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Review

Antimicrobial Peptides from Frogs of the Glandirana Genus

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Abstract: Glandirana is a genus of frogs that includes *G. rugosa*, *G. emeljanovi*, *G. minima*, *G. tientaiensis*, *G. susurra*, *G. nakamurai* and *G. reliquia*. These frogs produce antimicrobial peptides (AMPs), which are endogenous antibiotics that possess antibacterial, antifungal, antiviral and anti-endotoxin activity and help keep the hosts free from infections. In these activities, microbial death is promoted by membranolytic mechanisms that are mediated by the cationic charge and amphiphilic α-helical structures of these peptides. In general, these peptides are selective for microbes, showing low levels of hemolytic and cytotoxic activity, as well as possessing other biological activities, including anticancer, antioxidative and insulinotrophic action. In this review, a brief overview of AMPs with a focus on those from amphibians is provided, along with the phylogeny and nomenclature of frogs and AMPs from the Glandirana genus. This review then provides a comprehensive, in-depth description of the antimicrobial and other biological activities of all AMPs produced by known frogs of the Glandirana for the period 1994 to 2024. This description includes a detailed discussion of the structure/function relationships and mechanisms involved in the membrane interactions that drive these biological activities, with comparisons between AMPs from the same frog and between frogs across the genus. Based on their biological properties, AMPs from frogs of the Glandirana genus have been proposed for investigation as potential therapeutic agents, such as in the treatment of cancers and diabetes, as well as antimicrobial agents in areas, including crop protection, the food industry and oral hygiene.

Keywords: antimicrobial peptides (AMPs); Glandirana genus; *G. emeljanovi*; amphiphilic α-helix; membranes; bacteria; fungi; viruses; cancers; stapled peptides

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1. Introduction

It was generally believed that the production of antimicrobial peptides (AMPs) as a defence strategy was unique to unicellular organisms [1]; however, this belief began to be discredited by a number of studies in the later decades of the 1900s [2,3]. Notable is the work of Hirsch in 1956 and that of Spitznagel in 1966 showing that cationic proteins were responsible for the ability of human neutrophils to kill bacteria via oxygen-independent mechanisms, clearly not an activity associated with the adaptive immune system [4,5]. Subsequent investigations [2,3] eventually led to the work of Boman and colleagues in 1980, who were the first to fully isolate and characterize eukaryotic AMPs, which were derived from *Hyalophora cecropia* (the silk moth) [6] and today are known as cecropins [7]. This work also helped answer the longstanding question as to why insects, other invertebrates and plants, which are dispossessed of adaptive immune responses, remain free from infections for most of the time [1,2]. Interestingly, although beyond the scope of this review, more recent studies have provided increasing evidence for the existence of acquired immunity functions in invertebrates and plants [8,9].

Today, it is recognized that AMPs are key components of the innate immunity of creatures across Eukarya, possessing direct, potent activity against bacteria, viruses, fungi and parasites [10–13]. In relation to higher eukaryotes, amphibians played a central role in the discovery of AMPs when a series of landmark studies across the late 1980s and 1990s [2] led to the isolation of magainins from *Xenopus laevis* (the African clawed frog) [14]. These peptides were identified in the skin secretions of this frog by Zasloff and colleagues in 1987, who described them as a '. . . previously unrecognized, vertebrate antimicrobial host defence system.' [15]. The characterization of magainins showed that they carried a net positive charge [14], which appears to be a general feature of AMPs and is thought to allow these peptides to target microbial cells, which carry a net negative charge [16]. The positive charge carried by AMPs is also a primary determinant of their selectivity, which allows them to distinguish microbial cells from mammalian cells, which possess no overall electrical charge [17]. However, in 1997, further studies on *X. laevis* identified the PYL peptide, which was the first major example of anionic AMPs to be described in higher eukaryotes [18]. Since this study, anionic AMPs have been reported in other amphibians, [19], as well as a variety of other eukaryotes [20–22], and appear to target and interact with microbial membranes using a variety of strategies [19–22]. It is generally believed that these peptides serve to synergize the action of positively charged AMPs and may represent relics from the early evolution of defence molecules involved in host—pathogen interactions [19].

There have been numerous studies on the antimicrobial action of AMPs and the general, essential properties of these peptides are the possession of a cationic charge along with a hydrophobic and/or amphiphilic molecular architecture that, in combination, mediate their ability to interact with lipids [16,23]. In the case of α-helical AMPs, the study of these molecular architectural characteristics was greatly enhanced by the landmark studies of Eisenberg et al., who in 1982 developed the concept of the hydrophobic moment, $<\mu H>$ [24,25]. This concept is essentially a mathematical model that is able to quantify the structured spatial partitioning of hydrophilic and hydrophobic residues about the long axis of α-helical AMPs and hence provides a measure of their amphiphilicity [26,27]. This methodology then takes the hydrophobicity of α-helical AMPs to be the mean hydrophobicity, $<H>$, of their sequences, thereby providing a measure of the affinity of these peptides for the membrane interior [28,29]. Numerous studies based on this methodology have shown that there are no general, close correlations between the cationic charge/amphiphilicity/hydrophobicity and the ability of α-helical AMPs to selectively kill microbes [23,30,31]. However, these studies revealed a tendency for more amphiphilic peptides to seek interaction with the surface of membranes, which led to a number of methodologies designed to guide the structure/function analyses of AMPs and thereby the prediction of their modes of membrane interaction, as illustrated in Section 5.2.2 [26,27,32,33].

The ability of AMPs to interact with lipids allows these peptides to partition into the cytoplasmic membrane (CM) of bacteria, which is generally their primary site of action and leads to the death of the target organism through the lysis of the CM [16,23], although some of these peptides traverse the CM and attack intracellular targets [34,35]. In the case of AMPs that target Gram-negative bacteria, lipid interactivity also promotes their passage across the barrier posed by the outer membrane (OM) to gain access to the CM [36,37]. Most commonly, this passage is facilitated by the 'self-promoted pathway' [38] in which AMPs target and bind lipopolysaccharides (LPS) [37,39], which are anionic and the major component of the OM [40,41]. These binding events competitively displace Ca^{2+} and Mg^{2+} ions that help maintain cell surface stability and lead to the disruption of the OM, which allows further peptides to migrate to the CM and inflict lethal damage [37,39,42]. Based on these observations, a variety of models have been proposed to describe the antimicrobial action of AMPs, general variants of the barrel stave pore and toroidal pore models, which involve membrane disruption via discrete channel formation, and carpet-type and tilted-type mechanisms, which involve non-specific solubilization [16,30,39,42]. The multiplicity of their target sites and the relatively non-specific nature of their action has led to only a low incidence of microbial resistance to these AMPs, which is generally due to inherent

rather than adaptive mechanisms [36,43,44]. It is believed that microbial resistance to these peptides is unlikely to approach that of conventional antibiotics, endowing these AMPs with a major advantage [44–46], although this has been disputed [47,48]. Microbial resistance to antibiotics, particularly that of multiple drug-resistant (MDR) strains, is now recognized as a major threat to human health and a cause of mortality on a global scale [49,50]. The potential of AMPs for development as medically relevant antimicrobials was first recognized for magainins [51,52], and numerous derivatives of these peptides have been used for purposes ranging from spermicides to anti-biofilm agents [14]. To date, the most successful of these derivatives is pexiganan (MSI-78) [53], which is an analogue of magainin 2 with potent activity towards a broad range of Gram-positive and Gram-negative bacteria [54–56]. The major therapeutic potential shown by pexiganan is for topical application as an antibacterial agent [53,54], which led to the peptide becoming the first of AMPs to undergo commercial development [57,58]. In clinical trials for the topical treatment of diabetic foot ulcers [53,54,59], pexiganan was well tolerated and achieved clinical cure or improvement in the vast majority of patients [60]. Based on the success and lessons learnt from the development of pexiganan [14], a number of other compounds based on AMPs have undergone clinical trials for a variety of conditions [61–63]. A major example of amphibian AMPs in clinical trials is buforin II, which is derived from *Bufo gargarizans* (the Asiatic toad) and appears to exert its antibacterial activity through membrane translocation to attack DNA and other intracellular targets [34,35]. This peptide is in phase I clinical trials as a broad-spectrum agent for the treatment of bacterial infections [62] and, in particular, those due to *Acinetobacter baumannii* [64], which is currently classed as a critical priority pathogen by the World Health Organization (WHO) [65]. Currently, there is an urgent requirement for novel agents to combat infections due to *A. baumannii*, which is an invasive MDR pathogen that is associated with high mortality and is one of the major causes of nosocomial infections, which range from pneumonia and septicemia to meningitis and surgical site infections [66–68].

According to the APD3 database (https://aps.unmc.edu/), currently over a thousand AMPs, just over one third of all those known, have been identified in anurans [69] and it is generally accepted that the richest source of AMPs is the skin secretions of these creatures [70,71]. Amphibian skin possesses a highly plastic, cutaneous exocrine apparatus, which includes numerous granular (serous) glands that release AMPs in response to a microbial attack, to both kill these microbes and to assist in wound repair [72,73]. Research into AMPs and other bioactive compounds produced by amphibian skin secretions has been largely focused on anurans [73,74] and, currently, peptides from a wide variety of frogs, toads and salamanders are under investigation as therapeutically relevant antibacterial, antiviral, antifungal and antiprotozoan agents [19,70,73,74].

In particular, the true frogs, Ranidae family, have been a promising target in the search for novel AMPs as they are the most diverse and widely distributed group of anuran amphibians worldwide, occurring on all continents except Antarctica [74,75]. The Ranidae family has undergone many taxonomic reorganizations and phylogenetic studies [76–79] and, currently, the online database, AmphibiaWeb, lists 22 genera with 407 species for this family [80]. The biggest of these genera is the Rana with more than 100 species occurring from North America to the northern half of South America as well as across Europe and Asia [79,80]. In contrast, one of the smallest genera of the Ranidae is the Glandirana, which is found in eastern China, Korea, Japan and possibly the Russian Far East and, currently, includes five species with two pending [80]. Here, we present an overview of changes in the phylogeny of frogs from this genus and an update on the nomenclature, biological properties and mechanisms of action used by their AMPs.

2. The Phylogeny and Nomenclature of Frogs and AMPs from the Glandirana Genus

Glandirana means 'glandular frog' and this genus has had a convoluted taxonomic history [81]: it currently includes *G. rugosa*, *G. emeljanovi*, *G, minima*, *G. tientaiensis* and *G. susurra* [75] and with the exception of the latter frogs [82], each of these species have been

included in *Rana*, as well as other genera [81]. In the case of *G. emeljanovi*, this frog was initially recognized as a species separate to *G. rugosa* based on an analysis and comparison of the AMPs in the defence repertoires of these amphibians. In the mid-1990s, a family of AMPs were isolated from the skin secretions of *G. rugosa* found in Korea and named gaegurins [83,84], whilst around the same time, three AMPs named rugosins were identified in the skin secretions of a frog found in Japan that was also believed to be *G. rugosa* [85]. Nonetheless, although high levels of sequence homology existed between rugosins and gaegurins, differences were observed that would not be expected for peptides from exactly the same species [76,86]. This apparent discrepancy was resolved by recent advances in the phylogenetic analysis of the family Ranidae, which showed that '*Rana rugosa*' from Japan and Korea were similar, but different species of frogs that belonged to a new genus, Glandirana, and were reclassified as *Glandirana rugosa* and *G. emeljanovi*, respectively [76,80,86,87]. However, *G. emeljanovi* is found not only in Korea, but also in northeastern China, and an unanswered question was the taxonomic relationship between these frogs [88]. In response, the mitochondrial genomes of these frogs, as well as that of *G. rugosa*, were recently sequenced [89–92]. Tthe analysis of these mitogenomes showed that not only were *G. emeljanovi* from Korea and China the same species, but that they were a different species to *G. rugosa*, which also strongly supported the monophylogeny of the *Glandirana* genus [87,89]. *G. susurra* is the most recently characterized species of frog to be included within the Glandirama genus and it had previously been believed to be the *R. rugosa* found in most of mainland Japan and neighboring islands, including Sado Island [75,82]. However, although the frog inhabiting Sado Island was genetically close, it was postzygotically isolated and distinguishable from *R. rugosa*, both morphologically and in relation to its advertisement call [82,93]. Based on these observations, it was proposed that the frog inhabiting Sado Island had evolved from an ancient form of *R. rugosa* and, after a long separation, had speciated on this island and become endemic [82,94]. To take account of the genetic and phylogenetic differentiation between the frog of Sado Island and *R. rugosa* inhabiting other Japanese locations, the former frog was renamed *Rugosa susurra* and later as *G. susurra* when placed in the Glandirana genus [93]. Currently, both *G. susurra* and *G. rugosa* are found on Sado Island, although in distinct non-overlapping locations, and it is believed that speciation by *G. susurra* preceded the invasion of the Island by the present-day *G. rugosa* lineage [82,94]. It is not known how long *G. susurra* has resided on Sado Island [82], but analogous cases of separation involving Australian frogs have indicated that evolutionary change can be effected in a relatively short time in evolutionary terms, of the order of 10,000 years [95]. Most recently, a number of taxonomic studies have identified groups of frogs in the east and center of Japan that appear to be distinct from *G. rugosa* and represent new species in the Glandirana genus, including *G. reliquia* and *G. nakamurai* [87,96,97].

G. reliquia: taken from [80].

G. nakamurai: taken from [80].

Currently, there appear to have been no investigations into the capacity of *G. reliquia* and *G. nakamurai* to produce AMPs [87,96,97] and, historically, gaegurins were the first AMPs to be isolated from frogs of the Glandirana genus [83]. These peptides were also the first to be isolated from the skin of frogs in Korea, which led to them being named after the Korean word for frog, Gaegury [83]. However, more recently, there have been attempts to derive a consistent nomenclature for AMPs of the Ranidae [76,98,99] and using the system

proposed by Conlon, these peptides are assigned to one of fourteen families on the basis of sequence similarity [76]. Accordingly, AMPs from *G. susurra* have been named using this system and the gaegurins and rugosins [83,85] renamed, with the result that most of the known peptides produced by frogs of the Glandirana genus are now members of the brevinin, esculentin, ranatuerin and granuliberin families of AMPs (Figure 1) [76,86,100]. A summary of the phylogeny and nomenclature of frogs and AMPs from the Glandirana Genus is given below in Box 1.

Immediately after their discovery, AMPs from this genus were the focus of numerous studies, but no review of these peptides has been presented in over a decade and a half, although there has been much progress in elucidating their antimicrobial action and other biological activities, as well as deciphering the mechanisms underpinning these properties [55,86].

Host amphibian		AMPs	Aligned sequences	Family of AMPs
G. emeljanovi		B1EMb	FLPLLAGLAANFLPTII-CKISYKC	Brevinin 1
		B1EMa	FLGALFKVASKVLPSVK-CAITKKC	
		B1EMa'	FLGALFKVASKVLPSVF-CAITKKC	
G. sussura		B1SSc	FLPLLAGLAANFLPTII-CKLSKKC	
		B1SSd	FLPLLAGLAANFLPKII-CKIARKC	
	[1]	GSSa*	FIFLPIFRRPVSG*IPQACKISRKC	
G. sussura		B1SSa	FLGSLLGGINWVKNHV-NH_2	Acyclic
		B1SSb	FLGSLLGGISWVKNHV-NH_2	Brevinin 1
G. rugosa		B2Ra	GLLNTFKDWAISIAKGAGKGVLTTLSCKLDKSC	Brevinin 2
G. emeljanovi		B2EMb	GIMSIVKDVAKNAAKEAAKGALSTLSCKLAKTC	
		B2EMb'	GIMSIVKDVAKTAAKEAAKGALSTLSCKLAKTC	
G. sussura		B2SSc	SFLSSLKDWAISVAKGAGKGVLTTVACKLDKSC	
		B2SSd	SFLNSLKDWAISVAKGAGKGVLTTVACKLDKSC	
G. rugosa		B2Rb	SLFSLIKAGAKFLGKNLLKQGAQYAACKVSKEC	
G. emeljanovi		B2EMa	SLFSLIKAGAKFLGKNLLKQGACYAACKASKQC	
G. sussura		B2SSa	SFLSLIKAGAKFLGKNLLKQGAQYAACKVSKEC	
		B2SSb	SFLSLIKAGAKFLGKNMLKQGAQYAACKVSKDSENVNWKS	
G. rugosa		E2R	GILDSFKQFAKGVGKDLIKGAAQGVLSTMSCKLAKTC	Esculentin 2
G. emeljanovi		E2EM	GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLAKTC	
		E2EM'	GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLALTC	
G. sussura		E2SSa	GILDSFKQFAKGVGKDLIKGAAQGVLSTVSCKLAKTC	
G. sussura		R2SSa	GLISTIWNTASNVAGTLTDSVKCKFKKC	Ranateurin 2
G. rugosa		GR	FGFLPIYRRPAS-NH_2	Granuliberin
G. sussura	[1]	GSSa	FIFLPIFRRPVS-NH_2	
G. rugosa		BR	RPPGFTPFRIAPEIV	Bradykinin
G. sussura		BSSa	RPPGFSPFRIAPEIV	

Figure 1. Sequences and homology of AMPs from *G. rugosa*, *G. emeljanovi* and *G susurra.* Figure 1 was derived from [83,85,101]. [1] GSSa of the granuliberin family appears to have resulted from mutations in genes encoding brevinin-1 peptides produced by *G. sussura* that led to the insertion of a premature stop codon in their cDNA. The full, predicted sequence of GSSa is shown aligned with these brevinin-1 peptides (enclosed in a blue box) and the premature stop codon is indicated by *. Cysteine residues are shown aligned in red boxes and form the disulfide bond of the Rana box motif possessed by the parent peptides. In the case of R2SSa, this C-terminal Rana box motif was formed from six residues and in that of B2SSb, the second cysteine residue of this motif has been substituted by serine and an extra seven residues added to the sequence.

Box 1. The Phylogeny and Nomenclature of Frogs and AMPs from the Glandirana Genus.

- Frogs from the Glandirana genus, which is one of the smallest in the family Ranidae, are found in eastern China, Korea, Japan and possibly the Russian Far East.
- Glandirana means 'glandular frog' and currently, this genus includes *G. nakamurai* and *G. reliquia*, which are new species added over the last decade, and the established species *G. rugosa*, *G. emeljanovi*, *G. minima*, *G. tientaiensis* and *G. susurra*.
- AMPs from frogs of the Glandirana genus are are assigned to one of fourteen families on the basis of sequence similarity and most are now members of the brevinin, esculentin, ranatuerin and granuliberin families (Figure 1).

3. AMPs from *G. minima*, *G. tientaiensis* and *G. rugosa*

G. minima (the Fujian Frog) is endemic to the Fujian province, China, and has been known since 1979, whilst *G. tientaiensis* (the Tiantai frog) is only found in Zhejiang and Anhui provinces, China, and was first reported in 1933 [75]. However, currently, no AMPs appear to have been reported for these species of frog, as evidenced by searches in the Swissprot protein database [102] and APD3 database, which are the major repository of amphibian peptides [69]. Interestingly, a significant number of frog species appear to be devoid of AMPs, and although beyond the scope of this review, this observation has led to the view that antimicrobial function may not be the primary role of these peptides in anurans [74].

G. minima: taken from [80].

G. tientaiensis: taken from [80].

G. rugosa (the Japanese wrinkled frog) was first identified in 1838 [75] and a suite of three AMPs were isolated from this amphibian [85] that were later renamed brevinin-2 Ra (B2Ra: GLLNTFKDWAISIAKGAGKGVLTTLSCKLDKSC), brevinin-2 Rb (B2Rb: SLFSLIKAGAKFLGKNLLKQGAQYAACKVSKEC) and esculentin-2 R (E2R: GILDSFKQFAKGVGKDLIKGAAQGVLSTMSCKLAKTC) from rugosin A, rugosin B and rugosin C, respectively (Figure 1) [76,86,100]. A limited characterization of these AMPs showed that B2Ra and B2Rb were both 33 residues long and possessed net charges of +4 and +5, respectively, whereas E2R possessed 37 residues and had a net charge of +5. All three peptides carried a C-terminal Rana box motif [85,86], which is a post translational modification comprising a cysteine-stabilized, heptapeptide, loop-like fold, so called because it is conserved across many ranid AMPs (Figure 1) [71,73,86,103]. These studies also showed that B2Rb possessed broad-range antibacterial activity, whereas B2Ra possessed potent activity against Gram-positive bacteria, but weaker activity against Gram-negative bacteria (Table 1). E2R showed activity against Gram-positive bacteria, which was not quantified, and no further research into these peptides appears to have been undertaken [85]. A summary of AMPs from *G. minima*, *G. tientaiensis* and *G. rugosa* is given below in Box 2. Currently, the best characterized AMPs produced by frogs in the Glandirana are those identified in *G. susurra* [101] and *G. emeljanovi* [55,86].

G. rugosa: taken from [80].

Box 2. AMPs from G. minima, G. tientaiensis and G. rugosa.

- *G. reliquia* and *G. nakamurai* are found in Japan, whilst *G. minima* and *G. tientaiensis* occur in China and, currently, the production of AMPs by these frogs has not been reported.
- *G. rugosa* occurs in Japan and Korea and produces the cationic AMPs brevinin-2 Ra (B2Ra), brevinin-2 Rb (B2Rb) and esculentin-2 R (E2R), which are cationic and carry a C-terminal Rana box motif (Figure 1).
- B2Rb possesses broad-range antibacterial activity, whereas B2Ra possesses potent activity against Gram-positive bacteria, but weaker activity against Gram-negative bacteria (Table 1).

