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# Prevention of cancer and inflammation by soybean protease inhibitors

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## 1. ABSTRACT

Several plant-based nutrients and non-nutrients that can inhibit mutagenesis and cell proliferation have been identified. Some of the most promising compounds identified as chemopreventive and anti-metastatic agents include soybean-derived protease inhibitors (PIs), Bowman-Birk Inhibitor (BBI) and Kunitz-Trypsin Inhibitor (KTI). A growing body of evidence suggests that BBI could act as anti-carcinogenic agent and KTI is considered to prevent cancer invasion and metastasis. These inhibitors are non-toxic, are of low cost and can be taken orally or as a part of the daily diet. PIs are undergoing investigation in the clinical setting as potential agents for chemoprevention and anti-metastasis. A complex scenario about the interaction between PIs and cell signaling has been emerging. Soybean PIs are not just anti-proteolytic proteins, but can also be modulators of cell signal transduction. Cancer and inflammatory treatment strategies modulating signal transduction need further investigation.

## 2. THE VALUE OF SOYBEAN PROTEINS IN HUMAN HEALTH

Cancer is one of the leading causes of death worldwide, generally exceeded only by cardiovascular disease in the developed countries. It has been estimated that 30-40% of all kinds of cancer can be prevented with a healthy lifestyle and dietary measures (1). A large number of soybean proteins have diverse biological activities, which include hormonal, immunological, bacteriological and digestive effects (2). The beneficial effects of these compounds include lowering of cholesterol, anticarcinogenic effects, and protective effects against obesity, diabetes, bone loss, coronary heart disease and stroke, menopausal symptoms, irritants of the digestive tract, and kidney diseases (3,4). Dietary bioactive components include phytochemicals (soy isoflavones and genistein) and nutraceuticals (lunasin, lectins, BBI and KTI).

There is much evidence based on animal and epidemiological studies suggesting that some soybean compounds can prevent cancer in many different organ systems (5). Early epidemiologic (6) and rodent (7) studies showed associations between soy intake and reduced risk of breast and mammary cancer, respectively. It is believed that supplementation of human diets with certain soybean products could reduce the risk of hormonally dependent and independent cancers and cancer mortality rates (5,6,8). Adverse nutritional and other effects following consumption of raw soybean PIs have been attributed to the inhibition of functions of endogenous digestive enzymes (3) and a frequent cause of food-induced allergic or hypersensitivity reactions. KTI might be an occupational inhalant allergen (9).

## 3. FUNCTION OF SOYBEAN-DERIVED PROTEASE INHIBITORS (PIS)

Plant PIs are classified in different families, according to their primary structure (http://www.ba.itb.cnr.it/PLANT-PIs). This paper reviews the two major classes of soy proteins-derived PIs (10), KTI and BBI. The evidences reviewed here represent the novel opportunities in preventive or medical applications, in particular, beneficial effects of PIs in suppression of carcinogenesis, growth and metastasis, and inflammation in many different *in vitro* and *in vivo* assay systems and have been extensively investigated in the preclinical and clinical studies (11). The PIs act not only against digestive proteases of insects and mammals but also against other bacterial and fungal enzymes. The recent experiments prompted us to speculate that PIs are not just defensive proteins, but can also be modulators of cell signal transduction. Although there remain to be many unresolved subjects, the control of signal transduction pathway may represent an important cellular target for PIs.

### 3.1. Anticarcinogenic activity of Bowman-Birk inhibitor (BBI)

Soybean-derived Bowman-Birk Inhibitor (BBI), a serine protease inhibitor, is a 71-amino acid protein (8 kDa) with seven disulfide bonds which stabilize its active conformation and has a double head structure with the well-characterized trypsin inhibitory N-terminal domain (active site, $Lys^{16}$-$Ser^{17}$) on one head and the chymotrypsin inhibitory C-terminal domain (active site, $Leu^{43}$-$Ser^{44}$) on the other (12). In *in vitro* experiments, purified BBI and an extract of soybeans enriched in BBI called BBI concentrate (BBIC) potentiated the cell-killing effects induced by cisplatin in lung (13) and ovarian (14) cancer cells. BBIC treatment led to significantly enhanced cell killing by cisplatin in combination with radiation treatment in the lung (13), ovarian, breast, cervical, and head and neck cancer cells (15). It has radio-protective properties in normal cells (16). In addition, BBI also inhibited the cell proliferation and invasion of human prostate cancer cells (17). In animal experiments, both BBI and/or BBIC (~1% g of BBI/g of body weight) have the suppressive effects on carcinogen-induced transformation in colon (18,19,20), oral cavity (21), prostate (22), and lung (23). The carcinogens used are dimethyl-benzanthracene (22), 3-methylcholanthrene (23), and dimethylhydrazine (20). In addition, BBIC prevents sunlight-induced skin damage and reduces the risk of skin tumor formation and progression (24). Of interest is the observation that BBIC has no genetic toxicity (25,26). These results demonstrate that both BBI and BBIC prevent and suppress carcinogenesis and cancer progression without toxicity in many experiments (5,27).

