Therapeutic Potential of Host Defense Peptides in Antibiotic-resistant Infections

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Abstract: The emergence of infections caused by multi-drug resistant (MDR) pathogens pose a major burden to modern healthcare. Exacerbating this issue is the substantial decline in development of new classes of antibiotics by pharmaceutical companies. This has led to renewed interest in the therapeutic potential of natural anti-infective agents such as host defense peptides (HDPs). The broad antimicrobial and immunomodulatory activities of HDPs and their synthetic derivatives, coupled with the fact that they do not readily induce microbial resistance, makes them extremely valuable leads in the development of new treatment strategies for MDR infections. This review examines our knowledge of the mechanisms behind multi-drug resistance as well as the properties of HDPs and their therapeutic potential, especially in the case of MDR infections. Challenges to their development as new therapeutics are also discussed.

Keywords: Host-defense peptide, multi-drug resistant bacteria, immunomodulatory, immunity, infection, therapeutic, chemoattractant, inflammation.

INTRODUCTION

Major medical advances in prevention and treatment of infectious diseases have vastly improved quality of life and significantly increased life expectancy. This is largely due to the discovery and development of antibiotics. The discovery of penicillin in 1928 ushered in the golden age of antibiotic innovation (1940s-1960s), during which the majority of antibiotic families in use today were identified [1]. Widespread and often inappropriate exploitation of these antibiotics has resulted in the emergence of multi-drug resistant (MDR) pathogens. Infections caused by MDR pathogens are a major burden to modern healthcare as a result of high morbidity/mortality rates, and the higher treatment cost of MDR infections [2]. Compounding this issue is the fact that the current rate of antibiotic development has declined substantially, with only six new antibiotics, none of which were truly innovative, approved since 2003 [3]. Pharmaceutical drug discovery and development is a lengthy (8-12 years) and expensive ($400–$800 million per approved agent) process, with many regulatory hurdles and low success rates [1, 4-7]. Anti-infective agents are generally used in short-course treatment of acute infections and there are significant market restrictions placed on newly developed anti-infectives. Thus, pharmaceutical companies looking for the greatest return on investment have shifted their focus to the development of drugs for chronic conditions such as hypertension, depression and arthritis [4].

Recent antibiotic research, especially target-based research, utilizing genomics, combinatorial chemistry and high-throughput screening, has not been as productive as expected. This led to a push to re-examine the therapeutic potential of natural compounds and their derivatives and to explore new concepts and approaches. Cationic host defense peptides (HDPs) and their synthetic derivatives constitute one such class of compounds, exhibiting broad-spectrum anti-infective activity through both direct and adjunctive (immunomodulatory) action.

HDPs are produced by virtually all forms of life and are an evolutionarily ancient and apparently essential component of the host innate immune response to infectious agents. There have been more than 1200 HDPs identified or predicted, approximately 940 of which are found in eukaryotic organisms. A comprehensive list of HDPs can be found at http://aps.unmc.edu/AP/main.php [8, 9]. The broad range anti-infective activity of HDPs stems from the diversity of their functions including direct microbial killing; endotoxin neutralization; immune cell recruitment; modulation of pro-cytokine/chemokine production; suppression of potentially harmful inflammation; induction of cell differentiation; and enhancement of adaptive immune responses, cell survival, wound healing and angiogenesis. Interestingly, antimicrobial HDPs have retained their anti-infective activities over millions of years with few pathogens developing resistance mechanisms to them, while the broad immunomodulatory activities are not liable to resistance development, making them extremely attractive candidates for novel therapeutics targeting MDR pathogens. This review explores our current knowledge of HDPs and their synthetic derivatives as well as their potential use as novel therapeutics to combat MDR pathogens. Challenges facing their development are also discussed.

MULTI-DRUG RESISTANT BACTERIA

Until the 1960’s, numerous classes of antibiotics (both natural and synthetic) were introduced into clinical practice, including sulfonamides, β-lactams, phenyl propaniods, polypeptides, aminoglycosides, macrolides, and quinolones. However, the rapid development of resistance to these drugs upon their introduction was under appreciated. Less than a year after the first-generation penicillin was used to treat Staphylococcus infections, penicillin-resistant Staphylococcus aureus strains were discovered. With few exceptions, the introduction of each new antibiotic has been followed, within a few years, by the first cases of resistance.

Over the last two decades, an alarming number of MDR pathogens have been identified [10]. For example, methicillin-resistant S. aureus (MRSA) is not only resistant to methicillin but often to aminoglycosides, macrolides, tetracycline, chloramphenicol and lincomycin [11]. MRSA is currently a major source of hospital-acquired infections associated with significant morbidity and mortality [12]. Although MRSA infections are treatable with vancomycin, vancomycin resistant-MRSA strains have been reported [13]. Strains of several other pathogens exhibit resistance to multiple or essentially all available antibacterial agents, notably Enterococcus faecium, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.; known as the ESKAPE’ pathogens [10]. Data published in 2004 by the US National Nosocomial Infection Surveillance (NNIS) System reported a substantial increase, from 1992 to 2004, in the resistance rate and prevalence of multidrug resistant Gram-negative pathogens among healthcare-associated infections, especially ICUs. The NNIS also reported that 5.8% and nearly 21% of Escherichia coli and K. pneumoniae strains were multiresistant. These trends are of great concern and underline the urgency of developing alternative therapeutic strategies to combat drug-resistant infections.