Table 1. Antimicrobial and hemolytic activity of AMPs from *G. rugosa* and *G. emeljanovi*.

Bacteria	B2Ra	B2Rb	B2EMa	B2EMb	B2EMb′
			MIC (μM)		
S. aureus	1.8	1.8	ND	ND	ND
M. luteus	7.2	0.5	1.4	0.8	0.8
S. epidermidis	ND	ND	14.0	3.0	3.0
B. subtilis	3.6	1.8	14.0	3.0	3.0
S. pyogenes	14.4	3.6	ND	ND	ND
K. pneumoniae	ND	ND	28.0	7.5	7.6
S. dysenteriae	ND	ND	7.0	7.5	7.6
P. putida	ND	ND	28.0	15.0	15.2
P. aeruginosa	>28.0	28.8	28.0	15.0	15.2
E. coli	28.8	3.6	7.0	22.5	22.8
P. mirabilis	ND	ND	>56.0	>60.0	>60.0
S. marcescens	ND	ND	>56.0	>60.0	>60.0
S. typhimurium	ND	ND	>56.0	45.0	45.6
Fungi			MIC (μM)		
C. albicans	ND	ND	>56.0	45.0	>60.0
S. cerevisiae	ND	ND	>28.0	22.5	>60.0
Hemolysis			Maximal levels (%)		
Human erythrocytes	ND	ND	<1.5	<1.5	<1.5

Table 1 was compiled from [83,85] and shows the minimum inhibitory concentration (MIC, μM) of B2Ra (GLLNTFKDWAISIAKGAGKGVLTTLSCKLDKSC) and B2Rb (SLFSLIKAGAKFLGKNLLKQGAQYAACKVSKEC) from *G. rugosa*, and B2EMa (SLFSLIKAGAKFLGKNLLKQGACYAACKASKQC), B2EMb (GIMSIVKDVAKNAAKEAAKGALSTLSCKLAKTC) and B2EMb′ (GIMSIVKDVAKTAAKEAAKGALSTLSCKLAKTC) from *G. emeljanovi*, against a series of bacteria and fungi. Also shown is the activity of these peptides against human erythrocytes as the maximal % hemolysis achieved. ND denotes 'not determined'.

4. AMPs from *G. susurra*

G. susurra (the Sado wrinkled frog) was only identified in 2012 [75] and derives its name from 'susurrus', which in Latin means 'whispering' and reflects the fact that the advertisement call of *G. susurra* is much quieter than those of other anuran species in the same locality [82,104]. Most recently, in a major study, cDNA clones were amplified from the total RNA obtained from the skin of *G. susurra* and their deduced sequences revealed a tripartite organization, comprising an N-terminal signal sequence, followed by a spacer region rich in glutamic and aspartic acids, which was terminated at the C-terminus by the mature peptide [101]. This tripartite organization is typical of prepropeptides for amphibian AMPs, which are initially synthesized in the granular glands of the skin through ribosomal translation. Post-translational processing then occurs to yield the mature biologically active peptide, which is stored in the large granules of the glands for inducible release [73,74]. The bioinformatic analyses of the cDNAs from *G. susurra* showed that they encoded homologs of precursor polypeptides of a series of AMPs and bioactive peptides [101]. Bradykinin-SSa (BSSa) was the only non-AMP identified in *G. susurra* [101], and within the Glandirana genus, these peptides have only previously been reported to occur in *G. rugosa* [105], although they are known to be present in anurans from genera across the Ranidae, Hylidae, Ascaphidae and Bombinatatoridae [70]. Bradykinins (BKs) and bradykinin-related peptides (BRPs) are myotropic agents that are released in amphibian skin secretions and serve as antipredator defence agents. Essentially, these peptides act on receptors in predators to produce a diverse range of effects, including the induction of hypotension, vasodilatation, pain, inflammation and smooth muscle contraction [70,106]. For example, it has been proposed that these peptides may induce spasms in the smooth muscles that surround the digestive tract of a predator's gastrointestinal system, leading to vomiting and other adverse reactions that cause the predator to avoid the host amphibians [74,106].

G. susurra: taken from [80].

In general, the sequences of AMPs identified in *G. susurra* were found to be highly conserved across this species, *G. rugosa* and *G. emeljanove* (Figure 1), which supported earlier work suggesting that these frogs are very closely related but are not conspecific [82]. A number of the AMPs from *G. susurra* were found to be typical of the established families of anuran peptides, possessing a net positive charge and the C-terminal, heptapeptide, Rana box motif [101], as carried by peptides from *G. rugosa* and *G. emeljanove* [85,86]. These AMPs from *G. susurra* included brevinin-1SSc (B1SSc), brevinin-1SSd (B1SSd), brevinin-2SSb (B2SSb), brevinin-2SSd (B2SSd) and esculentin-2SSa (E2SSa) (Figure 1) [101]. However, other AMPs identified in *G. susurra* were atypical of these families and included brevinin-1SSa (B1SSa) and brevinin-1SSb (B1SSb), which were cationic, acyclic peptides carrying a C-terminal amide moiety (Figure 1). C-terminal amidation is a common post-translational modification found in amphibian AMPs [70] that appears to play a variety of roles in their biological activity, ranging from the stabilization of the α-helical structure to promoting microbial targeting [107]. None of the foregone AMPs were further characterized and the focus of these investigations was on the three remaining, atypical peptides produced by

G. susurra, namely brevinin-2SSb (B2SSb), ranatuerin-2SSa (R2SSa) and granuliberin-2SSa (GSSa), which are shown in Figure 1.

4.1. The Biological Activity of Brevinin-2SSb, Ranateurin-2SSa and Granuliberin-SSa

R2SSa (GLISTIWNTASNVAGTLTDSVKCKFKKC) was the first peptide of the ranatuerin-2 family to be discovered in the Glandirana genus and the initial analyses showed that this peptide and B2SSb (SFLLIKAGAKFLGKNMLKQGAQYAACKVSKDSENVNWKS) possessed net charges of +5 and +3, respectively, and appeared to be predominantly formed from the α-helical structure that carried modified C-terminal Rana box motifs (Figure 1) [101]. In the case of R2SSa, this modified C-terminal Rana box motif was formed from six residues and in that of B2SSb, the C-terminal cysteine residue of this motif had been substituted by serine and an extra seven residues added to the sequence (Figure 1). Similar variations of the Rana box motif, as well as those that possess additional disulfide bonds, have been reported for other frogs and it is believed that these observations reflect evolutionary divergence by amphibian AMPs [71,73,103]. In contrast to B2SSb and R2SSa, GSSa (FIFLPIFRRPVS-NH_2) exhibited a net charge of +3 and appeared to be primarily formed from a β-sheet structure that carried a C-terminal amide moiety [101]. Interestingly, the bioinformatic analysis suggested that GSSa resulted from mutations in the genes encoding brevinin-1 peptides produced by *G. sussura* which led to the insertion of a premature stop codon in the cDNA of these peptides (Figure 1) [101]. Similar cases have been reported for other phyla; for example, θ-defensin, produced by rhesus macaques, is heterodimeric and formed from initial monomers that are the product of a mutated α-defensin gene containing a premature stop codon in its mature AMP domain [108].

B2SSb, R2SSa and GSSa were found to possess a variety of biological activities (Table 2) [101], which is consistent with the multifunctionality of AMPs in general [2,109–111], although the functional diversity of amphibian peptides appears to be especially broad with roles ranging from insulin and corticotropin release to protease and neuronal nitric oxide synthase inhibition [70,100,112]. For example, B2SSb and R2SSa were found to be antioxidant peptides (AOs) [101], which has been reported for other amphibian AMPs and bioactive peptides and is believed to help protect the host anurans from oxidative stress and UV irradiation [111]. Endogenous antioxidants are key for the survival and adaptation of animals to the environment [113] and due to a variety of factors, such as the fragile nature of the stratum corneum, it is essential for amphibians to protect their skin against endogenous and exogenous oxidative insults far more than other vertebrates [111,114]. Typically, *Odorrana andersonii* (the golden cross band frog) lives in high-altitude environments with elevated UV radiation, and the skin of *O. andersonii* was shown to be highly tolerant to this radiation, primarily due to the presence of a rich diversity of AOs with a potent ability to scavenge free radicals [115].

B2SSb and R2SSa showed potent anticancer activity, reducing the viability of cell lines representing human liver cancer (HepG2) by over three quarters at low peptide concentrations (<12.0 μM). However, GSSa only achieved similar reductions in the viability of these cells at concentrations circa seven-fold higher than those of B2SSb and R2SSa, indicating much lower activity against HepG2 cells [101]. The ability to kill cancer cells is common to not only amphibian peptides, but AMPs in general [116–118] and is discussed in more detail in Sections 5.1.1 and 5.1.2 in relation to B1EMa and B1EMb from *G. emeljanovi* [119–122]. This ability appears to be underpinned by the fact that the CM of cancer cells resembles those of microbial cells by carrying a net negative charge [117,118,123]. In general, this resemblance in the membrane surface charge also allows AMPs to selectively target cancer cells over normal eukaryotic cells, which, as described in Section 1, carry no overall electrical charge [117,118,123]. However, B2SSb, R2SSa and GSSa showed cytotoxicity to normal mammalian cells, namely the monkey kidney cell line, COS7, and the calf pulmonary artery endothelium (CPAE) cell line, that was comparable to that of their anticancer activity [101]. These observations clearly made these peptides undesirable for therapeutic and biotechnical use, and it was proposed that they could be modified to reduce their cytotoxicity

and improve their selectivity [101]. The modification of AMPs to optimize their biological properties is a well-established strategy, and the properties most commonly varied are their net positive charge, hydrophobicity and amphiphilicity [124–127]. As described in Section 1, the net positive charge carried by AMPs is a primary determinant of their ability to selectively kill microorganisms; however, this ability is also known to be modulated by a complex interplay between interconnected peptide and membrane properties. For example, conformational transitions and self-assembly equilibria modulate the effective hydrophobicity of AMPs and thereby their ability to penetrate membranes, whilst kinetic processes can play a key role in promoting the selective killing of microorganisms by these peptides in the presence of host cells [128–130]. Based on these observations, a variety of strategies to reduce the cytotoxicity and improve the microbial selectivity of B2SSb, R2SSa and GSSa can be envisaged, ranging from the site-directed sequence mutation(s) to chemical modifications, such as lipidation and glycosylation [124–127].

Table 2. Antimicrobial activity of AMPs from *G. susurra*.

Bacteria	B2SSb	R2SSa	GSSa
		MIC (μM)	
S. aureus	>30.0	>44.0	>86.0
B. cereus	>30.0	>44.0	>86.0
C. michiganensis	3.8	44.0	3.8
P. aeruginosa	30.0	>44.0	>86.0
E. coli	30.0	>44.0	>86.0
S. enterica	30.0	>44.0	>86.0
X. oryzae	0.9	>44.0	>43.0
Fungi		MIC (μM)	
C. albicans	30.0	30.0	30.0
P. oryzae	30.0	>44.0	30.0

Table 2 was compiled from [101] and shows the minimum inhibitory concentration (MIC, μM) of B2SSb (SFLSLIK-AGAKFLGKNMLKQGAQYAACKVSKDSENVNWKS), R2SSa (GLISTIWNTASNVAGTLTDSVKCKFKKC) and GSSa (FIFLPIFRRPVS-NH_2) from *G. susurra* against a series of bacteria and fungi.

The antimicrobial efficacy of B2SSb, R2SSa and GSSa was evaluated against a panel of bacterial and fungal pathogens, which showed that these peptides had varying antimicrobial spectra and levels of activity (Table 2). An evaluation against human pathogens showed that R2SSa and GSSa were ineffective against all the bacteria assayed and B2SSb was ineffective against Gram-positive bacteria (Table 2). However, B2SSb showed moderate activity towards Gram-negative bacteria, which included *Escherichia coli* and *Pseudomonas aeruginosa* whose MDR strains are currently classed as amongst the world's most dangerous bacterial pathogens [65]. B2SSb, R2SSa and GSSa also exhibited moderate activity towards *Candida albicans* [101] and invasive infections by this fungus are a major cause of morbidity and mortality, especially in immunocompromised and critically ill patients [131,132], which has exacerbated the emergence of MDR strains of the organism [133,134]. Based on these observations, it was proposed that, appropriately modified to reduce their high cytotoxicity, B2SSb, R2SSa and GSSa may have the potential for development to treat infections due to Candida and Gram-negative bacteria (Table 2) [101]. An evaluation of the activity of B2SSb, R2SSa and GSSa against plant pathogens was a particular focus of the studies on these peptides (Table 2) [101]. The application of AMPs in the protection of plants and crops is an increasingly important use that receives relatively little attention in the literature [135,136]. These pathogens included the Gram-negative bacterium, *Xanthomonas oryzae*, which causes a serious blight of rice [137]; the Gram-positive bacterium, *Clavibacter michiganensis*, which causes ring-rot disease in potatoes [138]; and the fungus, *Pyricularia oryzae* (Magnaporthe oryzae), which causes rice blast disease (Table 2) [139]. Notably, B2SSb showed very high efficacy against *X. oryzae*, whilst both this peptide and GSSa showed similar potent activity towards *C. michiganensis* which was accompanied by moderate activity against *P. oryzae* in

both cases (Table 2). In contrast, R2SSa showed weak activity against *C. michiganensis* and was ineffective against both *X. oryzae* and *P. oryzae* (Table 2) [101]. These studies did not evaluate the toxicity of B2SSb, R2SSa and GSSa to host plant cells, which is clearly a requirement for the use of these peptides in crop protection and other plant-related uses. However, the toxicity of AMPs to mammalian cells is not necessarily indicative of phytotoxicity [140], as shown for a variety of these peptides [141,142]. Based on these observations, it was suggested that, appropriately modified, B2SSb, R2SSa and GSSa may have the potential for development as novel agents to address a number of increasingly problematic issues related to crop protection [101]. As major instances, AMPs have been investigated as biopesticides [143,144] in the search for 'natural' alternatives to pesticides in order to reduce the use of these environmentally damaging chemical agents [135,145]. AMPs have also been investigated for heterologous expression in plants to combat MDR phytopathogens [136,146], which are currently an ongoing, serious issue in agriculture [147,148].

4.2. Structure/Function Studies on Brevinin-2SSb, Ranateurin-2SSa and Granuliberin-SSa

B2SSb was found to exhibit strong antioxidant and free-radical scavenging activity whilst R2SSa was found to possess this activity at lower levels [101], and it is generally accepted that antioxidant activity is related to a variety of factors, including the secondary structure, amino acid arrangement and amino acid composition of AMPs/AOs [149,150]. Most importantly, the sequences of these peptides were rich in hydrophobic and aromatic residues such as tyrosine, tryptophan, methionine, proline and cysteine that have the ability to promote the scavenging of free radicals [151,152]. In particular, peptides with free cysteines are strongly antioxidative due to the highly reductive mercapto group possessed by these residues [149,150], which was proposed to help explain the higher levels of the antioxidative activity shown for B2SSb compared to R2SSa [101]. However, despite possessing two proline residues, which are one of the most common residues found in AOs [149,150], no antioxidative activity was detected for GSSa, indicating the importance of other factors to this activity [101]. For example, the amphiphilicity of AMPs/AOs appears to enhance their radical-scavenging activities by increasing their solubility and facilitating interactions and proton exchanges with radical species [149,150]. Based on the observation that amphibian AMPs and AOs show precursor similarity and that many AMPs have antioxidant activity whilst many AOs have antimicrobial activity, it has been suggested that these two peptide classes may have a common evolutionary origin [153,154]. Interestingly, most recently, AOs were identified in the skin secretions of *Salamandra salamandra* (the European fire salamander) [155], including salamandrin-1, which appeared to be derived from CFBD-1, a β-defensin first identified in *Cynops fudingensis* (the Fuding fire belly newt) [156]. Salamandrin-1 showed no antimicrobial or cytotoxic activity, and it was proposed that after glandular extrusion, this peptide was proteolytically cleaved from CFBD-1, thereby representing a novel amphibian strategy for generating AOs from AMPs [155].

To gain insight into mechanisms underpinning other biological activities of B2SSb, R2SSa and GSSa, scanning electron microscopy (SEM) was used to study the effects of these peptides on the morphology of various cells and microbes [101]. These AMPs were found to induce a range of surface abnormalities in the cells of *Staphylococcus aureus*, *C. michiganensis*, *E. coli*, *X. oryzae* and *C. albicans*. These effects included corrugations, blebbing and a loss of membrane integrity consistent with membranolytic action, although no clear correlation with the specificity and level of the antimicrobial action shown by B2SSb, R2SSa and GSSa could be discerned. However, these observations clearly suggested that these peptides interacted with the cell surface components of these various microorganisms, which was strongly supported by data showing that these peptides bound to both LPS and lipoteichoic acid (LTA) [101]. The ability to either promote or inhibit the action of AMPs is well established for LTA [157,158], which is a major, anionic and surface component found in the cell wall of Gram-positive bacteria [159,160], and LPS [37,39], which, as described above in Section 1, is the major anionic component of the OM possessed by Gram-negative bacteria [40,41]. Based on the affinity of B2SSb and R2SSa for LTA, it was also suggested that

these peptides may have the potential to act as anti-inflammatory agents by neutralizing the endotoxic activity of the molecule [101]. In addition to its cell wall functions, LTA is believed to translocate into systemic circulation and function as an endotoxin by activating the host immune system to induce sepsis [161,162]. However, this function of LTA has been the subject of considerable debate [163,164] and recent studies have suggested that after LPS, lipoprotein is the most potent pro-inflammatory endotoxin of bacterial cell walls [162]. In contrast, B2SSb and R2SSa showed a much higher affinity for LPS than LTA [101], and it is well established the former molecule acts as an endotoxin when released into systemic circulation by bacteria, which can lead to the induction of pro-inflammatory cytokines, sepsis and septic shock [161,165,166]. Based on these observations, it was proposed that B2SSb and R2SSa possessed a strong potential to act as anti-inflammatory agents in the treatment of LPS-mediated sepsis, although through apparently different mechanisms [101]. The ability of R2SSa to bind LPS was accompanied by a lack of activity towards Gram-negative bacteria (Table 2) and similar results have been reported for other amphibian AMPs [167], such as tigerinins, which are found in frogs from a number of families [168]. In contrast, B2SSb was able to both bind LPS and kill Gram-negative bacteria (Table 2) and, in combination, these observations are consistent with the view that the anti-endotoxin activity and antibacterial action of AMPs do not necessarily correspond and can represent different properties of these peptides [169]. Indeed, B2SSb would appear to fit a number of the general criteria required for this dual mode of action [169], including the possession of a strong positive charge and the ability to form an amphiphilic structure [101]. The capacity to exert antibacterial and anti-endotoxin activity similar to that described for B2SSb (Table 2) [101] has been reported for both other amphibian peptides [167,170,171] and AMPs from other sources [169,172–175]. This capacity gives these AMPs an advantage over many conventional antibiotics, such as β-lactams, which can promote the release of endotoxins and augment the severity of sepsis [161,174–177]. Nonetheless, efforts to introduce AMPs into sepsis therapy have generally failed [173–175] and currently, the only potential candidate in clinical trials would appear to be the synthetic peptide, EA-230, which, for example, has been shown to attenuate LPS-mediated systemic inflammation in experimental human endotoxemia [178,179]. Accordingly, it would seem that B2SSb and R2SSa merit further investigation as anti-sepsis agents, given that that there is no effective, safe drug to treat the disease [173] and sepsis is considered to be the most common cause of mortality in intensive care units [180,181].

SEM was also used to investigate the effect of B2SSb, R2SSa and GSSa on mammalian cells, which showed that these peptides induced high levels of membrane destruction when directed against cells of the COS7 and HepG2 cell lines [101], indicating a membranolytic mode of action that was suggestive of a carpet-type mechanism [16,30,39,42]. In the case of HepG2, the use of membranolytic action to promote killing cancer cells is typical of not only amphibian AMPs, but of these peptides in general [116–118], which is discussed in detail below in Sections 5.1.1 and 5.1.2 in relation to B1EMa and B1EMb from *G. emeljanovi* [119–122]. However, as described above in Sections 4.1 and 4.2, the ability of B2SSb, R2SSa and GSSa to induce the lysis of both microbial cells and normal mammalian cells [101] is atypical of the selectivity generally shown by AMPs [23,42]. Indeed, such a lack of specificity is more characteristic of cytolytic toxins found in the venom of stinging insects within the order, hymenoptera [182,183], which are generally produced as a response to a perceived threat [184]. One of the most studied examples of these cytolytic toxins is melittin from *Apis mellifera* (the European honey bee), which is the major cause of pain to humans and animals within the venom of these insects and serves a primary role in defending bee colonies from predators [184,185]. Interestingly, recent studies have suggested that some AMPs may serve roles in amphibian antipredator defence systems by promoting the permeabilization of a predator's epithelial tissue to facilitate the delivery of co-secreted BKs and BRPs, such as caeruleins [74,186,187]. These neuropeptides have been isolated from the skin secretions of a number of anurans [106,188] and it has been proposed that the cytolytic action of AMPs promotes their delivery to the endocrine and nervous systems of predators, thereby causing

pain and other adverse effects that deter predation [71]. Given the ability of B2SSb, R2SSa and GSSa to efficiently permeabilize mammalian cells, these peptides could potentially serve such roles in *G. susurra* by promoting the uptake by predators of BSSa or other myotropic agents produced by this frog [101]. Indeed, it was proposed that the cytotoxic activity of the amphibian AMPs involved in these delivery systems, per se, may provide a secondary and fast-acting effect on some predators; for example, by causing irritation or pain on oral mucosa [74,186]. It seems likely that delivery mechanisms based on tissue permeabilization may be used by other creatures, and mechanisms of this type would appear to be of particular significance in that they command a reappraisal of the textbook distinction between poisonous and venomous animals [186]. A summary of AMPs from *G. susurra* is given below in Box 3.

