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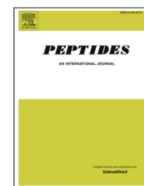

Immunomodulatory peptides—A promising source for novel functional food production and drug discovery

Milica Pavlicevic [a], Nelson Marmiroli [b], Elena Maestri [b,*]

[a] Institute for Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Serbia
[b] University of Parma, Department of Chemistry, Life Sciences and Environmental Sustainability, and Interdepartmental Center SITEIA.PARMA, Parco Area delle Scienze 11/A, 43124 Parma, Italy

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ABSTRACT

Immunomodulatory peptides are a complex class of bioactive peptides that encompasses substances with different mechanisms of action. Immunomodulatory peptides could also be used in vaccines as adjuvants which would be extremely desirable, especially in response to pandemics. Thus, immunomodulatory peptides in food of plant origin could be regarded both as valuable suplements of novel functional food preparation and/or as precursors or possible active ingredients for drugs design for treatment variety of conditions arising from impaired function of immune system. Given variety of mechanisms, different tests are required to assess effects of immunomodulatory peptides. Some of those effects show good correlation with *in vivo* results but others, less so. Certain plant peptides, such as defensins, show both immunomodulatory and antimicrobial effect, which makes them interesting candidates for preparation of functional food and feed, as well as templates for design of synthetic peptides.

1. Introduction

Plants have long been an invaluable source of bioactive peptide exhibiting plethora of activities [1–3]. Some widely used plants of commercial interest such as wheat (*Triticum* spp.), rice (*Oryza sativa* L.), maize (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) have been extensively studied for their antioxidative, antihypertensive, opioid, hypolipidemic, immunomodulatory properties, as well as for their ability to prevent diseases prevalent in modern-day (such as diabetes) [1,2,4–6]. Recently, plants who have been traditionally used in “folk medicine” to treat numbers of conditions (such as amaranth, *Amaranthus hypochondriacus* L., quinoa, *Chenopodium quinoa* Willd., chia, *Salvia hispanica* L., etc) [7–10] and have been unjustly neglected, have been entering into focus.

From all of the bioactive peptides, immunomodulatory peptides represent group that is both the most diverse and the most complex [11]. However, due to their applicability in food industry, medicine, cosmetics, pharmacology, etc. immunomodulatory peptides, of both plant and animal origin, are currently a major focus of research. Interest in immunomodulatory peptides stems not only from their diverse effects on both innate and adaptive immunities, but also from their potential for treatment of various autoimmune illnesses.

In the age of COVID-19, plants as sources of antiviral and immunomodulatory compounds have become a hot topic [12,13]. Peptide-based vaccines, and use of immunomodulatory peptides as adjuvants, are not new concepts [14,15], but have gained fresh attention. Given that peptide-subunits based vaccines provoke relatively weak immune response [16,17], plant-derived compounds in the vaccines are usually used as adjuvants. However, plant based adjuvants normally belong to classes other than peptides such as saponines, polysaccharides, terpenes and flavones [16,18–20]. Two classes of plant proteins proved to be efficient in improving both B cell proliferation and antibody production – lectins and heat shock proteins [20,21]. Plant heat shock proteins, especially Hsp90, can therefore be used as carriers as well as immunostimulants in vaccines [20]. Plants can also be used as bioreactors for expression of vaccine antigens [22]. For example, barley (*Hordeum vulgare* L.) and maize have been used for expression of HIV (human immunodeficiency virus) antigen, potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) for expression of hepatitis B antigen [22]. This led to development of so-called edible vaccines that could, due to their low cost, also be used in developing countries [22,23]. However, a problem with so-called edible vaccines is that efficacy can be diminished through changes in stereochemistry and folding (as a result with interaction with plant enzymes involved in immune response) as

* Corresponding author.
E-mail address: elena.maestri@unipr.it (E. Maestri).

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well as the lack of standardization [22,23].

In this context the recently constructed tool VaxinPAD [24] can be very useful, given that it combines current knowledge of immunomodulatory peptides with a bioinformatics approach and thus predicts their effects *in vivo*. For example, Hossain et al. used this vaccine to design multi-epitope vaccine against Saint Louis Encephalitis Virus [25]. However, this approach cannot predict stability of immunomodulatory peptides or their interactions with other components in the organism. Although this path is promising it should be approached carefully, given that most immunomodulatory peptides influence signal transduction to some extent and can also enhance production of proinflammatory cytokines.

Additionally, given the rising popularity of so-called personalized medicine and the increase in incidence of autoimmune diseases (such as psoriasis, celiac disease, type 1 diabetes, rheumatoid arthritis, lupus, *etc*.), application of multifunctional immunomodulatory peptides and/or anti-inflammatory and antimicrobial peptides in vaccines has become more significant [26–30]. For example, the peptide QQPQDAVQPF isolated from wheat serves as an antagonist for peptide from α- gliadin and therefore could be used in a treatment of celiac disease [31]. Gershteyn and Ferreira [32] established the so called Gershteyn-Ferreira (GF) index that was designed to illustrate correlation between diet and incidence of autoimmune diseases. According to this analysis, five crops (wheat, rye, soybean, quinoa and rice) have much lower GF indices compared to animal products used in the study and therefore are less likely to be causes of autoimmune disease. However, it should be pointed out that this study did not account for gliadin peptides modified by tissue transglutaminase, which are main cause of celiac disease, or for citrullinated proteins that aggravate symptoms of rheumatoid arthritis. Cyclotides, a class of cyclic plant peptides, were found to modulate autocrine T-cell proliferation which is the main cause of multiple sclerosis [33]. So-called cystine knot peptides, protease inhibitors found in many plant classes, were also shown to have protective effect against neurodegenerative diseases [34,35].

However, when testing immunomodulatory peptides *in vivo*, side effects have been observed, ranging from mild allergic reactions to organ failure [36]. Tables 1–3 lists some of the best-known immunomodulatory peptides with proven *in vivo* effect.

Additionally, due to the interest in functional food, production of food-derived bioactive peptides, either by hydrolysis, fermentation or technological processing, has garnered a lot of attention [37–41]. When considering plants as source of peptides with medicinal effects, traditional crops such as cereals, pseudocereals and legumes have been used for some time [42–45]. More recently, novel plant sources, including model species and tropical plants have been employed both to elucidate mechanisms of action and to discover new classes of compounds that could serve as blueprints for design of synthetic peptides [46–49].

The possibility of using food waste to produce peptides with medicinal effect [50,51] is especially interesting. Utilising waste as a source of immunomodulatory peptides would have both economic and ecological benefits, thus making food production more sustainable. Additionally, it could lead to discovery of peptides with different mechanisms of action.

In this review we provide an overview of mechanisms of immunomodulatory activity, compare different methods used for assessing immunomodulatory effects, discuss problems of reliability of *in vitro* testing, and provide some guidelines for further research. Special attention is paid to food-derived immunomodulatory peptides of plant origin.

2. Complexity of mechanisms of immunomodulatory activity

The term immunomodulation is a relatively broad one, since it encompasses any change in innate and/or adaptive immunity. Some authors have further expanded this definition by linking immunomodulation to oxidative stress [52]. Antimicrobial peptides, although interacting with many mechanisms involved in the innate immune response [37,53,54] are usually classified as a separate group and are therefore not included in this review. Although these two responses use different strategies to recognize and eliminate foreign materials, some cell types (neutrophils, dendritic cells, natural killer cells, *etc*.) can be involved in both types of immune response [55–60]. Therefore, immunomodulatory peptides are often multifunctional – exhibiting more than one effect on defence systems. Generally speaking, peptides can be described as immunosuppressive or immunostimulating, based on whether they inhibit or activate immune response [36,61]. More specifically, based on the process affected, immunomodulatory effects can be classified as proliferative/antiproliferative, proinflammatory/antiinflammatory and cytoprotective/cytotoxic (Fig. 1). Proliferative/antiproliferative peptides are those that are involved in stimulation (proliferative) or prevention (antiproliferative) of metastasis and spreading of tumor mass (thus, they are sometimes referred to as carcinogenic or anticancer peptides); proinflammatory/antiinflammatory peptides respectively exacerbate or prevent/alleviate inflammation, while cytoprotective/cytotoxic peptides are involved in regulation of apoptosis, and therefore, they are often described as apoptotic or anti-apoptosis peptides [62–64].

Although in some cases only either the innate or the adaptive response is activated, usually both are triggered by immunomodulatory peptides. For example, anticancer peptides often exhibit their effect via changes in cell-to-cell interaction and concentrations of cytokines, interleukins and growth factors, which are consequences of interaction between innate and adaptive components of immunity [65–67].