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Multidrug resistance in bacteria is generated by various mechanisms, including acquisition of resistance plasmids, transposons or integrons with genes encoding for resistance to specific antimicrobial agents, and/or induction due to mutation of regulatory loci controlling for example increased expression of multidrug efflux pumps that extrude a wide range of drugs [11]. Owing to the target specificity of each antibiotic, bacteria can become resistant through mutations that make the target less susceptible. For example, mutations in target enzymes, such as DNA topoisomerases, have been frequently identified in fluoroquinolone-resistant bacteria [15]. Resistance can also develop for non-proteinaceous targets, whereby an altered substrate structure is found in the resistant strain. For example, vancomycin binds to the lipid-linked disaccharide-pentapeptide, which is a precursor of cell wall peptidoglycan and consequently inhibits bacterial cell wall biosynthesis. In some vancomycin-resistant bacteria the substrate for vancomycin (the terminal two amino acids of the stem pentapeptide), D-Ala-D-Ala, is changed to D-Ala-D-lactic acid, reducing the binding affinity of vancomycin to its substrate [16]. In addition, bacteria may acquire, from other species, genes that encode less susceptible target proteins [17]. For example, MRSA expresses an alternative methicillin-resistant penicillin binding protein called PBP-2A. The gene for PBP-2A is located in the chromosome on a so-called genomic island that may have originated from *Staphylococcus sciuri* [18]. Bacteria may also acquire plasmids with genes encoding antibiotic inactivating enzymes; β-lactams such as penicillins and cephalosporins can be inactivated by plasmid-borne or derepressed chromosomal β-lactamases [19]. Additional resistance mechanisms include reduced drug access to the target by acquiring genes encoding for drug-specific active efflux pumps [20]. As well, porin deficiency has been identified in some Gram-negative bacteria and appears to prevent influx of many antibiotics [21].

Multidrug resistance is not limited to bacteria, but can also be observed in other pathogens including parasites, viruses, and fungi. There are reports of widespread resistance to earlier generation antimalarial drugs, such as chloroquine and sulfadoxin-pyrimethamine in most malaria-endemic countries [22]. The emergence of resistance is also an increasing concern for treatment of HIV infections [23]; and due to acquired resistance to antifungal therapies, higher mortality and/or morbidity rates from invasive mycosis have been observed [24, 25].

The return to the pre-antibiotic era is rapidly becoming a reality in many parts of the world [26] and new strategies to prevent the spread of potentially lethal multi-drug resistant pathogens are urgently needed. One strategy garnering a lot of attention is the development of enhanced derivatives of the natural anti-infective molecules, HDPs, as novel therapeutics.

**PHYSICAL PROPERTIES OF HOST DEFENSE PEPTIDES**

**Structure**

Essentially all species of life produce HDPs, with individual organisms producing not only different classes of peptides but also a number of variants within each class. These peptides exhibit enormous diversity in sequence, size and secondary structures, however there are some common physical characteristics that define HDPs. They are generally between 12 and 50 amino acids in length with a predominance of basic over acidic amino acids, conferring a net positive charge of +2 to +9 [27]. In addition, approximately 50% of the amino acids are hydrophobic. The presence of basic and hydrophobic amino acids promotes folding of linear HDPs into amphipathic secondary structures upon contact with lipid bilayers [28]. The secondary structures adopted by HDPs are used as the basis of their classification with four structural classes having been established. The two most common classes are β-sheet (e.g. defensins) and α-helical (e.g. cathelicidins, magainin) peptides. Less common are looped peptides (e.g. bactenecin) and extended structures rich in arginine, glycine, histidine, proline, and/or tryptophan (e.g. indolicidin) [8, 29]. Structure-function studies suggest that certain biological properties of HDPs are dependent on specific structural characteristics.

The biological activities of HDPs are initiated through their interaction with cellular membranes. In their antibacterial action, first, they bind to the negatively charged surface of bacterial membranes then, through hydrophobic and electrostatic interactions, insert into the membrane. The charge, hydrophobicity and amphipathicity are important to HDP function and explain why initial HDP structure-function studies focused on these characteristics in relation to *in vitro* antimicrobial activity [30-34]. In addition, the disulfide bonds found in the β-sheet and β-hairpin classes are essential for biological activity through stabilization of secondary structure [30, 35, 36]. For example the chemotactic and antimicrobial activity of the human β-defensin 3 (HBD-3) appears to be dependent on correct disulfide bond formation and peptide folding [30, 35]. It should be stated that the *in vitro* antimicrobial activity examined in these studies is likely to often have a limited physiological role due to the high inhibitory mono- and di-valent cation concentrations and polyanionic glycosaminoglycans and mucins found in biological fluids *in vivo* [37-39]. Nevertheless in circumstances in which these peptides are present in very high concentrations such as in phagocytic granules, in the immediate vicinity of degranulating phagocytes, and in the crypts of the intestine it seems likely that their direct antimicrobial activity could influence the outcome of infections. At lower concentrations the immunomodulatory activities might predominate as these are less affected by physiological salt concentrations.

To gain greater insight into the structure-function relationships behind HDP antimicrobial activity, Cherkasov et al. used the bovine cathelicidin bacitracin as the initial template to iteratively generate two large 9-amino acid peptide libraries with enhanced biological activity [40]. Experimental data generated from these two libraries was used in conjunction with machine learning quantitative structure activity (QSAR) approaches (discussed below), to relate atomic-resolution physical-chemical descriptors of HDPs to antimicrobial activity. Using this approach, the antimicrobial activities of 100,000 virtual peptides were effectively identified. Based on this study it is clear that the structure-function relationship is far more complex than once thought as the peptides predicted to be the most active were nearly identical in charge, hydrophobicity and amphipathicity to the inactive peptides.

There have been far fewer *in vitro* studies into the structural requirements for immunomodulation, likely owing to the large number of immunomodulatory activities each HDP possesses. One *in vitro* study identified the N-terminal portion of the human cathelicidin LL-37 (residues 1-13) as associated with its chemotactic activity and ability to oligomerize [41, 42]. QSAR methodology has also been exploited to generate synthetic HDPs with enhanced immunomodulatory activities, known as Innate Defense Regulators (IDRs). For example, IDR-1, IDR-1002 and IDR-1018 were loose derivatives of Bac2A, a linear derivative of bacitracin that possesses both antimicrobial and immunomodulatory activities [43, 44]. Structure-function studies revealed that IDRs such as IDR-1018 adopt different conformations, depending the makeup of the membrane of target cells. In the presence of bacterial model membranes and mammalian model membranes, IDR-1018 adopts a β-turn and an α-helical conformation, respectively. In the presence of mammalian model membranes Bac2A also adopts an α-helical conformation however, its mean residue ellipticity differs from IDR-1018, suggesting that IDR-1018 is capable of stronger membrane interactions and is therefore likely to be more effective at translocating into cells [44, 45]. This may explain why IDRs are better immunomodulators, since many immunomodulatory activities depend on the ability of HDPs to traverse the plasma membrane and...
interact with specific intracellular targets and receptors such as sequestosome-1, a key intracellular receptor of IDR-1 [46]. Further structural studies are required to determine if the ability to adopt α-helical conformations is a major factor in immunomodulatory activity and to identify other physical characteristics that regulate the immunomodulatory properties of peptides.