Box 3. AMPs from *G. susurra*.

- *G. susurra*, which occurs in Sado Island, Japan, produces brevinin-1SSc (B1SSc), brevinin-1SSd (B1SSd), brevin-in-2SSb (B2SSb), brevinin-2SSd (B2SSd) and esculentin-2SSa (E2SSa), which are cationic and possess a C-terminal, heptapeptide and Rana box motif (Figure 1).
- *G. susurra* produces seven atypical ranid-AMPs, of which three were characterized, namely, ranatuerin-2SSa (R2SSa), brevinin-2SSb (B2SSb) and granuliberin-2SSa (GSSa). All of these peptides are cationic, with the first two carrying modified C-terminal Rana box motifs and the third possessing a C-terminal amide moiety (Figure 1).
- B2SSb and R2SSa exhibit antioxidant activity and intrinsic antioxidants are essential for amphibians to protect their skin against both endogenous and exogenous oxidative insults.
- B2SSb, R2SSa and GSSa show varying levels of efficacy towards cancer cells but are cytotoxic to normal mammalian cells. Modified to reduce their cytotoxicity, B2SSb, R2SSa and GSSa show the potential for development to treat various cancers.
- B2SSb, R2SSa and GSSa exhibit varying levels of efficacy towards fungi and Gram-negative bacteria that are pathogenic to humans. Modified to reduce their cytotoxicity, B2SSb, R2SSa and GSSa show the potential for development to treat infections due to these microbes (Table 2).
- B2SSb, R2SSa and GSSa show varying levels of efficacy towards fungi, Gram-positive bacteria and Gram-negative bacteria that are pathogenic to plants, indicating the potential for development as crop protection agents (Table 2).
- B2SSb and R2SSa bind strongly to endotoxins and show the potential to act as anti-inflammatory agents.
- B2SSb and R2SSa appear to be predominantly formed from an α-helical structure, whereas GSSa appears to be primarily formed from a β-sheet structure, and the antibacterial, antifungal, anticancer and cytotoxic action of these AMPs appears to involve membranolytic mechanisms.

5. AMPs from *G. emeljanovi*

G. emeljanovi (the Imienpo Station frog or Rough-Skinned frog), which was first identified in 1913 [75], appears to be the archetypal species of Glandirana and is the best characterized in relation to its AMPs [86]. The initial characterization showed that there were six of these peptides that each possessed a C-terminal Rana box motif and could be considered to fall into three groups (Figure 1). The first of these groups was formed by brevinin-2EMa (B2EMa: SLFSLIKAGAKFLGKNLLKQGACYAACKASKQC), brevinin-2EMb (B2EMb: GIMSIVKDVAKNAAKEAAKGALSTLSCKLAKTC) and brevinin-2EMb′ (B2EMb′: GIMSIVKDVAKTAAKEAAKGALSTLSCKLAKTC), which were previously known as gaegurin 1, gaegurin 2 and gaegurin 3 (gaegurin 2′), respectively (Figure 1). B2EMb′ is generally regarded as a variant of B2EMb rather than a homologue as these peptides only differ by a N → T substitution at sequence position 12 (Figure 1). Each of these AMPs exhibited very low levels of hemolysis and possessed potent activity against Gram-positive bacteria, but weaker activity against Gram-negative bacteria and fungi (Table 1), and since their initial characterization, no further work on any of these brevinins appears to have been conducted. The remaining two major groups of AMPs from *G. emeljanovi* are formed from brevinin-1 EMa (B1EMa: FLGALFKVASKVLPSVKCAITKKC) and brevinin-1 EMb (B1EMb: FLPLLAGLAANFLPTIICKISYKC), previously known as gaegurin 5 and gaegurin 6, respectively, and esculentin-2 EM (E2EM: GILDTLQAFAKGVGKDLVK-

GAAQGVLSTVSCKLAKTC), formerly called gaegurin 4 (Figure 1) [83,86]. During the initial characterization of B1EMa and E2EM, a minor inconsistency in the sequence of each peptide was revealed, which was subsequently corrected; namely K17 → F17 and L35 → K35 substitutions, respectively [86]. These variants are generally referred to as B1EMa' (FLGALFKVASKVLPSVFCAITKKC) and E2EM' (GILDTLQAFAKGVGKDLVKGAAQGVL-STVSCKLALTC) and the accepted sequences of all known AMPs from *G. emeljanovi*, as listed in the Swissprot protein database [102], are shown in Figure 1.

G. emeljanovi: Taken from [80].

5.1. Brevinin-1 EMa and Brevinin-1 EMb

The initial characterization of B1EMa and B1EMb showed that these peptides were each 24 residues in length and possessed net charges of +5 and +2, respectively (Figure 1), [83] and, subsequently, the complete cDNA encoding B1EMa was isolated from a library constructed with mRNAs from the skin of *G. emeljanovi* [84]. The sequence of the B1EMa precursor polypeptide was deduced from this clone, which revealed a tripartite organization that was similar to that of precursor polypeptides from AMPs of *G. susurra* [101]: an N-terminal signal sequence followed by an acidic spacer region that was terminated at the C-terminus by the mature peptide [84]. The alignment of the sequences of B1EMa and B1EMb showed that they were highly homologous, paralleling the brevinin 1 peptides and other families of AMPs produced by *G. rugosa* and *G. susurra* (Figure 1). It is believed that amphibians produce homologous AMPs as a strategy to diversify and optimize their defence capabilities [70,71] and, consistent with these observations, B1EMa and B1EMb were found to serve a variety of biological roles (Tables 3–5) [70,100,112].

5.1.1. The Biological Activity of Brevinin-1 EMa, Brevinin-1 EMb and Their Derivatives

B1EMa and B1EMb were first recognized for their antimicrobial activity [83] and particularly, their strong preference for Gram-positive bacteria (Tables 3 and 4) [83,86,121,189–191]. There is an urgent need for novel agents to combat infections caused by these bacteria and their MDR forms, which are a major global cause of morbidity and mortality [192,193]. In response, numerous derivatives of B1EMa and B1EMb were produced in an effort to maintain or improve the antibacterial efficacy of the parent AMPs (Tables 3 and 4) [86] and it was found that reduced or linearized B1EMa retained the same profile and preferences for antimicrobial activity as the parent peptide [189]. In contrast, the linear form of B1EMb showed a complete loss of antibacterial and antifungal activity, but when this analogue underwent S → C substitutions at positions 18 and 24, the antimicrobial activity was comparable in profile and the preferences to B1EMb were restored [190]. It was proposed that both B1EMb and its serine substituted analogue showed the potential for development as novel antimicrobials; for example, these peptides exhibited potent activity towards *Mycobacterium smegmatis* [190]. This organism is generally used as a non-pathogenic model to assay AMPs for potential activity against *M. tuberculosis*, which is the causative agent of tuberculosis [194,195] and a leading cause of death from an infectious disease among adults worldwide [196]. Currently, *M. tuberculosis* is classed as of critical priority by the WHO and there is an urgent requirement for novel agents to combat infections due to this organism [65], which suggests that potentially, B1EMb and its serine substituted analogue

could be developed to serve in this capacity [190]. Nonetheless, the potential of B1EMa, B1EMb and their derivatives to serve as AMPs in a therapeutic context only appears to have been followed up in a few cases [197,198].

In relation to B1EMa, in what would appear to be the first major report on the antiviral potential of AMPs from *G. emeljanovi*, or indeed, the Gladirana genus, a recent study investigated the activity of peptide B against a series of viruses [199]. This peptide (FLGWLFKVASKVL-NH_2) [199], which was essentially the N-terminal sequence of B1EMa (1–13) with a V → W substitution at position 4 [120,191], exhibited activity against enveloped viruses but not non-enveloped viruses [199]. Viruses can be divided into two main categories: enveloped viruses, which are characterized by the possession of a membrane that is derived from the host cell and encapsulates the virion, and non-enveloped viruses, which lack a membrane surrounding their protein capsid [200,201]. The viruses inactivated by peptide B included hepatitis C virus, herpes simplex virus and notably, retrovirus and lentivirus [199]. Retroviruses can cause an array of malignancies, immunodeficiencies and neurologic disorders, whilst lentivirus, which is a sub-type of retrovirus, can cause chronic and deadly diseases, including AIDS [202]. Peptide B showed similar levels of potent activity against retrovirus and lentivirus that were accompanied by comparable levels of cytotoxicity to human keratinocytes (HFK) and, in the case of retrovirus, these levels of activity were quantified using a variety of infection models (Table 3) [199].

Peptide B also showed activity against a range of Gram-positive and Gram-negative bacteria, notably methicillin-resistant *S. aureus* (MRSA) and in both this case and the peptide's antiviral activity [199], the underlying mechanisms appeared to involve the formation of an amphiphilic α-helical structure and membranolytic mechanisms (Table 6) [199], which was consistent with previous studies [120,191]. However, given the peptide's significant cytotoxicity to human cells (Table 3), efforts to enhance both its selective antibacterial and antiviral activity were undertaken and stapled analogues were produced, which is described below in Section 5.3 [199]. A major example of an antibacterial derivative from B1EMa was GA-K4AL (FAKWAFKWLKK-NH_2), which was essentially the N-terminal sequence of B1EMa (1–11), with multiple residue substitutions (Table 3) [197]. This peptide was developed from lead molecules identified in earlier work that showed antibacterial activity at levels consistent with the therapeutic application but also exhibited strong hemolytic activity [191], which is clearly undesirable for such an application [203]. However, the systematic engineering of these lead molecules through multiple residue substitutions that effectively modulated their hydrophobicity and amphiphilicity generated GA-K4AL, which showed negligible hemolysis, but retained antibacterial activity with action against both Gram-positive and Gram-negative bacteria [197], contrasting to B1EMa [83] (Table 3). Notably, GA-K4AL also showed superior antibacterial efficacy to omiganan [197], which is a broad-spectrum analogue of the bovine AMP, indolicidin [204]. Omiganan has been clinically trialled for the topical treatment of a variety of conditions, such as atopic dermatitis [205], and most recently has been developed to combat skin infections due to MRSA [206]. Based on these observations, it was proposed that GA-K4AL may serve as a useful lead molecule for the development of novel antibiotics and merited investigation for its clinical and commercial potential [197].

In relation to B1EMb, one study investigated the antimicrobial activity of a C-terminally amidated isoform of the peptide, B1EMb-NH_2 (FLPLLAGLAANFLPTIICKISYKC-NH_2), and it was found that it possessed levels of activity against a range of bacteria and fungi [190] that were similar to those of B1EMb (Table 4). These observations suggested that C-terminal amidation had not greatly affected the antimicrobial activity of the peptide [190]. It is well established that this structural modification can have a variable effect on the antimicrobial efficacy of AMPs, promoting either decreases or increases in efficacy in some cases and having no effect on efficacy in other cases [207]. However, an important result from these studies on B1EMb-NH_2 was that the peptide showed activity against MRSA (Table 4) which was observed to be the most potent here for AMPs from *G. emeljanovi* [190] (Tables 1–4, 6 and 7). Although most MRSA infections, such as those of skin and subcutaneous tissues, are relatively harmless,

others, such as bacteremia, endocarditis and pneumonia, can be life-threatening and are associated with high levels of morbidity and mortality [208–210]. Currently, there is an urgent requirement for novel agents to combat infections due to MRSA, which is currently classed by the WHO as a high-priority pathogen [65] and based on these observations, it was suggested that B1EMb-NH_2 may have the potential to serve in this capacity [190]. Other major examples of antibacterial derivatives from B1EMb were PTP6 (FLKLLKKLAAKLF) and PTP7/PTP12 (FLGALFKALSKLL), which are essentially analogues of its N-terminal region, B1EMb (1–13), with multiple residue substitutions and deletions [121,198]. Similar to B1EMa, B1EMb has a preference for Gram-positive bacteria [83], which was also shown by PTP7/PTP12 who exhibited potent action against organisms such as *S. aureus*, but much weaker activity towards bacteria such as *E. coli* (Table 4) [211]. Using PTP7/PTP12 and derivatives, these latter authors showed that there was a strong correlation between the activity of these peptides against Gram-positive bacteria, their membranolytic action and their hydrophobicity [211], which, as described above in Section 1, is a major determinant in the activity of AMPs [16,23]. However, this correlation was not observed for Gram-negative bacteria and it was suggested that peptide characteristics in addition to hydrophobicity may be important to facilitating diffusion through the outer membrane of these bacteria and thereby, membranolytic antibacterial action [211]. Interestingly, these correlations were observed when the peptide hydrophobicity was quantified as the retention time in reverse-phase high-performance liquid chromatography, but not when measured as their mean hydrophobicity or <*H*>, as defined in Section 1 [211]. It was proposed that this experimental technique provided a better representation of peptide hydrophobicity because this property also depends upon peptide α-helicity, which is not taken into account by a simple mean of residue hydrophobicity [32,211]. Based on their preference for Gram-positive bacteria, B1EMb, PTP6 and PTP7/PTP12 were assayed against a panel of oral streptococci and each of these peptides was found to possess potent activity against *S. mutans*, *S. sobrinus*, *S. sanguis* and *S. gordonii* (Table 4) [198], which are major oral pathogens, causing diseases such as dental caries and periodontitis [212]. Safety considerations showed that these peptides were not toxic to normal eukaryotic cells and appeared to be non-immunogenic, suggesting that they would be safe for use in the gastrointestinal tract [198]; the induction of an immune response is a potential hazard associated with the therapeutic use of AMPs [213,214]. B1EMb, PTP6 and PTP7/PTP12 were also able to promote synergistic antibacterial effects with chlorhexidine or xylitol [198], which are conventional oral antimicrobials [215,216]. A similar synergistic action has been reported for other AMPs in combination with chlorhexidine against *S. mutans*, oral pathogens in plaque biofilms [217,218] and strains of *P. aeruginosa* involved in canine otitis externa, the chronic inflammation of the external ear canal [219]. This synergistic ability is well established for AMPs and appears to be based on their capacity to induce a loss of barrier function in microbial membranes, thereby facilitating the uptake of traditional antibiotics by target microbes [220]. Based on these observations, it was proposed that B1EMb, PTP6 and PTP7/PTP12, either alone or in combination with established oral antimicrobials, might be effective in the treatment of cariogenic oral streptococci [198,221]. Other AMPs that have been investigated for use as oral antimicrobials include C16G2 [222], which is in clinical trials as a mouth rinse component selective for *S. mutans* [179]. A major example of these AMPs is kappacins, which are cleaved from milk proteins and [223] serve as components in commercially available mouthwash and other dental care products to combat gingivitis and dental plaque [224].

Table 3. Antimicrobial, cytotoxic and hemolytic activity of B1EMa and its derivatives.

	B1EMa	A4W-B1EMa	V8W-B1EMa	GA-K4AL	Peptide B
Bacteria			MIC (μM)		
B. subtilis	<4.0	4.9	9.9	4.4	ND
M. luteus	<3.0	19.6	39.5	4.4	ND
S. aureus	1.3	2.5	9.9	4.4	ND
MRSA	ND	ND	ND	ND	12.5
S. epidermidis	<5.5	9.8	19.8	4.4	ND
E. coli	<20.0	19.6	19.8	8.8	ND
S. dysenteriae	<20.0	9.8	19.8	8.8	ND
S. typhimurium	>40.0	39.2	79.0	16.6	ND
K. pneumoniae	<20.0	9.8	9.9	4.4	ND
P. putida	19.4	ND	ND	ND	ND
P. aeruginosa	>35.0	78.4	158.0	8.8	ND
P. mirabilis	>75.0	>155.0	>158.0	ND	ND
S. marcescens	>77.0	ND	ND	ND	ND
Fungi			MIC (μM)		
C. albicans	19.4	ND	ND	ND	ND
S. cerevisiae	19.4	ND	ND	ND	ND
Viruses			EC_{50} (μM)		
Retrovirus	ND	ND	ND	ND	<11.0
Cytotoxicity			CC_{50} (μM)		
Human keratinocytes	<1.5	ND	ND	ND	>10.0
Hemolysis			Maximal levels (%)		
Human erythrocytes	<1.5	11.9	6.7	<1.0	ND

Table 3 was compiled from [83,191,197,199] and shows the minimum inhibitory concentration (MIC, μM) of B1EMa (FLGALFKVASKVLPSVK-CAITKKC) and its derivatives, A4W- B1Ema (FLGWLFKVASK), V8W-B1EMa (FLGALFKWASK), GA-K4AL (FAKWAFKWLKK-NH_2) and peptide B (FLGWLFKVASKVL-NH_2) from *G. emeljanovi* against a series of bacteria and fungi. Also shown is the activity of these peptides against viruses as the half maximal effective concentration (EC_{50}, μM), against human foreskin keratinocytes as the half maximal cytotoxicity concentrations (CC_{50}, μM) and against erythrocytes as the maximal % hemolysis achieved. ND denotes 'not determined'.

Similar to AMPs from *G. susurra* [101], in addition to antimicrobial activity, B1EMa and B1EMb were found to exhibit other biological properties [119–122,225]; for example, anticancer activity was demonstrated for both B1EMa and several analogues of its N-terminal sequence, B1EMa (1–11) (Table 5) [120]. These analogues, A4W-B1EMa (FLGWLFKVASK) and V8W- B1EMa (FLGALFKWASK) (Table 5) were essentially B1EMa (1–11) with A → W and V → W substitutions at positions 4 and 8 of the peptide's sequence, respectively (Table 5) [120]. Similarly to B1EMa [83], these analogues have been shown to possess potent antibacterial activity (Table 3), clearly illustrating the multifunctionality of these three peptides [191]. B1EMa, A4W-B1EMa and V8W-B1EMa demonstrated activity against a range of cancer cell lines, including those representing cancers of the lung (A549), breast (MCF-7), prostate (PC-3) and colon (HCT116) [120] (Table 5), which, globally, are the top four causes of cancer mortality [226]. More recently, another strongly antibacterial derivative of B1EMa [197] which was found to possess anticancer action was GA-K4 (FLKWLFKWAKK-NH_2), which is essentially B1EMa (1–11) with A → W and V → W substitutions at positions 4 and 8 of the peptide's sequence [119]. GA-K4 was found able to kill a spectrum of cancer cells that was similar to that of A4W-B1EMa and V8W- B1EMa, but with a potency that was generally up to tenfold stronger than the latter two peptides (Table 5) [119,120]. GA-K4 was also able to synergize the activity of doxorubicin [119], which is a frontline anticancer drug that inhibits DNA synthesis and function through alkylation and is extensively used in combination chemotherapy [227,228]. For example, the co-administration GA-K4 and doxorubicin was circa nine times more effective against cells of kidney cancer (A498) and

five times more effective against those of lung cancer (A549) compared to doxorubicin alone [119]. It was suggested that GA-K4 may kill cancer cells using membranolytic mechanisms and that the synergistic effect uptake of doxorubicin by these cells may be promoted by the ability of the peptide to compromise the integrity of their CM [119], as shown for other AMPs [229–231]. These observations reinforce the general view that AMPs promote the uptake of anticancer agents using mechanisms generally similar to those used in the case of antibiotics, as discussed above in this section [232]. GA-K4, along with B1EMa, A4W-B1EMa and V8W-B1EMa, showed low levels of hemolysis (Table 3) and no cytotoxicity to normal eukaryotic cells (Table 5), which led to the suggestion that they were worthy of further investigation in the production of therapeutically relevant, anticancer agents [119,120]. Reinforcing the potential of A4W-B1EMa and V8W-B1EMa to serve as both anticancer and antimicrobial agents, these peptides and their derivatives have been patented in both capacities [233,234].

Table 4. Antimicrobial and hemolytic activity of B1EMb and its derivatives.

Bacteria	B1EMb	B1EMb-NH_2	PTP6	PTP7/PTP12
		MIC (μM)		
B. subtilis	3.8	4.8	ND	ND
M. luteus	1.0	1.2	ND	2.1
S. aureus	ND	1.2	ND	3.5
MRSA	ND	4.8	ND	ND
S. epidermidis	3.8	2.4	ND	6.4
M. smegmatis	1.2	4.8	ND	ND
C. diphteriae	ND	1.6	ND	ND
S. mutans	<5.0	ND	4.1	4.4
S. sanguis	2.4	ND	<8.0	4.4
S. sobrinus	2.4	ND	4.1	4.4
S. gordonii	1.2	ND	8.2	4.4
P. vulgaris	ND	>38.0	ND	ND
E. coli	>9.0	2.4	4.1	>70.0
P. putida	57.60	ND	ND	ND
S. dysenteriae	19.20	ND	ND	ND
S. typhimurium	>19.0	ND	16.3	>70.0
K. pneumoniae	>9.0	ND	16.3	17.6
P. mirabilis	>75.0	ND	ND	ND
P. aeruginosa	57.60	19.2	ND	ND
S. marcescens	>75.0	ND	ND	ND
S. flexneri	ND	9.6	ND	ND
Fungi		MIC (μM)		
C. albicans	19.2	4.8	ND	ND
S. cerevisiae	19.2	ND	ND	ND
Hemolysis		Maximal levels (%)		
Human erythrocytes	<1.0	ND	<1.0	<4.0

Table 4 was compiled from [83,121,190,198,211] and shows the minimum inhibitory concentration (MIC, μM) of B1EMb (FLPLLAGLAANFLPTIICKISYKC) and its derivatives, B1EMb-NH_2 (FLPLLAGLAANFLPTIICKISYKC-NH_2), PT6 (FLKLLKKLAAKLF) and PTP7/PTP12 (FLGALFKALSKLL), from *G. emeljanovi* against series of bacteria and fungi. Also shown is the activity of these peptides against human erythrocytes as the maximal % hemolysis achieved. ND denotes 'not determined'.

