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The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells

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Abstract The process of carcinogenesis is complex and not easy to eliminate. It includes the initial occurrence of genetic alterations which can lead to the inactivation of tumor-suppressor genes and further accumulation of genetic alterations during tumor progression. Looking for food and food components with biological properties, collectively called nutraceuticals, that can hinder such alterations and prevent the inactivation of tumor-suppressor genes is a very promising area for cancer prevention. Proteins and peptides are one group of nutraceuticals that show potential results in preventing the different stages of cancer including initiation, promotion, and progression. In this review, we summarized current knowledge on the use of nutraceutical proteins and peptides in cancer prevention and treatment. We focused on the role of plant protease inhibitors, lactoferrin and lactoferricin, shark cartilage, plant lectins, and lunasin in the apoptosis, angiogenesis, and metastasis of cancer cells. Also included are studies on bioavailability and clinical trials conducted on these promising proteins and peptides.

Keywords Nutraceuticals · Proteins · Peptides · Apoptosis · Angiogenesis · Metastasis

1 Overview

The term nutraceutical was coined by Stephen DeFelice 21 years ago combining the words nutrition and pharmaceutics [1–3], both of which are important in human health. It encompasses any food or its components that, in addition to nutrition, provide health benefits including the prevention and treatment of malignancies. Thus, a nutraceutical is different from a dietary supplement as it must not only complement the diet but should aid in the prevention and/or treatment of a disease or a disorder. In addition, it is represented as a conventional food in the diet or as the sole item of a meal [2].

Food proteins are considered not only as nutrients for the proper maintenance of body functions but also as a source of important peptides with known biological activities. Biologically active peptides and proteins are either naturally occurring or produced by enzymatic digestion or fermentation. Bowman–Birk-type and Kunitz-type trypsin inhibitors are examples of naturally occurring proteins with known nutraceutical functions, while products of protein enzymatic digestion or hydrolysates are sources of biologically active peptides. Moreover, certain amino acids like arginine and glycine have demonstrated nutraceutical activities. In addition, food proteins, in general, upon gastrointestinal digestion can generate peptides with certain biological activities. In fact, not all dietary proteins are converted to individual amino acids and most of the ingested proteins are absorbed in the form of small peptides primarily in the form of dipeptides and tripeptides. In addition, small quantities of large peptides and proteins are absorbed [4]. This then results in the modulation of cellular activities, as peptides and proteins play a major role in different systems of the body such as the immune and endocrine systems. Therefore, food proteins can be considered as a source of nutraceutical peptides and amino acids which can exert biological functions to promote health and prevent disease, including cancer.

Cancer is considered a major public health problem not only in the United States (US) but also in many parts of the

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world. In the US, one of four human deaths is associated with cancer [5]. Moreover, it is also the leading cause of death in the population <85 years of age [5]. As expected, the survival rate of patients suffering from cancer is dependent on the stage on which the cancer is diagnosed. In general, a 5-year survival rate of at least 90% is associated with initial stages of cancer where metastasis is not involved, and the survival rate decreases to approximately 8% at later stages of the disease where tumor invasion of other organs and regional lymph nodes and distant metastases are involved [6–8]. As such, metastasis of tumors from their primary origin is difficult to manage and results in a very poor prognosis of the patient. It is, therefore, important to search for naturally occurring food components or products of their digestion that can mediate the process of metastasis and angiogenesis. Several studies have shown the anticancer potential of dietary proteins, peptides, and amino acids, either naturally occurring, product of fermentation, enzymatic hydrolysis, or gastrointestinal digestion, by mediating apoptosis and angiogenesis, important steps to control tumor metastasis.

Apoptosis, also known as programmed cell death, is characterized by a series of morphological cell changes [9] which includes loss of specialized surface structure, reduction in volume, conservation of cytoplasmic organelles, condensation of nuclear chromatin, and phagocytosis [10, 11]. Apoptosis can occur via two distinct signaling pathways, the intrinsic and the extrinsic. The intrinsic pathway targets the mitochondrial membrane in response to stresses like lack of growth factors and DNA injury [12], while the extrinsic pathway is associated with binding of apoptosis-inducing ligands to cell surface receptors such as Fas-associated death receptor, tumor necrosis factor-α (TNF-α) receptor, and TNF-related apoptosis-inducing ligand receptors [13]. The ability to escape apoptosis is one of the hallmarks of cancer cells [14] and can lead to tumor progression.

Angiogenesis, the formation of new blood vessels, is one of the requirements for tumor growth and metastasis [15]. Angiogenesis of cancer cells starts from the tumor itself by producing molecules that signal normal tissue to produce proteins necessary for the formation of new blood vessels. It provides oxygen and necessary nutrients for cancer cells to grow and eventually spread to other parts of the body. The members of the vascular endothelial growth factors (VEGF) and their receptors are currently recognized as regulators of angiogenesis. VEGF-A and its receptor, VEGFR-2, are the major players [16]. The attachment of VEGF-A to its receptors leads to signaling cascades that eventually favor the process of angiogenesis. As such, this step in the process of tumor progression is a target for the development of anticancer drugs to prevent the spread of tumors [17–19].

Metastasis is defined as the ability of cancer cells to infiltrate into lymphatic and blood vessels, travel through the bloodstream, and disseminate to other parts of the body. This process is mostly mediated by the activity of the matrix metalloproteinase (MMP) family of enzymes which degrade the extracellular matrix (ECM), a crucial step in tumor invasion and metastasis [20].

Thus, these three important processes, apoptosis, angiogenesis, and metastasis, are the main focal points for the study of carcinogenesis. Table 1 summarizes some physiological factors that regulate apoptosis, angiogenesis, and metastasis of cancer cells that can be targeted by nutraceutical proteins and peptides which may lead to cancer prevention and treatment. The objective of this review is to discuss the role of nutraceutical proteins and peptides in carcinogenesis including apoptosis, angiogenesis, and metastasis.

2 Plant protease inhibitors

Protease inhibitors are found in plant tissues, particularly from legumes. Epidemiological studies have shown that legumes can act as possible protective agents against several types of cancer such as breast, colon, and prostate. This has led to a series of extensive *in vitro* and *in vivo* studies and clinical trials on the chemopreventive effects of legumes and their components. To date, several investigations have shown that plant protease inhibitors can inhibit different stages of carcinogenesis including initiation, promotion, and progression.

There are several groups of plant protease inhibitors such as serine protease inhibitors, potato inhibitors, carboxypeptidase inhibitors, cystatin (cysteine protease inhibitors), and mustard and squash inhibitors. Table 2 summarizes some of the biological activities of plant protease inhibitors associated with carcinogenesis.

One of the most extensively studied plant protease inhibitor in the field of carcinogenesis is the soybean-derived Bowman–Birk inhibitor (BBI); other BBI from lentil and pea have also shown similar biological activities. The role of BBI in carcinogenesis was evaluated either as a purified BBI or as BBI concentrate (BBIC) [21–23]. Saito et al. [24] showed that BBI was able to control the growth of human osteosarcoma cells (U2OS) via the induction of a tumor-suppressor gene called connexin 43 (Cx43). They showed that BBI (200 μg/ml during 8 days) had a negative growth effect on U2OS cells, with concomitant cytostatic effect at the G_1 phase of the cell cycle and an increase in apoptosis, observed by an augmented number of cells at the sub-G_1 phase. Moreover, they showed that BBI restored the expression of tumor-suppressor Cx43 mRNA level in a time-dependent manner. This event was linked to the