**Expression**

HDPs are encoded as individual genes, with HDP families often clustered together within the genome. For example, the human α- and β-defensin genes co-localize to chromosome 8p21-23, while the sole cathelicidin LL-37 is found on chromosome 3p21.3 [9]. Regulation of gene expression varies by individual peptide, species, tissue, cell type, and differentiation state of a cell. Their expression is coordinately regulated at the transcriptional and post-translational levels and can be constitutive or up-regulated by inflammatory/immune stimuli such as immune mediators (e.g. cytokines) pathogens, or pathogen components (e.g. bacterial lipopolysaccharide). HDPs are synthesized as inactive pro-forms, likely for intracellular storage purposes. During or after secretion they are proteolytically cleaved, releasing the biologically active peptide; thus the activity of HDPs is not only dependent on gene expression but also on the presence of specific proteases at the site of HDP secretion [9].

Despite substantial variation in HDP expression patterns, sequence, size, secondary structures, and anatomical site of production, all HDPs possess one or both of the two major functions discussed below, immunomodulation or direct microbicidal activity.

**Mechanisms of Action**

**I. Antimicrobial Activities**

When investigating the direct antimicrobial activity of HDPs it is appropriate to refer to them as antimicrobial peptides (AMPs); which represent a promising lead for new, effective treatments for microbial diseases. AMPs initially attracted attention as alternative antibiotic candidates due to their ability to destabilize bacterial, fungal and possibly viral membranes [27], but the complexity of these molecules and their heterogeneity of action are now increasingly appreciated Fig. (1). Thus AMPs have been called ‘dirty drugs’ since they disrupt many biological processes in the target microbe with modest potency rather than acting on a specific target with high-affinity, reducing the selective pressure for de novo resistance development [47]. Virtually all AMPs are directly antimicrobial in vitro under appropriate conditions (e.g. in buffer or dilute medium). However, under physiological conditions, the direct bacterial activity is often antagonized, as discussed above[48]. For example, LL-37 is protective against bacterial infections in vivo and exhibits direct bactericidal activity in phosphate buffer but does not reduce the bacterial load in physiologically relevant tissue culture medium [49].

Despite identification of an ever-growing number of AMPs (both natural and synthetic), their mode of action is only slowly being elucidated. The direct antimicrobial activity of HDPs is largely attributed to their ability to fold into structures that have both cationic and hydrophobic domains [47], although studies with synthetic peptides have revealed that the physical determinants of activity are very complex [40]. The overall net positive charge ensures initial attraction with and accumulation at the polyanionic microbial cell surface and insertion into anionic membranes of the Gram-negative and Gram-positive bacteria [47]. To reach the cytoplasmic membrane of bacterial cells, HDPs must cross the outer membrane and peptidoglycan of Gram-negative bacteria or the thicker peptidoglycan that contains associated anionic polymers such as lipoteichoic acid in Gram-positive bacteria. Since the interaction of cationic peptides with the outer membrane of Gram-negative bacteria is better understood, it will be described here. Following the initial binding of HDPs to the outer membrane, the peptides initiate their own passage across the outer membrane via the ‘self-promoted uptake’ mechanism [50]. The peptides possess greater affinity for the negatively charged surface LPS than native divalent cations, such as Ca²⁺ and Mg²⁺, and as a result, the divalent cations are displaced, causing areas of disruption in the outer membrane. This enables the translocation across the outer membrane of the peptides, which can then associate with the phospholipids of the cytoplasmic membrane. Initially, the peptides insert at the interface between phospholipid head groups of the outer leaflet and the fatty acyl tails in parallel to the plane of the membrane [51]. Once the number of interacting peptides reaches a critical level, the peptides are proposed to aggregate or associate, and perturb the membrane bilayer. There is substantial disagreement as to what happens next; several hypotheses, including the barrel stave, torroidal pore, carpet and aggregate models (see refs [52-54] for an overview of these mechanisms), aim to explain peptide action on bacterial membranes in terms of lysis or membrane busting. However there is an increasing appreciation that although membrane busting does occur in model systems at high enough peptide concentrations, it is unlikely to be the actual mechanism in many cases [47].

Indeed we propose that membrane perturbation likely leads to one of (at least) three possible fates: (a) perturbation of membrane associated functions such as peptidoglycan biosynthesis, [55] energy generation, [56] cell division etc, (b) disruption of the physical integrity of the bilayer, through membrane thinning, transient poration and/or disruption of the barrier function [57] and/or (c) translocation across the cytoplasmic membrane to act on various cyto-

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**Fig. (1).** Antimicrobial activity of host defence peptides (HDPs). The figure represents the different antimicrobial mechanisms of action exhibited by HDPs, such as disruption of the cytoplasmic membrane (1) or interaction with intracelllar targets and disruption of cellular processes including DNA/RNA synthesis (2), interfering with protein synthesis (3), protein folding (4) and cell wall synthesis.
plasmic targets [47]. While the various models can explain the ability of cationic peptides to disrupt the cytoplasmic membrane, the aggregate model further describes how the peptides can cross the membrane to act on cytoplasmic targets [47]. It is worth mentioning that many peptides are also able to freely translocate across the membranes of eukaryotic cells and have properties that strongly resemble cell penetrating peptides [58].

An increasing number of HDPs have been suggested to act on internal targets, either as their major mechanism of action or in addition to membrane perturbation [47]. For example, HBD-3 and plecasin, a fungal defensin, act by binding to the bacterial cell-wall precursor Lipid II to inhibit cell wall biosynthesis [55, 59]. Other proposed cytoplasmic and/or membrane targets of peptides include DNA/RNA synthesis, condensation of intracellular DNA, protein synthesis/folding, cell wall synthesis/integrity and cell division [47, 60]. Pyrrocycin and its analogues interact with DNAK, a heat-shock protein involved in chaperone-assisted protein folding, inhibiting its ATPase activity, which results in accumulation of misfolded proteins and cell death [61].