Consistent with the multifunctionality of AMPs [2,109–111], B1EMb was shown to possess anticancer activity (Table 5) [121] and to act directly on pancreatic β Rin5mf cells, stimulating insulin secretion [225]. As described above in Section 5, B1EMb is highly prevalent in the skin of *G. emeljanovi* [12], and its ability to stimulate an insulin release suggested that the skin of the frog stores some components of insulin secretagogues [225], as reported for other frogs and their AMPs [70,235,236]. Based on these results, it was proposed that B1EMb should be investigated for its effect on insulin-related disorders to aid the design

of anti-diabetic drugs; for example, to combat type 2 diabetes mellitus [225], which is a rapidly increasing global problem that is closely linked to the current, burgeoning epidemic of obesity [237]. In relation to anticancer activity, B1EMb, its derivative, PTP7/PTP12 and other analogues were found to have activity against a range of cancer cell lines, including those representing breast cancer (MCF-7) and its MDR variant (MCF-7/DOX) (Table 5) [121]. This latter cell line shows resistance to doxorubicin through the possession of the P-glycoprotein [238,239], which is a member of the ABC transporter family that is found in many MDR cancer cells and acts as an efflux pump, eliminating a wide range of drugs and other substrates from these cells [240,241]. The highest efficacy of the tested peptides was shown by B1EMb and PTP7/PTP12, which showed potent activity against all the cell lines tested, including MCF-7 and MCF-7/DOX (Table 5) [121]. These results clearly showed that B1EMb and PTP7/PTP12 were not P-glycoprotein substrates and, taken with their lack of toxicity to normal eukaryotic cells, led to the proposal that these peptides could be developed as therapeutic agents to treat MDR cancers [121]. Breast cancer is rapidly becoming the leading cause of oncologic morbidity and mortality among women on a global scale [226], in part due to the lack of drugs able to prevent or reverse MDR mediated by P-glycoprotein and other efflux transporters [242,243].

Table 5. The anticancer activity of B1EMa, B1EMb and their derivatives.

Cancer Cells	B1EMa	A4W-B1EMa	V8W-B1EMa	GA-K4	B1EMb	PTP6	PTP7/PTP12
				IC_{50} (μM)			
A498	54.1	56.5	169.1	21.5	ND	ND	ND
A549	57.1	82.0	329.9	14.5	2.5	9.1	5.6
HCT116	44.4	23.5	113.9	14.8	ND	ND	ND
MCF-7	72.0	59.6	156.9	ND	1.9	11.5	3.7
MCF-7/DOX	ND	ND	ND	ND	1.8	ND	3.7
MKN45	13.7	63.8	112.6	22.5	ND	ND	ND
PC-3	17.1	95.5	137.4	29.1	2.0	8.7	3.5
SK-MEL-2	18.6	23.5	ND	22.3	ND	ND	ND
NCl-H630	16.4	58.6	102.2	ND	ND	ND	ND
Hep 3B	ND	ND	ND	ND	1.9	9.8	3.8
SK-OV-3	15.0	71.1	118.7	12.6	ND	ND	ND
293	ND	ND	ND	ND	2.2	9.1	4.7
Cytotoxicity				IC_{50} (μM)			
MCF-10a	ND	240.5	343.4	ND	ND	ND	ND

Table 5 was compiled from [119–121] and shows the half inhibition concentration (IC_{50}, μM) of B1EMa (FLGALFKVASKVLPSVKCAITKKC) and its derivatives, A4W- B1EMa (FLGWLFKVASK), V8W-B1EMa (FLGALFKWASK) and GA-K4AL (FAKWAFKWLKK-NH_2), along with B1EMb (FLPLLAGLAANFLPTIICKISYKC) and its derivatives, PTP6 (FLKLLKKLAAKLF) and PTP7/PTP12 (FLGALFKALSKLL), from *G. emeljanovi* against a range of cancer cell lines. These included A498 (kidney), A549 (lung), HCT116 (colon), MCF-7 (breast), MCF-7/DOX (breast resistant to doxorubicin), MKN45 (stomach), PC-3 (prostate), SK-MEL-2 (skin), NCl-H630 (liver), Hep 3B (liver), SK-OV-3 (ovary), U937 (lymphoma) and 293 (kidney). Also shown is the IC_{50} (μM) of these peptides against MCF-10a, which is a non-transformed breast cell line. ND denotes 'not determined'.

5.1.2. Structure/Function Relationships of Brevinin-1 EMa, Brevinin-1 EMb and Their Derivatives

B1EMa and B1EMb were unstructured, or random coil, in aqueous solution but in a membrane mimetic environment and each peptide adopted an amphiphilic α-helical architecture that stretched between residues 3–20 and residues 4–24 of B1EMa and B1EMb, respectively (Figures 2D,E and 3) [189,244,245]. This conformational behavior is typical of α-helical AMPs and it is well established that these random coil to α-helix transitions are a major force in the binding and insertion of these peptides into membranes [246]. The possession of an amphiphilic α-helical structure by B1EMa and B1EMb was found to be essential for their membrane interactions and thereby their antimicrobial activity,

anticancer action and insulinotropic properties [86,120,121,189,244]. However, a series of structure/function studies on these peptides showed that similarities and differences between their α-helical structures influenced their capacity for membrane interactions and biological activity [86]. Both B1EMa and B1EMb were found to possess a proline residue at position 14 of their sequences (Figures 1 and 2D,E), which caused a kink in their α-helical structure, endowing it with a curvature of circa 25° (Figure 3) [86,189,244,245]. Similarly located, internal proline residues have been reported for many other α-helical AMPs [69] and it is well established that this residue can either break or kink an α-helix due to the inability of its amino acid side chain to donate an amide hydrogen bond and the steric interference caused to the α-helix formation by this side chain [117,247]. Essentially, both B1EMa and B1EMb formed a continuous, curved, amphiphilic α-helix with hydrophilic and lysine residues located on its convex face and hydrophobic residues such as valine and isoleucine situated on its concave face (Figures 2D,E and 3) [86,189,244,245]. A similar molecular architecture has been reported for a number of other α-helical AMPs [69,248] and, in general, the presence of their proline kink has been shown to be relevant to the activity of these peptides [248–251]. These AMPs are prevalent in amphibians [69–71], with prototypic examples including buforins and their derivatives [34,35] and maculatin 1.1 from *Litoria genimaculata* (the Green-Eyed Tree frog) [252,253]. Nonetheless, it is known that the presence of a proline kink in an α-helical structure can have a variable effect on the biological activity of AMPs [248,251,254–256] and, in response, the role of this residue in the biological action of B1EMa and B1EMb was probed [189,244]. P → A substitutions at position 14 of these peptides had a generally similar effect on their overall structures, generating molecules that showed a low level of intrinsic curvature of circa 10° or less, but lacked the prominent kink at position 14 of the parent AMPs [86,189,244]. However, in contrast, P → A substitutions at position 14 of these peptides promoted differing effects on their selectivity and biological action [86,189,244]. In the case of B1EMa, this substitution had no significant effect on the antimicrobial activity but led to greatly enhanced hemolytic activity [189]. Similar results have been reported for other α-helical AMPs [248] and for both, these peptides and B1EMa [189], a primary driver of this effect, appeared to have a disruption to their compact, curved structures and, hence, their ability to shield their apolar residues from the aqueous phase [189,248]. This loss of shielding ability effectively increased the hydrophobicity of these peptides and thereby their affinity for zwitterionic lipids [189,248], which are the primary constituents in the outer leaflet of the erythrocyte CM [23,257]. In contrast, a P → A substitution at position 14 of B1EMb led to a significant decrease in the antimicrobial activity, but had little effect on the hemolytic activity [244]. Similar results were reported for the corresponding substitutions in maculatin 1.1 [258–260], which led to the suggestion that there may be similarities in the structure/function relationships of the proline residues in these two AMPs [86]. A P → A substitution at position 15 of maculatin 1.1 appeared to promote a decrease in the antibacterial activity by disrupting a membrane-interactive, amphiphilic wedge formed by this proline and flanking charged and hydrophobic residues [252,261]. However, it has been shown that the resulting loss of the amphiphilic structure by maculatin 1.1 promotes an increase in the hemolytic activity [252,258,260], which contrasts to B1EMb and suggests that the antibacterial action of the latter peptide is affected differently by a P → A substitution than is maculatin 1.1 [244]. Indeed, B1EMb would seem to have no capacity to form an amphiphilic wedge similar to that of maculatin 1.1, given that the proline residue at position 14 of B1EMb is flanked on either side by strongly hydrophobic four-residue segments (Figure 1). There is some evidence to suggest that the internal proline of both B1EMb and other α-helical AMPs may contribute to their bacterial selectivity by promoting a preferential affinity for anionic lipids, although the mechanisms by which the residue mediates this lipid preference are unclear [86,244,257]. Currently, it is generally believed that the underlying role of the proline residue at position 14 of both B1EMb and B1EMa is to stabilize and maintain the arrangements of the amphiphilic α-helical structure that optimize their membrane interactivity and, thereby, their capacity for selectivity and biological action [86,189,244,245].

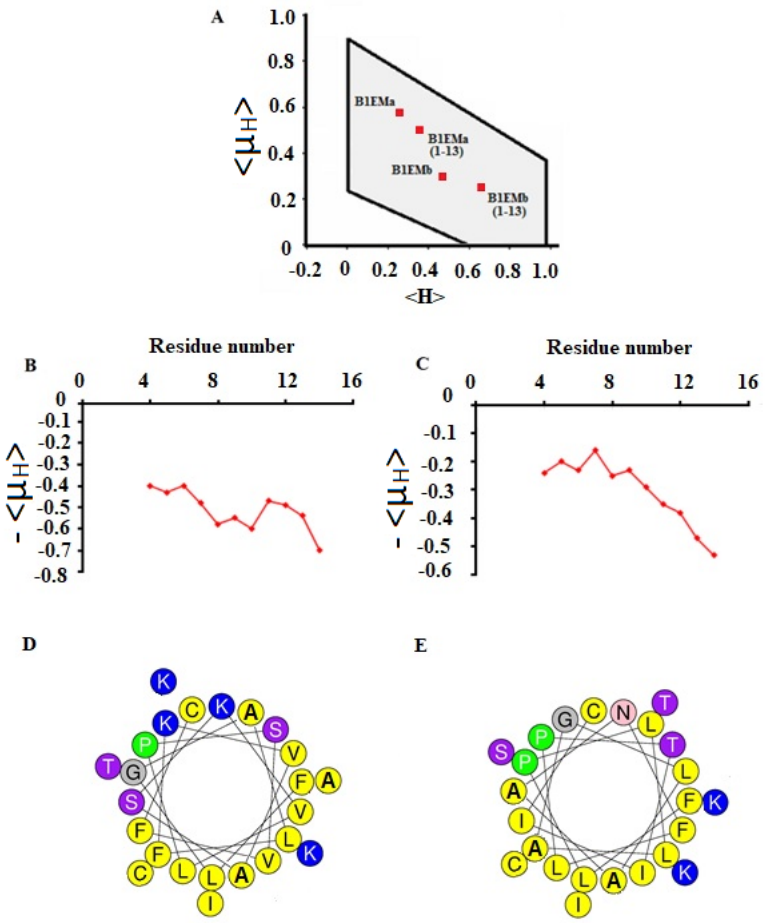

Figure 2. The potential of B1EMa (FLGALFKVASKVLPSVKCAITKKC) and B1EMb (FLPLLAGLAAN-FLPTIICKISYKC) to form tilted, amphiphilic α-helical structure. The potential of B1EMa (1–13) and B1EMb (1–13) to form tilted α-helical structure was determined using extended hydrophobic moment methodology, as previously described [33]. Essentially, this a statistically based methodology based on the amphiphilicity (<μH>) and hydrophobicity (<H>) of α-helical AMPs, as defined in Section 1. In (**A**), this analysis yielded values of <μH> = 0.52 and <H> = 0.36 for B1EMa (1–13) and <μH> = 0.26 and <H> = 0.65 for B1EMb (1–13). These values of <μH> and <H> were then plotted on the extended hydrophobic moment plot diagram, which showed that the data points representing both peptides lay in the shaded area, indicating candidacy to form tilted segments. In (**B**,**C**), the potential of B1EMa (1–13) and B1EMb (1–13) to form a hydrophobicity gradient, which is characteristic of tilted α-helical architecture, was visualized by amphiphilic profiling, which essentially plots <μH> along a peptide's sequence, as previously described [262]. In both cases, these peptides possessed putative hydrophobicity gradients that extended from residue 4 to residue 13 and increased in the N→ C direction. (**A**–**C**) also shows that, in comparison to B1EMb (1–13), B1EMa (1–13) is much more amphiphilic (<μH> = 0.52 versus <μH> = 0.26) and far less hydrophobic (<H> = 0.36 versus <H> = 0.65). These structural differences are reflected by the fact that the hydrophobicity gradient of B1EMa (1–13) (**B**) possesses lower overall hydrophobicity than that of B1EMb (1–13) (**C**). In (**D**,**E**), B1EMa and B1EMb were modeled as a two-dimensional axial projection using the software at [263]. These analyses revealed that both peptides formed amphiphilic α-helices with hydrophobic face of circa 200° and 220°, respectively, that primarily comprised the apolar residues alanine, phenylaniline, valine, leucine and isoleucine (yellow circles). These α-helices also possessed hydrophilic face of circa 160° and 140°, respectively, that was predominantly formed from charged residues, including lysine (blue circles); polar residues, including serine and threonine (purple circles); and uncharged residues, including glycine (grey circles) and proline (green circles).

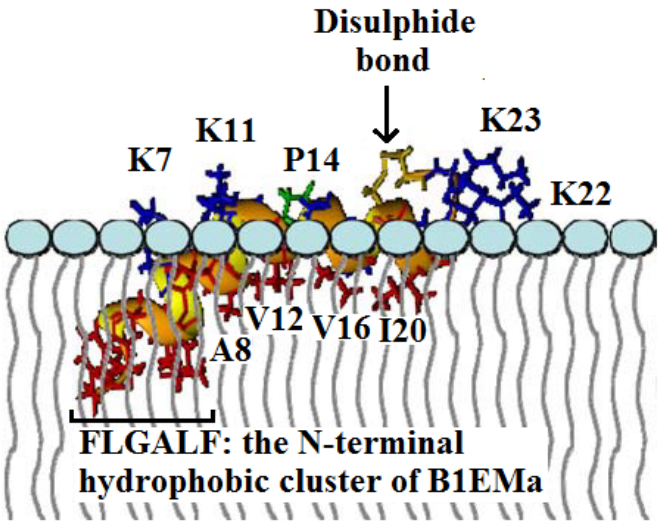

Figure 3. The membrane orientation of B1EMa from *G. emeljanovi*. Figure 3 was revised from [86] and shows that B1EMa (FLGALFKVASKVLPSVKCAITKKC) forms a continuous amphiphilic α-helix (Figure 2), with a curvature of circa 25°, primarily due to the presence of a proline residue at position 14 of the peptide. This amphiphilic α-helix possesses lysine and other hydrophilic residues located on its convex face and hydrophobic residues such as valine, alanine and isoleucine situated on its concave face. The lysine residues possessed by B1EMa, at sequence positions, 7, 11, 22 and 23, would allow the convex, hydrophilic face of the peptide to associate with the lipid head group region (Figure 2) and it is well established that these residues are able to promote the membrane partitioning of AMPs via the snorkeling mechanism, as described in the text [98,245]. In this membrane orientation, B1EMa (15–24) is partitioned parallel to the bilayer surface, which anchors and stabilizes the oblique insertion of B1EMa (1–13) such that its hydrophobic face penetrates the acyl chain region of the membrane at an angle of circa 45° (Figure 3) [242]. This mode of oblique membrane insertion would be consistent with the use of tilted structure and B1EMa (1–13) was predicted to form a hydrophobicity gradient that extended from residue 4 to residue 13 and increased in the N→ C direction (Figure 2). This may be particularly relevant to lysine residues at sequence positions 7 and 11, which lie in B1EMa (15–24) at the surface-associated end of the putative tilted α-helix formed by B1EMa (1–13) (Figures 1 and 2); snorkeling by similarly located lysine residues has been shown to promote the oblique insertion of a number of other amphibian AMPs with tilted characteristics [241,247]. It has been predicted that B1EMb from *G. emeljanovi* will utilize a similar mode of membrane interaction.

Structure/function studies on B1EMa and B1EMb also showed that the proline residue at position 14 of their sequences effectively divided each of these peptides into two regions, formed by residues 1–13 and residues 15–24, respectively (Figues 2D,E and 3) [86,189,244,245]. The region delineated by residues 15–24 of B1EMa (B1EMa (15–24)) and B1EMb (B1EMb (15–24)) includes the C-terminal, heptapeptide and Rana box motif of these peptides (Figure 1) [83]. Showing structural similarities to the AMPs produced by *G. rugosa* [85], this Rana box motif was formed by a loop-like, α-helical fold that was stabilized by a disulfide bond between their cysteine residues at positions 18 and 24 of the peptide, respectively (Figure 3) [83]. The possession of an intact Rana box region by B1EMb appeared to be required for anticancer action [121] and insulinotrophic activity [225] and there is evidence suggesting that this is also the case for the antimicrobial activity of the peptide [86]. Studies on both B1EMb and B1EMb-NH_2 showed that the loss of the disulfide bond between their cysteine residues led to decreased insulinotrophic and antimicrobial activity, respectively, that was attributed to the reduced ability of their C-terminal regions to stabilize their lipid interactive α helical structure [190,225]. In contrast, it has been shown that the reduction in the disulfide bond between the cysteine residues at positions 18 and 24 of B1EMa led to no significant loss of either the α-helicity or antibacterial activity, suggesting that an intact Rana box region was not necessary for the biological activity of

the peptide [189]. Similar results have been reported for the membranolytic antibacterial peptide, thanatin, from *Podisus maculiventris* (the Spined soldier bug) [264], that shows homology and structural similarities to B1EMa, including the possession of a C-terminal Rana box-type motif comprising an eight-residue loop stabilized by a disulfide bridge [265]. In further contrast to B1EMb [86], C → S substitutions in the Rana box region of both linear B1EMa and thanatin led to decreased antimicrobial activity, which led to the suggestion that these cysteine residues, but not their linkage, helped maintain the secondary structure and hydrophobicity/amphiphilicity balance required by these peptides for biological action [189,266]. Interestingly, B1EMa also shares significant homology with a number of cytolytic AMPs, including melittin, as well as ponericin W and Css54, which are found in the venom of the ant, *Pachycondyla goeldii* and *Centruroides suffuses* (the Mexican scorpion), respectively, although the functional significance of these observations was unclear [267–269].

The region delineated by residues 1 to 13 of B1EMa (B1EMa (1–13)) and B1EMb (B1EMb (1–13) comprises an amphiphilic α-helix that is flanked at the N-terminus by a hydrophobic segment that is formed by the residues, FLGALF and FLP, respectively (Figues 2D,E and 3), and appears to be required for the biological action of these AMPs [86,121,191,225]. In combination, the structural characteristics of the proline-linked N-terminal α-helix and Rana box region of B1EMa and B1EMb facilitate the ability of these AMPs to form pores in target membranes, which appears to be the fundamental mechanism underlying the biological activity of these peptides (Figure 3) [86,225]. In relation to the antimicrobial activity of B1EMa and B1EMb, precise details of these pore-forming mechanism(s) were not elucidated, but it was predicted that there would be general similarities to those used by other AMPs [86]. Essentially, the lysine residues carried by these peptides would facilitate the targeting of microbial cells via electrostatic attraction to anionic components of the target cell membrane. The amphiphilic characteristics of the α-helical structure formed by B1EMa and B1EMb would then drive the partitioning of these peptides into the membrane, such that their hydrophobic surfaces interact with the apolar core of the membrane, and their hydrophilic surfaces engage in electrostatic associations with the head group region of the membrane [270,271]. It was predicted that the curved α-helical structure of both peptides would adopt similar membrane orientations, which in the case of B1EMa is illustrated in Figure 3. In this membrane orientation, B1EMa (15–24) is partitioned parallel to the bilayer surface, which anchors and stabilizes the oblique insertion of B1EMa (1–13) such that its hydrophobic face penetrates the acyl chain region of the membrane at an angle of circa 45° (Figure 3). A similar membrane orientation has been reported to facilitate the antibacterial action of other AMPs with a proline-induced, curved α-helical structure [257,272]. For these AMPs, it was suggested that the main driver of membrane penetration for the hydrophobic termini of these AMPs was the bending potential introduced into their the α-helical structure by the presence of their proline residues [257,272].