Table 1 Regulators of apoptosis, angiogenesis and metastasis in carcinogenesis

Apoptosis
Pro-apoptosis
Bcl-2 family of proteins: Bax, Bad, Bak, Bid
Fas-L
TNF
TRAIL
Cytochrome *c*
p53
PTEN
Anti-apoptosis
Bcl-2 family of proteins: Bcl-2, Bcl-X_L
XIAP
Survivin
c-IAP2
c-FLIP-L
NF-κB
PI3K/Akt
Angiogenesis and metastasis
Growth factors (GF)
Vascular endothelial GF
Acidic fibroblast GF
Basic fibroblast GF
Placental GF
Hepatocyte GF
Granulocyte colony stimulating GF
Platelet-derived GF
Platelet-derived epidermal GF
Epidermal GF
Transforming GF
Inflammatory molecules
Cyclooxygenase-2
Prostaglandin E_2
Interleukin-1
TNF-α
Interleukin-6
Interleukin-8
Integrins and other adhesion molecules
Alpha v beta 3
Alpha v beta 5
Fibronectin
Cadherins
Catenins
Selectins
Proteases
Urokinase form of plasminogen activator
Matrix metalloproteinases
Cathepsins
Others
Angiopoietin-1
Angiogenin
Oncogenes (c-*myc*, *ras*, c-*jun*)
Osteopontin
Leptin
Hypoxia-inducible factor 1

capability of BBI to restore gap junction intercellular communication as measured by the mean number of cells reached by Lucifer yellow dye from 0.2 (untreated) to 5.5 (BBI-treated). They also showed that the negative growth, controlled by BBI, was Cx43-dependent as introduction of antisense oligonucleotides (ASO) resulted in 50% increase in saturation density, compared to the cells treated with BBI and no ASO. Another study by the same group also showed the ability of BBI to negatively control the growth of M5067 ovarian sarcoma in mice by decreasing tumor weight and proliferating cell nuclear antigen and increasing expression of Cx43 [25]. These studies demonstrated the ability of BBI to control the growth of cancer cells via increasing the expression of the tumor-suppressor molecule Cx43. Chen et al. [26] showed that BBI was able to suppress proteasomal chymotrypsin-like activity in MCF-7 breast cancer cells. The decrease in the proteasome chymotrypsin-like activity in MCF-7 cells was in conjunction with the increase in the expression of proteasome target proteins $p27^{KIP1}$ and $p21^{Cip1/WAF1}$. The increase in the expression of these cyclin-dependent kinases resulted in the arrest of the cell cycle at the G_1/S phase. This study also reported the ability of BBI to inactivate ERK1/2 phosphorylation but this effect was abrogated by the presence of phosphatase inhibitor, suggesting that BBI inactivated ERK1/2 by involvement of a specific phosphatase. The dephosphorylation of ERK1/2 was correlated with an increase in the induction of the MAP kinase phosphatase-1 in a dose-dependent manner. However, the study was not able to show the inhibitory effect of BBI on epidermal growth factor (EGF)-stimulated activation of ERK1/2 and Akt. Du et al. [27] showed the ability of BBI and its palmitic acid conjugate to inhibit chemical-induced carcinogenesis using cultured mouse mammary glands. They showed that BBI (20 μg/ml) and its palmitic acid conjugate (20 μg/ml) were able to inhibit 7,12-dimethylbenz[a] anthracene (DMBA)-induced transformation of mouse mammary glands during the developing period. It was demonstrated that only its palmitic acid conjugate was able to inhibit DMBA-induced transformation before the promotion period. This indicates that increased hydrophobicity improved the biological activity of BBI possibly due to the increase in tissue retention. It was also shown that BBI conjugated with palmitic acid had an increased residence time and decreased kidney clearance, thus increasing its activities in target tissues and organs [28].

Table 2 Nutraceutical properties of plant protease inhibitors associated with carcinogenesis

Plant protease inhibitors	Biological activity (dose, model)	References
Bowman–Birk inhibitor	Controlled growth of sarcoma cells through upregulation of Cx43 (200 μg/ml, *in vitro* and *in vivo*)	[24, 25]
	Suppressed proliferation of breast cancer cells through accumulation of MAPK phosphatase-1 and induction of apoptosis and lysosome membrane permeabilization (20 and 200 μM, *in vitro*)	[26, 29]
	Induced apoptosis in prostate cancer cells through upregulation of Cx43 (500 μg/ml and 2000 mg/kg/day, *in vitro* and *in vivo*)	[31]
	Antiproliferative effects on human colon cancer cells (19–125 μM, *in vitro*)	[33–35]
Kunitz trypsin inhibitor	Suppressed cancer invasion and metastasis through inhibition of uPA expression (10 μM and 50 g/kg diet, *in vitro* and *in vivo*)	[40, 42]
	Suppressed LPS-induced and UV-induced cytokine production (5 μM, *in vitro*)	[163, 164]
	Inducted apoptosis in rat lymphoma cells (5 μg/ml, *in vitro*)	[49]
Buckwheat trypsin inhibitor	Induced apoptosis in leukemia cells (0.5–100 μg/ml, *in vitro*)	[45, 47]
	Induced apoptosis in human solid tumor cells (6.25–50 μg/ml, *in vitro*)	[48]
Cysteine proteinase inhibitor	Inhibited murine B16 melanoma cell invasion (1 μg/ml, *in vitro*)	[51]
Carboxypeptidase inhibitor	Inhibited EGF/TGF-α antagonist A 431 and MDA-MB-453 cells (10–50 μg/ml, *in vitro*)	[53]

Cx43 connexin 43, *LPS* lipopolysaccharide, *UV* ultraviolet, *uPA* urokinase plasminogen activator, *MAPK* mitogen-activated protein kinase

A Bowman–Birk protease inhibitor from *Vigna unguiculata* seeds, called the black-eyed pea trypsin/chymotrypsin inhibitor (BTCI), induced apoptosis and lysosome membrane permeabilization in MCF-7 breast cancer cells [29]. BTCI caused a dose-dependent cytotoxic effect to MCF-7 breast cancer cells (IC_{50}=200 μM) without affecting the viability of normal breast cells (MCF-10A). The mechanism involved in the cytotoxic effect was found to be related to cell cycle arrest at the G_2/M and S phases as well as induction of apoptosis. The apoptotic process was associated with changes in the morphology of the nucleus and mitochondria, increased number of cells with reduced mitochondrial membrane potential, cells undergoing DNA fragmentation, and cells with altered plasma membrane integrity.

BBI has also demonstrated its ability to prevent the process of carcinogenesis in prostate cancer cells. Kennedy and Wan [30] demonstrated that BBI (100 μg/ml) affected the growth and clonogenic survival of different human prostate epithelial cells and prostate cancer cells. Moreover, they showed that BBI can inhibit invasion of LNCaP prostate cancer cells when PC-3 conditioned medium was used as a chemoattractant. In another study, Tang et al. [31] demonstrated the ability of BBI and BBIC to induce apoptosis in *in vitro* and *in vivo* prostate cancer cells, respectively. BBI caused a dose-dependent decrease in the viability of LNCaP, resulting in the complete growth inhibition at 500 μg/ml treated for 24 h. Also, BBI increased the expression of Cx43 and cleaved caspase-3 at 500 μg/ml with a peak at 9 h of treatment. These *in vitro* results were further confirmed by *in vivo* experiments using the transgenic rats developing adenocarcinoma of the prostate (TRAP) model fed with 3% BBIC. BBI also demonstrated its ability to inhibit the process of colorectal carcinogenesis. Kennedy et al. [32] showed that BBI (0.1% of the diet for 6 months) in its purified and BBIC (0.1% of the diet for 6 months) forms were able to inhibit rat colon carcinogenesis induced by dimethylhydrazine while the autoclaved BBIC with no protease inhibitor activity showed no suppressing activity, demonstrating the importance of the protease inhibitor activity in the anticancer potential of BBI. Furthermore, BBI from other legumes such as pea and lentils demonstrated their ability to inhibit proliferation of human colon cancer cells *in vitro*. BBI from lentils inhibited the growth of HT-29 colon cancer cells with an IC_{50} of 32 μM without affecting the growth of human normal colon fibroblast CCD-33Co [33]. In addition, BBI protease inhibitor from pea (PPIC) and its recombinant form showed similar growth inhibitory properties on HT-29, showcasing that the inhibitory property was independent of apoptosis, as the addition of caspase-3 did not abolished cell growth [34].