Additionally, certain cationic HDPs at sub-inhibitory concentrations can also prevent bacterial biofilm formation, which is strongly inhibited by the development of chronic infections. Established biofilms protect bacteria from host defense mechanisms and antibiotic therapy [62, 63]. Studies by Overhage et al. and Hall et al. demonstrated that at concentrations well below its minimum inhibitory concentration, LL-37 strongly inhibited biofilm formation and had an effect on existing P. aeruginosa and S. epidermidis biofilms [64, 65]. Overhage et al. tested a range of peptides and found this selective action against biofilms occurred only with certain peptides, e.g. with bovine indolicidin but not polymyxin B or bovine bactenecin.

HDPs have also been studied for their antiviral activity against a wide spectrum of viruses. Peptides can inhibit viruses through a wide variety of mechanisms involving almost every part of the viral life cycle [66]. For example, Dermaseptin directly interacts with the HIV virion, disrupting its membrane [67]. Antiviral peptides such as bovine and human lactoferricin can block viral entry by interacting with glycosaminoglycans on mammalian cell surfaces, specifically heparin sulfate, which are important for viral attachment [68, 69]. The spread of virions from one cell to a neighboring cell across tight junctions can also be inhibited by antiviral peptides such as the rabies virus inhibitor NP-1, which inhibits cell-to-cell spread of HSV [70]. Antiviral peptides have also been demonstrated to block viral entry or attachment by interacting directly with specific viral receptors on the host cell and glycoproteins in the viral envelope. For example, the polyphemusin analogue T22 binds to the chemokine receptor, CCR4, the T-cell surface protein CD4 and the viral envelope protein gp120 to inhibit binding and fusion of the HIV envelope with the host cell membrane [71, 72]. Antiviral peptides may also interact with and permeabilize the host cell membrane to interfere with viral entry. For instance, an eight-residue cyclic DL-α-peptide lowers the pH in endocytic vesicles, which inhibits entry of adenovirus particles through this pathway [73]. One major mechanism for the action of such peptides was revealed by studies that showed that antiviral peptides, such as LL-37 and human and bovine lactoferricin, can translocate across the plasma and nuclear membranes of host cells [74, 75]. Consequently, the internalized peptides could enhance host cell antiviral mechanisms [49] or block viral gene/protein expression.

Magainins and cecropins were among the first HDPs reported to display anti-parasitic activities [76], with magainin-2 causing swelling and eventual rupture of Paramecium caudatum [77]. Since then, anti-parasitic activities have been reported for numerous natural and synthetic HDPs [78, 79]. The underlying mechanism appears to be membrane disruption [80]. Due to differences in membrane composition, anti-parasitic peptides can exhibit a higher affinity for the membranes of infected erythrocytes than for normal erythrocytes [81, 82]. Hemolytic peptides induce hemoglobin leakage and parasite starvation while non-hemolytic peptides enter infected host cells and bind to the parasitic membrane [22]. Once bound, the peptides can disrupt the plasma membrane of the parasite modifying the parasite’s membrane properties (e.g. charge and fluidity of lipid and protein components) to interfere with their normal functions and following internalization, disrupt essential biological processes [80, 82, 83].

An increasing number of antifungal peptides have been reported that induce cell lysis, interfere with cell wall synthesis, inhibit cellular energization and depolymerize the actin cytoskeleton [66]. Moerman et al. demonstrated that α-helical pore-forming peptides isolated from scorpion venom exhibited antifungal activities via permeabilization of fungal membranes [84]. Pn-AMP1, a small cysteine-rich peptide, caused depolymerization of the actin cytoskeleton in Saccharomyces cerevisiae and C. albicans [85]. Antifungal peptides can also enter the cytoplasm; the cidal activity of the glycine-rich peptide tenezin-3 is not a result of membrane permeabilization or depolarization but involves peptide entry into the cytoplasm [86].

II. Immunomodulatory Activities

The innate immune system is the first line of defense against microorganisms and is evolutionarily quite conserved among invertebrate and vertebrate animals. It is recognized as the most ancient arm of the immune system and its effectiveness is characterized by a rapid and effective response, intended to quickly eliminate threats. HDPs are an important component of the innate immune system. They are produced by many cells including epithelial and immune cells [87-89], acting at the site of infection in an autocrine and/or paracrine manner. HDPs can exhibit robust immunomodulatory activities and/or have direct antimicrobial activity Fig. (2). However, as mentioned above, under physiological conditions their antimicrobial activities are considerably dampened which is not the case for their selective immunomodulatory activities, suggesting that these are the more likely explanation for their protective effects against microbes in vivo [37, 49]. These selective immunomodulatory capabilities make HDP the starting point for novel therapies against multi-drug resistant infections, acting on the host to boost protective immunity while modulating excessive inflammation [90]. Some of the most relevant immunomodulatory functions displayed by HDP are described below.

Chemotactic Activity

The presence of microorganisms, induces the local production and/or release of chemo-attractant (chemotactic) agents such as chemokines and HDPs, which are capable, with varying efficiency, of recruiting immune cells to the site of infection. HDPs also induce the expression of a broad range of chemokines such as CXCL8/IL8, CCL2/MCP1 by neutrophils, monocytes and other immune cells [91, 92]. These direct and indirect chemotactic activities are a conserved ability among HDPs, making this a strategic tool for screening and selecting for improved synthetic immunomodulatory HDPs (IDRs). This method was recently used to select three IDRs developed in our laboratory, IDR-1, IDR-1002 and IDR-1018 [93], which demonstrated and enhanced ability to induce chemokines in human peripheral blood mononuclear cells (PBMC). These activities also appear to underlie their protective effects in bacterial infections including those caused by MDR strains [94, 95].

Anti-endotoxin Activity

Lipopolysacharide (LPS), the outer membrane component of Gram-negative bacteria also known as endotoxin, is a classical inducer of systemic inflammation and deadly syndromes like sepsis [96]. Many antibiotics stimulate the release of endotoxin, enhancing the occurrence of systemic inflammation and consequent sepsis [97]. HDPs possess the capacity to dampen production of endotoxin-induced pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α) by blocking or modulating toll like receptor
(TLR) signaling pathways, and in some cases partly by direct LPS binding [98-100]. These activities may underlie the profound anti-inflammatory activity evident in infection models treated with IDR peptides [43, 94], as well as in sterile inflammation models.