The oblique insertion of B1EMa (1–13) into membranes (Figure 3) resembles the mode of insertion used by AMPs with a tilted structure, which is characterized by a hydrophobicity gradient along the α-helical long axis [273]. This structural arrangement causes tilted AMPs to penetrate membranes at a shallow angle of between 20° and 80°, thereby promoting a range of membrane-destabilizing effects [42,262], including pore formation [274]. For example, the antimicrobial action of maculatin 1.1, as described above in this section, is believed to involve tilted membrane penetration, driven by the presence of a hydrophobicity gradient in the segment, maculatin 1.1 (1–15) [272,273,275]. Here, the theoretical analysis of B1EMa (1–13) showed that the peptide possessed the potential to form a tilted structure (Figure 2A) with a hydrophobicity gradient that increased in the N → C direction over residues six to fourteen (Figure 2B). These structural characteristics resembled those of the tilted structure formed by maculatin 1.1 (1–15) and could promote the oblique penetration of B1EMa (1–13) into membranes (Figure 3) [272,273]. As described above in this section, flanking the putative tilted structure formed by B1EMa (1–13), at the extreme N-terminus of the peptide, is a segment formed from the strongly hydrophobic residues,

FLGALF (Figures 1 and 3). Earlier studies showed that a complete loss of antimicrobial activity resulted from the deletion of this cluster of residues, from both B1EMa [86] and its putative tilted region, B1EMa (1–13), in isolation [191]. These observations suggested that this strongly hydrophobic N-terminal segment would help drive the tilted insertion of B1EMa into membranes, with its loss reducing the capacity of the peptide to engage in the pore-forming mechanisms that lead to membranolytic and antimicrobial function (Figure 3). The membrane orientation of B1EMa shown in Figure 3 would also allow the charged residues on the convex, hydrophilic face of the peptide, K7, K11, K22 and K23, to associate with the lipid head group region (Figure 3), and it is well established that these residues are able to promote the membrane partitioning of AMPs via the snorkeling mechanism [117,276]. According to this mechanism, the α-carbons of lysine residues are able to reside in the membrane core region whilst their long alkyl side-chains extend, allowing the positively charged moieties of these residues to engage in electrostatic interactions with the lipid head-group region [277]. This may be particularly relevant to K7 and K11, which lie at the surface-associated end of the putative tilted α-helix formed by B1EMa (1–13) (Figures 2B and 3); snorkeling by similarly located K residues has been shown to promote the oblique insertion of a number of other amphibians with tilted characteristics [273,278].

In relation to the anticancer action of B1EMa and B1EMb [119–122], as described in Section 4.1, the ability of AMPs to kill cancer cells seems to be underpinned by the fact that the CMs of these cells resemble those of microbial cells by carrying a net negative charge [117]. This structural resemblance also appears to underpin the fact that AMPs kill cancer cells using mechanisms that are generally similar to those used by these peptides to kill microbes [279]. The use of these mechanisms allows AMPs to kill cancer cells through the induction of apoptosis/necrosis via CM disruption and/or translocation to attack internal targets, such as mitochondria [118,123,280]. The action of B1EMb and derivative peptides against cancer cell lines appeared to promote the blebbing of the CM and the fragmentation of DNA [121], which is consistent with an attack on mitochondrial membranes and the induction of apoptosis in these cells [281,282]. Mechanisms underpinning the anticancer action of B1EMa and B1EMb do not seem to have been investigated [119–122], but it is interesting to note that the internal proline possessed by buforin IIb is essential for the non-membranolytic anticancer action of the peptide [283]. Buforin IIb targets gangliosides on the surface of cancer cells and then uses proline-mediated membrane translocation via the formation of transient toroidal pores to enter these cells, which leads to the induction of apoptosis via mitochondrial-dependent pathways [284,285]. The insulinotrophic activity of B1EMb appeared to be selective for pancreatic cells although the mechanism(s) underpinning this specificity were not elucidated; presumably, it would involve the targeting of some anionic component(s) of the CM possessed by these cells [225]. The peptide appeared to stimulate insulin secretion from pancreatic cells via pore formation in their CM and the induction of an increased Ca^{2+} influx into these cells, which was proposed to constitute a novel mode of action for stimulating insulin release by AMPs [225]. In the case of other known AMPs, stimulating the secretion of the hormone from pancreatic cells appears to involve either the depolarization of their CM with a significant increase in intracellular Ca^{2+} or a Ca^{2+} independent pathway whose precise mode of action has yet to be elucidated [235,286].

No detailed model appears to have been presented for the mechanism(s) of membrane pore formation used by either B1EMb or B1EMa, although it has been suggested that internal proline kinks have a general tendency to disrupt the formation of barrel stave pores by AMPs, but to stabilize the construction of toroidal pores by these peptides [251]. It has previously been shown that a tilted structure can help promote pore formation by AMPs [274] and here, we have shown that B1EMb (1–13) possesses the potential to form this structure (Figure 2A) with a hydrophobicity gradient that increased in the N → C direction over residues seven to fourteen (Figure 2C). As described above in this section, flanking this putative tilted structure, at the extreme N-terminus of B1EMb (1–13), is a segment formed from the strongly hydrophobic residues, FLP (Figure 1), whose absence led to a complete

loss of antimicrobial, anticancer and insulinotrophic action by the peptide [121,225]. A loss of antimicrobial activity was also reported for the deletion of the N-terminal triplet of residues, FLP, from brevinin 1E from *Rana esculenta* (the common water frog) [287], which shows high levels of sequence and structural similarity to B1EMb [288]. In the case of the insulinotrophic action of B1EMb, this loss of biological activity also appeared to be related to a reduced capacity to form an α-helical structure, thereby inhibiting membrane pore formation and Ca^{2+} influx into pancreatic cells [225]. These observations suggest that this FLP segment may help drive the tilted membrane insertion of B1EMb, both directly and indirectly, by stabilizing its formation of an α-helical and tilted structure; by analogy, it is tempting to speculate that a similar function may be served by the N-terminal segment, FLGALF, of B1EMa [191]. A summary of B1EMa and B1EMb from *G. emeljanovi* is given below in Box 4.

Box 4. Brevinin-1 EMa and brevinin-1 EMb from *G. emeljanovi*.

- *G. emeljanovi*, which occurs in Korea, produces brevin-in-2EMa (B2Ema), brevinin-2EMb (B2EMb) and brevinin-2EMb' (B2EMb'), which are cationic and possess a C-terminal, heptapeptide and Rana box motif (Figure 1).
- B2Ema, B2EMb and B2EMb' exhibit very low levels of hemolysis and possess potent activity against Gram-positive bacteria, but weaker activity against Gram-negative bacteria and fungi (Table 1).
- *G. emeljanovi* produces brevinin-1 EMa (B1Ema), brevinin-1 EMb (B1EMb) and esculentin-2 EM (E2EM), which are cationic and possess a C-terminal, heptapeptide and Rana box motif (Figure 1).
- B1Ema and B1EMb show very low levels of hemolysis and cytotoxicity to normal human cells and exhibit potent activity towards Gram-positive bacteria, showing the potential for development to treat infections due to these microbes. These peptides show weaker activity against fungi and Gram-negative bacteria (Tables 3 and 4).
- B1EMb shows the ability to stimulate insulin release and shows the potential for development to treat insulin-related disorders and to aid in the design of anti-diabetic drugs.
- Derivatives of B1Ema, including A4W- B1Ema, V8W- B1Ema, GA-K4AL and peptide B, show varying levels of hemolysis and cytotoxicity. These peptides show potent activity towards Gram-positive bacteria and weaker activity towards Gram-negative bacteria and enveloped viruses. Modified, as appropriate, to reduce toxicity to human cells, A4W- B1Ema, V8W- B1Ema, GA-K4AL and peptide B show the potential for development to treat infections due to these microbes (Table 3).
- Derivatives of B1EMb, including B1EMb-NH2, PTP6 and PTP7/PTP12, exhibit low levels of hemolysis and potent activity towards Gram-positive bacteria, showing the potential for development to treat infections due to these microbes. These peptides show weaker activity against fungi and Gram-negative bacteria (Table 4).
- B1Ema, B1EMb and their derivatives, including A4W- B1Ema, V8W- B1Ema, GA-K4, PTP6 and PTP7/PTP12, exhibit low cytotoxicity and varying levels of efficacy against a spectrum of cancers, indicating the potential for development to treat these disorders, including those with MDR (Table 5).
- B1EMa and B1EMb form continuous, curved amphiphilic α-helices with a hydrophobicity gradient and this structural arrangement appears to be the primary driver of the membranolytic action that underpins the antimicrobial, antifungal, anticancer and insulinotrophic action of these peptides (Figure 3).
- B1EMb and B1Ema possess a central proline residue that appears to help promote the stability of their amphiphilic α-helical structure and the efficacy of their membranolytic biological action (Figure 3).
- B1EMa and B1EMb B1EMb appears to require an intact Rana box to stabilize membrane interactions involved in its antimicrobial, anticancer and insulinotrophic action, whereas this does not appear to be a requirement for the biological activity of B1Ema.

5.2. Esculentin 2EM

Currently, E2EM (GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLAKTC) is the best characterized of the AMPs produced by *G. emeljanovi* [55,86,289,290] and earlier studies showed that the peptide was 37 residues in length and possessed a net charge of +4 (Figure 1) [83]. In the subsequent work, the complete cDNA-encoding E2EM was isolated from a library constructed with mRNAs from the skin of *G. emeljanovi* and, similarly to B1EMa [84], this clone was a single open reading frame that encoded the precursor polypeptide of E2EM [84]. The deduced sequence of this precursor polypeptide comprised a tripartite organization that included an N-terminal signal sequence, followed by an acidic spacer region and the mature peptide at the C-terminus by [84]. Later studies elucidated the organization of the gene encoding E2EM and suggested that the expression of the peptide was induced in response to a microbial challenge by regulatory mechanisms that were similar to those used by insects and mammals for the induction of AMPs [291]. In contrast to B1EMa and B1EMb, E2EM does not appear to have been investigated for its anticancer action and insulinotrophic activity, although these properties have been recently demonstrated for other esculentin peptides [70,235]. Major examples of these peptides include esculentin-2Cha from *Lithobates chiricahuensis* (the Chiricahua leopard frog) and esculentin-2 HYba1 and 2 from *Hydrophylax bahuvistara* (Wide-spread fungoid frog) [292–294]. Currently, E2EM only appears to have been studied for its antimicrobial function in the defence of *G. emeljanovi*.

5.2.1. The Antimicrobial Role of Esculentin 2EM and Its Derivatives

Similarly to B1EMa and B1EMb, E2EM showed weak activity towards Gram-negative bacteria, fungi and protozoa, but potent efficacy towards a panel of Gram-positive bacteria (Table 6) [83,289,295,296], which suggested the potential for the development of this peptide and its derivatives as novel agents against these latter organisms [55,86,100,289,297,298]. A number of studies have shown that the reduced or linearized form of E2EM (E2EM-lin) has a preference for Gram-positive bacteria [172,295,299,300], which is highly similar to that of E2EM in relation to its efficacy and target spectrum (Table 6) [83,289,295,296]. For example, both E2EM and E2EM-lin killed *Staphylococcus epidermidis* at levels that were medically relevant (Table 6) [83,299] and comparable to those reported for other AMPs [301], such as temporin-1DRa and its homologues, which are derived from *Rana draytoni* (the California red-legged frog) [302]. Although generally considered a beneficial commensal organism of the human skin, increasingly, *S. epidermidis* is being recognized as a major nosocomial pathogen in early-onset neonatal sepsis, catheter-related bloodstream infections and other biomedical device-related infections [303,304]. In addition to its antibacterial activity [295,299,305], it has also been demonstrated that E2EM and E2EM-lin possesses similar activity against *C. albicans* and *Saccharomyces cerevisiae*, which suggests that these peptides have the potential for development as novel antifungal agents (Table 6) [83,172,289]. For example, as described above in Section 1, there is an urgent need for novel drugs to combat emerging infections caused by *Candida* spp [131,132] and, in particular, *C. auris*, which is increasingly responsible for invasive diseases with unusually high mortality rates across the globe [306,307]. It has also been shown that E2EM-lin is highly thermostable in the presence of fungal membranes [172] and a similar thermostability has been demonstrated for the peptide in the presence of bacterial membranes [290]. These observations reinforce the suggestion that the peptide has the potential for development as an agent against bacterial and fungal pathogens in the food industry [290,299]. This is the most prevalent use of AMPs and thermostability is essential for these peptides, given their involvement in processing procedures at temperatures of up to 90 °C [308–310]. However, many AMPs do not exhibit thermostability; for example, aurein 2.5, which is from *Litoria aurea* (the Green and Golden Bell frog), showed a high rate of denaturation at temperatures approaching 90 °C in the presence of bacterial membranes, whilst E2EM-lin maintained its structure under similar conditions [311].

A number of studies have been undertaken with the aim of both producing E2EM and derivatives of the peptide with antibacterial activity and addressing major problems associated with the development of AMPs (Table 6) [289,296,312–314]. It is well established that a variety of factors such as toxicity, a lack of structural stability, proteolytic susceptibility and high manufacturing costs have slowed the progress in realizing the therapeutic potential of AMPs [16,315–317]. One earlier investigation assessed the feasibility of expressing E2EM as a glutathione S-transferase (GST) fusion protein in an *E. coli* host, which showed that the purified recombinant peptide possessed a similar target specificity and antibacterial activity to native E2EM. No toxicity to host *E. coli* cells was detected, which appeared to be blocked by the presence of the GST moiety, and the peptide was produced in a high yield, apparently due to the preclusion of proteolysis [318]. This heterologous expression system has since been used in a number of other studies on E2EM [295], for example, the production of recombinant hybrids formed from the latter peptide and the human AMP, LL-37, which were found to possess potent activity against both Gram-positive and Gram-negative bacteria [314]. *E. coli* is the most frequently used bacterial host for the expression of AMPs and gene fusion technology has been widely used for the production and/or purification of these peptides in this host, offering decreased time and production costs as well as easy scale up [136]. Other heterologous expression systems used to produce E2EM and functionally characterize its antibacterial activity include Xenopus kidney epithelial cells and Xenopus oocytes [291].

In a series of studies, C-terminally truncated E2EM was used in an attempt to produce analogues with minimal sequences that maintained the antibacterial activity of the native peptide [289,296,312]. Currently, length is a major issue in the therapeutic development of AMPs, both in terms of their commercial cost of production and other factors, such as the efficient loading of drug delivery systems [16,315–317]. In general, C-terminally truncated analogues of E2EM with sequences longer than E2EM (1–23) were found to show some level of antimicrobial activity, whereas those with sequences equal to, or shorter than, E2EM (1–23) showed a complete loss of this activity [289,296,312]. However, the analogue, 23D16W, (GILDTLKQFAKGVGKWLVKGAAQ-NH_2), which was essentially E2EM (1–23) with a D16 → W16 substitution possessed a spectrum of selective antibacterial activity that was similar to that of E2EM (Table 6) [296,312]. This analogue represented a reduction in the size of E2EM of around two fifths and was used as a basis for more recent studies, which attempted to enhance the proteolytic and chemical stability of shortened E2EM analogues through stapling, as discussed below in Section 5.3 [313].

5.2.2. Structure/Function Relationships of Esculentin 2EM and Its Derivatives

E2EM was largely unstructured in aqueous solution, but in a membrane mimetic environment, the peptide adopted an α-helical structure, although there was some dispute as to the precise location of this molecular architecture [289,295,319]. Several studies suggested that E2EM formed a structure comprising two amphihilic α-helices, which extended from residues 2 to 10 and from residues 16 to 32 and were connected by a flexible loop spanning residues 11 to 15 [295,319]. However, an alternative α-helical structure has been suggested for E2EM [289], which appears to be consistent with other experimental data and is the currently accepted topology for the peptide [55,86]. In this topology, E2EM adopts a structure comprising a long amphiphilic α-helix formed by residues 2–23 and a shorter amphiphilic α-helix consisting of residues 25–34 (Figure 4C,D). These two α-helices are connected by a glycine residue at position 24 of E2EM, which is responsible for a flexible hinge that allows the independent movement of both helices with a bending angle that was predicted to be from 60° to 150° (Figure 5D,E) [289]. It is well established that this residue possesses high conformational flexibility due to its lack of a side chain that enables it to introduce a break or kink into the α-helical structure of AMPs [117,320]. Similarly located internal glycine residues have been reported for many other α-helical AMPs [69], including those from amphibians [69–71], and it is known that the presence of a glycine kink in an α-helical structure can have a variable effect on the biological activity of AMPs [321–325].

In response, several truncation studies have shown that the presence of this residue is essential to both the antimicrobial and hemolytic action of E2EM [289,296] and interestingly, these studies also showed that potential or observed breaks in the α-helical structure of other AMPs is often also mediated by glycine or proline residues that are situated at, or near, sequence position 24 [289].

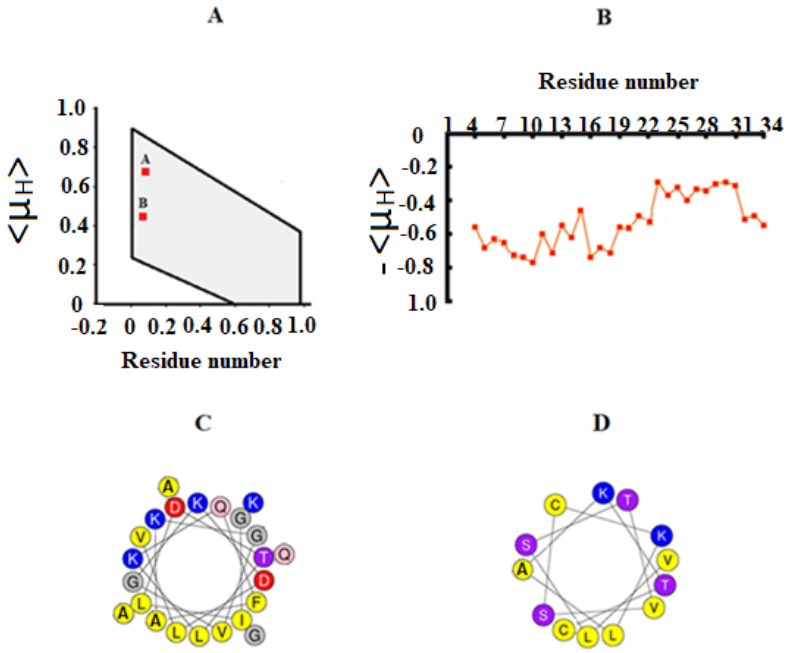

Figure 4. The potential of E2EM-lin (GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLAKTC), which is E2EM with no disulfide bond, to form tilted, amphiphilic α-helical structure. The potential of E2EM-lin (1–23) and E2EM-lin (25–37) to form tilted α-helical structure was determined using extended hydrophobic moment plot methodology, as previously described [33]. Essentially, this a statistically based methodology based on the amphiphilicity (<*μH*>) and hydrophobicity (<*H*>) of α-helical AMPs, as defined in Section 1. In (**A**), this analysis yielded values of <*μH*> = 0.67 and <*H*> = 0.09 for E2EM-lin (1–23), and <*μH*> = 0.43 and <*H*> = 0.06 for E2EM-lin (25–37). These values of <*μH*> and <*H*> were then plotted on the extended hydrophobic moment plot diagram, which showed that the data points representing both peptides lay in the shaded area, indicating candidacy to form tilted segments. In (**B**), the potential of E2EM-lin to form a hydrophobicity gradient, which is characteristic of tilted α-helical architecture, was visualized by amphiphilic profiling, which essentially plots < *μH* > along a peptide's sequence, as previously described [262]. E2EM-lin (1–23) possessed a putative hydrophobicity gradient that extended from residue 10 to residue 23 and increased in the C→ N direction (**B**). In contrast, E2EM-lin (25–37) formed no discernable hydrophobicity gradient, which suggested that this region does not form tilted structure (**B**). In (**C**,**D**), E2EM-lin (1–23) and E2EM-lin (25–37) were modeled as a two-dimensional axial projection using the software at [263]. These analyses revealed that E2EM-lin (1–23) formed an amphiphilic α-helix with a hydrophobic face of circa 140°, primarily comprised of the apolar residues phenylaniline, valine and leucine (yellow circles). This α-helix also possessed a hydrophilic face of circa 220° that was predominantly formed from charged residues, including lysine (blue circles) and aspartic acid (red circles), and uncharged residues, including glycine (grey circles) and glutamine (mauve circles) (**C**). E2EM-lin (25–37) formed an amphiphilic α-helix with an apolar face of circa 200°, primarily comprised of the apolar residues alanine, valine and leucine (yellow circles). This α-helix also possessed a hydrophilic face of circa 160° that was predominantly formed from charged residues, including lysine (blue circles) and uncharged residues, including serine and threonine (purple circles) (**D**).