Immunomodulatory peptides can express their effects at many different levels in the cell (Fig. 2). Antiproliferative peptides can be involved in suppression of reactive oxygen species [62]. For example, Amar et al. [68] found that the extract of rosemary (*Rosmarinus officinalis* L.) that exhibited antiproliferative activity against U937 and CaCo-2 cells also inhibited production of H_2O_2 by gamma-glutamyl transpeptidase and decreased concentration of both free radicals. At the same time antiproliferative peptides could promote formation of tubulin-peptide complexes through conformational changes of tubulin [69] which in turn prevents formation of microtubules and division of cells. Additionally, antiproliferative peptides could inhibit the activity of topoisomerase II (also the key target for several anticancer drugs), thus preventing replication of DNA and continuation of cell cycle [70]. Proinflammatory/antiinflammatory peptides act by regulating expression of interleukins [either main proinflammatory interleukins, such as interleukin 1-beta (IL-1β), interleukin 2 (IL-2) or interleukin 6 (IL-6) (although interleukin 6 (IL-6) could "behave" also like antiinflammatory signal) [71]] or main antiinflammatory interleukins, such as interleukin 4 (IL4) and interleukin 10 (IL10) [72,73]), and cytokines such as tumor necrosis factor-α (TNF-α) [61,66,71–74] and interferon (IFN)-γ interfering with signal transduction by modulating concentrations of cytosolic Ca^{2+} [75] and through inhibition of neutrophil aggregation and changes in concentration of adhesion molecules on monocyte and leukocyte membranes [76,77]. Signal transduction pathway that will be activated/deactivated during inhibition/activation of inflammation depends from the type of chemokine secreted: for example, proinflammatory cytokines, such as, TNF-α influences activity of components involved in mitogen-activated protein kinase (MAPK), IL-1β modulates G-protein-coupled receptors pathway, IL-6 changes pathway involved in activation/deactivation of transcription factor nuclear factor (NF)-kB [78–80]. Cytoprotective/cytotoxic peptides can interfere with signal transduction in the cell [81] and with protein synthesis [82] as well as influencing expression of genes coding for caspases and nuclear factor of activated T cells (NFAT) [83,84]. In the terms of signalling transduction, main "targets" of cytotoxic/cytoprotective peptides are pathways involved in regulation apoptosis process, such as c-Jun N-terminal kinase (JNK) pathway, NF-κB pathway and signal transducer and activator of transcription 1 (STAT1) pathway, but, due to the release of Ca2+ ions from their storage in endoplasmatic reticulum (ER) mitochondria,

Table 1
Antiinflammatory/proinflammatory peptides from food plants, source, and bioactivity demonstrated in vitro or in vivo. Where available, the common name of the peptide is indicated. Data were recovered from PlantPepDB and BIOPEP database and integrated with literature searches.