**Immune Cell Differentiation**

Immune cell differentiation is essential to the proper development of immune responses. HDPs appear to have a direct link with this event as well. For example, LL-37 induces dendritic cell (DC) and bone forming-like cell differentiation [34, 101]. Similarly, hLF11, a lactoferrin-derived HDP well known for its in vivo protective effects on MDR infections, promotes the differentiation of a macrophage subset with pro and anti-inflammatory capabilities that are highly effective against bacterial pathogens [102]. Peptides also have very distinct activities when interacting with classical (M1) and alternatively activated (M2) macrophages [103].

**Wound Healing and Angiogenesis**

Wound healing involves the re-growth of epithelial layers and the formation of new blood vessels (angiogenesis), which is necessary for the wounded site to return to homeostasis. HDPs play an important role in this process by acting directly on epithelial and endothelial cells, inducing promoting re-epithelialization and angiogenesis, effects that have been demonstrated both in vivo and in vitro [104]. HDPs also induce wound healing indirectly through their chemotactic effects on epithelial cells and the induction of metalloproteinases [105, 106]. In fact, a lack of HDPs is linked to impaired re-epithelialization of chronic wounds [107, 108].

**Other Functions**

Enhancing cell survival and polarization of the adaptive immune system (adjuvant activity) are among other immunomodulatory activities attributed to HDPs [109, 110]. The molecular mechanisms by which these and other HDPs functions occur are complex and slowly being discovered. Mounting evidence suggests that HDPs target multiple processes within a given cell, with the responses depending on the nature of the peptide and the target cell type. In the case of LL-37, it appears to engage multiple receptors including the P2X7 receptor, formyl peptide receptor like-1 (FPRL-1) and other unknown Gi-protein coupled receptors, as well as GAPDH, an intracellular receptor [111-113].Some of the key signaling pathways that play a role in HDP immunomodulation include the mitogen-activated protein kinases (MAPK) p38, JNK, and extracellular signal-regulated kinase-1/2 (ERK1/2), as well as the Src-family kinases, NF\(^{\kappa}\)B (transiently) and PI3 kinase pathways [114].

The immunomodulatory activities displayed by HDPs have been demonstrated in animal models and appear to be important for pathogen clearance. Their role has been highlighted in models where the lack of HDPs leads to a reduced ability to clear infections, such as in transgenic mice lacking β-defensin 1 and cathelicidin-related antimicrobial peptide (CRAMP) or in human patients...
lacking LL-37 and α-defensin 3. The reduced pathogen clearance is likely due to dysregulation of the immune response and/or decreased peptide mediated-killing leading to increased susceptibility to infections [115-118]. The immunomodulatory and antimicrobial properties of HDPs make them excellent candidates to treat infections; especially those caused by MDR pathogens, particularly in combination with other antimicrobial therapies.

THERAPEUTIC POTENTIAL OF HDPS

Infectious diseases, particularly those caused by MDR bacteria and parasites, are a leading cause of death, accounting for approximately one third of deaths worldwide [119]. HDPs and their synthetic derivatives possess several attributes that make them attractive candidates for novel anti-infectives targeted to MDR pathogens. Variants with enhanced antimicrobial activity can kill a broad range of pathogens acting on components of several essential pathways, which makes the development of effective resistance to them much less likely. As outlined above, most HDPs favorably modulate the innate immune response, which responds to and counteracts a broad range of infectious agents. Thus the anti-infective activity of immunomodulatory HDPs is very broad and not limited to a single class of pathogen [120, 121]. Furthermore, the ability to target the immune response rather than the pathogen also limits the selective pressure on the pathogen. The following sections explore HDPs in preclinical development or clinical trials, as well as peptides recently identified as potential lead compounds Table I.

HDPs in Clinical Trials

I. Directly Antimicrobial Peptides

Direct antimicrobial activity has been characterized for numerous synthetic peptides, several of which were introduced into clinical trials as topical anti-infectives. Migenix (www.migenix.com; now renamed BioWest Therapeutics) completed two separate Phase III clinical trials with Omiganan (MX-226/CSL-001), demonstrating safety in more than 3,000 patients and for the first time statistically significant efficacy in decreasing catheter colonization and reducing microbiologically confirmed tunnel infections (p<0.02). However, Omiganan is a first generation peptide of modest activity derived from bovine indolicidin, and likely for this reason missed its primary endpoint of physician-called infections (p<0.08). Nevertheless it was also statistically significantly effective in Phase II clinical trials against severe acne and Rosacea, but as an anti-inflammatory agent rather than a direct antimicrobial and should go into Phase III trials for Rosacea within the next 12 months.

Pexiganan acetate (MSI-78), a synthetic magainin derivative was tested in Phase III trials as an anti-infective for diabetic foot ulcers. Pexiganan completed Phase III clinical trials but in 1999 was denied approval by the food and drug administration because it did not prove more efficacious than standard fluoroquinolone therapy [122]. It was recently announced, by a new company Dipexium, that Pexiganan would re-enter Phase III trials to enable resubmission of a new drug application (http://www.dipexium pharmaceuticals.com/newsrel.html). In addition, it has been reported that second generation antimicrobial peptides based on Pexiganan have been developed that exhibit greater efficacy in vitro and hold the promise of greater efficacy in vivo.

Only a small number of HDPs with direct antimicrobial activity are in clinical trials for systemic use. Novozyrne Inc.’s lead compound, currently in preclinical development, is a derivative of plectasin (NZ2114), a fungal defense exhibits broad spectrum antimicrobial activity, especially against systemic, drug-resistant Gram positive infections including MRSA. Unlike many of the antimicrobial HDPs that cause pore formation, plectasin inhibits cell wall synthesis via interaction with Lipid II [59]. It has excellent activity both in vitro and in vivo, minimal toxicity, is recombinantly produced by fermentation thus reducing cost of goods, and as a disulfide bonded defense molecule is quite protease resistant. PMX-30063 (Polymedix), a defensin mimetic made up of β-amino acids, was developed for the treatment of MRSA infections. PMX-30063 has shown potent broad-spectrum antibacterial activity against Gram positive and negative bacteria in vitro and in vivo and in Phase I trials demonstrated ex vivo antibacterial activity and that it was well tolerated systemically (www.polymedix.com/pdf/PMX-30063_fact_sheet.pdf).