Additional research, including clinical trials, has continued to examine and elucidate the therapeutic effects, nutritional benefits, and toxic consequences of orally ingested BBI or BBIC. BBIC has achieved Investigational New Drug status from the FDA in 1992, and studies to evaluate BBIC as an anticarcinogenic agent in human populations began (27). In the phase I clinical trial, BBIC was administered as a single oral dose to patients with oral leukoplakia (28,29). Administration of BBIC as a single-dose tablet in doses ranging from 25–800 Chymotrypsin Inhibitory Units/day (20-1000 mg of BBIC/kg of body weight) for 4 weeks in a total of 24 subjects resulted in no clinically observed toxicities (29). No laboratory evidence of toxicity was also observed (29). Pharmacokinetics studies in patients showed that BBI was taken up rapidly, and a metabolite of BBI was excreted in the urine. In 2000, BBIC has been shown to be a valid suppressor of oral leukoplakia in the human phase IIa clinical trial (30). Large-scale clinical trials to evaluate its potential anticarcinogenic and chemopreventive properties are underway in cohorts with oral leukoplakia (30), prostate disease (hyperplasia and cancer) (31), and head and neck cancer (32), supporting that BBI and BBIC could be a useful agent for treatment of human cancer without significant adverse side effects.

Besides the preventive effect against cancer, BBI has anti-inflammatory properties: a) inhibition of nephrotoxicity induced by the antibiotic gentamicin (33); b) inhibition of superoxide anion radical generation in polymorphonuclear leukocytes and HL-60 cells with neutrophil-like characteristics (34), which may reduce the likelihood for free radical DNA damage and transformation to malignant phenotypes; c) prevention of Dengue fever and allergic disorders (35); and d) protection against space radiation-induced cytotoxicity and phenotypic change associated with transformation of human normal epithelial cells (36). BBI is recommended as a radioprotector (37). Furthermore, there are other functions in BBI, including improvement of voiding and sexual functions, relaxation of urethral and corporal smooth muscle, and prevention of skeletal muscle atrophy (38,39).

From the literature, it is not clear the underlying mechanism of BBI activities as anticarcinogenic agent. Some of the mechanisms are reported (20,40). Firstly, BBI can function as an anticarcinogen, possibly through interaction with a cellular serine protease, that play an important role for cell proliferation (2,41,42). A chymotrypsin-like protease may act as a potential target, whereas trypsin inhibitory activity is not essential (11,43,44). In contrast, protease inhibitory activity is not required for radioprotection (16). Secondly, the Her-2/neu