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
Seed glutenin fragment 51	YG	*Amaranthus hypochondriacus* L.	proinflammatory	In vitro	Stimulate proliferation rate for 90%	Proliferation of peripheral blood lymphocyte	[117]
Seed glutenin fragment 54	GFL	*Amaranthus hypochondriacus* L.	proinflammatory	In vitro	Increased adhesion in dose-depended manner starting from concentration of 0.2 μM	Increased adhesion of monocytes and macrophages and activation of polymorphonuclear leukocytes	[117]
Seed glutenin fragment 64	EAE	*Amaranthus hypochondriacus* L.	proinflammatory	In vitro	Stimulate proliferation rate for 25%	Proliferation of peripheral blood lymphocyte	[118]
Seed glutenin fragment 65	KRP	*Amaranthus hypochondriacus* L.	proinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Proliferation of peripheral blood lymphocyte	[118]
Seed glutenin fragment 66	YGG	*Amaranthus hypochondriacus* L.	proinflammatory	In vitro	Stimulate proliferation rate for 33%	Proliferation of peripheral blood lymphocyte	[118]
Seed glutenin fragment 73	KEEAE	*Amaranthus hypochondriacus* L.	antiinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Proliferation of peripheral blood lymphocyte	[118]
cliotide T28	GGSIPCGESCVFLPCFLPGCSCKSSVCYLN	*Clitoria ternatea* L.	antiinflammatory	In vitro	Secretion of IL-6 and IL-8 1.6 fold increase; secretion of TNF-α 1.2 fold increase	Increase secretion of IL-6, IL-8 and TNF-α in RAW 264.7 macrophages	[119]
cliotide T33	GFNSCSEACVYLPCFSKGCSCFKRQCYKN	*Clitoria ternatea* L.	antiinflammatory	In vitro	Secretion of IL-6 and IL-8 1.6 fold increase; secretion of TNF-α 1.2 fold increase	Increase secretion of IL-6, IL-8 and TNF-α in RAW 264.7 macrophages	[120]
cliotide T32	GDLFKCGETCFGGTCYTPGCSCDYPICKNN	*Clitoria ternatea* L.	antiinflammatory	In vitro	Secretion of IL-6 and IL-8 1.6 fold increase; secretion of TNF-α 1.2 fold increase	Increase secretion of IL-6, IL-8 and TNF-α in RAW 264.7 macrophages	[121]
	VIK	*Glycine max* (L.) Merr.	antiinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7 macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	[122]
	VPY	*Glycine max* (L.) Merr.	antiinflammatory	In vitro	Secretion of TNF-α and IL-8 reduced in dose-dependent manner, starting from concentration 2 mM	Reduced secretion of TNF-α and IL-8 in RAW 264.7 macrophages	[123]
	RQRK	*Glycine max* (L.) Merr.	antiinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7	[122]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
					bioactivity and its activity wasn't determined	macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	
	YPFVVNA	*Glycine max* (L.) Merr.	proinflammatory	In vivo	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7 macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	[122]
Soymetide 9	MITLAIPVN	*Glycine max* (L.) Merr.	proinflammatory	In vivo	IC_{50} 25 μM	Antagonist for fMLP	[122]
Soymetide 13	MITLAIPVNKPGR	*Glycine max* (L.) Merr.	proinflammatory	In vitro	IC_{50} 50 μM	Antagonist for fMLP	[124]
	GGRKQGQHQQEE	*Glycine max* (L.) Merr.	proinflammatory	In vivo	At concentration 100 μM increased number of CD8+ cells for 17%, CD11b+ cells for 20% and CD49b+ cells for 29%	Increases the number of cytotoxic lymphocytes, natural killer cells and different types of leukocytes	[125]
	GRGDDDDDDDDD	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7 macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	[122]
	GVNLTPCEKHIMEKIQ	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7 macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	[122]
	SKWQHQQDSCRKQKQ	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of lipopolysaccharide-induced inflammation in RAW 264.7 macrophages by inhibiting production of NO, secretion of TNF-α and IL-1ß and expression of iNOS and COS	[122]
	YFVP	*Helianthus annuus* L.	antiinflammatory	In vitro	Inhibition 25% at concentration 500 pg/mL	Inhibition of IL-1β mediated activation of NF-κB	[126]
	SGRDP	*Helianthus annuus* L.	antiinflammatory	In vitro	Inhibition 25% at concentration 500 pg/mL	Inhibition of IL-1β mediated activation of NF-κB	[126]
	MVWGP	*Helianthus annuus* L.	antiinflammatory	In vitro	Inhibition 40% at concentration 500 pg/mL	Inhibition of IL-1β mediated activation of NF-κB in monocytes	[126]
	TGSYTEGWS	*Helianthus annuus* L.	antiinflammatory	In vitro	Inhibition 40% at concentration 500 pg/mL	Inhibition of IL-1β mediated activation of NF-κB in monocytes	[126]
	YG	*Hordeum vulgare* L.	proinflammatory	In vitro	Inhibition 25% at concentration 500 pg/mL	Inhibition of IL-1β mediated activation of NF-κB in monocytes	[127]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
Labatidin	AGVWTVWGTI	*Jatropha multifida* L.	antiinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Inhibition of human complement activation through the classical pathway	[128]
	HY	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration of for 19.65 % and PGE 2 concentration for 27.95%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	PY	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration of for 16.98% and PGE2 concentration for 17.88%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	YW	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 24.57% and PGE2 concentration for 34.86%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	ANP	*Juglans regia* L.	proinflammatory	In vivo	At concentration 0.1 mM increased NO concentration for 12.56% and PGE 2 concentration for 6.61%	Increase of NO and PGE2 concentration in BV-2 cells	[129]
	GGW	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 17.59% and PGE2 concentration for 30.49%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	LPF	*Juglans regia* L.	antiinflammatory	In vivo	At concentration of 0.1 mM increased expression of IL-6 2-fold; expression of IL-1β 5-fold; expression of TNF-α 3-fold	Increase in expression of IL6, IL-1β and TNF-α in mouse spleen cells	[129]
	NLQ	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 13.78% and PGE2 concentration for 19.8%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	VYY	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased concentration of NO for 22.86% and PGE2 concentration for 31.73%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
	GVYY	*Juglans regia* L.	antiinflammatory	In vivo	At concentration of 0.1 mM decreased expression of IL-6 3-fold; expression of IL-1β 4-fold; expression of TNF-α 4-fold	Decrease in expression of IL-6, IL-1β 4-fold; expression of TNF-α 4-fold in BV-2 cells	[129]
	LGGW	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 25.07% and PGE2 concentration for 33.08%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	SACV	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 19.42% of and PGE2 concentration for 26.41%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	APTLW	*Juglans regia* L.	antiinflammatory	In vivo	At concentration of 0.1 mM increased expression of IL-6 3-fold; expression of IL-1β 4-fold; expression of TNF-α 3-fold	Increase in expression of IL-6 IL-1β and TNF-α in BV-2 cells	[129]
	CTLEW	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 4 mg/mL decreased concentration of NO for 20%	Decrease of NO concentration in spleen cells	[130]
	AAFAATY	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased concentration of NO and PGE2 in BV-2 cells for 16.39% and 22.46%, respectively	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
	FDLIYSV	*Juglans regia* L.	antiinflammatory	In vivo	At concentration 0.1 mM decreased NO concentration for 6.74% and PGE2 concentration for 14.82%	Decrease of NO and PGE2 concentration in BV-2 cells	[129]
cyclolinopeptide D	PFFWIMLL	*Linum usitatissimum* L.	proinflammatory; cytotoxic	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Increased expression of TNF-α, IL-1β, and CCL2 and decreased expression of IL-10 in macrophages	[131]
cyclolinopeptide G	PFFWIMLM	*Linum usitatissimum* L.	proinflammatory; cytotoxic	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Increased expression of TNF-α, IL-1β, and CCL-2 and decreased expression of IL-10 in macrophages	[132]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
	GPETAFLR	*Lupinus angustifolius* L.	antiinflammatory	In vitro	At concentration 100 μg/mL IL-1β expression decreased for 40%; CCR-2 increased 2-folds; CCL-2 expression decreased for 50%; TNF-α expression decreased 30%; at concentration 500 μg/mL IL-6 expression decreased 60%	Increase in expression of IL-1β and CCR-2 and decrease in expression CCL-2, IL-6 and TNF-α in macrophages	[133]
	YGIYPR	*Oryza sativa* L.	antiinflammatory; proliferating	In vitro	Decrease of IL-1ß amount for 10%, proliferation in dose-dependent manner in range of 12.5–100 μg/mL	Decrease of IL-1ß amount and increase of proliferation of and macrophage RAW 264.7 cells	[134, 135]
LR13	LLPPFHQASSLLR	*Oryza sativa* L.	antiinflammatory	In vitro and in vivo	In vitro-at concentration 25 μg/mL amount of IL-1β released in stimulated RAW 264.7 cells decreased for 40%; in vivo (mouse brain and spleen) CD 4+ cells increased 53%; CD 25+ cells increased 136%; IL-10 secretion increased 33% and IL-4 secretion increased 3-fold; IL-17 secretion decreased 82% and IFN-γ secretion decreased 45%	Decrease in IL-1β in RAW 264.7 cells in vitro; in vivo increase in number of CD 4+ cells CD 25+ cells and secretion of antiinflammatory cytokines (namely, IL-10 and IL-4) and decrease in secretion of proinflammatory cytokines (namely, IL-17 and IFN-γ)	[135]
PEP1	GIAASPFLQSAAFQLR	*Oryza sativa* L.	antiinflammatory	In vitro and in vivo	In vitro at concentration 25 μg/mL amount of IL-1β released in stimulated RAW 264.7 cells decreased for 20%; in vivo (mouse brain and spleen) CD 4+ cells increased 35%; CD 25+ cells increased 103%; IL-10 secretion increased 21% and IL-4 secretion increased 3-fold; IL-17 secretion decreased 67% and IFN-γ secretion decreased 29%	Decrease in IL-1β in RAW 264.7 cells in vitro; in vivo increase in number of CD 4+ cells CD 25+ cells and secretion of antiinflammatory cytokines (namely, IL-10 and IL-4) and decrease in secretion of proinflammatory cytokines (namely, IL-17 and IFN-γ)	[135]
	AEMIDLAAKMLSEGRG	*Oryza sativa* L. subsp. Japonica	antiinflammatory	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Decrease in secretion of TNF-α and IL-1ß in macrophage	[136]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
	RGAVLH	*Pinus koraiensis* Siebold & Zucc.	proinflammatory	In vitro	At concentration 1 mg/mL increased rate of phagocytosis in for 30% and NO production from 2.5 to 15 μM	Increase in phagocytosis rate and NO production in RAW 264.7 cells	[137]
	RMVLPEYELLYE	*Salvia hispanica* L.	antiinflammatory	In vitro	At concentration 100 μM decreased concentration of PGE2 for 50%, secretion of TNFα for 40%, secretion of IL-6 for 34.26% and secretion of IL-10 for 15.95%	Decrease in PGE-2 concentration and secretion of TNFα, IL-6 and IL-10 in 3T3L-1 adipocytes	[138]
	EDDQMDPMAK	*Setaria italica* (L.) P.Beauv.	antiinflammatory; antiproliferative	In vitro	At concentration 100 μM decreased secretion of TNF-α for 42.29% and IL-6 secretion for 56.59%	Decrease in TNF-α and IL-6 secretion in RAW 264.7 cells	[139]
	QNWDFCEAWEPCF	*Setaria italica* (L.) P.Beauv.	antiinflammatory; antiproliferative	In vitro	At concentration 100 μM decreased TNF-α secretion for 44.07% and IL-6 secretion for 43.45%	Decrease in TNF-α and IL-6 secretion in RAW 264.7 cells	[139]
	IF	*Solanum tuberosum* L.	antiinflammatory	In vivo	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Decrease in TNF-α and IL-6 secretion in RAW 264.7 cells	[140]
	pyroEL	*Triticum* spp.	antiinflammatory	In vitro	Decreases expression of TNF-α mRNA for 2 folds at concentration 1.6 mM	Decrease in TNF-α expression in rat hepatocytes	[141]
	ECFSTA	*Triticum* spp.	proinflammatory	In vitro	At concentration 20 μg/mL decreases expression of iNOS for 62.5%, expression of IL-6 for 66.67% and expression of TNF-α for 63%	Decrease in expression of iNOS, IL-6 and TNF-α	[142]
	QQPQDAVQPF	*Triticum durum* Desf.	antiinflammatory; antiproliferative	In vivo	At concentration 10 μg/mL decreases IL-10 and IFN-γ secretion for 30%	Decrease in IL-10 and IFN-γ secretion by human blood lymphocytes	[143]
	PPYCTIVPFGIFGTNYR	*Triticum durum* Desf.	antiinflammatory; antiproliferative	In vivo	At concentration 10 μg/mL decreases secretion of TNF-α for 60% decreases secretion of IFN-γ for 40%	Decrease in secretion of TNF-α and IFN-γ by human blood lymphocytes	[143]
	PFNQL	*Zea mays* L.	antiinflammatory	In vitro	At concentration 0.5 mM inhibited secretion of IL-6 for 46.2%	Decrease in secretion of IL-6 in human macrophage-like U 937 cell line	[144]

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Table 1 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Event and examined cell type	Reference
	FLPPVT	*Zea mays* L.	antiinflammatory	In vitro	At concentration 0.5 mM inhibited secretion of IL-6 for 19.5%	Decrease in secretion of IL-6 in human macrophage-like U 937 cell line	[144]
	FLPFNQL	*Zea mays* L.	antiinflammatory	In vitro	At concentration 0.5 mM inhibited secretion of IL-6 for 57.7%	Decrease in secretion of IL-6 in human macrophage-like U 937 cell line	[144]
	PFNQLAG	*Zea mays* L.	antiinflammatory	In vitro	At concentration 0.5 mM inhibited secretion of IL-6 for 13.4%	Decrease in secretion of IL-6 in human macrophage-like U 937 cell line	[144]
	SQLALTNPT	*Zea mays* L.	antiinflammatory	In vitro	At concentration 0.5 mM inhibited secretion of IL-6 for 10.4%	Decrease in secretion of IL-6 in human macrophage-like U 937 cell line	[144]

Abbreviations and markings: IL-6- interleukin 6; IL-8- interleukin 8; IL-1β-interleukin-1 beta; NO- nitric oxide; iNOS- inducible nitric oxide synthase; COX-2- cyclooxygenase 2; PGE 2- prostaglandin E; TNF-α- tumor necrosis factor alpha; NFkB- activated B cell kappa light chain enhancer nuclear factor; IFN-γ-interferon gamma; GLP- alcohol-soluble protein fraction of durum wheat; CCL2- chemokine (C-C motif) ligand 2; CCR2- C-C chemokine receptor type 2; fMLP- N-Formylmethionyl-leucyl-phenylalanine.

secretion of cyclic AMP (CAMP) as secondary messenger was also observed [84–87].