BBI isoinhibitors, IBB1 (with trypsin and chymotrypsin inhibitory properties) and IBBD2 (trypsin inhibitory property only), purified from soybean BBI also showed cytotoxic effect on HT-29 colon cancer cells [35]. These isoinhibitors inhibited the growth of HT-29 colon cancer cells (IC_{50}=39.9 μM for IBB1 and IC_{50}=48.3 μM for IBBD2) with concomitant increase in the number of cells in the G_0–G_1 phase of the cell cycle. The same study demonstrated the noncytotoxic effect of BBI when its protease inhibitor activity was removed. This protease inhibitor also demonstrated its *in vitro* chemopreventive effect on other types of cancer such as oral cavity [36, 37] and lung cancer [38, 39]. In contrast to previous studies, a

recent study in MDA-MB-231 breast cancer xenograft mouse model showed that BBI given at 20 mg/kg body weight for 2 months at a frequency of three times per week was not able to reduce tumor incidence in mouse [155]. Also, BBI was not able to suppress urokinase-type plasminogen activator (uPA) and cell invasion of ovarian cancer cells *in vitro* [40]. These conflicting results can be explained by the purity of BBI used in these studies and the possible presence of other bioactive compounds in BBIC. Clinical studies on the use of BBIC showed that it was well-tolerated by the patients and led to promising results for prostate and oral carcinomas.

Kunitz trypsin inhibitor (KTI) is another protease inhibitor originally isolated from soybean [41]. In contrast with BBI, KTI only has a trypsin inhibitory property and devoid of chymotrypsin inhibitory activity. The biological significance of KTI in carcinogenesis is mainly attributed to its ability to suppress invasion and metastasis of cancer cells. In an *in vitro* study conducted by Kobayashi et al. [42], KTI was able to suppress ovarian cancer cell invasion by blocking urokinase upregulation. They showed that KTI was able to suppress uPA upregulation in both unstimulated and transforming growth factor (TGF)-β1-stimulated HRA human ovarian cancer cell line, as well as its mRNA expression. This reduction in uPA cell-associated activity was in conjunction with its ability to block TGF-β1-stimulated phosphorylation of ERK1/2 at 3 μM. Moreover, this is also implicated with the ability of KTI, but not BBI, to reduce invasiveness of HRA ovarian cancer cells. Also, dietary supplementation of soybean KTI (5 to 50 g/kg diet) inhibited spontaneous metastasis but not experimental metastasis of Lewis lung carcinoma cells in C57BL/6 mice [40]. Also, dietary supplementation of KTI resulted in 40% reduction in total tumor burden in peritoneal metastasis using HRA ovarian cancer cell line implanted in C57BL/6 mice. The antimetastatic potential of KTI was associated with its ability to specifically reduce the expression of uPA protein and inhibit the phosphorylation of MAP kinase and PI3 kinase proteins including MEK, ERK1/2, and Akt and also the inhibition of Src-dependent signaling pathways. Inagaki et al. [43] reported that KTI was able to inhibit the Src signaling pathway as measured by the expression of phosphorylated Src, ERK1/2, and Akt in wild-type c-Src-transfected cells but not in constitutively active c-Src-expressing cells. It was concluded that upstream target(s) of Src were involved in KTI inhibited invasion and metastasis in ovarian cancer. Also, a Kunitz-type trypsin inhibitor isolated and purified from soybean showed *in vitro* cytotoxic and apoptosis-inducing properties to different cancer cell lines including nasopharyngeal cell lines CNE-1 and CNE-2 and human hepatocellular carcinoma cell line HepG2 [44], but the mechanism of action involved is yet to be elucidated.

Another class of protease trypsin inhibitor was found in buckwheat, a dicotyledonous plant widely cultivated in Asia, Eastern Europe, North America, and Australia. Park and Ohba [45] isolated two buckwheat protease inhibitors, BWI-1, which is a member of the potato inhibitor I family and inhibited trypsin, chymotrypsin, and subtilisin, and BWI-2a with homology to the vicilin family that only inhibited trypsin. BWI-1 and BWI-2a showed antiproliferative effect on T-acute lymphoblastic leukemia cells, Jurkat cells, and CCRF-CEM cells, without affecting the growth of human normal blood lymphocytes. The cytotoxic effect was attributed to their pro-apoptotic function as assessed by DNA laddering. This study also showed that the trypsin inhibitory activity was responsible for the cytotoxic and pro-apoptotic functions as chemical modification of the inhibitors, by blocking its arginine residue, resulted in the loss of these activities. The cytotoxic effect was also seen in the recombinant form of BWI-1 (rBTI) on multiple myeloma IM-9 cells at 200 μg/ml [46] and myeloid leukemia K562 cells at 12.5 to 200 μg/ml [47] via induction of apoptosis. Li et al. [48] demonstrated that the mechanism involved in the cytotoxic effect and pro-apoptotic properties of rBTI was through the action of caspases as well as the increased permeabilization of the mitochondria. In this study using solid tumor cells such as human esophagus (EC9706), human hepatoma (HepG2), and human cervical (HeLa) carcinoma cells, they demonstrated that rBTI was able to cause cytotoxicity in a time-dependent and dose-dependent manner. The mechanism involved in the cytotoxic effect was found to be related to mitochondrial pathways of apoptosis as evidenced by increased DNA fragmentation, increased expression of cytosolic cytochrome *c*, a concomitant reduction in the expression of cytochrome *c* in the mitochondria, increased mRNA and protein expressions of pro-apoptotic Bax and Bak, decreased mRNA and protein expressions of anti-apoptotic proteins Bcl-2 and Bcl-xl, upregulation of caspase-3 and caspase-9 activities, and disruption of the mitochondrial transmembrane potential. This study revealed a mechanistic insight on how rBTI induced apoptosis in solid tumors, indicating the importance of the trypsin inhibitory property as the cytotoxic effect of the inactivated rBTI was very minimal (approximately 10%), even at the highest concentration used (50 μg/ml).

Other proteinase inhibitors that showed potential in cancer prevention and therapy included the trypsin inhibitor isolated from *Peltophorum dubium*, with similar amino acid sequence to soybean KTI, that showed induction of apoptosis in rat lymphoma cells [49]. The trypsin inhibitor from sweet potato at 100 μg/ml induced apoptosis in NB4 promyelocytic leukemia cells through induction of Bax, p53, mitochondrial cytochrome *c* release, and caspase-3 activity and reduction in Bcl-2 expression [50]. Also, the

cysteine proteinase inhibitor from potato (PCPI 8.7) inhibited cell invasion of murine B16 melanoma cells [51]. The potato carboxypeptidase inhibitor (PCI) showed a potential to reduce tumor cell growth, invasion, and metastasis as it acted as an antagonist of the EGF. PCI was found to compete with EGF for EGF receptor (EGFR) binding which resulted in the inhibition of EGFR activation and cell proliferation in human pancreatic cancer cell lines Panc-1, Capan-1, and HIT [52]. Inhibition of EGFR signaling was demonstrated by a reduction in tyrosine transphosphorylation and a decrease in the kinase activity of EGFR. Moreover, the antiproliferative effect of PCI was demonstrated *in vivo* by a reduction in the growth of Capan-1 pancreatic tumors implanted subcutaneously in nude mice. In addition, Sitja-Arnau et al. [53] reported the mechanism of action by which PCI acted as an EGF blocker. They showed that PCI has an inhibitory effect on EGFR dimerization induced by EGF or TGF-α in A431 human vulvar epidermoid cell. Also, EGFR tyrosine phosphorylation was inhibited in MDA-MB-453 breast cancer cells and A431 cells when treated with PCI at concentrations ranging from 50 to 300 μg/ml. In addition, PCI inhibited ErbB-2 phosphorylation in BT474 breast cancer cells, indicating that PCI blocked the signaling pathway that drives epithelial cell proliferation.