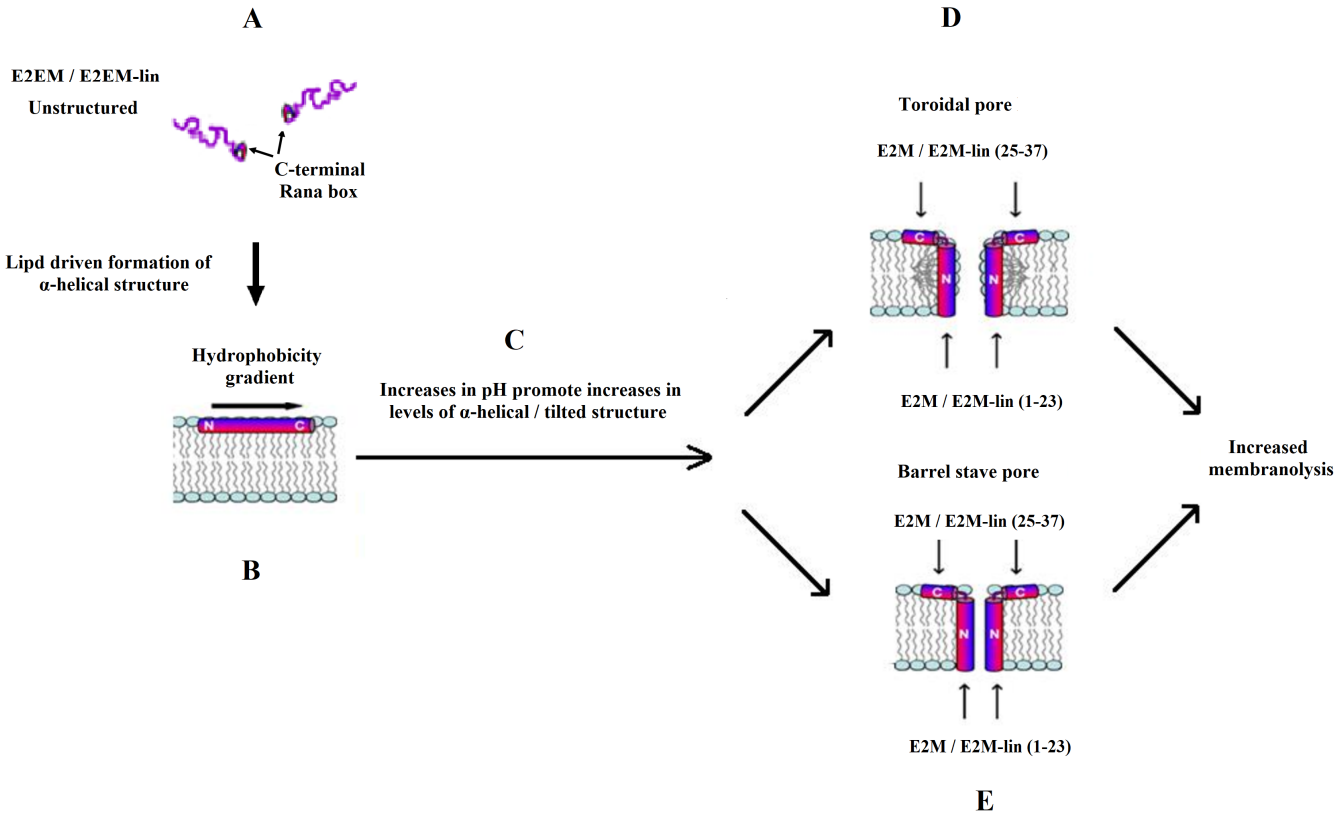

Figure 5. A model for the pH-dependent, antibacterial action of E2EM/E2EM-lin (GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLAKTC)**. Figure 5** was revised from [86,299,300] and shows a schematic representation of the pH-dependent antimicrobial action proposed for E2EM/E2EM-lin. Initially, the unstructured peptide (**A**) interacts with the bacterial membrane surface and forms α-helical structure (represented as cylinders) with a hydrophobic surface (red) and a hydrophilic surface (blue) (**B**). The α-helical structure formed by E2EM/E2EM-lin (1–23) is driven by the presence of bacterial lipids and possesses a hydrophobicity gradient (**B**). The levels of this structure are enhanced by increasing pH (**C**), which then promotes pore formation by E2EM/E2EM-lin via membrane insertion and the adoption of a transmembrane orientation, which is stabilized by the surface interactions of E2EM/E2EM-lin (25–37) (**D**,**E**). Potentially, E2EM/E2EM-lin can form a toroidal pore (**D**) or a barrel stave pore (**E**) and the major difference between these pore types is that in the former pore, the membrane leaflets deform to allow the lipid head-group region to remain in contact with the hydrophilic face of the E2EM/E2EM-lin membrane spanning region, which is not observed in the latter pore [42]. In both cases, increasing pH promotes higher levels of pore formation and membranolysis, which are maximal under alkaline conditions and lead to microbial cell death (**D**,**E**); for clarity, two monomers of E2EM/E2EM-lin are shown in this pore-forming process, but it has been predicted that between five and ten monomers are involved [290].

The glycine residue at position 24 of E2EM effectively divided the peptide into two regions, formed by residues 1–23 (E2EM (1–23)) and residues 25–37 (E2EM (25–37)), respectively (Figues 4C,D and 5D,E) [55,86,289,297,299,300]. E2EM (25–37) included the C-terminal heptapeptide that constitutes the Rana box motif of the peptide and similar to B1EMa and B1EMb, formed a cysteine-stabilized, loop-like fold [83]. This loop-like fold was essentially an α-helical structure that was stabilized by a disulfide bond between cysteine residues at positions 31 and 37 of the peptide (Figure 1 and [289,295,319]. The presence of E2EM (25–37) appeared to be required for the optimal antibacterial efficacy of E2EM, playing a key role in maintaining the pore-forming activity of the peptide, although not directly participating in the pore formation itself [289,295,305]. It has been recently shown that E2EM (25–37) possesses an approximately constant distribution of

hydrophobicity along the α-helical long axis over residues 25 to 37, which is indicative of an orientation parallel to the surface (Figure 4B) [300] and consistent with the studies, showing that this segment serves as a membrane anchor to stabilize pore formation by E2EM (Figure 5D,E) [289,295,305]). Similar observations have been made for nicomicin-1 from the small polychaeta *Nicomache minor*, which shows homology and structural similarities to E2EM, including a C-terminal Rana box-type motif comprising a six-residue loop stabilized by a disulfide bridge. Nicomicin-1 showed potent activity against Gram-positive bacteria and the region, including its Rana box-type motif, appeared to stabilize the membrane interactions of its N-terminal α-helical segment, which promoted the peptide's membranolytic, antibacterial activity [326]. A number of studies have also investigated the role of the disulfide bond possessed by E2EM (25–37) and showed that the reduction of this bond to generate E2EM-lin (25–37) led to no significant loss of α-helicity in E2EM and had no discernible effect on either the membrane anchoring or antibacterial action of the peptide [295,299,305]. E2EM-lin also showed levels of α-helicity and efficacy against fungi [172] that were very close to those obtained for E2EM [83,289], which led to the suggestion that the disulfide bond possessed by E2EM (25–37) was not essential for either the antifungal activity of E2EM [172] or its broader antimicrobial action [86,300].

E2EM (1–13) formed an N-terminal amphiphilic α-helix, which possessed the potential to form a tilted peptide with an hydrophobicity gradient that extended in the C → N direction over residues 10 to 23 (Figure 4B) [299]. Many of these residues had high α-helix-forming propensities and it was predicted that the possession of these residues would promote a variety of intramolecular interactions that not only enhanced the structural stability of the peptide's tilted architecture, but also contributed strongly to the thermostability of E2EM-lin [290]. The formation of the tilted structure by E2EM (1–23) was also proposed to help explain the results of several other studies on the membranolytic action of the parent peptide. As described above in Section 5.2.1, in isolation, E2EM (1–23) shows no antibacterial activity, but a D16 → W16 substitution to form 23D16W endowed the peptide with activity against Gram-positive bacteria and weaker action towards the Gram-negative (Table 6) [296,312]. It was proposed that this effect was promoted by the abilities of tryptophan to both anchor the analogue to the surface regions of the membrane and to stabilize its α-helical conformation [296], abilities that are well established for this residue [117]. However, it has also been proposed that this tryptophan substitution may have helped orientate and promote the formation of tilted structure by E2EM (1–23) [290], thereby contributing to the restored antibacterial ability of the peptide [296]. Similarly, it was also proposed by these latter authors that the stapling of E2EM (5–19) derivatives into an α-helical conformation may have promoted the formation of a tilted structure [290], thereby endowing these peptides with activity against Gram-positive bacteria [313,327].

In combination, the structural characteristics of the glycine-linked E2EM (1–13) and E2EM (25–37) facilitate the ability of E2EM to form pores in membranes, which appears to be the fundamental mechanism underlying the biological activity of the peptide (Figure 5D,E) [55,86,289,297,299,300]. Mechanisms underpinning the antifungal action of E2EM do not seem to have been investigated, but E2EM-lin appeared to kill *C. albicans* using a mode of action that involves the permeabilization of the organism's CM via the adoption of a lipid-interactive α-helical structure [172]. Indeed, it is generally believed that the membranolytic mechanisms used by AMPs to kill fungi are similar to those used by these peptides to kill bacteria [23,42]. For example, the adoption of a lipid-interactive, α-helical structure is a strategy used by aurein 2.5 to disrupt membranes and kill both prokaryotic [311,328] and eukaryotic microbes [329,330]. In relation to the antibacterial activity of E2EM, earlier studies suggested that the peptide may be able to form pores in bacterial membranes [331], which was confirmed by work showing that this ability underpinned the action of the peptide against a variety of bacteria [297,305]. Higher efficacy in this ability appeared to drive the strong preference of E2EM for Gram-positive bacteria over Gram-negative bacteria and related to the differing compositions and topologies of membranes from these two bacterial classes [297,305]. The direct visualization of the effects

of the E2EM action on cells of the Gram-positive bacterium, *Micrococcus luteus*, using transmission electron microscopy (TEM) clearly showed the leakage of intracellular contents through pore-like structures in the cell wall and CM of the organism [297]. The cell wall is a relatively porous mesh that does not represent a permeability barrier to most AMPs the size of E2EM (3.74 kDa [331]) and allows these peptides to access the CM of Gram-positive bacteria with their effective concentration not significantly reduced [157,158]. Upon accessing the CM of *M. luteus*, the high levels of anionic lipid in this membrane appeared to drive electrostatic interactions with E2EM that promoted pore formation and membrane permeabilization by the peptide, resulting in cell death [297,305]. In general, anionic lipids form around 70% or more of the lipid in the CM of Gram-positive bacteria [157,158,332] and it was proposed that the mechanism used by E2EM to kill *M. luteus* may represent the peptide's general mode of action against bacteria [297,305].

TEM was also used to visualize the effects of E2EM on cells of the Gram-negative bacterium *E. coli*, which revealed bleb-like structures that appeared to be the outer membrane (OM) of the organism separating and distending from the damaged CM [297]. The disruptive action of E2EM on the OM of *E. coli* showed some similarities to that of other AMPs that cross the barrier posed by the CM to access and permeabilize the CM of target organisms [333–335], such as derivatives of porcine lactoferricin (LFs) [333] and cecropin B from *H. cecropia* [37,336]. However, TEM revealed no apparent formation of pores in the CM of *E. coli* by E2EM, although the peptide was clearly able to access and disrupt these membranes, which suggested that the peptide acted sequentially on the OM and then the CM [297]. Based on these observations it was proposed that a major factor underpinning the preference of E2EM for Gram-positive bacteria over Gram-negative bacteria was the barrier function of the OM reducing the effective concentration of the peptide able to access the CM [297]. A minor contribution to this preference appeared to come from the lower levels of anionic lipids found in the CM of *E. coli* and other Gram-negative bacteria [297], which form around 30% or less of the lipids in these membranes [23,332,337]. This relative decrease in anionic lipids appeared to reduce the capacity of E2EM to engage in electrostatic interactions with the CM of Gram-negative bacteria as compared to those of Gram-positive bacteria [297]. The process by which E2EM crossed the OM of *E. coli* was not determined, but a number of possibilities would appear to exist; for example, targeting LPS of the *E. coli* OM and uptake by the self-promoted pathway, which appears to be used by LFs [333] and cecropin B [37,336]. Other targets of AMPs appear to be OM protein 1 and OM lipoprotein of *E. coli* and other Gram-negative bacteria, which promote the uptake of both these peptides and their target proteins, resulting in the permeabilization of the CM and an attack on intracellular targets [334,335].

Attempts to characterize membrane pores formed by E2EM have shown that the peptide forms transient, heterogeneous channels with different sizes [305,331] and that the process of pore construction appears to involve the assembly of between five and ten monomers [86,298,312]. The peptide was sufficiently long to span a membrane in an α-helical conformation [86], which requires a minimum of circa 22 residues [42,338], and it was observed that E2EM possessed the potential to form barrel-stave pores (Figure 5E) [86,298]. According to this model, assembled monomers of E2EM would form a transmembrane pore with an orientation that lay approximately perpendicular to the bilayer surface. In this orientation, the hydrophobic residues of these assembled monomers interact with the lipid core of the membrane, forming the outer surface of the pore, whilst their hydrophilic residues line the pore interior (Figure 5E) [16,30,39,42]. The pore formed by E2EM showed some structural similarities to that described for alamethicin [298], which is an AMP that is produced by the fungus, *Trichoderma viride,* and is generally taken to be the prototypic former of barrel stave pores [339,340]. However, the ability to form barrel stave pores has only been conclusively demonstrated for a relatively small number of peptides other than alamethicin [341]; notably, pardaxin from fish [342], ceratoxins from insects [342] and DCD-1L from humans [343,344]. It has also been suggested by a recent computational study based on the activity determinants of α-helical AMPs that E2EM may utilize the carpet

mechanism in its antimicrobial action [345], although this mechanism can be considered as a multiple toroidal pore formation [42]. Based on these observations, it was proposed that E2EM was more likely to form toroidal-type pores (Figure 5D) [86,289], which are known to be transient in nature and heterogeneous in size, and their formation is the most common membranolytic mechanism used by AMPs to kill microbes [16,30,39,42]. Indeed, it has been suggested that, similarly to AMPs with proline kinks, the glycine kink of peptides such as E2EM have a general tendency to disrupt the formation of barrel stave pores, but to stabilize the construction of toroidal pores [251]. Using the toroidal pore model, E2EM would insert perpendicularly into the bilayer, but in contrast to the barrel stave pore formation, the peptide would remain in close association with the membrane lipid head groups (Figure 5D). This form mode of insertion causes the membrane surface to cavitate inwards and to ultimately form a pore that is lined by polar lipid head groups and hydrophilic surfaces of E2EM (Figure 5D) [16,30,39,42]. Schemes to describe the use of both the barrel stave pore formation and toroidal pore formation in the antimicrobial action of E2EM have been presented [86,289], which have been updated by more recent work on E2EM-lin (Figure 5D,E) [172,290,299,300].

Based on the redundancy of the disulfide bond possessed by E2EM (25–37) and other results, a series of studies on E2EM and E2EM-lin have shown that these peptides use the same model of pore formation in their membrane interaction and antimicrobial activity and, for clarity, when describing these models, we refer to E2EM/E2EM-lin [86,172,290,295,299,300,305]. A model based on earlier studies proposed that in the initial stages of this model, the strong positive charge of unstructured E2EM/E2EM-lin targets negatively charged components of the bacterial cell membrane, including phosphatidylglycerol (PG) and cardiolipin (CL) (Figure 5A) [86,299], which are the major, negatively charged lipids in bacterial membranes [23,42]. The localization of the peptide to the interface of these membranes then promotes the adoption of a strongly amphiphilic α-helical structure, which facilitates the partitioning of E2EM/E2EM-lin into these membranes to engage in electrostatic associations with the lipid head-group region and hydrophobic interactions with the membrane acyl chain core (Figure 5B). In the next stages of this model, E2EM/E2EM-lin (24–37) lies on the membrane surface, anchoring the parent peptide, whilst the conformational flexibility provided by G(24) allows E2EM/E2EM-lin (1–23) to realign and adopt a transmembrane orientation. The association of these transmembrane regions of E2EM/E2EM-lin then leads to pore formation, membranolytic action and the death of the target bacteria [299], which is illustrated in Figure 5D,E using two monomers of the peptide for clarity, although, as described above in this section, it is believed that higher-order oligomers of E2EM/E2EM-lin participate in this process [86,298].

The model based on earlier studies also showed that the major peptide-based structure/function relationship underpinning the membranolytic action of E2EM/E2EM-lin appeared to be the formation of a tilted structure within E2EM/E2EM-lin (1–23). The formation of this tilted structure resulted from the adoption of an α-helical structure by the peptide (Figure 5B) and appeared to drive the transmembrane realignment of this N-terminal region of E2EM/E2EM-lin to engage in pore formation (Figure 5D,E) [86,299]. However, the hydrophobicity gradient associated with E2EM/E2EM-lin (1–23) appeared to decrease with the increasing depth of the angled membrane insertion (Figure 4B), which is in contrast to B1EMa, B1EMb (Figure 2B,C) and most tilted peptides and suggested functional relevance [262]. It has previously been proposed that in addition to promoting membrane insertion, the tilted structure possessed by E2EM/E2EM-lin (1–23) could play a more direct role in pore formation by the peptide [299] and it is known that a tilted structure is able to promote protein–protein interactions [262]. Interestingly, the first ten N-terminal residues of E2EM/E2EM-lin (1–23): GILDTLKQFA (E2EM/E2EM-lin (1–10)), which were predicted not to form a tilted structure (Figure 4B), showed significant homology with the N-terminal α-helical segment of hadrurin, [346]. Hadrurin is a membranolytic toxin found in the venom of the scorpion, *Hadrurus aztecus* [346], and by analogy, it is tempting to speculate that the strongly hydrophobic nature of E2EM/E2EM-lin (1–10) may help drive

membrane insertion by the parent peptide (Figure 5). In this respect, E2EM/E2EM-lin would show similarities to B1EMa and B1EMb, which, as described above in Section 5.1.2, possess strongly hydrophobic N-terminal segments that appear to be required for the membranolytic and biological action of these AMPs [86,121,191,225].

Consistent with previous work [297,305], the model based on earlier studies showed that the membranolytic action of E2EM/E2EM-lin also depended upon membrane-based factors, namely the lipid composition of target microbial membranes [86,299]. Essentially, the ability of E2EM/E2EM-lin to form an α-helical/tilted structure was primarily mediated by PG in the case of Gram-positive bacteria and PE in that of Gram-negative bacteria (Figure 5B) [86,299]. It is well established that PG is generally the major component in membranes of the former organisms, whilst phosphatidylethanolamine (PE) is the predominant lipid in membranes of the latter bacteria [23,42]. Moreover, PG induced higher levels of an α-helical/tilted structure in E2EM/E2EM-lin than PE, which appeared to underpin the general preference shown by the peptide for action against Gram-positive bacteria over Gram-negative bacteria [86,299]. In contrast, CL is found in the membranes of both these bacterial classes to varying degrees [23,42] and although the lipid was able to induce an α-helical/tilted structure in E2EM/E2EM-lin with an efficacy that was similar to that of PG, the corresponding levels of lysis shown by the peptide were greatly reduced [86,299]. These results contrasted strongly with AMPs that specifically target CL to induce membranolysis [347] and it was proposed that CL may reduce the membranolytic action of E2EM/E2EM-lin by restricting but not abolishing the insertion of the peptide into target microbial membranes through high-affinity electrostatic binding [86,299]. Similar mechanisms involving electrostatic binding to PE have also been shown to inhibit the membranolytic action of a number of AMPs [348], for example, maximin H5 from the toad, *Bombina maxima* (the Giant Fire-Bellied toad) [349]. There is also the possibility that CL promotes the antibacterial activity of E2EM-lin through recently reported non-lytic mechanisms where the interactions of AMPs with CL-rich lipid microdomains perturbs the functional organization of bacterial membranes, thereby promoting lethal effects on cell metabolism [350,351]. However, currently, the mechanisms underpinning the contribution of CL to the antimicrobial action of E2EM-lin are an open question and await further investigation [86,299]. In relation to the membranolytic antifungal action of E2EM/E2EM-lin, the role of individual lipids was not investigated; however, it was observed that this action was generally comparable in the level and mode [172] to that observed when the peptide was directed against Gram-negative bacteria [299,300]. The CM of fungi resembles those of Gram-negative bacteria in that they are predominantly formed from PE and other zwitterionic lipids [23,42] and it was speculated that the PE induction of an α-helical/tilted structure in E2EM/E2EM-lin may feature in the antifungal action of the peptide [299]. There is also the possibility that changes to the structural characteristics of lipids could contribute to the membranolytic action of E2EM/E2EM-lin: it is well established that these intrinsic properties are primary determinants in the membrane interactions of AMPs [352]. In the case of PG, which is a lamellar lipid, it was concluded that changes to the structural characteristics of the lipid were unlikely to contribute to the membranolytic action of E2EM/E2EM-lin [86,299]. In the case of CL and PE, it is well established that these lipids are able to adopt non-lamellar structures and that this ability is able to influence the membranolytic action of AMPs in multiple ways [348,353], as previously described in relation to E2EM/E2EM-lin [86,299,300]. A full discussion of this ability is beyond the scope of this review; however, as an example, it has been shown that the cone shaped molecule formed by PE is able to promote negative membrane curvature and enhance the capacity of tilted AMPs to destabilize and permeabilize membranes [354–356]. In contrast, the ability of the cone-shaped molecule formed by CL to promote negative membrane curvature has been shown to counter the tendency of AMPs to induce positive membrane curvature and thereby inhibit pore formation by these peptides [357,358].