As previously stated, many plant peptides are multifunctional. For example, the peptides GRGDDDDDDDDD, GVNLTPCEKHIMEKIQ and SKWQHQQDSCRKQKQ from soybean [88], QNWDFCEAWEPCF and EDDQMDPMAK from foxtail millet (*Setaria italica* (L.) P.Beauv.) [89], QQPQDAVQPF and PPYCTIVPFGIFGTNYR from wheat [90] all showed both antiproliferative and antiinflammatory activity, while PFFWIMLL and PFFWIMLM from flaxseed (*Linum usitatissimum* Griseb.) exhibited both proinflammatory and cytotoxic activity [91,92]. In certain cases, seemingly uncorrelated activities such as antimicrobial and antiproliferative could be exhibited by the same peptides. For example, the peptide limyin (sequence KTCENLATYYRGPCF) isolated from lima bean (*Phaseolus limensis* Macfad.) showed both antifungal and antiproliferative activity *in vitro* [93]. In this case, the reason behind this multifunctionality is structural similarity of limyin to defensins which allows to penetrate the cell through specific binding to receptors on the cell membrane.

As could be seen from Tables 1–3, many immunomodulatory peptides are isolated from plant species of big economic significance, such as soybean, wheat and maize, while plants with less prominent use have not been sufficiently examined. Two additional problems are that often only a hydrolysate or extract is characterized and used in *in vivo* studies, without isolation of individual peptides [94–97] and that peptides are being tested for only one aspect of immunomodulatory activity, without examining for all of them, which might lead to many useful peptides being discarded.

3. How to test for immunomodulatory effects?

Because immunomodulatory peptides achieve their effects by many different mechanisms, determination of immunomodulatory activity is difficult. It is impossible to test different aspects simultaneously, thus leading to the possibility of false negative and/or false positive results. Determination of immunomodulatory activity is further complicated by the inability to assess separately the effects of innate and adaptive immunities, since many cells types (natural killer cells, dendritic cells, neutrophils etc.) as well as small cellular messengers such as cytokines, present linkage between innate and adaptive responses [62,98,99]. Therefore, the most effective way to check for immunomodulatory activity is to test for each specific effect individually.

The most reliable way to test cytotoxic activity *in vitro* is to determine cell viability and proliferation by employing assays for uptake of dyes, such as MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay or neutral red (NR) uptake assay, or by measuring activity of specific enzymes that activate in response to oxidative stress (for example, acid phosphatase or lactate dehydrogenase) [100–103]. Cytotoxic activity *in vivo* is tested by measuring cytokine profiles, gene expression of genes coding for proteins that involved in apoptosis process (such as caspases) and monitoring histological changes in liver and thymus [101–106]. Apoptosis is usually measured *in vitro* by either assessing mitochondrial function (transmembrane potential or caspase concentration) or by determining level of DNA oxidation [103,107,108]. The common in vivo method for testing anti-inflammatory activity is the measurement of degree of swelling (paw edema test), while in vitro methods include determination of macrophage activity (through changes in respiratory burst), measuring concentrations of cytokines, and/or degree of adhesion of monocytes to endothelium [109–112]. Proliferative/antiproliferative activity is usually determined *in vitro* by the methyl-[3H]-thymidine incorporation assay and lymphocyte proliferation assay and in vivo by observing histological changes in affected cells and/or by lymphocyte proliferation assay (that showed good correlation with *in vitro* results) [113–116, Fig. 2].

Fig. 2 summarizes the main methods for assessing 3 principal modes of immunomodulatory activity *in vitro* and *in vivo* and gives correlation of *in vitro* with *in vivo* results. Given that only few papers provide comparison of *in vitro* and *in vivo* results, such correlation requires further testing. Problems with correlation of in vitro and in vivo results are further discussed in Chapter 6.

4. Examples of bioactive peptides with different bioactivities

Here we give examples of peptides of plant origin exhibiting one (or more) of immunomodulatory activities. Table 1 lists peptides with proven antiinflammatory/proinflammatory activity; Table 2 contains peptides with proven antiproliferative/proliferative activity and Table 3 shows peptides with proven cytotoxic/cytoprotective activity. As can be observed, peptides which exhibited multiple activities were counted per each activity and thus can be present in more than one table.

As can be seen from Tables 1–3 and as discussed in previous chapters, immunomodulatory peptides exploit different mechanisms to achieve their effect and are thus characterized by different values representing their activity. Two major problems are evident from Tables 1–3: 1. single peptide can (and often does) exhibit more than one effect and thus

Table 2
Antiproliferative/proliferative peptides from food plants, source, and bioactivity demonstrated in vitro or in vivo. Where available, the common name of the peptide is indicated. Data were recovered from PlantPepDB and BIOPEP database and integrated with literature searches.

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Examined cell line or enzyme	Reference
	NHAV	*Cannabis sativa* L.	antiproliferative	In vitro	At concentration 10 μg/mL increased survival by 60%	PC12 cells	[145]
	HVRETALV	*Cannabis sativa* L.	antiproliferative	In vitro	At concentration 10 μg/mL increased survival by 60%	PC12 cells	[145]
cliotide T1	GIPCGESCVFIPCITGAIGCSCKSKVCYRN	*Clitoria ternatea* L.	antiproliferative; cytotoxic	In vitro	HD_{50} 7.1 μM; IC_{50} 0.6 μM	HeLa cells	[146]
cliotide T6	SIPCGESCVYIPCLTTIVGCSCKNSVCYSN	*Clitoria ternatea* L.	antiproliferative; cytotoxic	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HeLa cells	[120]
cliotide T10	GIPCGESCVYIPCTVTALLGCSCKDKVCYKN	*Clitoria ternatea* L.	antiproliferative; cytotoxic	In vitro	IC_{50} 0.7 μM	Lung cancer cells	[147]
	RKYVD	*Curcuma longa* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Dalton's lymphoma ascites cell	[148]
	APVLQIKKTGSN	*Fagopyrum esculentum* Moench	proliferative	In vitro	At concentration 10 μg/mL, increased percentage of lympoblasts 3.61 folds	HL-60 cells	[149]
	VFDGEL	*Glycine max* (L.) Merr.	antiproliferative	In vitro	IC_{50} 7.9 mM	Inhibitor of topoisomerase II	[70]
	MLPSYSY	*Glycine max* (L.) Merr.	antiproliferative	In vitro	At concentration 1 mg/mL decreased number of cells in G2/M phase for 17%	P388D1 cells	[150]
	AEINMPNY	*Glycine max* (L.) Merr.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HeLa cells	[151]
	FEITPEKNPQ	*Glycine max* (L.) Merr.	antiproliferative	In vitro	IC_{50} 2.4 mM	Inhibitor of topoisomerase II	[70]
	IETWNPNNKP	*Glycine max* (L.) Merr.	antiproliferative	In vitro	IC_{50} 4.0 mM	Inhibitor of topoisomerase II	[70]
	GRGDDDDDDDDD	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	MCF-7 cells; Caco-2 cells; HepG2 cells	[122]
	GVNLTPCEKHIMEKIQ	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	MCF-7 cells; Caco-2 cells; HepG2 cells	[122]
	SKWQHQQDSCRKQKQ	*Glycine max* (L.) Merr.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	MCF-7 cells; Caco-2 cells; HepG2 cells	[122]
curcacycline A	GLLGTVLL	*Jatropha curcas* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	MCF-7 cells; Caco-2 cells; HepG2 cells	[122]
curcacycline B	PILLGILGS	*Jatropha curcas* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HeLa cells	[152]
	CTLEW	*Juglans regia* L.	antiproliferative; cytotoxic	In vitro	At the concentration of 1.0 mg/mL increased number of cells in sub-G1 phase to 18.24% (control- 3.79%); increased the apoptosis	MCF-7 cells	[130]

(continued on next page)