All these studies support the hypothesis that plant protease inhibitors can be used as nutraceuticals to halt the process of carcinogenesis in humans at different stages, including initiation, promotion, and progression.

3 Lactoferrin and lactoferricin

Lactoferrin (Lf), one of the components of milk proteins, is an iron-binding protein originally found and isolated from breast milk. Its structure confers high resistance to degradation by proteolytic enzymes thus it can pass through the gastrointestinal tract intact, resulting in its internalization via a unique receptor-mediated mechanism [54, 55] thereby affecting gene transcription [54, 56]. Lf has a wide range of biological activities including antimicrobial properties, improvement of iron status, anti-inflammation, cell proliferation promotion, and improvement of immune function [57, 58]. Earlier studies suggest that the possible mechanism by which Lf acts as an antitumorigenic molecule is through innate ability to bind iron [59], since iron can promote oxidation thereby damaging nucleic acid structure. However, this suggested mechanism has not yet been fully delineated. Meanwhile, other potential mechanisms have been suggested regarding the role of Lf in the process of carcinogenesis [59–61] including induction of programmed cell death, prevention of angiogenesis, and regulation of cell cycle protein expression.

Table 3 offers an overview of different cancer types that can potentially be prevented or treated by Lf and lactoferricin (Lfcin). Human lactoferrin (hLf) promoted growth arrest at the G_1 to S transition of the cell cycle in breast cancer cells [62]. This arrest was accompanied by modification of the activity and expression of cyclin-dependent kinases including decreased expression and activity of Cdk2 kinase and increased expression of Cdk inhibitor p21 but not p27. It has also been shown that hLf induced cell cycle arrest at the G_0–G_1 checkpoint in three oral and neck cancer cells. The mechanism involved in the inhibition of cell cycle was found to be related to p27/cyclin E-dependent pathways and may be mediated by changes in Akt phosphorylation as hLf-treated cells showed a marked decrease in phosphorylated Akt [63]. In another *in vitro* study using a variety of human cancer cells [64], hLf demonstrated its ability to regulate cell growth by controlling the level of retinoblastoma protein (Rb), a key tumor suppressor involved in cell cycle progression. The investigators showed that hLf mediated cell growth arrest by maintaining Rb in its hypophosphorylated form with concomitant increase in the expression of p21, but not p27, and blockage of E2F1-responsive genes. In murine and human head and neck squamous cell carcinoma, hLf demonstrated its ability to arrest cell cycle at the G_1–G_0 phase with downregulation of cyclin D1 [65].This study also demonstrated the ability of hLf to inhibit cellular release of pro-inflammatory and prometastatic cytokines. Moreover, Lf demonstrated its chemopreventive effect in a wide range of cancer types. Mohan et al. [66] demonstrated the ability of bovine Lf (bLf) to modulate DMBA-induced hamster buccal pouch carcinogenesis. Also, bLf-fed hamsters painted with DMBA showed a significant reduction in the frequency of bone marrow micronucleated polychromatic erythrocytes when compared with just DMBA-painted group. The chemopreventive mechanism involved is the potential of bLf to modify the activities of enzymes involved in phase I and phase II metabolism. In bLF-fed hamster, the activity of cytochrome P450 (phase I enzyme) was reduced while the activities of glutathione *S*-transferase and DT-diaphorase (phase II enzymes) were increased when compared to DMBA-treated hamsters. This observation was consistent with both the buccal pouch and liver of the animals. In addition, bLf-treated hamsters showed lower lipid peroxidation than just the DMBA-painted hamsters. In C3h/HeJ mice implanted with SCCVII tumors, orally delivered (40 to 80 mg/kg/day for 10 days) hLf inhibited tumor growth (75%) and increase in the number of lymphocytes, compared with the control group [65]. In another study using female A/J mice, orally fed bLf showed post-initiation chemopreventive effect on lung tumorigenesis induced by 4-methylnitrosoamino-1-3-pyridyl-1-butanone [67]. Zemann et al. [68] recently showed that bLf (50 μg/

Table 3 Nutraceutical activities of lactoferrin and lactoferricin associated with carcinogenesis

Cancer type	Mechanism of anticancer action (dose, model)	References
Breast	Cell cycle arrest at G_1, decrease in cyclin-dependent kinases, increased expression of p21, maintaining pRb in hypophosphorylated form in MDA-MB-231 cells (50 μg/ml, *in vitro*)	[62]
	Cell cycle control via persistent hypophosphorylated form of Rb in H1299 cells (50 μg/ml, *in vitro*)	[64]
Cervix	Growth arrest through induction of TGF-β/Smad-2 signaling in HeLa cells (50 μg/ml, *in vitro*)	[68]
Colon	Cell cycle arrest at S phase through downregulation of cyclin E1 in Caco-2 cells (2 μM, *in vitro*)	[90]
	Induction of caspase-1 and IL-18 (30–300 mg/kg for 7 days, *in vivo*)	[73]
	Increase production of $CD4^+$, $CD8^+$, and IL-18 (30–300 mg/kg for 7 days, *in vivo*)	[72]
Head, neck, and oral	Reduction of DMBA-induced carcinogenesis through modulation of metabolizing enzymes and cellular redox status (0.2% of basal diet for 14 weeks, *in vivo*)	[66, 78]
	Tumor inhibition through direct cellular inhibition and immunomodulation (20–250 μM and 40–80 mg/kg/day for 10 days, *in vitro* and *in vivo*)	[65]
	Cell cycle arrest through downregulation of cyclin-dependent kinases and upregulation of p27 protein expression in head and neck cancer cell lines (10 μM, *in vitro*)	[63]
	Induction of apoptosis through JNK/SAPK activation in SAS cells (2 mg/ml, *in vitro*)	[87]
Leukemia	Cytotoxicity via caspase-mediated and cathepsin B-mediated mechanism in T-leukemia cells (31 μM, *in vitro*)	[91]
	Induction of apoptosis through activation of caspase and release of cytochrome *c* in T-leukemia cells (200 μg/ml, *in vitro*)	[88]
Lung	Inhibition of NNK-induced tumorigenesis via apoptosis (2%, *in vivo*)	[67]
	Cell cycle control via persistent hypophosphorylated form of Rb (50 μg/ml, *in vitro*)	[64]
Nerve	Inhibition of tumor growth and induction of apoptosis through activation of caspases in neuroblastoma cells (40 μg/ml and 1 and 2 mg/day for 3 days, *in vitro* and *in vivo*)	[89]

pRb phosphorylated retinoblastoma, *DMBA* 7,12-dimethylbenz[a]anthracene, *NNK* nitrosamine ketone

ml) inhibited the growth of HeLa cells through the activation of the TGF-β/Smad-2 pathway but not MAP kinase/ERK1/2 activation. Several studies have demonstrated that Lf played an important role in the process of angiogenesis and metastasis of cancer cells. In a study done in rats, orally administered bLF inhibited VEGF-165-induced angiogenesis as measured by the vascularized area, total microvascular length, microvessel intersection, and intersection in mesenteric windows [69]. This study also showed that bLf was able to decrease the proliferation rate of endothelial cells *in vitro*. Also, bLF inhibited angiogenesis in chick embryo chorioallantoic membrane (CAM) as shown by a larger area of vascularization [70]. This study also demonstrated the ability of bLf to inhibit cell proliferation of endothelial cells induced by VEGF and human basic fibroblast growth factor. Other studies have shown that bLf posed a property to inhibit metastasis of cancer cells including colon carcinoma metastasis in the lungs through increase in the number of cytotoxic cells in white blood cells [71], increase in the production of IL-18 in intestinal epithelial cells [72, 73], serum, and peritoneal macrophages [70], increase in caspase-1 activity and interferon-γ [73], and activation of interferon-α/IL-17 effector pathways. It has, therefore, been suggested that the multiple functions of Lf are associated with its ability to interact with receptors; this mechanism is not yet fully understood. Sakamoto et al. [74] showed, for the first time, that hLf interacts with proteins with the arginine–glycine–aspartic acid (RGD) motif, being bound to RGD-containing human ECM fibronectin and vitronectin.