II. Immunomodulatory Peptides

There have been a number of peptides developed that exhibit immunomodulatory activity. Some are solely immunomodulatory, like IMX942, while others possess a certain degree of antimicrobial activity, like hLF1-11 and talactoferrin. Inimex Pharmaceuticals has focused its research on the development of IDRs. Their lead peptide is IMX942, a 5-amino acid derivative of a bovine indolicidin, which was developed for the treatment of infections, especially in neutropenic patients, and displays efficacy in animal models against infections by a number of antibiotic resistant bacteria [94]. IMX942 has completed Phase IA clinical trials. The hormone peptide α-melanocyte-stimulating hormone (α-MSH) has become a lead for therapeutic development at Action Pharma owing to its extensive modulation of pro- and anti-inflammatory responses [123]. A derivative, AP214 is undergoing Phase IIB clinical trials for prevention of sepsis-induced kidney failure (http://www.actionpharma.com/index.dsp?page=73).

hLF1-11 developed by AM-Pharma is a human lactoferrin derivative intended to prevent bacteremia and fungal infections in immunocompromised individuals. It enhances macrophage-media- ted phagocytosis and killing of C. albicans and S. aureus [102]. hLF1-11 has completed Phase I trials and the side effects were found to be much milder than many of the marketed antibiotics with no serious adverse events; however currently development is suspended. Talactoferrin, developed by Agennix (www. agennix.com/) is derivative recombinant form of human lactoferrin that possesses broad immunomodulatory activity including immune cell recruitment, activation of dendritic cells and enhancement of adaptive immune responses [124-126]. A double-blind, placebo-controlled Phase II trial evaluated talactoferrin versus placebo in 190 adult patients with severe sepsis enrolled at 24 leading centers across the U.S. The Phase II trial was reported at the American Thoracic Society International Conference to have achieved its primary endpoint of a reduction in 28-day all-cause mortality (12.5% absolute reduction, 46.5% relative reduction). Apparently exploratory analyses suggested that talactoferrin might be effective in reducing the levels of certain cytokines and chemokines that are important in the initiation and propagation of the inflammatory response in severe sepsis.

New Developments in Potential Lead HDPs and Alternative Design Methods

Many research groups continue to focus on identifying novel HDPs from natural sources that are already effective against MDR pathogens, several of which are listed in Table I. Many of these HDPs were discovered in phylogenetically old organisms such as the cyanobacterium, Hydra magnipapillata, which rely on rudimentary immune systems to survive infections [127, 128]. Although many of the novel HDPs are reported to have potent antimicrobial activity, these are often demonstrated under non-physiological conditions, therefore more in vitro and in vivo studies are required to assess their potential as leads compounds in the development of therapeutics.

New tools such as robotics and machine learning are being developed to facilitate rational design of peptides, minimizing the number and amount of peptides synthesized and tested. Natural and synthetic HDPs with desirable biological activities are used as templates to generate novel peptide libraries consisting of thousands of synthetic peptides through random amino-acid substitution, scrambling and truncation. These novel peptides are then tested in vitro to identify those with enhanced antimicrobial activity, which go on to
<table>
<thead>
<tr>
<th>Drug/peptide</th>
<th>Description</th>
<th>Intended use</th>
<th>Activity against MDRs</th>
<th>Progress</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial/Immunomodulatory</strong></td>
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<tr>
<td>Omiganan [MX-226/CSL-001]</td>
<td>Indolicidin derivative</td>
<td>Prevention of central venous catheter infections, skin anti-sepsis, Rosacea</td>
<td>MRSA, VRE and ESBL Escherichia coli [146]</td>
<td>Phase IIIb/II</td>
<td>NCT 00231153, NCT 00608959</td>
</tr>
<tr>
<td>Pexiganan acetate [MSI-78]</td>
<td>Maganin derivative</td>
<td>Topical antibiotic- diabetic ulcers</td>
<td>several resistant strains [147]</td>
<td>Phase III</td>
<td>NCT 00563394, NCT 00563433</td>
</tr>
<tr>
<td>LTX-109 (Lytx Biopharma)</td>
<td>Peptidomimetic</td>
<td>Topical antibiotic</td>
<td>MRSA, VRE, MDR-P. aeruginosa</td>
<td>Phase I/IIa</td>
<td><a href="http://www.lytxbiopharma.com/?a=3&amp;sub=48">http://www.lytxbiopharma.com/?a=3&amp;sub=48</a></td>
</tr>
<tr>
<td>PAC-113 (Pargen biopharmaceuticals)</td>
<td>Histatin derivative</td>
<td>Antifungal</td>
<td>MDR-Candida albicans</td>
<td>Phase Ib</td>
<td>NCT 00659971</td>
</tr>
<tr>
<td>XOMA-629</td>
<td>BPI derivative</td>
<td>Impetigo</td>
<td>MRSA</td>
<td>Phase Ila</td>
<td><a href="http://www.xoma.com">www.xoma.com</a></td>
</tr>
<tr>
<td>HB-1345 (Helix Biomedix)</td>
<td>Lipohexapeptide</td>
<td>Acne</td>
<td>drug resistant P. acnes</td>
<td>Pre-phase I</td>
<td><a href="http://www.helixbiomedix.com">www.helixbiomedix.com</a></td>
</tr>
<tr>
<td><strong>Immunomodulatory/antimicrobial</strong></td>
<td></td>
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<tr>
<td>hLF1-11 (AM-Pharma)</td>
<td>Lactoferrin derivative</td>
<td>Prevention of bacteraemia and fungal infections</td>
<td>MRSA, MDR-Acinetobacter baumannii, fluconazole-resistant C. Albicans [150]</td>
<td>Phase I/I</td>
<td>NCT 00509938</td>
</tr>
<tr>
<td>Talactoferrin (Agennix)</td>
<td>Lactoferrin derivative</td>
<td>Non-small cell lung cancer, diabetic ulcers and renal cancer, sepsis, cancer</td>
<td></td>
<td>Phase I/II/III</td>
<td>NCT 00923741, NCT 00630656, NCT 00095186</td>
</tr>
<tr>
<td>Opebacan [rBPI21] (XOMA)</td>
<td>BPI derivative</td>
<td>Endotoxemia in patients receiving stem cell transplants</td>
<td>MDR-A. bauman- ni[151]</td>
<td>Phase I/I</td>
<td>NCT 00462904</td>
</tr>
<tr>
<td>RDP58 (Genzyme)</td>
<td>HLA class-I derivative</td>
<td>Inflammatory bowel disease</td>
<td></td>
<td>Post phase II</td>
<td><a href="http://www.genzyme.com/corp/licensing/genz_p_rdp_58_login.asp">www.genzyme.com/corp/licensing/genz_p_rdp_58_login.asp</a></td>
</tr>
<tr>
<td>AP214 (Action Pharma)</td>
<td>α-MSH derivative</td>
<td>Sepsis-induced kidney failure</td>
<td></td>
<td>Phase Ib</td>
<td>NCT 00903604, NCT 01256372</td>
</tr>
</tbody>
</table>
become templates for the synthesis of new libraries. Machine learning tools are used in conjunction with the experimental data generated from these libraries to generate predictive models of peptide activity. One example is QSAR analysis, which relates a series of quantifiable chemical and physical characteristics to a specific biological activity; thus the biological activity of a compound can be predicted based on its physical properties [129]. This approach facilitates rational large-scale design of novel therapeutic candidates because it allows for rapid and efficient virtual interrogation of large compound libraries. The pharmaceutical industry already utilizes QSAR during the small compound screening phase of drug discovery and in toxicological studies. QSAR analysis, relating the atomic-resolution physical-chemical descriptors to antimicrobial activity, combined with artificial intelligence/neural network approaches was recently employed, based on a large training dataset to predict the activity of 100,000 virtual 9-mer peptides [40]. In vitro, the peptides predicted to have the greatest antimicrobial activity were equally or more effective against a wide array of MDR bacteria than conventional antibiotics and were also protective in a murine S. aureus infection model. This demonstrates that unbiased computational approaches such as QSAR analysis coupled to artificial neural networks are viable, inexpensive and rapid methods for the discovery and development of novel HDPs with greater therapeutic potential.