Table 2 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Examined cell line or enzyme	Reference
					rate- 4.45 times; IC_{50} 0.60 ± 0.17 mg/mL		
	EQRPR	*Oryza sativa* L.	antiproliferative	In vitro	At concentration 600-700 µg/mL inhibited growth of colon cancer cells 84%, breast cancer cells growth 80% and liver cancer cells growth 84%	Colon cancer cells (Caco-2, HCT-116); breast cancer cells (MCF-7, MDA-MB-231); liver cancer cells (HepG-2)	[153]
	FRDEHKK	*Oryza sativa* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	PBMC cells	[154]
Oryzatensin	GYPMYPLPR	*Oryza sativa* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	PBMC cells	[155]
TK17	TPMGGFLGALSSLSATK	*Oryza sativa* L.	antiproliferative	In vitro	At concentration 100 g/mL decreased proliferation for 20%	PBMC cells	[135]
GBP1	NSVFRALPVDVVANAYR	*Oryza sativa* L.	antiproliferative	In vitro	At concentration 100 g/mL decreased proliferation for 30%	PBMC cells	[155]
	GLTSK	*Phaseolus vulgaris* L.	antiproliferative	In vitro	IC_{50} 134.6 µM; at concentration 100 µM number of cells in G2 phase increased to 54.1% and the S phase was reduced to 15.6% (control 39.9% in G1, 40.4% in S and 19.5% in G2 phases)	HCT116 cells	[156]
	LSGNK	*Phaseolus vulgaris* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HCT116 cells	[156]
	MTEEY	*Phaseolus vulgaris* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HCT116 cells	[156]
	GEGSGA	*Phaseolus vulgaris* L.	antiproliferative	In vitro	IC_{50} 156.7 µM; at concentration 100 µM number of cells in G2 phase increased to 63.6% and the S phase was reduced to 2.4% (control- 39.9% in G1, 40.4% in S and 19.5% in G2 phases)	HCT116 cells	[156]
	MPACGSS	*Phaseolus vulgaris* L.	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HCT116 cells	[156]
limyin	KTCENLATYYRGPCF	*Phaseolus limensis* Macfad.	antiproliferative	In vitro	At concentration 50 µM inhibited growth of for 60%	BEL-7402 cells and SHSY5Y cells	[157]
	RGPPP	*Pseudostellaria heterophylla* (Miq.) Pax	antiproliferative	In vitro	At concentration 100 µg/mL exhibited stimulation index 1.27	Caco-2 cells	[158]
	RGVGV	*Pseudostellaria heterophylla* (Miq.) Pax	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Caco-2 cells	[158]
	PCNHSFR	*Pseudostellaria heterophylla* (Miq.) Pax	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity	Caco-2 cells	[158]

(*continued on next page*)

Table 2 (*continued*)

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Examined cell line or enzyme	Reference
					and its activity wasn't determined		
	QRFRALASAR	*Pseudostellaria heterophylla* (Miq.) Pax	antiproliferative	In vitro	At concentration 50 μg/mL exhibited stimulation index 1.2	Caco-2 cells	[158]
	VDVWFKNVER	*Pseudostellaria heterophylla* (Miq.) Pax	antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	Caco-2 cells	[158]
	EDDQMDPMAK	*Setaria italica* (L.) P.Beauv.	antiinflammatory; antiproliferative	In vitro	At concentration 100 μg/mL decreases viability for 15%	Caco-2 cells	[139]
	QNWDFCEAWEPCF	*Setaria italica* (L.) P.Beauv.	antiinflammatory; antiproliferative	In vitro	At concentration 100 μg/mL decreases viability for 10%	Caco-2 cells	[158]
	QQPQDAVQPF	*Triticum durum* Desf.	antiinflammatory; antiproliferative	In vivo	At concentration 10 μg/mL decreased proliferation for 40%	PBMC cells	[143]
	PPYCTIVPFGIFGTNYR	*Triticum durum* Desf.	antiinflammatory; antiproliferative	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	PBMC cells	[143]
VarvA	GETCVGGTCNTPGCSCSWPVCTRNGLPVC	*Viola arvensis* Murray	antiproliferative; cytotoxic	In vitro	IC_{50} 6 μM	U-937 GTB cells	[159]
VarvB	TCTLGTCYTAGCSCSWPVCTRNGVPICGE	*Viola arvensis* Murray	antiproliferative; cytotoxic	In vitro	IC_{50} 4 μM	U-937 GTB cells	[159]
cycloviolacin O2	GIPCGESCVWIPCISSAIGCSCKSKVCYRN	*Viola odorata* L.	antiproliferative; cytotoxic	In vitro and in vivo	IC_{50} 3.27 μM; lethal to mouse in the dose: 2 mg/kg	U-937 GTB cells	[160, 161]
Viphi A	GSIPCGESCVFIPCISSVIGCACKSKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	IC_{50} 4.91 μM (MM96L) IC_{50} 15.5 μM (HeLa) IC_{50} 3.19 μM (HFF-1) IC_{50} 1.75 μM (BCG-823)	MM96L cells; HeLa cells; HFF-1 cells; BCG-823 cells	[162]
Viphi F	GSIPCGESCVFIPCISAIIGCSCSSKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	IC_{50} 6.35 μM	HeLa cells	[162]
Viphi G	GSIPCEGSCVFIPCISAIIGCSCSNKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	IC_{50} 1.03 μM (MM96L) IC_{50} 6.35 μM (HeLa) IC_{50} 1.76 μM (HFF-1) IC_{50} 2.91 μM (BCG-823)	MM96L cells; HeLa cells; HFF-1 cells; BCG-823 cells	[162]
Vitri A	GESCVWIPCITSAIGCSCKSKVCYRNGIPC	*Viola tricolor* L.	antiproliferative; cytotoxic	In vitro	IC_{50} 0.6 μM	U-937 GTB cells	[163]

Abbreviations and markings: HD_{50}- concentration of peptide that causes hemolysis in 50% of examined cells; IC_{50}- concentration of peptide that causes inhibition of growth in 50% of examined cells or concentration of peptide that causes 50% enzyme inhibition; PBMC- peripheral blood mononuclear cells.

classification of peptides into distinctive groups presents a problem; 2. to save time and cost, effect of peptides is often assessed *in vitro* and usually as a part of extract, which makes determining of activity of individual peptides very difficult. These two issues and reasons behind them are further discussed in Chapters 5 and 6.

5. Is there connection between structural characteristics and activity?

As evidenced in Tables 1–3, the highest percentage of immunomodulatory peptides isolated from plants exhibit antiproliferative and antiinflammatory activity (Fig. 3). Due to the small number of peptides exhibiting proliferative activity, results obtained for this peptide class should be taken with care.

To assess if there is difference and a correlation between types of amino acid (AA) and bioactivity of the peptides, we compared observed and expected frequencies for each type of AA residues (aliphatic, aromatic, polar, and positively and negatively charged) using the chi-square test [167]. For analysis we have used collection of 109 peptides which sequences are listed in Tables 1–3.

The expected frequencies were calculated using this formula:

Expected frequency = (the number of amino acids in a given group × the total number of peptides with a particular effect) / 20

Percentages of individual types of amino acids residues in peptides of different activity are presented in Table 4, considering the set of peptides listed in Tables 1–3. Results of chi square test also showed significant differences for observed and expected frequencies of AA residues in peptides with different activity ($\chi 2$ (1,4) = 38.52; p = 0.0012).

As it can be observed from Table 4, both antiinflammatory and proinflammatory peptides contain high percentage of positively charged and hydrophobic (aliphatic and aromatic) AA residues. This is in accordance with results obtained for antiinflammatory peptides isolated both from animal and plant food [168–171, Table 1]. For example, RQRK isolated from soybean [88], RGAVLH isolated from Korean nut pine (*Pinus koraiensis* Siebold & Zucc.) [171], MVWGP isolated from sunflower (*Helianthus annuus* L.) [172], all are either hydrophobic or positively charged. The reasons behind these structural requirements are

Table 3
Cytotoxic/cytoprotective peptides from food plants, source, and bioactivity demonstrated in vitro or in vivo. Where available, the common name of the peptide is indicated. Data were recovered from PlantPepDB and BIOPEP database and integrated with literature searches.