Combination of Lf with other chemopreventive dietary components and other peptides against cancer has also been studied. The adjuvant effect of human neutrophil peptide-1 and Lf against oral squamous cell carcinoma (OSCC) showed that their combination had a potential cooperative effect in causing cytotoxicity [75]. Also, combination of Lf with tea polyphenols showed synergistic effect in causing cytotoxicity to CAL-27 oral carcinoma cells [76] and preventing hamster buccal pouch carcinogenesis [77, 78]. In contrast, Haba et al. [79] were not able to demonstrate the preventive effect of bLf in 2-amino-1-methyl-6-phenyl-imidazo-4,5,6-pyridine-induced breast carcinogenesis in rats. A potential explanation might be the difference in processing conditions that affected the biological activity of bLf.

Talactoferrin alpha (TLf) is a recombinant human Lf isolated from *Aspergillus niger* var. *awamori* and is structurally and functionally similar to the native hLf in all aspects except for the nature of glycosylation [80]. Its anticancer activity is very similar to hLf and bLf and has been evaluated in clinical trials against solid tumors, renal metastatic cancer, and non-small cell lung cancer (NSCLC). The US Food and Drug Administration granted a fast-track designation for TLf for the clinical development of first-line

NSCLC treatment which was based on the substantial improvement in response rate and improvement in median progression-free survival (mPFS) by 2.8 months [81, 82]. In the phase I trial of oral TLf, TLf was found to be very well-tolerated with no toxicities reported. Although TLf was undetectable in circulation, its pharmacodynamic indicator IL-18 was found to be increased and 88% of the patients experienced a decrease in tumor growth rate (TGR) [83]. Moreover, three of the patients with NSCLC survived for at least 1 year after the start of TLf monotherapy. In a follow-up phase IB trial, TLf was again well-tolerated by patients with metastatic cancer. NSCLC patients (n=12) experienced 87% relative reduction in TGR and renal cell carcinoma (RCC) patients (n=7) experienced a 98% relative reduction in TGR [84]. In a phase 2 clinical trial of TLf in RCC, Jonasch et al. [85] reported that TLf given at a dose of 1.5 g twice daily (12 weeks on, 2 weeks off) resulted in no toxicities to 44 patients with RCC, 6.4 months mPFS, 21.1 months median overall survival, and a 77% 1-year survival rate. The effect of orally administered bLF was also evaluated clinically in patients with ≤5 mm colorectal polyps. The clinical trial showed that ingestion of 3 g of bLf daily significantly reduced the growth of adenomatous polyps in patients 63 years old or younger compared to the group who received placebo.

Bovine lactoferricin (bLfcin) is a cationic peptide produced by acid–pepsin hydrolysis of Lf obtained from cow's milk [86]. The role of bLfcin in carcinogenesis has been shown to be related to its ability to induce apoptosis in different cancer cell lines. Sakai et al. [87] showed that lactoferrin peptide (Lf-p) generated by pepsin digestion at 2 mg/ml induced apoptosis in human OSCC as evidenced by cleavage of caspase-3 and poly(ADP-ribose) polymerase. The mechanism involved was through activation of the JNK/SAPK pathway via phosphorylation of ERK1/2 and JNK and loss of Lf-p-induced apoptosis in the presence of JNK/SAPK inhibitor. Another mechanism by which bLfcin induced apoptosis in cancer cells was through activation of the mitochondrial pathway involving the activation of caspases. In an *in vitro* study conducted in leukemia and different carcinoma cell lines, bLfcin caused cytotoxicity to these cancer cells without affecting the viability of untransformed human lymphocytes, fibroblasts, or endothelial cells [88]. Also, bLcfin (200 μg/ml) was able to activate caspase-2, caspase-3, and caspase-9 and cause mitochondrial swelling, leading to release of cytochrome *c* in the cytosol of Jurkat leukemia cells. A structure–function relationship study showed that the 10-amino acid peptide FKCRRWQWRM exhibited the same cytotoxic effect as the whole bLfcin, demonstrating that this sequence is the one responsible for the apoptosis-inducing property of bLfcin. A similar activation of apoptosis was observed in neuroblastoma cells (40 μg/ml) accompanied by reduced tumor growth in a neuroblastoma cells-xenografted rat model when bLfcin was given at 1 and 2 mg/day for 3 days [89]. Another study also showed the capability of bLfcin at 2 μM to affect cell cycle progression through arresting the S phase of the cell cycle accompanied by reduced expression of cyclin E in human colon cancer cells [90]. However, the antimicrobial core fragment of bLfcin with amino acid sequence RRWQWR was not able to kill T-leukemia cells and breast cancer cells [88, 91]. The cytotoxic effect was seen only when the antimicrobial fragment was delivered internally through fusogenic liposomes involving caspase-mediated and cathepsin B-mediated pathways. Another synthetic peptide derived from bLfcin with amino acid sequence WKKWDipKKWK at 0.5 mg/50 μL saline showed antitumoral effect dependent of T cells when injected intratumorally in lymphoma-implanted BALB/C mice [92]. Yoo et al. [93] demonstrated that subcutaneous administration of bLfcin (0.5 mg/mouse) inhibited liver and lung metastases of lymphoma and melanoma cells in experimental and spontaneous metastasis models. The same study also demonstrated the ability of bLfcin to inhibit tumor angiogenesis as shown by the reduced number of tumor-induced blood vessels and suppression of tumor growth in the early stage of carcinogenesis. Mader et al. [94] also showed the ability of bLfcin to inhibit angiogenesis both *in vitro* and *in vivo*. Angiogenesis induced by bFGF and VEGF-165 in C57BL/6 mice was inhibited by bLfcin treatment involving heparin-binding growth factors. *In vitro* experiments supported the observed results *in vivo* as bLfcin inhibited the proliferation and migration of human umbilical vein endothelia cells (HUVEC).

4 Shark cartilage

The idea that shark cartilage (SC), a collagenous protein, might be used as an anticancer substance arose from wrong assumptions that sharks do not develop cancer because their skeleton is made up of cartilage accounting for about 6% of their total body weight. Several *in vitro* and *in vivo* studies show that SC plays an important role in preventing the spread of cancer by its ability to inhibit angiogenesis. The proposed mechanisms of action involved in this anti-angiogenic effect of SC included inhibition of bFGF-induced angiogenesis in the Matrigel mouse model [95], prevention of collagenase-induced collagenolysis [96], increase T cell infiltration into the tumor [97, 98], and inhibition of cell adhesion through modification of focal adhesion proteins [99]. The anti-angiogenic property of SC was also demonstrated by several studies using the CAM *ex ovo* model [100–103]. Moreover, SC demonstrated anti-metastatic and antitumor growth effects in various *in vivo*