Most recently, the model for pore formation by E2EM/E2EM-lin was extended when it was shown that the membranolytic action of the peptide was pH-dependent with an alkaline optimum (Figure 5) [300], contrasting to the vast majority of pH-dependent AMPs so far reported, which show acid optima [223]. A major example of these latter AMPs is maximin H5, which would appear to be the only other known instance of pH-dependent AMPs using a tilted structure to drive their membranolytic action [223,359]. Indeed, a pH-dependent membranolytic action with an alkaline optimum places E2EM/E2EM-lin in a very small group of established AMPs [172]. For example, AWRK6, which is a derivative of Dy2 from the frog, *Rana dybowskii* (the Dybowski's frog,) exhibited potent antibacterial activity under alkaline conditions [360] and has therapeutic potential for treating diabetes [361] and endotoxin-induced inflammatory responses [362]. An alkaline pH appeared to enhance the membranolytic action of E2EM/E2EM-lin by reducing the positive charge carried by the peptide, which is typical of pH-dependent AMPs with alkaline optima [290], as in the case of Dy2 and AWRK6 [360]. It was proposed that these lower levels of a positive charge would effectively increase the hydrophobicity of E2EM/E2EM-lin and its capacity for membrane insertion, as well as reducing the energetically unfavorable impact of repulsive electrostatic interactions between E2EM/E2EM-lin molecules involved in pore formation [300]. However, the primary mechanism by which an alkaline pH enhanced the membranolytic action of E2EM/E2EM-lin appeared to be to increase the levels of an α-helical/tilted structure adopted by the peptide (Figure 5C) [300] and analogous results have been shown for Dy2 and AWRK6 [360]. Similar to the work described above in this section that was conducted under neutral pH conditions [86,299], the ability of E2EM/E2EM-lin to adopt an α-helical/tilted structure under alkaline pH conditions was primarily mediated by PG in the case of Gram-positive bacteria and PE in that of Gram-negative bacteria (Figure 5B) [300]. Moreover, PG induced higher levels of an α-helical/tilted structure in E2EM/E2EM-lin than PE, indicating that the general preference shown by the peptide for action against Gram-positive bacteria over Gram-negative bacteria was maintained under alkaline pH conditions [300]. There is also the possibility that pH-related changes to the structural characteristics of lipids could contribute to the membranolytic action of E2EM/E2EM-lin, as previously suggested [223]. In the case of PG and PE, it was concluded that pH-related changes to their headgroup charge and morphology were unlikely to contribute to the enhanced membranolytic action of E2EM/E2EM-lin at an alkaline pH [300]. However, in the case of CL, the charge on the lipid increases from − 1 to − 2 under these pH conditions [363,364], which led to the suggestion that this pH-dependent charge effect could enhance the initial electrostatic interaction between E2EM/E2EM-lin and bacterial membranes [300]. Indeed, it was also proposed that this pH-dependent charge effect could help to compensate for the decreased net-positive charge of the peptide at an alkaline pH, as well as contributing to the increased binding affinity of E2EM/E2EM-lin for bacterial membranes under these pH conditions [300]. The morphology of CL also changes under alkaline conditions, resulting in electrostatic repulsion effects between its headgroups that promote looser lipid packing in membranes [363,364], which led to the suggestion that this ability could promote the access of E2EM/E2EM-lin to bacterial membranes [300]. In combination, these observations clearly suggest that changes to the intrinsic properties of CL have the potential to contribute to the pH-dependent membranolytic activity of E2EM-lin against both Gram-positive and Gram-negative bacteria. A summary of E2EM from *G. emeljanovi* is given in below in Box 5.

Table 6. Antimicrobial and haemolytic activity of E2EM and its derivatives.

Bacteria	**E2EM**	**E2EM-lin**	**23D16W**
	MIC (μM)		
S. aureus	ND	3.1	4.4
M. luteus	0.7	<3.0	1.0
S. mutans	ND	3.1	ND
S. epidermidis	2.7	3.1	ND
B. subtilis	2.7	6.3	20.2
S. pyogenes	ND	6.3	ND
K. pneumoniae	6.7	>6.0	10.3
K. aerogenes	ND	200.0	ND
S. dysenteriae	6.7	ND	20.6
P. putida	26.8	ND	ND
P. aeruginosa	28.7	75.0	51.5
E. coli	20.6	>20.0	10.3
P. mirabilis	>53.0	>50.0	ND
S. marcescens	>53.0	ND	>82.0
S. typhimurium	53.6	ND	51.5
Fungi		MIC (μM)	
C. albicans	53.6	60.0	ND
S. cerevisiae	53.6	60.0	ND
Hemolysis		Maximal levels (%)	
Human erythrocytes	<2.0	<2.0	<1.0

Table 6 was compiled from [83,172,289,295,296,299,327] and shows the minimum inhibitory concentration (MIC, μM) of E2EM (GILDTLQAFAKGVGKDLVKGAAQGVLSTVSCKLAKTC) and derivatives, E2EM-lin (E2EM with no disulfide bond) and 23D16W (GILDTLKQFAKGVGKWLVKGAAQ-NH_2), from *G. emeljanovi* against a series of bacteria and fungi. Also shown is the activity of these peptides against human erythrocytes as the maximal % hemolysis achieved. ND denotes 'not determined'.

Box 5. Esculentin 2EM from *G. emeljanovi*.

- *G. emeljanovi* produces esculentin-2 EM (E2EM), which is cationic and possesses a C-terminal, heptapeptide and Rana box motif. E2EM and its derivatives have also been produced by heterologous expression systems.
- E2EM and derivatives, including E2EM-lin and 23D16W, exhibit very low levels of hemolysis and are generally ineffective against Gram-negative bacteria, but exhibit potent activity towards the Gram-positive, showing the potential for development to treat infections due to these microbes (Table 6).
- E2EM and E2EM-lin are thermostable and exhibit moderate activity against fungi, indicating the potential for development as antimicrobial agents in the food industry (Table 6).
- E2EM/E2EM-lin exert their antibacterial and antifungal action using pH-dependent, membranolytic mechanisms that are enhanced by alkaline pH conditions (Figure 5).
- E2EM appears not to require an intact Rana box for its membranolytic action and E2EM/E2EM-lin forms two juxtaposed, amphiphilic α-helical segments that are connected by a central glycine residue which promotes the molecular flexibility required for this action (Figure 5).
- The membranolytic action of E2EM/E2EM-lin appears to be underpinned by the formation of a tilted/α-helical structure in its N-terminal region that promotes pore formation and is primarily mediated by PG in the case of Gram-positive bacteria and PE in that of Gram-negative bacteria (Figures 4 and 5).

5.3. Stapled AMPs from G. emeljanovi

As described above in Section 5.1.1, peptide B, which was essentially the N-terminal sequence of B1EMa (1–13) with residue substitutions, was shown to possess antibacterial and antiviral activity [199]. In attempts to enhance this antimicrobial activity, isoforms of peptide B were generated with α-helical structures stabilized by hydrocarbon stapling (Figure 6A) [199], which has been increasingly used to generate novel AMPs since its introduction in 2000 [313,365–367]. In general, the stapling of AMPs involves the incorporation of two Cα-methyl, Cα-alkenyl residues with subsequent macrocyclization, which effectively covalently links the sidechains of these residues by hydrocarbon bridges [313,366]. In the case of peptide B isoforms, this stapling was achieved using oct-4-enyl bridges between residue sidechains at positions *i* and *i* + *4* of their α-helical conformations (Figure 6B) [199], which is regarded as the most effective crosslink for this purpose [368,369]. It was found that this stapling was able to enhance the biological activity of peptide B isoforms; however, this effect was strongly dependent on the location of staples and did not always correlate with staple-mediated α-helix stabilization [199]. This variation is commonly associated with stapled, α-helical AMPs and arises primarily from the hydrophobic nature of hydrocarbon staples and its effects on the levels and distribution of hydrophobicity and amphiphilicity on the surface of these peptides [365,367].

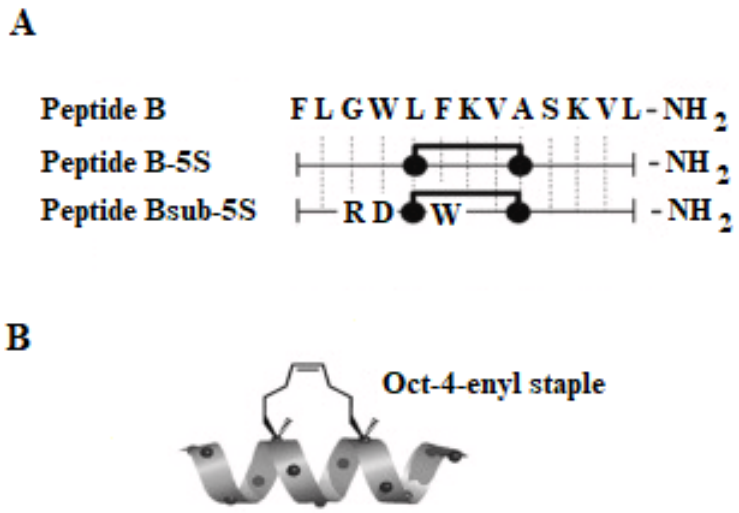

Figure 6. The stapling of AMPs from *G. emeljanovi*. Figure 6 was derived from [199] and shows a schematic representation of the residue stapling involved in the generation of peptide B-5S and peptide B-sub5S from peptide B, which is derived from B1EMa. (**A**) shows the sequences of peptide B-5S (FLGWLFKVASKVL-NH_2) and peptide B-sub5S (FLRDLWKVASKVL-NH_2) where the two black dots connected by a bold line represent residues that are cross-linked via an oct-4-enyl staple. (**B**) schematically illustrates this stapling for peptide B-5S and peptide B-sub5S when in an α-helical conformation and shows that residues linked by oct-4-enyl staples in (**A**) are located at positions i and *i* + *4* of these conformations. Similar stapling arrangements were used in the generation of E2EM15W-S1, E2EM15W-S2 and E2EM15W-S3 from 23D16W, which is derived from E2EM [327].

The stapled isoforms of peptide B, peptide B-5S and peptide B-sub5S were scanned for activity against hepatitis C virus, herpes simplex virus, retrovirus and lentivirus, which revealed an antiviral efficacy that was higher than that of peptide B [199]. In particular, peptide B-5S and peptide B-sub5S showed similar levels of potent activity against retrovirus and lentivirus that were accompanied by comparable levels of cytotoxicity to the human keratinocyte cells, HFK. In the case of retrovirus, the quantification of these levels of activity using a variety of infection models (Table 7) [189] showed them to be comparable to that of many other strongly antiviral AMPs [10]. Relative to peptide B, peptide B-5S was linked at positions L5 and A9 (FLGW<u>L</u>FKV<u>A</u>SKVL-NH_2), whilst peptide B-sub5S

(FLRDLWKVASKVL-NH_2) was both conjoined between positions L5 and A9 and possessed a number of residue substitutions (in bold) (Figure 6A) [199]. These peptides have been proposed as novel, alternative antiviral treatments [370] and an urgent need for agents to combat enveloped viruses is clear given three global pandemics due to highly pathogenic coronaviruses within the last two decades [371,372]. Studies on mechanisms underpinning the antiviral activity of peptide B, peptide B-sub5S and peptide B-5S indicated that a viral entry into host human cells may be a major site of action for these peptides [199]. It is well established that AMPs can target multiple steps in the replication cycle of viruses and host cell entry, which is an early step in this cycle and a common site of action for the membranolytic action of AMPs against enveloped viruses [10,373–375]. For example, the de novo designed peptide, brilacidin and the vespine peptide, MP7-NH_2, were reported to induce the lysis of target viral envelopes, thereby blocking membrane fusion and the entry of these viruses into host cells [376,377]. In the case of peptide B, peptide B-sub5S and peptide B-5S, the disruption of the viral envelope appeared to lead to the aggregation of viral particles and, thereby, a loss of infectivity [199], which has been reported for other antiviral AMPs [10,378,379]. However, these observations clearly suggested that the antiviral action of peptide B, peptide B-sub5S and peptide B-5S was primarily driven by membranolytic mechanisms, which was strongly supported by their lack of activity against non-enveloped viruses [199]. It is generally accepted that due to the lack of a lipid envelope, non-enveloped viruses are either resistant to membranolytic AMPs or show susceptibility to these peptides via non-lytic mechanisms [10,379]. For example, α-defensins inhibited the activity of human papillomavirus by interfering with a post-binding step before endosome escape, whilst θ-defensins inhibited this activity by preventing viral binding and uptake by host cells [380,381].

Peptide B-sub5S and peptide B-5S were strongly membrane-interactive and appeared to inactivate enveloped viruses using membranolytic mechanisms that were driven by the amphiphilicity of their α-helical structures [199], which is the mode of action most commonly used by AMPs against these viruses [10,373,378,379]. However, compared to peptide B, peptide B-5S and peptide subB-5S possessed significantly higher levels of an α-helical structure and amphiphilicity, which corresponded to their generally higher antiviral efficacy (Tables 3 and 7) [199]. These structural enhancements appeared to drive higher levels of interaction with viral envelopes and resulted primarily from the presence of oct-4-enyl staples within the structures of peptide B-5S and peptide subB-5S. Essentially, these staples increased the stability and levels of α-helical structures possessed by these peptides, whilst the hydrophobic properties of these staples promoted changes to the distribution of hydrophobicity on the surface of these structures [199], which is consistent with studies on other stapled AMPs [367]. In the case of peptide B-sub5S, higher levels of amphiphilicity were also promoted by residue substitutions designed to generate a more even balance between the distribution of hydrophobicity and hydrophilicity on the surface of the peptide's α-helical structure [199]. As a result of these residue substitutions, the antiviral activity of peptide B-sub5S was enhanced in relation to peptide B-5S [199]; this form of 'fine-tuning' is a widely used strategy for optimizing the properties and biological activity of stapled AMPs [313,365,367].

Further studies on mechanisms underpinning the antiviral activity of peptide B, peptide B-5S and peptide B-sub5S compared this activity to the size of the target viruses, which suggested that the optimal size for the action of these peptides was circa 200 nM; however, these peptides were ineffective against the vaccinia virus, whose size is around 360 nM [199]. These observations suggested that the ability of these peptides to perturb viral envelopes might be related to high membrane curvature [199] and similar results have been reported for other membranolytic, α-helical AMPs with antiviral activity [373,378,382–385]. Studies on the AH and C5A peptides have suggested that the high curvature of the viral envelope enhances the ability of α-helical AMPs to directly engage in electrostatic and hydrophobic interactions with these envelopes, which could be the case for peptide B, peptide B-5S and peptide B-sub5S [378]. Studies on the AH and C5A peptides have also suggested

that the perturbation of viral envelopes with high curvature by AMPs is promoted by two generic mechanisms involving membrane solubilization and/or pore formation, which, again, could be the case for peptide B, peptide B-5S and peptide B-sub5S [373]. Studies on the influenza virus [386] have suggested that AMPs are likely to solubilize viral envelopes using carpet-type mechanisms and form pores in these envelopes using toroidal pore-type models [16,30,39,42]; however, currently, the membranolytic mechanisms used by antiviral AMPs are poorly understood. [10,373,386]. In addition to their antiviral activity, peptide B-5S and peptide B-sub5S showed varying levels of activity against a variety of Gram-positive and Gram-negative bacteria, notably peptide B-sub5S killed MRSA at levels that were circa twofold higher (Table 7) than those of peptide B (Table 3) [199]. The antibacterial mechanisms of peptide B, peptide B-5S and peptide B-sub5S were not investigated, but given their membranolytic action against viruses, it would seem likely that their antibacterial mechanisms would also involve membrane disruption, as demonstrated for other stapled, α-helical AMPs [387,388].

Table 7. Antimicrobial and cytotoxic activity of stapled B1EMa and E2EM derivatives.

	Peptide B-5S	**Peptide B-sub5S**	**E2EM15 W-S1**	**E2EM15 W-S2**	**E2EM15 W-S3**
Bacteria			MIC (μM)		
B. subtilis	ND	ND	1.8	3.6	3.6
S. aureus	ND	ND	1.8	3.6	3.6
MRSA	>50.0	6.3	ND	ND	ND
S. epidermidis	ND	ND	>120.0	>120.0	60.0
E. coli	ND	ND	>120.0	60.0	60.0
S. dysenteriae	ND	ND	>120.0	30.0	30.0
S. typhimurium	ND	ND	>120.0	>120	>120.0
K. pneumoniae	ND	ND	>120.0	30.0	30.0
P. aeruginosa	ND	ND	>120.0	120.0	>120.0
P. mirabilis	ND	ND	>120.0	>120	>120.0
Viruses			EC_{50} (μM)		
Retrovirus	<8.0	<5.0	ND	ND	ND
Cytotoxicity			CC_{50} (μM)		
Human keratinocytes	>8.0	>6.0	ND	ND	ND

Table 7 was compiled from [199,327] and shows the minimum inhibitory concentration (MIC, μM) of stapled AMPs from *G. emeljanovi*, against a series of bacteria, including the B1EMa derivatives, peptide B-5S (FLGWLFKVASKVL-NH_2) and peptide B-sub5S (FLRDLWKVASKVL-NH_2), and the E2EM derivatives, E2EM15W-S1 (TLKQFAKGVGKWLVK-NH_2), E2EM15W-S2 (TLKQFAKGVGKDLVK-NH_2) and E2EM15W-S3 (TLKQWAKGVGKWLVK-NH_2). For each of these AMPs, stapled residues are underlined. Also shown for the same peptides is the half maximal effective concentration (EC_{50}, μM) of their antiviral activity and the half maximal cytotoxicity concentrations (CC_{50}, μM) for their cytotoxicity to keratinocytes. ND denotes 'not determined'.

Antibacterial activity has also been demonstrated for stapled derivatives of E2EM, namely those derived from 23D16W, which, as described above in Section 5.2.2, was E2EM (1–23) with a D16 → W16 substitution (Table 6) [296,312]. These stapled derivatives were truncated forms of 23D16W, essentially variants of E2EM (5–19) with stapling characteristics [389] similar to those described above in this section for peptide B isoforms [199]. These stapled E2EM (5–19) derivatives were linked at positions A6 and G10 and were E2EM15W-S1 (TLKQFAKGVGKWLVK-NH_2), E2EM15W-S2 (TLKQFAKGVGKDLVK-NH_2) and E2EM15W-S3 (TLKQWAKGVGKWLVK-NH_2). Compared to 23D16W, these peptides showed enhanced activity against Gram-positive bacteria, although no activity towards Gram-negative bacteria (Table 7) [327]. In particular, E2EM15W-S1 showed levels of activity against some Gram-positive bacteria (Table 7) that were over twenty-fold higher than that shown by 23D16W (Table 6) [327]. For example, this stapled peptide showed potent activity against *B. subtilis* (Table 7) [327] and although generally considered to be non-pathogenic, the resistance of the organism to conventional antibiotics is increasingly

being reported [390–392]. Currently, *B. subtilis*, with the potential for pathogenicity, is increasingly prevalent in hospitalized patients [393] and can cause serious conditions, such as bacteremia and meningitis in those with underlying diseases or an immunocompromised state [394,395]. The mechanisms underpinning the antibacterial activity of peptide B-5S, peptide B-sub5S and E2EM15W-S1 were not extensively investigated but appeared to show general similarities to their antiviral mechanisms. Essentially, in the stapling in peptide B-5S, peptide B-sub5S appeared to enhance the stability and amphiphilic characteristics of their α-helical structures, thereby promoting the enhanced levels of the membranolytic, antibacterial activity compared to peptide B [199,327].

Taken in combination, these studies showed that stapled derivatives of AMPs from *G. emeljanovi* have a number of characteristics that are desirable for their development as antimicrobial agents. For example, peptide B-5S and peptide B-sub5S target enveloped viruses on their entry into host human cells [199] and the accessibility of this site of action makes these peptides an attractive proposition for therapeutic intervention [10,373,374]. In relation to both the antiviral and antibacterial activity, stapling enhanced the resistance of these peptides to proteolysis by shielding proteolytic-susceptible sites and reducing the flexibility of their α-helical conformations required for proteolytic action [199,327]. Similar results have been reported for other stapled, α-helical AMPs [367,396] and it is well known that the general susceptibility of unstapled AMPs to enzymatic degradation has largely limited their clinical use to topical applications [61,397]. In contrast, a major disadvantage of peptide B-5S and peptide B-sub5S was their significant cytotoxicity towards human cells, which led to a therapeutic index that was too low for therapeutic application [199]. This poor selectivity is a general problem encountered in the development of antiviral AMPs [373,398] and arises predominantly from the fact that the membrane associated with enveloped viruses is derived from host eukaryotic cells [200,399]. In response, it was proposed that the selectivity of peptide B-5S and peptide B-sub5S for enveloped viruses could be achieved through the further modulation of their amphiphilic properties [199], as demonstrated for other stapled antiviral AMPs [365,366,370]. Indeed, it has been proposed that the stapled AMPs from *G. emeljanovi* studied here (Table 7) could serve as a sequence template for the rational design of antiviral and antibacterial AMPs [199,327,370]. This approach has been highly effective in identifying novel α-helical AMPs [313,365,367,370,373,398] and the structure/function relationships revealed in studies on stapled analogues of E2EM sequences were major drivers in its development [400–403]. Work on E2EM is generally regarded as pioneering the development of hydrocarbon-stapled AMPs and their use not only in an antimicrobial context, but also in other capacities [313,366], ranging from the treatment of cancers to serving as cell-penetrating peptides [313,365–367,370,373,398]. Indeed, an increasing number of stapled α-helical AMPs are being reported [404], including orthologues of E2EM [367], and, most recently, stapled β-hairpin peptides with potent antimicrobial activity have been described [51]. A summary of stapled AMPs from *G. emeljanovi* is given below in Box 6.

Box 6. Stapled AMPs from *G. emeljanovi*.