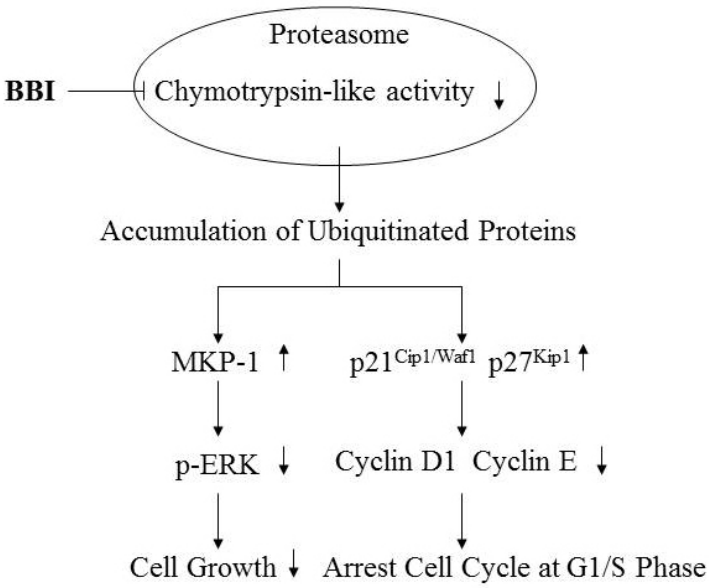


**Figure 1.** Proposed model of BBI in cell growth signaling cascade. Chen *et al.* (60) demonstrated that proteasome inhibition by BBI is associated with accumulation of ubiquitinated proteins and the proteasome substrates, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, accompanied with downregulation of cyclin D1 and cyclin E which could arrest cell cycle at G1/S phase. In addition, BBI abates proteasome function and results in upregulation of MKP-1, which in turn suppresses ERK1/2 activity and cell growth.

oncogene (also known as erbB2) encodes a transmembrane glycoprotein that belongs to the human epidermal growth factor receptor family. Up-regulation of this oncogene is frequently linked with increased metastasis and poor prognosis. BBI inhibits proteolytic cleavage of the extracellular domain of erbB-2, which results in the stabilization of erbB-2 pro-protein and prevention of conversion of the protein into a constitutively active conformation (45). Thirdly, BBI suppresses the expression of certain oncogenes (e.g., c-myc and c-fos), which results in abrogation of tumor aggressiveness (11). Fourthly, BBI induces negative growth control caused by the expression of connexin43 gene in mice with M5076 ovarian sarcoma (46). Fifthly, BBI inhibits matriptase, a member of the type II transmembrane serine protease class, that is of considerable interest for the development, homeostasis, and cancer invasion and metastasis of epithelial tissues (47). Finally, the precise mechanisms by which BBI suppresses carcinogenesis have been vigorously investigated by Chen *et al.* (40). The reliable explanation is that BBI inhibits the proteasomal chymotrypsin-like activity in MCF7 breast cancer cells, suggesting that BBI is an effective proteasome inhibitor (40). BBI strongly induced the mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) via suppression of the ubiquitin-proteasome pathway (Figure 1). MKP-1 inactivates ERK1/2 signaling involved in the control of cell proliferation, which was accompanied by the accumulation of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ and the consequent downregulation of cyclin D1 and cyclin E and possibly followed by dysregulation of cell cycle progression. This suggests that BBI might induce cell-cycle arrest at $G_1$/S phase. These results support the notion that proteasome inhibition by BBI is a novel mechanism that contributes to prevention of cancer (40).

There is no evidence to support the notion that BBI-mediated MKP-1 up-regulation is the only or ideal pathway target for cancer prevention. BBI has multifunctional properties. BBI-dependent overexpression of MKP-1 could represent one of the major pathways for suppression of MAPK-dependent signaling pathways. MAPK-NF-kB signaling pathways are involved in up-regulating COX-2 gene expression (48). The overexpression of COX-2 in many cancers provided the rationale for clinical trials with COX-2 inhibitors for cancer prevention or treatment. The epidemiological observation that nonsteroidal anti-inflammatory drugs (NSAIDs; COX inhibitors) prevent colon and possibly other cancers has spurred novel approaches to cancer prevention. These data allow us to speculate that BBI may down-regulate COX-2 expression through MKP-1-mediated inactivation of MAPK.

### 3.2. Anti-invasive/metastatic activity of Kunitz trypsin inhibitor, KTI

The following largely focuses on our current understanding of the apparent functions of soybean KTI. Soybean plants contain several KTI genes, which are differentially expressed during the soybean life cycle. KTI accumulates during seed development and is present in the embryonic axis and cotyledons (49). It is believed that KTI will be shown to play a major role in the prevention of several diseases including cancer and inflammation: the effect of KTI has been confirmed in an *in vivo* mouse model system. KTI (1.5% g of KTI/g of body weight) treatment in a peritoneal disseminated metastasis model of ovarian cancer cells resulted in a 40% reduction in total tumor burden when compared with control animals (50). In an *in vivo* spontaneous metastasis assay, the diet supplementation with KTI (1.5% w/w) for 28 days