studies [95, 96, 101, 104]. Bukowski [105] reported that AE-941, a SC extract, exhibited four different mechanisms of action including matrix protease inhibition, inhibition of VEGF binding to its receptor, induction of endothelial cell apoptosis, and stimulation of angiostatin production. Barber et al. [106], using a murine renal tumor model, showed that SC was not able to abolish carcinogenesis but delayed the development of papillary and solid tumors. In contrast, a study conducted by Horsman et al. [107] was not able to duplicate these results when SC-treated mice implanted with SCCVII carcinoma did not show any retarding effect on primary tumor growth and inhibition of lung metastasis. However, the positive *in vitro* and *in vivo* results led to clinical trials of SC against advanced cancer. The results of these trials were not promising. In the evaluation of SC safety and efficacy against advanced cancer given at 1 g/kg daily at three divided doses, several patients experienced gastrointestinal toxicity or intolerance to SC accompanied by 21 adverse events associated with SC intake [108]. Moreover, SC as a single agent was inactive in patients with advanced-stage cancer and had no improving effect on their quality of life. In a phase III trial evaluating the ability of SC (24 to 96 g/day divided into three to four servings daily) to improve overall survival of patients with advanced cancer, SC again failed to show any positive effect on survival rate when compared to a placebo group [109]. In addition, the quality of life of the SC group did not improve when compared to placebo. The study concluded that the SC product did not show any efficacy in patients with advanced colorectal and breast cancers. Recently, two more *in vitro* studies analyzing peptides derived from SC showed again potential as an anti-angiogenic agent via suppression of VEGF-induced migration and tubulogenesis of HUVEC [110] and inhibited microvessel outgrowth from rat aorta [111]. Moreover, a proteolytic digest of SC significantly moderated hyperuricemia in rats when given at 1.5% and 2.5% in the diet [112]. This discrepancy between *in vitro*/*in vivo* studies and clinical trials might be attributed to a lack of studies demonstrating the bioavailability of SC when taken orally as well as the advanced stage of cancer of the patients that underwent clinical trials.

5 Plant lectins

Plant lectins are proteins or glycoproteins that possess hemagglutinating properties. They are widely distributed in plants and can recognize and bind to sugars thereby eliciting several cellular processes [113]. There are seven families of structurally and evolutionarily related lectins [114], of which the three most studied are legume lectins, type II ribosome-inactivating proteins (RIPsII) and *Gallanthus nivalis*-related lectins [115–117]. Legumes are probably the most popular and the most intensively studied source of lectins. Table 4 summarizes the mechanisms suggested by which plant lectins can stimulate cell death of cancer cells.

A lectin preparation from *Bauhinia variegata* seeds that binds melibiose inhibited the proliferation of MCF-7 breast and HepG2 hepatoma cells [118] with IC_{50} of 0.18 and 1.4 μM, respectively. Moreover, this preparation also showed inhibition of the activity of HIV-1 reverse transcriptase. Notably, *B. variegata* lectin showed a weaker mitogenic response in mouse splenocytes than concanavalin A (Con A); however, the maximal response was achieved at the same concentration for both compounds. Lectin isolated and purified from *Phaseolus vulgaris* cv. (Anasazi beans) also showed potent inhibition on MCF-7 breast cancer cells, inhibited HIV-1 reverse transcriptase, and evoked mitogenic response in murine splenocytes [119]. Another lectin, called PCL, isolated from *Phaseolus coccineus* seeds showed anticancer potential that was sialic acid-specific. Chen et al. [120] reported that PCL showed potent antiproliferative effect towards L929 cells involving both apoptosis and necrosis. The cytotoxic effect of PCL was attributed to the activation of caspases, as addition of caspase inhibitors abrogated the antiproliferative effect of PCL. Con A, one of the most studied legume-derived lectin, also showed the same antiproliferative effect on A375 human melanoma cells [121]. The mechanisms involved were caspase-dependent apoptosis and mitochondrial membrane collapse, leading to the release of cytochrome *c* to the cytosol and activation of caspase-9 and caspase-3. It was also reported that the antiproliferative effect of Con A was linked to its sugar-binding activity as a decrease in hemagglutination was correlated to a decrease in its antiproliferative effect. It was also observed that its sugar-binding activity center overlapped its hemagglutinating active site.

The second group of the most extensively studied lectins (RIPsII) is commonly found in *Abrus* and *Viscum* genera. Peptide fractions derived from *Abrus* lectin by trypsin digestion showed *in vitro* immunostimulatory properties in tumor-bearing mice [122]. The peptide fractions produced (1 to 10 ng/ml) showed proliferative effects towards immune cells, such as thymocytes and splenocytes, being more active in the former. They also demonstrated the ability to activate natural killer (NK) cells. Ghosh and Maiti [123] showed that *Arbus* agglutinin was able to activate tumor-associated macrophages, resulting in increased production of nitric oxide and increased cytotoxicity towards tumor cells *in vitro*. Perhaps the most studied lectin from this group is the mistletoe (*Viscum album*) lectin. Mistletoe lectin has demonstrated a wide range of biological activities in tumor cells in *in vitro* and *in vivo* administered orally, locally, systemically, or subcutaneously [117]. In colon

Table 4 Mechanisms of cancer cell death induced by plant lectins

Cell death	Action (lectin type, dose, model)	References
Autophagy	Internalization to mitochondria leading to BNIP3-mediated autophagy (Con A, 20–40 mg/kg, *in vivo*)	[165]
	Mitochondrial generation of reactive oxygen species and activation of p38 and p53 (*Polygonatum cyrtonema*, 15 μg/ml, *in vitro)*	[166, 167]
	Induction of LC3-II generation, double-layer vesicle, BNIP3 and acidic vascular formation (Con A, 40 μg/ml and 20 mg/kg, *in vitro* and *in vivo*)	[168]
Apoptosis	Mitochondrial depolarization (mistletoe extract, 60–250 μg/ml, *in vitro*; *Polygonatum cyrtonema*, 15 μg/ml, *in vitro*)	[128, 166, 167]
	Cytochrome *c* release (*Polygonatum cyrtonema*, 15 μg/ml, *in vitro*)	[167, 168]
	Caspase activation (*Clematis montana*, 1 pM, *in vitro*; *Sophora flavescens*, 1 μg/ml, *in vitro*)	[134, 135]
	Decreased expression of NF-κB, X-linked inhibitor of apoptosis proteins, and Akt/kinase B; activation of TNF receptor 1 and caspase-8 (Korean mistletoe, 100 ng/ml, *in vitro*)	[124]
	Degradation of the cytoskeletal protein gelsolin (*Viscum album* agglutinin-I 1 μg/ml, *in vitro*)	[129]
	Increased expression of FasL and FADD proteins (*Polygonatum odoratum*, 25 μg/ml, *in vitro*)	[133]

BNIP3 BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, *FADD* Fas-associated protein with death domain

cancer cells (COLO), mistletoe lectin induced apoptosis via activation of caspases and decreased expression of anti-apoptotic proteins [124] attributed to apoptosis via TNF receptor 1. Administration of active Chinese mistletoe lectin (ACML) delayed colon cancer development in a mouse model through regulation of innate and adaptive immune responses [125]. ACML was able to enhance antigen-specific activation and proliferation of CD4+ and CD8+ T cells as well as NK and γδT cells. Moreover, this lectin showed *in vitro* ability to alter cytokine gene expression in human colonic carcinoma [126] and IEC-6 rat intestinal epithelial cells [127]. Seifert et al. [128] showed that mistletoe lectin was able to cause cytotoxicity to human acute lymphoblastic leukemia cells through activation of apoptosis. Also, the intraperitoneal administration of mistletoe lectin preparations improved the survival of mice injected with leukemia cells without any side effects. Lavastre et al. [129, 130] also showed the ability of mistletoe lectin to induce apoptosis and degradation of cytoskeletal proteins in human leukemia cells PLB-985 and X-CGD cell lines. In comparison to vincristine, a known drug for lymphoblastic leukemia patients, mistletoe lectin showed the same cytostatic and apoptosis-inducing effects to human B cell lymphoma WSU-1 cells [131]. Mistletoe lectin was able to decrease the growth and spread to the lungs of melanoma in an immunodeficient mice model xenografted with human melanoma cells [132]. At a low dose of 30 ng/kg, mistletoe lectin administered intraperitoneally induced apoptosis in primary melanoma tumors and increased the number of dendritic cells infiltrating these tumors, preventing the growth and spread of tumors through immunomodulatory actions.