Rational design of immunomodulatory peptides is more difficult largely because of their pleiotropic targets and effects and the relative novelty of the field. Thus rational design studies utilize specific immunomodulatory functions as markers of overall activity, such as suppression of TLR agonist mediated TNF-α production and induction of chemokines by PBMCs. Using this approach, innate defense regulator (IDR)-1, also called IMX-942, the first peptide designed to be purely immunomodulatory, was generated in our laboratory and proved protective in animal models of infection despite lacking direct antimicrobial activity [94]. The first generation synthetic immunomodulatory peptides are now being used as templates in an iterative design process, much like the one used for antimicrobial peptides, to generate new immunomodulatory peptides such as IDR-1002 with greater anti-infective activity [95].

In addition, a great deal of research now involves the development of alternative HDP design methods that improve efficacy while reducing cost, including ultrashort peptides, lipopeptides, peptides containing unnatural amino acids and self-assembling peptide nanoparticles [130-133]. Lipopeptides are potent antimicrobials (their mode of action is similar to directly antimicrobial HDPs) that are active against MDR bacteria. They are about 6 or 7 amino acids in length and can be linear or cyclic with a fatty acid moiety covalently linked to the N-terminus. N-terminal acylation of a number of natural and synthetic HDPs with C8-C18 long fatty acid moieties also enhances their antimicrobial activity, largely as a result of alterations to their hydrophobicity, oligomerization in solution and ability to interact with cellular membranes [134-136]. Makovitzkiet al. have exploited these properties by engineering ultrashort cationic lipopeptides (USLiP), peptides of two to four amino acids conjugated to a fatty acid (12-16 carbon) chain [137]. They are potent antimicrobials, with MICs similar to that of native HDPs, and unlike many native lipopeptides and HDPs they exhibit low or no hemolytic activity. The structure of these USLiPs is unique in that the amino acid sequence seems to determine target cell specificity, while the fatty acid chain compensates for the lack of hydrophobic amino acids in the peptide and promotes oligomerization. The potent antimicrobial activity of USLiPs coupled with their simple composition make them economically viable leads for therapeutic development. Liu et al. designed self-assembling nanoparticle micelles consisting of multiple HIV-1 TAT-derived peptides linked to cholesterol moieties. This design enhances the local density of cationic charge and peptide mass, thus improving the overall antimicrobial activity. The nanoparticles were effective against MRSA and VRE and in a rabbit model of S. aureus-induced

### Table 1: Contd....

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<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Imcroporin (I. maculates)</td>
<td>α-helical</td>
<td>Antibacterial</td>
<td>MRSA</td>
<td></td>
<td>[152]</td>
</tr>
<tr>
<td>Vejovine (Vaejovimesicatus)</td>
<td>α-helical</td>
<td>Antibacterial</td>
<td>Clinical isolates of MDR-P. aeruginosa, Klebsiella pneumoniae and A. baumannii</td>
<td></td>
<td>[153]</td>
</tr>
<tr>
<td>Hydramacin-1 (H. magnipapilliau)</td>
<td>α-helical/β-sheet</td>
<td>Antibacterial</td>
<td>ESBL- K. pneumoniae, K. oxytoca, E. Coli</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td>Arminin 1a (H. magnipapilliau)</td>
<td>defensin</td>
<td>Antibacterial/antifungal</td>
<td>P. aeruginosa, C. albicans</td>
<td></td>
<td>[128]</td>
</tr>
<tr>
<td>Oh-defensin (Ornithoctonus hainana)</td>
<td>defensin</td>
<td>Antibacterial</td>
<td>P. aeruginosa, C. albicans</td>
<td></td>
<td>[154]</td>
</tr>
<tr>
<td>Temporins (temporin-1Tb-1Tf) and esculetin [Eric 1-18 and 1-21] (Pelophylax tessano/ridibundus)</td>
<td>α-helical polypeptides</td>
<td></td>
<td>MDR- P. aeruginosa (in vivo)</td>
<td></td>
<td>[155]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDR-S. maltophilia, E. Faecium, A. baumannii, S. aureus (in vitro)</td>
<td></td>
<td>[156]</td>
</tr>
</tbody>
</table>

*International clinical trial registration number as indexed on www.clinicaltrial.gov

Abbreviations: BPI, bactericidal/permeability-increasing protein; ESBL, extended spectrum beta-lactamase; HLA, human leukocyte antigen; MSH, melanocyte-stimulating hormone; MRSA, methicillin-resistant Staphylococcus aureus, MDR, multidrug resistant; VRE, vancomycin-resistant enterococci; β-hCG, beta-human chorionic gonadotropin
mенингит, пересекает кровно-энцефалический барьер, подавляет бактериальный рост и ограничивает опасность инфекции [133].