- Covalently linking the sidechains of residues in α-helical AMPs by hydrocarbon bridges is a general strategy used to enhance their structural stability and selective, antimicrobial activity.
- α-Helical derivatives of B1Ema, peptide B-5S and peptide B-sub5S and E2EM, E2EM15W-S1, E2EM15W-S2 and E2EM15W-S3 were produced with conformations linked by oct-4-enyl staples at residue positions *i* and *i* + *4* (Figure 6).
- E2EM15W-S1, E2EM15W-S2 and E2EM15W-S3 are ineffective against Gram-negative bacteria, but show potent activity towards Gram-positive bacteria, indicating the potential for development to treat infections due to these microbes (Table 7).
- Peptide B-5S and peptide B-sub5S show moderate activity towards enveloped viruses, but also show significant cytotoxicity to human cells. Modified to reduce their cytotoxicity, these peptides show the potential for development to treat viral infections (Table 7).

There is evidence to suggest that the antibacterial action of E2EM15W-S1, E2EM15W-S2 and E2EM15W-S3 and the antiviral action of peptide B-5S and peptide B-sub5S are primarily driven by the amphiphilic properties of these AMPs and membranolytic mechanisms.

6. Discussion

Frogs of the recently established Glandirana genus are found in locations spread over East Asia and include well-established members that were discovered in the late 1800s and 1900s, namely, *G. rugosa*, *G. emeljanovi*, *G. tientaiensis* and *G. minima*. More recent additions to this genus include *G. susurra*, which was identified around a decade ago [82,93], and several potentially new species of frog, including *G. nakamurai* and *G. reliquia*, that were reported in 2020 [87,96,97].

In common with many species of amphibians, populations of those from the Glandirana genus are in decline, as indicated by the IUCN Red List of threatened species. According to this list, populations of *G. rugosa* and *G. emeljanovi* are cases for the least concern, whilst those of *G. tientaiensis*, *G. minima* and *G. susurra* are endangered. In the case of *G. nakamurai* and *G. reliquia*, these frogs are not currently present on the IUCN list of threatened species; presumably their situation in this context is unknown due to the newness of their discovery [405]. Frogs of the Glandirana genus inhabit a variety of aqueous environments, such as marshes, rivers and wetlands, and the major threats to their existence derive from invasive predation and the result of human activities, ranging from pollution to the habitat modification that drives the loss of wet environments [82,405–408]. Although strategies to minimize these declines in amphibian populations are in operation, their loss represents a major threat to global biodiversity [409,410] and the depletion of a rich and valuable source of bioactive peptides and AMPs [70,71].

There have been limited investigations into the AMPs produced by frogs of the Glandirana genus and currently, only those produced by *G. rugosa*, *G. susurra* and *G. emeljanovi* have been sequenced and, to varying degrees, characterized both functionally and phylogenetically. Based on sequence similarity, these peptides have been assigned to various families of AMPs, including brevinin 1, brevinin 2, esculentin 2, ranateurin 2, granuliberin and bradykinin (Figure 1). In the case of AMPs from *G. susurra* and *G. emeljanovi*, it has been shown that the prepropeptides of these peptides have a tripartite organization [84,101], which is typical of preprodermaseptins [411]. Studies on the molecular evolution of preprodermaseptin genes from ranid frogs, including those of B1EMa and E2EM, have suggested that genetic diversification, driven by positive selection, had occurred within the C-terminal, AMPs-coding region of these prepropeptides. Based on these studies, it has been proposed that this diversification of the mature AMPs may form part of a strategy to accelerate the adaptation of host frogs and their immune response to changing ecological niches and microbial predators [411,412]. On this basis, it seems likely that AMPs from *G. rugosa*, *G. susurra* and *G. emeljanovi* and possibly peptides from other frogs in Glandirana arose from an ancestral gene, believed to be circa 150 million years old, that diversified by several rounds of duplication and a subsequent divergence of loci [2]. Interestingly, there is evidence to suggest that the diversity of these AMPs may have, in part, resulted from random substitutions involving the operation of a mutagenic error-prone DNA polymerase [2].

Similar DNA polymerases are increasingly being identified in other eukaryotes as well as prokaryotes and it is becoming increasingly clear that errors are a natural part of DNA replication that provide organisms with the opportunity to accelerate the evolution of genes and their products, with the potential to offer a selective advantage [413–415]. Several characteristics of AMPs from the Glandirana genus could potentially have resulted from the function of error-prone DNA polymerases, for example, the insertion of a premature stop codon in genes encoding brevinin-1 peptides that led to the production of GSSa (Figure 1) [101]. The fact that this truncated brevinin-1 peptide has been retained by *G. susurra* clearly suggests that the peptide's biological activities have endowed the frog with a survival advantage. Another characteristic of AMPs from the Glandirana genus that could have involved error-prone DNA polymerases is their generation of suites of homologous peptides with diverse and multiple overlapping biological functions (Figure 1). These observations are consistent with the view that diversifying positive selection had accelerated the adaptation of these frogs and the suites of AMPs in their innate immune systems to changing environments. Indeed, the production of multiple homologous AMPs appears to be an adaptive strategy that is common to many amphibians [70,71] and a well-studied example is the two suites of peptides that were identified in the skin and brains of frogs belonging to *Bombina* spp. [19]. By analogy to these studies, it would be predicted that the production of suites of structurally related, multifunctional AMPs by *G. rugosa*, *G. susurra* and *G. emeljanovi* serves to maximize the breadth, efficacy and speed of their immune response to microbial threat and other stresses.

The biological activities exhibited by the AMPs and their derivatives obtained from *G. rugosa*, *G. susurra* and *G. emeljanovi* included antibacterial, antifungal, antiviral, anticancer, antioxidative, anti-endotoxin and insulinotrophic action (Tables 1–7). Several of these biological activities were non-antimicrobial and appeared to be related to the overall protection of the host amphibian's skin, namely the antioxidant activity of R2SSa [101] and the insulinotophic action of B1EMb [225]. In vertebrate evolution, amphibians were the first creatures to develop a keratinized tegument, which allowed them to permanently abandon the aquatic environment and become fully terrestrial [416]. Indeed, the skin of amphibians is vital to their survival, not only representing physical protection from both endogenous and exogenous insults, but also serving a wide variety of functions related to respiration, osmoregulation and thermoregulation that are essential to their existence in a terrestrial environment [114,417]. B1EMb is the only AMP from frogs in the Glandirana genus that has been shown to possess insulinotophic activity [225], although this activity has been shown for a variety of other brevinins [70,71,288]. However, a peptide, KC-19, isolated from *Rana saharica* (the Sahara frog) showed insulinotropic activity and circa 68% homology with B2Ra [418]. Our own search of the Swissprot database showed that KC19 also shared comparable levels of homology with E2R and E2EM and based on these observations, it may be fruitful to investigate other AMPs from *G. rugosa* and *G. emeljanovi* for the possession of insulinotrophic activity [102]. The in vivo significance of the insulinotophic action shown by B1EMb was not investigated [225] and the biological role of this action appears to be poorly understood for amphibian AMPs in general [70,235,236]. However, there is evidence to suggest that insulin may play a role in the amphibian wound-healing process, which occurs much faster in amphibians than other vertebrates and appears to be an evolutionary adaptation favorable for survival in their life-styles [114,419]. Consistent with this suggestion, animal studies have shown that both systemic and topical insulin can rapidly improve wound closure, reduce the wound healing time and improve wound remodeling through modifying inflammation, accelerating epithelialization and neovascularization [420,421]. In relation to the antioxidant activity of R2SSa, this is the only AMP from frogs in Glandirana that has been shown to possess this activity [101], although the occurrence of other AMPs with these properties within the genus is likely, given their importance in protecting the host from oxidative stress and UV irradiation [111,114]. For example, it has been suggested that UV radiation is able to breach the skin barrier of amphibians and induce immunosuppression, increasing their susceptibility to microbial

invasion and exposure to chemical contaminants, ultimately resulting in the death of the host creature [422,423]. Threats to amphibian skin from oxidative stress primarily come from reactive oxygen species [111], such as superoxide anions, peroxides, hydroxyl radicals and singlet oxygen, which are able to inflict damage on most biological molecules, including proteins, DNA, RNA and lipids, again ultimately leading to cell death [424,425]. Interestingly, studies on frogs and other amphibians led to the identification of a number of peptides that possessed antioxidant activity, but no antimicrobial activity, hemolytic action or cytotoxicity, and were found to promote wound healing, although the biological significance of these observations is currently unclear [426,427].

In relation to anticancer activity, B1EMa, B1EMb and a number of their derivatives were found to be non-toxic to mammalian cells and to kill a broad range of cancer cells (Table 5); notably, B1EMb and PTP7/12 showed potent activity against MDR cancer cells, suggesting the potential for development as anticancer agents (Tables 3–5) [121]. Currently, a number of AMPs are in clinical trials for the treatment of cancers [428]; for example, LL-37 has completed a phase I trial and LTX-315 is in a phase II trial to evaluate their efficacy against melanoma and solid tumors, respectively [429,430]. In contrast to B1EMa, B1EMb and their derivatives, B2SSb, R2SSa and GSSa were found to possess both anticancer activity and cytotoxicity to healthy mammalian cells (Table 2) and, in combination, these observations belie several major, open questions in relation to AMPs with anticancer activity. Essentially, these questions are what factors differentiate peptides with selectivity for cancer cells from those that do not and what factors endow AMPs with the ability to kill cancer cells? It is well established that not all AMPs possess anticancer activity [117,431,432]. Recent responses to these questions concluded that the anticancer action of AMPs depends upon varying contributions from each of a number of peptide-based and membrane-based factors, with no single factor alone responsible for this action [117,433]. Based on these observations, it was proposed that the anticancer action of AMPs parallels the "lock and key" model postulated for enzyme activity, where the molecular architecture of the peptide has to support its binding and insertion into a membrane of a given composition [117]. It would seem that, given their clear homology, AMPs from the Glandirana genus may be an appropriate paradigm to test this proposal. For example, the sequence of brevinine-1SSc (B1SSc) from *G. susurra* differs to that of B1EMb only in the C-terminal Rana box region (Table 1), which is believed to serve an anchoring function in the membrane interactions of the latter peptide (Figure 2). Currently, B1SSc has not been characterized and investigating this peptide for anticancer activity could illuminate the potential role and contributions of both its C-terminal region and that of B1EMb to such activity.

The vast majority of AMPs from *G. rugosa*, *G. susurra* and *G. emeljanovi* do not appear to have been investigated for antiviral activity, which has only been reported for derivatives of B1EMa from *G. emeljanovi* (Tables 3 and 7). However, these peptides possessed the potential for development as novel antiviral agents and, currently, AMPs are viewed as possible therapies for viral infections or for use as prophylactic agents to prevent viral spread [10]. In contrast, antifungal activity has been demonstrated for most major AMPs and several of their derivatives in the case of *G. rugosa*, *G. susurra* and *G. emeljanovi*, although, in general, this research has been limited (Tables 2–4 and 6). However, in the case of B2SSb, R2SSa and GSSa, it has been suggested that these the AMPs have the potential for development as novel agents to combat fungal phytopathogens in crop protection [101] and, in the case of E2EM-lin, it has been proposed that the peptide could be developed as a thermostable, antifungal agent in the food industry [290]. It is also interesting to note that another major cause in the global decline of amphibian species is infections due to *Batrachochytrium dendrobatidis* and this fungal pathogen has been reported throughout East Asia where many strains are believed to be endemic [434–436]. However, infections due to *B. dendrobatidis* appear to have only been reported for *G. rugosa* and *G. emeljanovi* and the prevalence of these infections is low [435,437]. Based on these observations, it is tempting to speculate that the antifungal AMPs and defence systems possessed by frogs of the Glandirana genus may help protect them from infection by *B. dendrobatidis*.

The vast majority of research on AMPs from frogs in Glandirana has focused on their antibacterial activity, which showed that peptides from *G. rugosa* and *G. emeljanovi*, including B2Ra, B2EMa, B2EMb, B1EMa, B1EMb, E2EM and their derivatives, generally possess a strong preference for Gram-positive bacteria and exhibit potent activity against a broad spectrum of these organisms (Tables 2–4 and 6). Based on this preference, potential uses for these peptides include serving as: oral antibacterial agents in mouthwash [198,221], antibacterial packaging components in the food industry [290,299], lead compounds in the development of hydrocarbon-'stapled' antibacterial and antiviral peptides [313,370] and antimycobacterial and anti-staphylococcal agents, including MRSA, in nosocomial settings [190,199] (Tables 2–4 and 6). Investigations into mechanisms underpinning the relative resistance of Gram-negative bacteria to AMPs from *G. rugosa* and *G. emeljanovi* appear to have been limited. However, studies on PTP7/12, E2EM and E2EM-lin have suggested that these mechanisms involve contributions from the barrier function of the OM and the decreased negative charge/levels of anionic lipids found in the CM of these organisms, as compared to Gram-positive organisms [211,297,300]. Studies on E2EM-lin have also suggested that potentially, the high affinity of the peptide for CL may attenuate its activity against both the latter organisms and Gram-positive bacteria, but in general, the ability of Gram-negative bacteria to resist the action of AMPs from frogs in Glandirana is poorly understood [300]. In contrast, a number of peptides from *G. susurra* and *G. rugosa*, including B2Rb and B2SSb, showed no particular bacterial preference and exhibited broad spectrum activity against both Gram-positive and Gram-negative bacteria (Tables 1 and 2). In the case of B2SSb, this broad-spectrum antibacterial activity was accompanied by an ability to neutralize the endotoxins of these respective bacterial classes, namely LTA and LPS [82], which is known to lead to an anti-inflammatory effect and promote the process of wound healing [169,174,438], as demonstrated for the AMPs of other amphibians [171,439]. In particular, B2SSb showed a high affinity for LPS, suggesting that the peptide may have the potential for development as an agent to combat infections due to pathogenic Gram-negative bacteria, which currently pose a dire threat to human health [440,441]. For example, the peptide showed activity against *P. aeruginosa* (Table 2), which is the main cause of morbidity and mortality in cystic fibrosis (CF) patients, as well as being a leading nosocomial pathogen [442,443]. Indeed, it has been shown that in CF, this organism synthesizes LPS with structures that are unique to the airway environment and are able to both promote resistance to conventional antibiotic AMPs and generate increased or unique inflammatory responses [443–445]. Currently, *P. aeruginosa* is classed as a high-priority pathogen by the WHO [65] and there is an urgent requirement for novel agents to combat infections due to this organism [443,446,447].

An investigation into the antimicrobial, anticancer and insulinotrophic activities of AMPs produced by frogs in Glandirana have primarily focused on B1EMa, B1EMb and E2EM (Tables 2–6) and have shown that these peptides exhibit similar functional organization: an N-terminal, a membrane-penetrating domain flanked by a C-terminal anchoring region [55,86,119–122,225]. In each case, this latter region included a Rana box motif and the integrity of this motif appeared to be necessary for the biological activity of B1EMb [86,121,225], but not B1EMa or E2EM [55,86]. A similar functional promiscuity has been reported to exist for the Rana box motifs of other ranid AMPs and, in combination, these results reinforce the view that the integrity of this motif is not a universal requirement for the biological action of these peptides [71,103]. The seven-residue structural arrangement for the Rana boxes of AMPs produced by *G. rugosa*, *G. emeljanovi* and most of those identified in *G. sussura* is the general case for ranid frogs. However, reflecting evolutionary divergence, *G. sussura* also produced AMPs with Rana box motifs that had shorter lengths and others that were devoid of this C-terminal moiety (Figure 1). Interestingly, as described above in Sections 5.1.2 and 5.2.2, Rana box-type sequences with functional and structural similarities to the Rana box regions of B1EMa and E2EM have been identified in AMPs produced by organisms in other phyla, namely, thanatin from insects [265] and nicomicin-1 from marine worms [326]. It has been proposed that this coincidence of structure/function

relationships arose through convergent evolution [448] and similar examples have been reported in other AMPs, including muscin from the house fly *Musca domestica* [449], oxyopinin 4a, from the lynx spider, *Oxyopes takobius*, [448] and kaliocin-1, which is derived from the human protein, lactoferrin [450,451].

The N-terminal, membrane-penetrating domain of B1EMa, B1EMb and E2EM showed some similarities in their functional organization, with each possessing a lipid-interactive α-helical structure that included a putative tilted segment and was terminated by a flanking cluster of strongly hydrophobic residues (Figure 3). In concert with their C-terminal anchoring regions, these N-terminal domains promoted the ability of B1EMa, B1EMb and E2EM to induce membrane lysis via pore formation and in each case, it has been suggested that these peptides may utilize a toroidal pore-type mechanism [86,251,289]. In the case of B1EMa and B1EMb, these peptides are believed to interact with membranes via the formation of a continuous, curved amphiphilic α-helix, primarily induced by a proline kink at position 14 of their sequences (Figure 3). In general, the mechanisms underpinning pore formation by B1EMa and B1EMb are poorly understood [86,119–122,225], although the differing structural characteristics of these peptides suggest that there may be differences between these mechanisms [86]. For example, compared to B1EMa (1–13), B1EMb (1–13) has much greater hydrophobicity and far lower amphiphilicity, which could clearly lead to differences in the ability of these peptides for tilted insertion and the formation of toroidal pores in membranes (Figure 2). In addition, the positive charge of both peptides results from the presence of lysine residues in their primary structure and the differing number and distribution of these residues in B1EMa and B1EMb could also lead to differences in the ability of these peptides to interact with membranes. B1EMb possesses three fewer lysine residues than B1EMa, which could reduce its relative ability to target and bind membranes, as well as decreasing the potential of the peptide to utilize the snorkeling mechanism compared to that of B1EMa (Figures 1–3).

In contrast to B1EMa and B1EMb, E2EM-lin forms two discontinuous amphiphilic α-helical domains that appear to interact with the membrane such that its N-terminal α-helical domain lines the lumen of toroidal pores (Figure 5D,E). The membrane interactions of E2EM-lin were enhanced by the alkaline pH via mechanisms that appeared to involve the formation of increased levels of a tilted structure in its N-terminal domain under these pH conditions [290,299,300]. Currently, the biological significance of this pH dependence is unclear, but one possibility may be that the peptide has a role in controlling wound-associated infections; it is well established that an alkaline pH promotes the microbial colonization of wound sites [452,453]. Indeed, it has previously been suggested that E2EM-lin has the potential for development as a therapeutic agent to treat not only wounds, but also other diseases and conditions that are associated with an alkaline pH [290], such as bacterial prostatitis, psoriasis, acne, atopic dermatitis and urinary infections [454–456]. The pH-dependent antibacterial activity shown by E2EM-lin (Figure 5) [299,300], taken with its antifungal activity and high thermostability [172], could also enhance the previously proposed potential of the peptide for development as an antimicrobial in the food industry [290,299]. A variety of fungi and Gram-positive bacteria with tolerance to a high pH and temperatures are known to act as food-spoilage organisms [457] and a number of AMPs with alkaline optima have been investigated for their ability to serve as antimicrobials within this context [172,458–461]. Based on these observations, it can be envisaged that E2EM-lin, or its derivatives, may find application in active packaging materials that are being developed by food preservation technology to help maintain the safety and quality of food. These packaging materials are formed from polymers which incorporate AMPs and are able to prolong the shelf life of the food product by inhibiting microbial growth on the surface of the product or the headspace inside the packaging [462,463]. Given that E2EM/E2EM-lin are members of the family of esculentin 2 peptides, and therefore show homology with other amphibian AMPs, it seems possible that peptides both within and without the Glandirana genus could show a similar pH dependency [70,76]. Within the Glandirana genus, E2R and E2SSa are the only other known esculentin 2 peptides and they

show a very high homology with E2EM/E2EM-lin (Figure 1). E2R and E2SSa also show a similar sequence organization to E2EM/E2EM-lin with an N-terminal domain and a C-terminal Rana box region that are separated by a glycine residue at position 24 (Figure 1), although, as described above in Sections 3 and 4, these peptide have yet to be fully characterized [85,86,101]. However, outside of this genus, the only known pH-dependent amphibian AMP with an alkaline optimum for antibacterial activity and homology to E2EM/E2EM-lin is FL9, which is a derivative of fallaxin, from *Leptodactylus fallax* (the Mountain Chicken frog) [459,460]. FL9 shows the potential to form a tilted structure that possesses a homology with the N-terminal tilted region of E2EM/E2EM-lin, which led to the suggestion that the antimicrobial action of FL9 may show similarities to that of E2EM/E2EM-lin [172,290]. Indeed, studies on E2EM-lin have clearly demonstrated and emphasized the importance of considering pH when characterizing the antimicrobial action of not only this peptide, but also AMPs in general [290,299], given that the data cited in the literature to describe the action of AMPs is usually determined under neutral pH conditions [118]. It is suggested that a more holistic approach to characterizing the antimicrobial action of AMPs would be appropriate and should include determining the optimal pH for the action of these peptides as a matter of course [464,465].

In summary, this review has shown that peptides from frogs of the Glandirana genus provide a comprehensive paradigm for the antimicrobial, anticancer and other biological activities of AMPs, as well as the potential therapeutic and biotechnical uses of these peptides. This review also includes extensive tabulated data on the homology and biological activities of AMPs from frogs of the Glandirana genus, allowing comparisons between the structure/function relationships of these peptides, both those within a given species and between those of multiple species. Potentially, these data may also aid the elucidation of structure/function relationships in AMPs from newly identified frogs, both those from Glandirana, such as *G. nakamurai* and *G. reliquia* (Section 2) [87,96,97], and those from other genera, such as recently reported members of the Boophis genus in Madagascar [466]. Indeed, currently, over 7700 species of frogs are known and well over half of these have been described since the mid-1980s [80], which is around the time that the first AMPs from frogs were reported by Zasloff and colleagues [15]. It would seem that not only frogs of the Glandirana genus, but anurans in general represent an untapped source of many more novel AMPs and biologically active peptides with the potential for medical and commercial exploitation.

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