Other lectins isolated and purified from other plant sources also showed the same promising effects against cancer. A lectin from *Polygonatum odoratum* induced apoptosis in murine fibroblast L929 cells through both death receptor and mitochondrial pathways [133]; a mannose-binding lectin from *Clematis montana* induced cytotoxicity to L929, Hela, MCF-7, and HepG2 cancer cells lines [134]; and a mannose-binding lectin from *Sophora flavescens* induced apoptosis in HeLa cells dependent of the caspase-mediated signaling [135]. Interaction studies between lectin and known anticancer drugs have also demonstrated positive effects. The proteasome inhibitor bortezomib enhanced the antiproliferative property of mistletoe lectin on human G631 melanoma cells [136]. Also, combination of doxorubicin and mistletoe extracts containing lectin caused cytotoxicity and induced DNA fragmentation to human leukemia Jurkat cells [137]. The use of mistletoe extracts in clinical trials has shown promising results. The dose-limiting toxicity, the assessment of its safety profile, and the maximum tolerated doses of the recombinant counterpart of natural mistletoe lectin, known as aviscumine, is underway [115].

6 RGD-containing peptide: lunasin

Lunasin is a RGD peptide, originally isolated from soybean. It is composed of 43 amino acid residues with unique amino acid sequences including the presence of a cell adhesion motif composed of RGD and a carboxylic acid tail composed of nine aspartic acid residues. Hernandez-Ledesma et al. [138] reported that the amino acid sequence 23–31 is responsible for lunasin to target histones H3 and H4; the RGD motif for the internalization of lunasin into the cells and the polyaspartic acid tail for direct binding of lunasin to core histones thereby affecting mitosis and eventually leading to cell death. Galvez and de Lumen [139] first reported the biological property of

lunasin, formerly known as a soybean cDNA encoding small subunit peptide of 2S soy albumin. They showed that transfection of lunasin plasmid into murine hepatoma cancer cells, murine fibroblast cells, and human cancer cells arrested cell division, caused abnormal elongation of spindle fiber, chromosomal fragmentation, and cell lysis. Moreover, treatment of synthetic lunasin showed preferential adherence of lunasin to chromatin, leading to disruption of kinetochore and inhibition of mitosis. These initial observations led to successive studies showing the possible role of lunasin in cancer prevention. Galvez et al. [140] demonstrated that exogenous application of synthetic lunasin prevented the transformation of murine fibroblast into cancerous foci through internalization via the RGD motif and affecting H3 and H4 histones acetylation *in vivo*. This study also showed, for the first time, the ability of lunasin applied at 250 μg/week to prevent tumorigenesis in SENCAR mouse skin cancer model. Also, topical application of lunasin modestly inhibited epidermal cell proliferation in mice [141]. The first biological property of lunasin isolated and purified from soybeans was reported by Jeong et al. [142]. They showed that lunasin partially purified from soybean by anion exchange chromatography and immunoaffinity chromatography inhibited colony formation induced by *ras* oncogene (1 to 10,000 nM) and inhibit core H3 histone acetylation (10 μM). Lunasin was then shown to be present in different plants including barley [143], wheat [144], *Solanum* family [145], rye [146], and amaranth [147]. Lunasin isolated and purified from barley inhibited colony formation of stably *ras* oncogene-transfected mouse fibroblast and inhibited histone acetylation in mouse fibroblast NIH3T3 and MCF-7 human breast cancer cells [143]. Lunasin from wheat also showed the same ability to inhibit H3 and H4 histone acetylation and lunasin isolated from liver of rats had the same biological activity [144]. Lunasin from *Solanum nigrum* L inhibited H3 and H4 histone acetylation, the activities of histone acetyl transferases, and phosphorylation of retinoblastoma proteins [145]. In addition, lunasin from this plant protected DNA from oxidative damage by suppressing the generation of hydroxyl radicals [148]. Lam et al. [149] showed that lunasin was able to suppress foci formation in E1A-transfected mouse fibroblast NIH 3T3 cells and resulted in fivefold increase in p21 expression. Our laboratory also showed the potential anticancer property of lunasin from soybean. We first showed the anti-inflammatory property of a mixture of lunasin (100 μM) and lunasin-like peptides from soybean through modulation of cyclooxygenase-2 (COX-2)/prostaglandin E_2 and inducible nitric oxide synthase (iNOS)/nitric oxide pathways [150]. Further purification of the mixture and determination of the mechanism of the anti-inflammatory action of lunasin showed that the 43-amino acid lunasin from soybean at 50 μM potently inhibited the COX-2 and iNOS pathways and blocked the nuclear translocation of the p50 and p65 subunits of nuclear factor of kappa B (NF-κB) [151]. Moreover, lunasin inhibited lipopolysaccharide (LPS)-induced NF-κB transactivation in transiently NF-κB transfected RAW 264.7 macrophages. This study also showed the ability of lunasin to downregulate LPS-induced production of IL-1β and IL-6. Our studies provided a mechanism by which lunasin can prevent the process of carcinogenesis through inhibition of

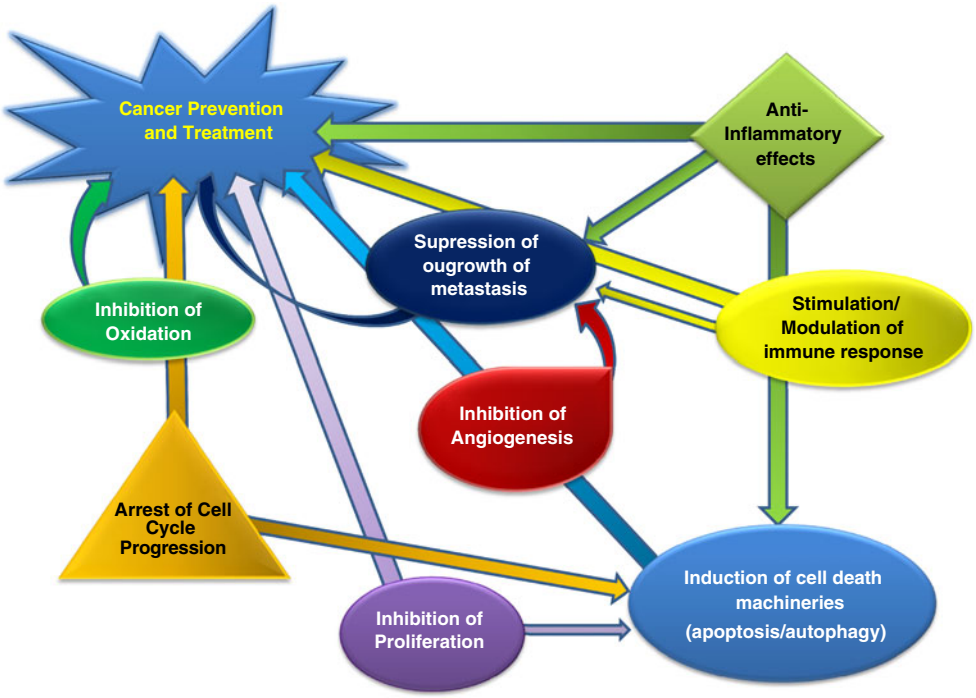

Fig. 1 Multifaceted mechanisms by which nutraceutical dietary proteins and peptides can modulate the process of carcinogenesis

Table 5 Summary of clinical trials using nutraceutical proteins and peptides in cancer treatment