Name	Sequence	Source	Effect	In vivo/ in vitro	Activity	Examined cell line or enzyme	Reference
cyclosquamosin D	PGGVLSYY	*Annona squamosa* L.	cytotoxic; antiinflammatory	In vitro	IC_{50} from 1.1-2.1 µg/mL (depending of cell type); IC_{50} 60 nM (COX-2)	COX-2; Hep G2, Hep 2; Hep 15, KB; CCM2 and CEM cells	[164, 165]
cyclosquamosin F	PALTTYGA	*Annona squamosa* L.	cytotoxic	In vitro	IC_{50} from 1.1-2.1 µg/mL (depending of cell type)	COX-2; Hep G2, Hep 2; Hep 15, KB; CCM2 and CEM cells	[164, 165]
cyclosquamosin E	PGGVLSYYY	*Annona squamosa* L.	cytotoxic	In vitro	IC_{50} from 1.1-2.1 µg/mL (depending of cell type)	COX-2; Hep G2, Hep 2; Hep 15, KB; CCM2 and CEM cells	[164, 165]
cliotide T1	GIPCGESCVFIPCITGAIGCSCKSKVCYRN	*Clitoria ternatea* L.	antiproliferative; cytotoxic	In vitro	IC_{50} 0.6 µM	HeLa cells	[146]
cliotide T10	GIPCGESCVYIPCTVTALLGCSCKDKVCYKN	*Clitoria ternatea* L.	antiproliferative; cytotoxic	In vitro	IC_{50} 0.7 µM	HeLa cells	[147]
cliotide T11	GIPCGESCVFIPCTITALLGCSCKDKVCYKN	*Clitoria ternatea* L.	cytotoxic	In vitro	IC_{50} 8 µM	HeLa cells	[121]
	CTLEW	*Juglans regia* L.	antiproliferative; cytotoxic	In vitro	At the concentration of 1.0 mg/mL increased the number of cells in sub-G1 phase to 18.24% (control 3.79%); increased the apoptosis rate 4.45 times; IC_{50} 0.60 ± 0.17 mg/mL	MCF-7 cells; HeLa cells	[130]
	AGA	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition 34.63%	CaMPDE	[166]
	QIAK	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition 36.63%	CaMPDE	[166]
	RWIQ	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition around 37%	CaMPDE	[166]
	AKLMS	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition around 17%	CaMPDE	[166]
	QQAKQ	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition around 35%	CaMPDE	[166]
	KQLSTGC	*Linum usitatissimum* L.	cytotoxic	In vivo	activity inhibition 34.21%	CaMPDE	[166]
cyclolinopeptide D	PFFWIMLL	*Linum usitatissimum* L.	proinflammatory; cytotoxic	In vitro	IC_{50} 39 µg/ml	HeLa cells	[131]
cyclolinopeptide G	PFFWIMLM	*Linum usitatissimum* L.	proinflammatory; cytotoxic	In vitro	IC_{50} 43 µg/mL	HeLa cells	[132]
VarvA	GETCVGGTCNTPGCSCSWPVCTRNGLPVC	*Viola arvensis* Murray	antiproliferative; cytotoxic	In vitro	IC_{50} 6 µM	U-937 GTB cells	[159]
VarvB	TCTLGTCYTAGCSCSWPVCTRNGVPICGE	*Viola arvensis* Murray	antiproliferative; cytotoxic	In vitro	IC_{50} 4 µM	U-937 GTB cells	[159]
cycloviolacin O2	GIPCGESCVWIPCISSAIGCSCKSKVCYRN	*Viola odorata* L.	antiproliferative; cytotoxic	In vitro and in vivo	IC_{50} 3.27 µM lethal dose to mouse: 2 mg/kg	U-937 GTB cells	[160, 161]
Viphi A	GSIPCGESCVFIPCISSVIGCACKSKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HeLa cells	[162]
Viphi F	GSIPCGESCVFIPCISAIIGCSCSSKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	IC_{50} 6.35 µM	HeLa cells	[162]
Viphi G	GSIPCEGSCVFIPCISAIIGCSCSNKVCYKN	*Viola philippica* Cav.	antiproliferative; cytotoxic	In vitro	Peptide was analyzed as part of hydrolysate showing bioactivity and its activity wasn't determined	HeLa cells	[162]
Vitri A	GESCVWIPCITSAIGCSCKSKVCYRNGIPC	*Viola tricolor* L.	antiproliferative; cytotoxic	In vitro	IC50 0.6 µM	U-937 GTB cells	[163]

Abbreviations and markings: IC50- concentration of peptide that causes inhibition of growth in 50% of examined cells (or in the case of enzyme concentration of peptide that decreases activity of enzyme for 50%); CaMPDE- calmodulin-dependent-phosphodiesterase.

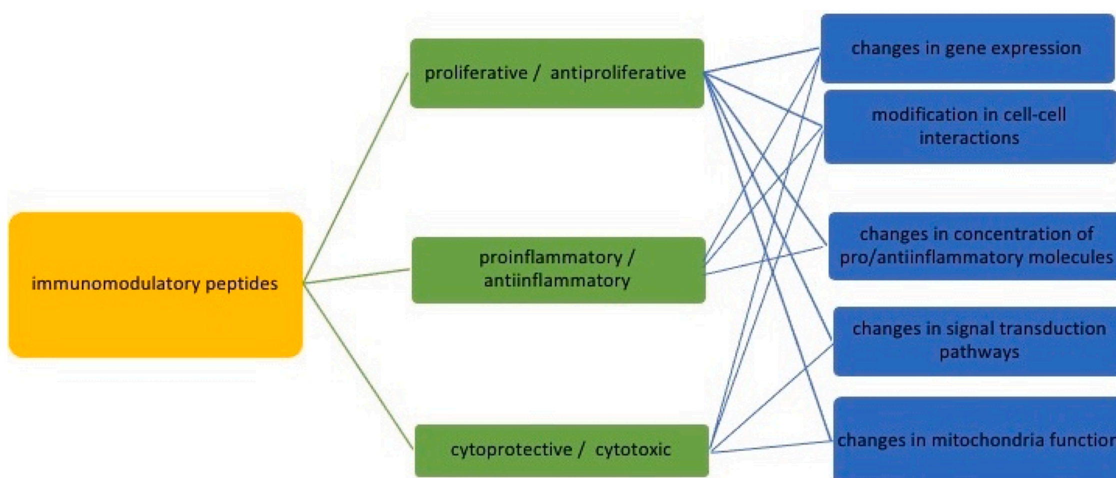

Fig. 1. Different activities involved in immunomodulation and the main mechanisms by which each effect is achieved.

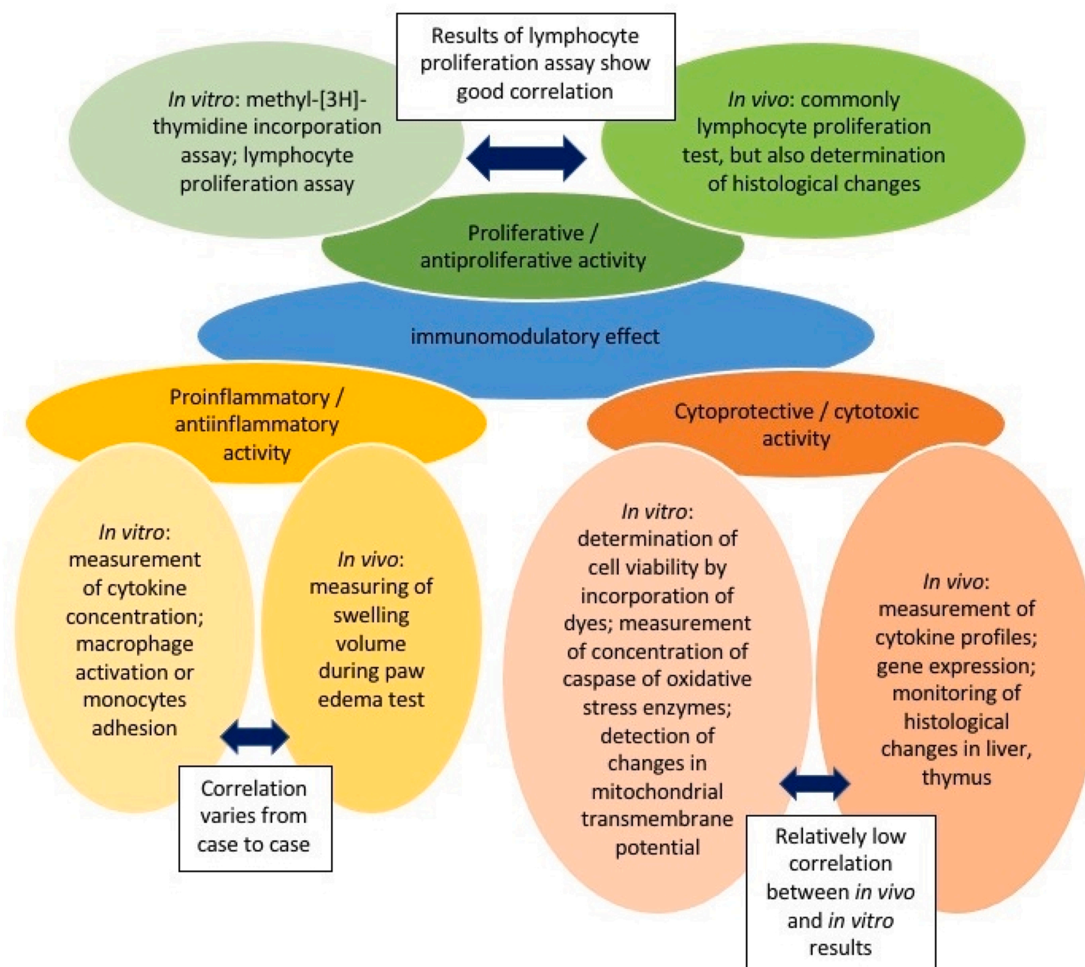

Fig. 2. Overview of assays most used for determining different aspects of immunomodulatory activity. Known correlations between in vivo and in vitro results are marked.