**Challenges Facing Therapeutic use of HDPs and Potential Solutions**

Considering the numerous advantages of HDPs as novel therapeutics it may seem quite surprising that so few peptides have made it into clinical trials and none as yet into the clinic. To further develop HDPs into novel therapeutics several issues must first be addressed.

**Cost of Manufacturing**

Ideally therapeutic peptides should have a low production cost to ensure affordability. Unfortunately, the current method of solid-phase synthesis using fluoromethylmethoxycarbonyl (FMOC) chemistry synthesis of HDPs is prohibitively expensive for large-scale production, although solution phase synthesis offers considerable promise. One approach to reducing cost is designing biologically active peptides with minimal length such as the 9-mer peptides of Cherkasov et al. or the USLiPs of Makovitzki et al., thus reducing the amount of costly chemical precursors required [40, 137]. A new platform, designed specifically for commercial-scale peptide production is also required. A number of groups are attempting to address this through recombinant synthesis. Novozyme Inc. uses a high-yield fungal expression system in order to mass-produce a highly pure form of its lead peptide, NZ2114 [138]. Recently, a method involving recombinant synthesis and easily cleavable fusion protein partners was utilized for high yield production of several HDPs including LL-37, IDR-1 and MX-226 [139]. During the purification process, a naturally occurring protease, sumoase, which is cloned upstream of the target peptide, cleaves immediately downstream of the sumoase sequence freeing the pure peptide. Because this method utilizes an intrinsic cleavage system and a simple two-step purification process, it can easily be scaled up for industrial use while maintaining a low manufacturing cost [139].

**Stability and Toxicity In vivo**

Peptides are sensitive to proteolytic degradation, which substantially decreases their half-life. Our own, as-yet unpublished, pharmacokinetic studies have revealed half lives in the blood of as little as 2 minutes. Methods developed to enhance peptide resistance to proteases generally involve altering peptide structure to improve in vivo stability, although the down side to these approaches is that almost all of these substantially increase the cost of goods and are incompatible with recombinant synthesis. One approach is synthesis of peptide complexes such as tetrabranched structures (four peptides linked by a lysine core) or nanoparticles [133, 140]. Retro-inverso peptide isomers, in which the L-amino acids are replaced D-amino acids in the reverse sequence order, have also been developed. Because human proteases cannot metabolize the D-amino acid peptides, these isomers have significantly enhanced stability but maintain the spatial positioning and biological activity of the original peptide [141, 142]. This method has been successfully used to generate a biologically active Bac2A isomer (RI-Bac2A) that was more resistant to proteolytic degradation and exhibited reduced toxicity [142].

A number of HDPs exhibit some degree of systemic toxicity, which may explain why most peptides in clinical trials are used topically. The toxicity may be because HDPs interact with numerous cell types in different ways resulting in a complex mode of action however, little research has actually been done to address this issue. Although toxicity was initially thought to be a result of membrane disruption leading to cytolysis, many HDPs interact with and translocate across eukaryotic membranes [75, 94, 113] without disrupting them. This is probably because eukaryotic membranes lack negatively charged lipids and the strong membrane potential gradient found in prokaryotic membranes, and contain cholesterol [57]. Anti-infective HDPs can and have been designed that are protective in animal models of infection with little or no associated toxicity as is the case for IDR-1 [94]. Toxicity and pharmacokinetic studies must be carried out for HDPs in ex vivo human tissue models and relevant animal models to determine the mechanism behind the toxicity during systemic administration as well as identify other potential side effects, the appropriate formulations and alternate routes of administration.

**Development of Resistance**

There was concern as to whether widespread use of directly antimicrobial HDPs would promote the development of peptide-resistance by pathogens to both the therapeutic and endogenous HDPs. Several bacterial species already possess HDP-resistance mechanisms such as secretion of peptidases and proteases and alteration of surface charge [143]. However, considering the broad target range exhibited by peptides it seems unlikely that the selective pressure placed on the pathogens would be great enough to incur resistance. Furthermore, there is no evidence that resistance mechanisms are easily acquired [143, 144]. Nisin a well recognized HDP has been used as food preservative for years with no effect on an individual’s ability to fight infections [144, 145]. Finally, the use of immunomodulatory peptides could circumvent this issue completely as they target the immune response rather than the pathogen [94, 95].

**CONCLUSION**

The emergence of MDR pathogens has resulted in an urgent need to develop novel antimicrobial therapies. As anti-infective agents, HDPs can be directly antimicrobial and/or immunomodulatory. Thus, HDPs are unlikely to promote microbial resistance because they disrupt multiple biological processes in the pathogen or target the host immune system rather than the pathogen. Furthermore, HDPs can suppress the potentially harmful inflammation associated with infection. These properties make HDPs an attractive alternative in the treatment of MDR infections. Thousands of natural and synthetic HDPs have been identified however, only a handful of HDPs have made into clinical trials and none approved for clinical use in part because of issues regarding cost of manufacturing, peptide stability and toxicity. Although more work is needed, substantial strides have been made in recent years to overcome these setbacks, and the future looks promising for the development and widespread use of peptides as therapeutics targeted to MDR pathogens.

**ACKNOWLEDGEMENTS**

The authors acknowledge funding from the Canadian Institutes for Health Research, Genome BC and the Grand Challenges in Global Health Research program for their own peptide research. R.H. holds a Canada Research Chair. N.J.A. holds a studentship from the Natural Sciences and Engineering Research Council of Canada. A.T.Y.Y. received studentships from the Cystic Fibrosis Canada and the Natural Sciences and Engineering Research Council of Canada. O.M.P. is the recipient of a University of British Colombia and a Killam Doctoral fellowship and a Canadian Institute of Health Research–Vanier Canada graduate scholarship.

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