Nutraceutical proteins and peptides		Results	References
Talactoferrin	Phase I (solid tumors)	Very well-tolerated at dose 1.5 to 9 g/day; no hematological, hepatic, or renal toxicities; increased circulating IL-18 (pharmacodynamic indicator of TLf activity); 88% of patients decreased tumor growth	[83]
	Phase IB (NSCLC, RCC)	Very well-tolerated; 50% disease control rate; mPFS of 4.2 months in NSCLC patients; mPFS of 7.3 months in RCC patients	[84]
	Phase II (RCC)	Very well-tolerated; no hematologic, hepatic, or renal toxicities reported; 14-week progression-free survival rate of 59%; median overall survival of 21.1 months	[85]
Shark cartilage		No difference in overall survival between patients receiving SC and patients receiving placebo; no suggestion of improvement of quality of life on patients receiving SC	[109]
Lectin	Fermented European mistletoe extract (melanoma)	3.3% patients developed systemic adverse drug reactions (ADR); 12.8% patients developed local ADR; no life-threatening ADR. ADR-related mortality and ADR-related tumor enhancement not observed; longer survival of population in the treatment group	[169]
	Phase II, viscum fraxini-2 (HCC)	13.1% patients presented complete response (mPFS=29 months); 8.1% patients presented partial response (mPFS=5 months); no hematologic toxicity; mild nonhematologic toxicity at 10,000 ng/ml (×2)	[170]
	Phase I, rViscumin (variety of cancer)	Clinical toxicities observed were fatigue, fever, nausea, vomiting, and allergic reactions; best responses were stable in 11 patients out of 41 enrolled; stimulated immune response; recommended a dose of 5,600 ng/kg twice weekly for further clinical trial	[171]
Bowman–Birk inhibitor concentrate	Phase I	No dose toxicity observed; reduction in serum prostate antigen (up to 43%); decreased serum triglycerides and prostate volume	[172]
	Phase I	Not toxic for patients with oral leukoplakia	[173]
	Phase IIa	31% patients achieved a clinical response, not toxic up to a dose of 1,066 units chymotrypsin inhibitory activity; decreased in total lesion area	[174]

NSCLC non-small cell lung cancer, *RCC* renal cell carcinoma, *HCC* hepatocellular carcinoma

inflammatory pathways. Hernandez-Ledesma et al. [152] confirmed the ability of lunasin to inhibit LPS-induced production of TNF-α and IL-6 in RAW 264.7 macrophages. Moreover, they showed that lunasin was able to inhibit the oxidation of reactive oxygen species production by macrophages and as a free radical scavenger. Our laboratory also demonstrated the ability of lunasin to induce apoptosis in cancer cell lines. Gonzalez de Mejia et al. [153] indicated that lunasin caused a G_2/M phase arrest of the cell cycle in a dose-dependent manner in L1210 leukemia cells. Moreover, lunasin caused apoptosis in L1210 leukemia cells through direct activation of caspase-3. Our study in HT-29 human colon cancer cells showed another mechanism by which lunasin can induce apoptosis in cancer cells [154]. Lunasin caused a dose-dependent inhibition of the growth of HT-29 colon cancer cells with an IC_{50} of 61.7 μM and resulted in G_2 arrest of the cell cycle. Lunasin caused cytotoxicity to HT-29 colon cancer cells by activating apoptosis through the mitochondrial pathway. There was evidence of the increase in the expression of pro-apoptotic Bax and decrease in the expression of the anti-apoptotic Bcl-2 proteins. This study showed, for the first time, that lunasin can alter the Bax/Bcl-2 ratio in such a way that the process of apoptosis will be activated. We also showed the ability of lunasin to increase the activity of caspase-3, an executioner of apoptosis, as well as increase the expression of the pro-apoptotic nuclear clusterin. Hsieh et al. [155] showed the ability of lunasin to prevent breast cancer tumorigenesis in a breast cancer xenograft mouse. They showed that intraperitoneal injection of lunasin at 4 and 20 mg/kg body weight in mouse subcutaneously implanted with MDA-MB-231 human breast cancer cells decreased tumor incidence by 33% and 49%, respectively. Tumors from lunasin-treated animals showed reduced cell proliferation and increased apoptotic rates.

An important property of any bioactive compound is its ability to escape gastrointestinal digestion and be absorbed through the bloodstream to be able to act on target tissues and organs. Bioavailability studies of lunasin in both animals and humans showed promising results. Lunasin from rye showed stability towards pepsin and pancreatin *in vitro* digestion and the liver, kidney, and blood of rats fed with lunasin-enriched rye showed the presence as detected by Western blot [146]. Moreover, lunasin extracted from these tissues was able to inhibit histone acetyl transferase, indicating that lunasin is bioavailable and bioactive. Hsieh et al. [155] reported that lunasin is bioavailable when orally administered in mouse and rat. Synthetic 3H-labeled lunasin was absorbed in CD-1 mice and distributed in various tissues including lung,

mammary gland, and prostate. They also found a significant amount of radiolabeled lunasin in the brain of mice. Moreover, lunasin extracted from the blood and liver of lunasin-enriched soy flour-fed rats was bioactive and able to suppress foci formation in the same concentration as synthetic lunasin. Our laboratory also conducted a bioavailability study of lunasin in humans [156]. We recruited healthy men and fed them with 50 g of soy protein for 5 days after a 1-week washout period. After 5 days of feeding, blood samples were collected 30 min and 1 h after soy protein ingestion. Analysis of plasma showed that lunasin was present at a concentration of 50.2 to 110.6 ng/ml of plasma. This represents 4.5% absorption of lunasin from 50 g of soy protein. These initial bioavailability studies are very important in supporting a clinical trial for lunasin.

7 Amino acids

Individual amino acids have also shown potential to inhibit carcinogenesis mainly by inhibiting angiogenesis. Yeh et al. [157] showed that arginine at 1,000 μmol/L can lower the production of VEGF, bFGF, and MMP-2 in SW480 human colon cancer cells. Moreover, they showed that supplementation of arginine in the diet of SW480 colon cancer cells xenograft mouse model resulted in lower MMP-2, MMP-9, and VEGF receptor levels in tumors and higher NO and spleen NK compared to a control group. Another study by the same group [158] also showed that HUVEC expression of cellular adhesion molecules CD11b and IL-8 was reduced by arginine treatment. Chou et al. [159] reported that the production of VEGF, bFGF, PGE_2, and MMP-2 was upregulated in endothelial cells in the presence of HeLa cells. Glycine also showed inhibition of tumor growth in animal models. Rose et al. [160] found that dietary glycine at 5% in the diet fed to mice 3 days prior to subcutaneous implantation of B16 tumors resulted in decreased tumor size by 50–75% and lighter tumor weight by 65% compared to a control group. Moreover, they also showed that glycine prevented the formation of small tumors (0–5 mm) by 23%, medium tumors (5–10 mm) by 64%, and large tumors (>10 mm) by almost 80% in the liver of male rats [161]. The gavage administration of branched-chain amino acids to obese diabetic rats have shown their ability to attenuate preneoplastic lesions development and inhibited angiogenesis by reducing VEGF expression [162].

8 Concluding remarks

Several bioactive proteins and peptides are now recognized for their health-promoting properties and the possibility of their use for the prevention and treatment of malignancies such as cancer. Figure 1 summarizes the mechanisms by which nutraceutical proteins and peptides can modulate the process of carcinogenesis, while Table 5 gives an overview of the results of some human clinical trials. However, a major impediment in these studies has been the susceptibility of proteins/peptides to gastrointestinal digestion thereby resulting in the reduction of their biological activities. Proteins and peptides such as Lf and lunasin have shown to be bioavailable, thus might exert their biological properties to target tissues and organs. This review highlighted the importance of including these proteins and peptides, as well as foods that contain them, in our daily dietary consumption for the prevention and possible treatment of diseases such as cancer. However, the major challenge on the use of bioactive peptides and proteins in treating various malignancies such as cancer would be the translation of *in vitro* and *in vivo* results into clinical outcomes.

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