTATCTCT CTGTGAGCAA GAGAGAGATT CCGATGATGA
     CCCTGGTAGA GGAATAGAGA GACACTCGTT CTCTCTCTAA GGCTACTACT

        D  Q  G  E  V  T  E  Q  V  V  K  R  S  V  I  G
101  AGATCAAGGG GAAGTTACAG AACAAGTTGT AAAAAGATCA GTCATTGGGT
     TCTAGTTCCC CTTCAATGTC TTGTTCAACA TTTTTCTAGT CAGTAACCCA

     C  W  T  K  S  I  P  P  R  P  C  F  V  K  G  *
151  GCTGGACCAA GAGTATACCA CCAAGGCCTT GTTTTGTAAA AGGATAAAAC
     CGACCTGGTT CTCATATGGT GGTTCCGGAA CAAAACATTT TCCTATTTTG

201  TTGAATTGGA AGTCATCTAA TTTGGAATAT CATTTAGCTA AATGCTAAAG
     AACTTAACCT TCAGTAGATT AAACCTTATA GTAAATCGAT TTACGATTTC
251  GTCTGATAAA AATAAAAATA TCACCCAAAA AAAAAAAAAA
     CAGACTATTT TTATTTTTAT AGTGGGTTTT TTTTTTTTTT
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Fig. 3. Nucleotide sequence of cloned skin secretion cDNA encoding prepro-HV-BBI. The putative signal peptide is double underlined, the single copy of HV-BBI is single underlined and the stop codon (TAA) is indicated by an asterisk.

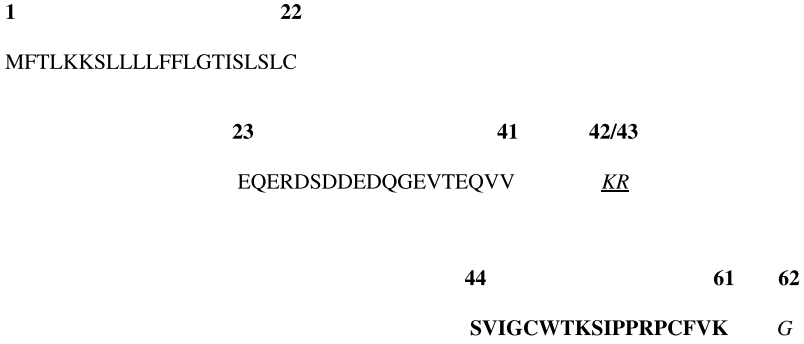

Fig. 4. Domain topography of prepro-HV-BBI. Residues 1–22 constitute the putative signal peptide. Residues 23–43 constitute the acidic spacer peptide region typified by classical -KR- (-Lys-Arg-) propeptide convertase processing sites (italicized and underlined). The single copy of mature HV-BBI (residues 44–61) are in bold typeface and the C-terminal glycyl (G) residue that donates the amide is in italics.

demonstrated its trypsin inhibitory activity, although the reported K_i was rather low (306 μM) for an inhibitor of this class suggesting a less than optimal assay or a less than optimal peptide.

Here we report the primary structure and precursor organization of a novel peptide of the pLR/ranacyclin family that we have named HV-BBI (*Huia versabilis* Bowman–Birk inhibitor). A synthetic replicate of the natural mature peptide, in contrast to the previous report on trypsin inhibition for ORB [21], displayed a K_i for trypsin of approximately 19 nM; that is 17,000-fold more potent than ORB. The only difference in primary structure within their respective inhibitory loops, is the substitution of the Lys (K) residue in the presumed P5′ position of ORB by Arg (R) in the case of HV-BBI. Although it is possible that this substitution has caused the large increase in potency it is highly unlikely, as pLR, the prototype peptide, also has a Lys residue in this position, yet displays a similar order of potency to HV-BBI, as indeed does sunflower trypsin inhibitor (SFTI), that has an Ile (I) residue at this site [25]. We focused attention on the P1 site occupied by a Lys (K) residue in the conserved canonical BBI motif, -TKSIPP-, and synthesized analogues of HV-BBI with an Arg (R) residue and a Phe (F) residue at this position. The Arg substituted analogue displayed reduced potency when compared to the wild-type peptide (54 nM versus 19 nM) but still displayed a 5000-fold higher potency than that reported for ORB. As expected, substitution of the Lys residue at P1 with Phe, completely abolished inhibitory activity against trypsin, although it did result in a molecule that exhibited modest inhibitory activity against chymotrypsin (K_i value of approximately 389 nM). Thus, from the limited structure/activity data presented here, it would appear that a Lys residue is preferred over an Arg at this site and that an aromatic residue is unacceptable for trypsin inhibitory activity. These data would agree strongly with those established for plant BBIs of trypsin [10,24–25].

What then could be the biological function of protease inhibitors in amphibian skin secretions? Protein-based protease inhibitors serve to protect the animal/plant host against the range of extrinsic proteases produced by invasive microorganisms [29]. Such enzymes play direct roles in the pathogenesis and virulence of most strains of opportunistic bacteria by damaging host tissues and evading host defences [30]. It has been speculated therefore, that the Kunitz-type trypsin inhibitor in the skin secretion of the Madagascan tomato frog, *D. guineti*, may be employed as a surface anti-infective agent, as this species does not apparently produce antimicrobial peptides [11]. However, HV-BBI was isolated from the skin secretion of a species, *H. versabilis*, which contains a plethora of potent antimicrobial peptides representing at least five different structural classes [8]. Thus, while the proposed antimicrobial role for a trypsin inhibitor in *D. guineti* skin secretion, in the apparent absence of additional abundant antimicrobial peptides, would appear to have merit, it is not a strong biological argument for *H. versabilis* or indeed for other species of ranid frog. It may be more realistic to propose that protease inhibitors in amphibian defensive secretions, that are for the most part essentially peptide based, perform some function in preventing proteolysis of labile peptides either by endogenous amphibian enzymes or by those encountered once the secretions enter the body or tissues of attacking predators.

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