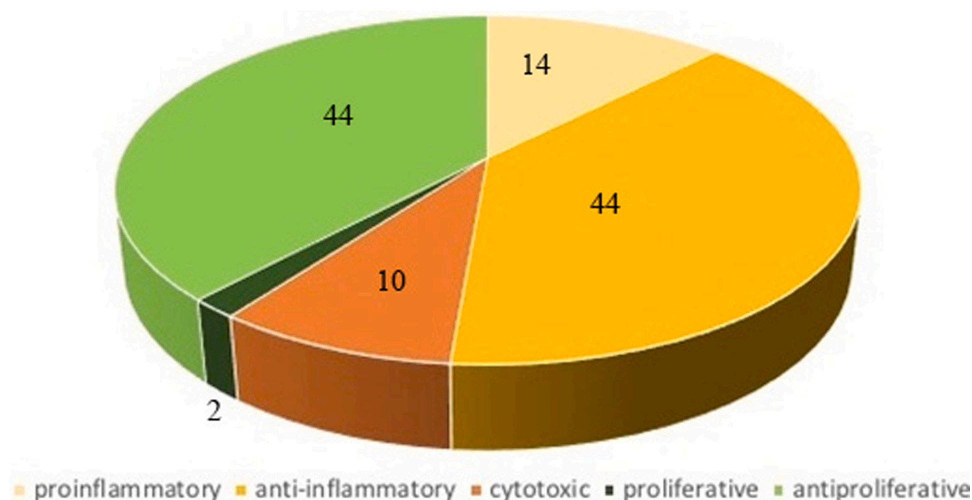

Fig. 3. Distribution of known plant immunomodulatory peptides according to the ascertained bioactivity. Peptides are listed in Tables 1–3.

Table 4
Percentage of positively charged, negatively charged, polar, aliphatic and aromatic AA residues in peptides of different activity.

Activity Type of AA	Proinflammatory (%)	Antiinflammatory (%)	Cytotoxic (%)	Proliferative (%)	Antiproliferative (%)
positively charged	9.89	8.05	4.04	25.00	10.36
negatively charged	5.49	10.84	2.02	8.33	7.77
polar	18.68	27.24	33.33	16.67	33.33
aliphatic	47.25	38.70	46.46	50.00	39.99
aromatic	18.68	15.17	14.14	0	9.25

two-folds: hydrophobicity allows for interaction between peptide and nonpolar components of cell membrane, which in turn modulate signaling pathways, usually mitogen-activated protein kinase (MAPK) pathway or nuclear factor-kappa light-chain-enhancer of activated B cells (NF-kB) pathway [168,169], while positive charge might allow peptide to act like chemokine, similarly to mechanism described for defensins [170,171]. However, there are exceptions to this positive/hydrophobic rule. For example, QEPQESQQ, QQQQQGGSQSQSQKG, GRGDDDDDDDDD from soybean all exhibit antiinflammatory activity while containing significant amount of polar and/or negatively charged AA residues [88,172–174]. Given that both proinflammatory and antiinflammatory peptides interact with same molecules either as activators or inhibitors [61,66,7–80,88,168–172], that could explain their similarity in type of AA present. As seen from Table 1, cytotoxic plant peptides possess high percentage of aliphatic and aromatic AA residues. This is the case, for example, of many of the peptides isolated from flaxseed that showed cytotoxic activity in vivo (AGA, QIAK, RWIQ, AKLMS, QQAKQ, KQLSTGC) [174, Table 3]. Such hydrophobicity is necessary because cytotoxic peptides form channels in membrane to achieve their cytotoxic effect [175]. Since proinflammatory peptides also require a degree of hydrophobicity to bind to cytokine receptors in membrane, like interleukin 1 receptor, interleukin 6 receptor [176,177], that could explain why peptides PFFWIMLL and PFFWIMLM isolated from linseed showed both proinflammatory and cytotoxic activity in vitro [178,179]. Additionally, Rekdal at al. [180] found that the presence of tryptophan near a positively charged residue could enhance cytotoxic activity, like for example, in the case of RWIQ peptide that exhibits high cytotoxic activity in vivo [175]. Dai et al. [181] suggested that positive charge is necessary for disruption of membrane integrity via snorkeling mechanism. Antiproliferative peptides also contain significant percentage of hydrophobic and positively charged AA residues (Table 4), which could explain why few of plant immunomodulatory peptides show both antiproliferative and antiinflammatory activity (Tables 1, 2). However, antiproliferative peptides also have significant number of polar AA residues. An explanation for why both polar and hydrophobic AA are present in high percentage in antiproliferative peptides was given by Dia and González de Mejia [182] on the example of lunasin from soybean. Such amphipathic composition is necessary for interaction of lunasin with integrins, namely, α5β1 integrin, which allowed it be internalized into the nucleus. Once in the nucleus, lunasin changed the expression of genes coding for key molecules involved in migration, adhesion and signal transduction in the cell: the main ones are genes for focal adhesion kinase (FAK), p50 nuclear factor enhancer of the kappa light chains of activated B cells (NF-κB), inhibitor of kappa B alpha (IκB-α) and topoisomerase [182,183]. Similarly, as with cytotoxic peptides, the presence of positively charged AA residues allows for better interaction of peptide with membrane of cancer cell [184,185].

Additionally, similarly to results obtained for peptides derived from animal sources [1], at least one proline residue was present in 59.64% of examined sequences. A reason behind such proline abundance is probably due to the fact that proline increases stability of peptide in gastrointestinal tract [186], making it less susceptible to degradation by proteases.

Giving that these characteristics of individual peptide classes (high hydrophobicity and positive charge of antiinflammatory/proinflammatory peptides; high number of aliphatic and aromatic residues in cytotoxic peptides with positive charge near N-terminus, as well as amphipatic character of antiproliferative peptides), bioinformatics and statistics can be employed to predict release of immunomodulatory peptides from proteins, as well as to predict effect of peptide based on its AA composition. In fact, new statistical methods for prediction of release of antiproliferative and anti-inflammatory peptides are being developed [187–189].

6. *In vitro* vs. *in vivo* activity

Tables 1–3 represents a collection of plant-derived immunomodulatory peptides, created from PlantPepDB [190] (http://14.139.61.8/PlantPepDB/index.php; last accessed on July 10th, 2021), BIOPEP

database [191] (http://www.uwm.edu.pl/biochemia/index.php/en/biopep; last accessed on July 18th, 2021) and our own literature search. As it can be observed from Tables 1–3, significantly more peptides are tested *in vitro* (using appropriate cell lines) than *in vivo* (using animal models and, to drastically less extent, human subjects).

Price, speed and ethical concerns are the main reasons why immunomodulatory activities of peptides are often determined *in vitro* or *ex vivo*. However, it is difficult to assess correlation between *in vitro* and *ex vivo* results and those from *in vivo* studies, because this depends on several factors:

1 Type of test used: *in vitro* and *in vivo* studies typically use different methods to determine the same bioactivity (Fig. 2). For example, anti-inflammatory activity is often determined *in vitro* by measuring cytokine profiles or macrophage activation, while *in vivo* cell-mediated immune and humoral immune responses are monitored directly or by determining spleen parameters [192,193]. The problem with using different tests is that they have different sensitivities and specificities. Even when the same methodology is used *ex vivo* and *in vivo*, results may vary [96]. For example, cytotoxicity assays that measure cytokine profiles show very low correlation with *in vivo* results, due to diminished sensitivity of tumor cells to such tests and inaccurate measurements of different cytokine subpopulation concentrations [194,195].
2 Stability of immunomodulatory peptides *in vivo*: there is a lack of correlation between gastrointestinal digestion and simulation of digestion *in vitro* in terms of both the structures and stabilities of resulting peptides. Such differences primarily stem from two factors: type and specificity of enzyme(s) applied for hydrolysis, together with the inability of digesters faithfully to imitate peristaltic action, changes in pH and ionic force, and the emulsification process in a living gastrointestinal tract [74,196,197]. During hydrolysis in the lab, pure enzymes or enzyme combinations are used and pH is kept constant. Such conditions do not exist in the gastrointestinal tract. Furthermore, the presence of small molecules that can act as activators or inhibitors for proteolytic enzymes is not replicated in laboratory settings. Sager et al. [198] proposed so called GastroDuo and the salivary tracer technique for tracking of drug release and metabolism during fasting conditions. Similar technique could be employed to monitor the fate of bioactive peptides in the gastrointestinal tract.
3 Matrix effects: this is an umbrella term used to encompass all interactions between individual food components. Certain constituents, such as lipids, can significantly decrease rates of hydrolysis by protecting peptides from degradation. This can have both positive and negative effects: peptide stabilities may be enhanced or alternatively less-active peptides can be formed or peptides that could activate an allergic response [1,196,199].

Low reproducibility of *in vitro* studies can also be due to low reproducibility in complex cell models [200]. Such low reproducibility in complex cell models can arise from several factors: incorrect selection of culture media, too high or too low cell density, usage of various test to report the same activity. Hirsch and Schildknecht [200] suggested several guidelines (such as correct selection of plasticware and culture media, better cell characterization, etc.) to enhance reproducibility when using complex cell models.

Although many studies using whole plant hydrolysates (from mung bean, *Vigna radiata* (L.) R.Wilczek, lousewort, *Pedicularis longiflora* Rudolph, wild garlic, *Allium carolinianum* Redouté, aloe, *Aloe vera* (L.) Burm.f., etc.) have shown comparable effect of those hydrolysates *in vitro* and *in vivo* [96,201–204], comparison of *in vitro* and *in vivo* effect of individual plant immunomodulatory peptides is rarely done. Two notable exceptions are lunasin from soybean and cyclotides, which are present in five plant classes: Rubiaceae, Solanaceae, Violaceae, Fabaceae and Cucurbitaceae. Both types of peptides possess unique structural features, namely disulfide bond and "molten globule like" structure in case of lunasin and disulfide bond and cyclic structure in the case of cyclotides, allowing them to remain relatively intact in the gastrointestinal tract and thus conserve their antiproliferative effect [204–208].

On the other hand, using animals as model systems for predicting results in humans has its difficulties. Given their small size, ease of breeding and speed by which they reach sexual maturity, mice and rats are commonly used as model system for human immune system [209–211]. Mice are used far more than rats, due the fact that their genome is easier to manipulate and also because currently few types of lab mice models are used for assessing the function of individual classes of immune cells - like, for example, β2M-knockout mice that served to elucidate role of NK cells in immune response [212] - or as models for specific disease: for example, functional human immune system (HIS) mouse engrafted with peripheral blood mononuclear cells as a model for cancer, severe combined immunodeficiency (SCID) mice as a model for autoimmune diseases [209–215]. Also, manipulations can be done to obtain mouse models for specific diseases, for example, induction of colitis in rats by addition of dextran sulfate [216–220] or induction of arthritis in rats by injection of Freund's complete adjuvant (FCA) or collagen [221–223]. These models are useful for elucidating the mechanism by which plant hydrolysates prevent these inflammatory diseases: for instance, Li et al. [149] found that addition of barley leaf in diet of mice in which colitis was induced by dextran sulfate activated peroxisome proliferator-activated receptor-γ (PPARγ) signaling and modulated gut microbiota. Similarly, Xuan-qing et al. [219] and Miao et al. [220] found that Baitouweng, traditional Chinese mixture comprised of multiple medicinal plants [220], alleviated symptoms of ulcerative colitis in mice by regulating microbiota, restoring intestinal epithelial barrier, modulating number of T cells subtypes (namely, T helper 17 (Th17) and T regulatory (Treg) cells) and activating interleukin 6/ signal transducer and activator of transcription 3 (IL-6/STAT3) pathway [219,220]. However, a common problem is that in all these instances the effect of hydrolysates and plant preparations rather than individual peptides was monitored [217,222,223].

However, there are several reasons why the mouse is not a good model for a human immune system:

1 Different signaling molecules: Vitamin D is the main molecule controlling immune system in humans [209–211,224,225]. Besides being involved in activation of innate immune response, primarily via activation of monocyte via vitamin D receptor (VDR) [226], modulation of cell differentiation and inhibition of cell, it is also involved in reduction of inflammation, primarily via inhibition of proinflammatory cytokines from macrophage [227] and expression of genes involved in antimicrobial response [226]. On the other hand, expression of VDR in mice is much lower [224] and the main signaling molecule in immune system of mouse is nitric oxide [210,211,218]. Therefore, in reactions where NO is a key signaling molecule, such as in inflammation [228,229], results obtained in mice should be taken with caution. Additionally, some signaling molecules such as granulysin, proinflammatory and cytolytic molecules present in granules secreted by natural killer (NK) cells and interleukin, are absent in mice [230,231].
2 Difference in enzyme concentration and/or activity: Concentration and/or activity of certain enzymes, mainly those involved in apoptosis like Caspase 8 and Caspase 10 [211] are also different in mice and humans.
3 Different amount of cell subtypes: Neutrophils, CD8 + T cells are present in higher concentration in humans, while lymphocytes are present in higher numbers in mice [209,211,231,232].
4 Different expression of receptors on surface of cell involved in immune response: Many of receptors that are involved in cell differentiation, cell to cell adhesion and "communication" between cells in humans, such as CD4, CD58, ICAM3, Toll-like receptors, interleukin-8 receptor, are absent in mice [209,231,232].

5 Different response to signaling molecules: Even in cases where there is no difference in concentration and/or activity of receptor, response of same cell subclass to same "trigger" might be different in mice and humans. For example, interferon-α (IFN-α) promotes differentiation of T helper 1 (Th1) cells in humans, but not in mice [231]. Activation of natural killer (NK) cells in mice and humans is also significantly different [233].
6 Age of the mouse (rat): Due to the lower cost, younger animals are often used for research [232]. This could be a problem because both production and maturation of B and T cells are continuing during the first 26 weeks of mouse life [232–234].
7 Different microflora: Impact of microbiota on modulation of immune response is well documented [235,236]. Microbiota of humans and all types of lab mice are significantly different, due to the sterile conditions in the lab [211,232]. Masopust et al. [211] found that microbiota of so-called "dirty" mouse (mouse from the wild) is much more similar to the microflora in human gastrointestinal tract. Thus, using "dirty" mouse as a model system might have some advantages.

Therefore, comparison of data obtained on mice or rats with those obtained from human volunteers is shaky at best.

Another thing to consider is great variability among human subjects, especially when examining inflammatory response [95,96]. Sometimes the effects of peptides can be either masked or exaggerated because of significant variation in the sample of human participants. Such differences stem from the fact that the immune response is influenced by a combination of genetic differences, hormonal status, age, body composition, level of stress, and other factors [237–242].

7. Defensins and cryptic peptides - a promising template for design of synthetic peptides

Two classes of immunomodulatory peptides are receiving increased attention – defensins and cryptic peptides [58,243–246]. Although both defensins and cryptic peptides are classified as antimicrobials, both also exhibit immunomodulatory effects [247,248]. Different classes of defensins have slightly different structures, but all defensins are cationic peptides rich in cysteine. The cysteine facilitates antioxidative effects and enables their regulation of reactive species production. Additionally, they can influence both innate and adaptive immune responses, through macrophage and neutrophil activation and participation in signal transduction pathways [248,249]. However, they can also act as immunosuppressors and/or result in production of proinflammatory cytokines [246]. Cryptic peptides are structurally less homogenous, but it is proven that they are produced from plant proteins in response to antigen [245]. They can also influence innate immunity through activation of natural killer cells [245]. Pearce et al. isolated from soybean leaves a peptide called GmSubPep [250]. It was derived from extracellular subtilisin-like protease (subtilase) and had the ability to initiate MAPK signalling cascade by binding to putative receptor in membranes [250]. CAP-derived peptide 1 (CAPE1) isolated from tomato has also been shown to enhance transcription of genes involved in antioxidative defence and modulate protein-protein interaction [251].

Given their multifunctionality, both defensins and cryptic peptides can serve as blueprints for synthetic peptide design and, after purification or synthesis, as active agents in vaccines or as integral components of functional food and feed. Keeping in mind the conserved structural characteristics of defensins (45–54 AA residues, three anti-parallel β-sheets and one α-helix stabilized by 4 disulfide bonds) [252], and the known sequences of isolated cryptic peptides [249,250], it is possible to synthesize peptide with similar characteristics that would then, based on previously discussed correlation between structure and function, exhibit similar effect *in vitro* and *in vivo*. For example, Shwaiki et al. [253] synthetized D-lp1, peptide whose sequence is based on defensins from barley and that showed strong antimicrobial activity towards spoilage yeast by causing overproduction of reactive oxygen species and changing membrane permeability. Therefore, such peptide could have a commercial application in food industry to prevent food spoilage.

8. What next?

Currently the main way to produce peptides that may be used in drugs, food or vaccine production is *in silico* analysis based on structural similarities with peptides that already exhibit the required *in vivo* effect [254]. Free online software, such as the recently introduced PepFun [255], is very useful. Although *in silico* analysis does not always yield peptides with the desired effect, it saves time and reagents and allow for a rapid discovery of stable peptides. These synthetic peptides can also be employed as agents in drug design, adjuvants in vaccines or as part of new functional foods.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors report no declarations of interest.

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