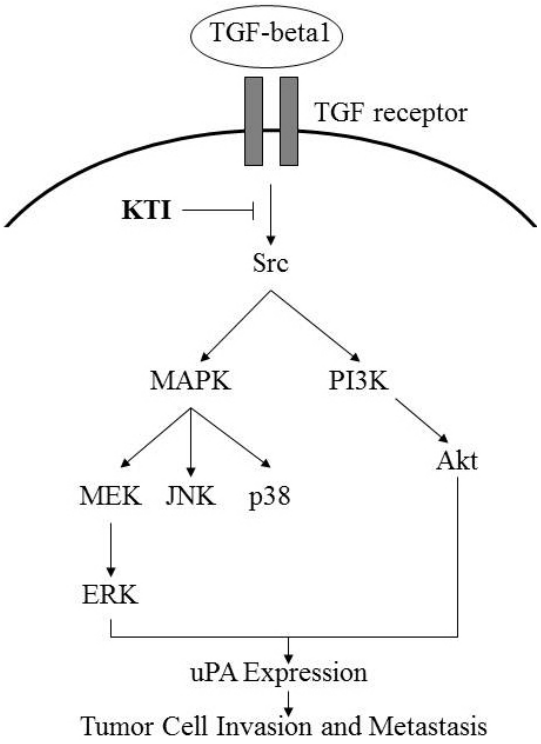


**Figure 2.** Proposed model of KTI in cell invasion signaling cascade. KTI suppresses TGF-beta1-stimulated uPA expression and promotion of invasion and metastasis through upstream target(s) of Src and acts as an anti-invasive and anti-metastatic agent.

immediately after subcutaneous tumor cell inoculation significantly inhibited the formation of lung metastasis in C57BL/6 mice (50). However, the diet supplementation with KTI did not reduce the number of lung tumor colonies (50). These results suggest that dietary supplementation of KTI more efficiently regulates the mechanism involved in the entry into vascular circulation of tumor cells (intravasation) than in extravasation during the metastatic process.

We have also identified novel findings regarding mechanisms by which KTI inhibits signaling pathways (51,52). KTI inhibited the transforming growth factor-beta1 (TGF-beta1)-induced upregulation of urokinase-type plasminogen activator (uPA) and subsequently suppressed the invasiveness in human ovarian cancer cells (50). TGF-beta1 directly activates Src kinase, which in turn activates mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt, the downstream targets of Src, for uPA up-regulation in human ovarian cancer cells. KTI inhibits TGF-beta1-stimulated activation of Src, ERK, and Akt, which results in suppression of uPA expression and invasion (Figure 2). Both the MEK inhibitor and PI3K/Akt inhibitor masked the effects of KTI upon uPA production and invasion. In cells expressing the constitutively active (CA)-c-Src and expressing CA-MEK or CA-Akt, KTI failed to reverse TGF-beta1-induced uPA up-regulation and invasion (51,52). These data allow us to speculate that KTI suppresses uPA expression and promotion of invasion possibly through upstream target(s) of Src and acts as an anti-invasive and anti-metastatic agent. KTI treatment may be beneficial for ovarian cancer patients with or at risk for peritoneal disseminated metastasis.

The anti-inflammatory effects of KTI have also been examined as intraperitoneal injection and dietary supplements on bacterial lipopolysaccharide (LPS)-induced lethality in a mouse infection model (53). KTI plays a role as a potent anti-inflammatory agent by inhibiting the LPS-induced MAP kinase activation, leading to the suppression of cytokine (interleukin-1beta and tumor necrosis factor-alpha) expression (53). More recent data indicate that KTI inhibits LPS-induced up-regulation of cytokine expression possibly through suppression of ERK1/2 and p38 kinase-mediated NF-kB activation. Furthermore, KTI inhibited ultraviolet-induced up-regulation of cytokine expression predominantly through suppression of JNK signaling pathway in primary human keratinocytes (54). KTI also suppressed LPS-induced cytokine production of gingival fibroblasts (55), possibly through suppression of MAP kinase activation. Thus, KTI could act as a modulator of Src-MAP kinase-dependent signaling cascade. These findings may identify anti-cancer and anti-inflammatory properties of KTI and may be relevant to the use of this compound in modulating cancer and inflammation (55).

## 4. DIRECTIONS FOR FUTURE RESEARCH

This review on soybean-derived PIs highlighted the excellent progress made to develop basic research as

well as robust preclinical and clinical models for prevention of carcinogenesis and cancer promotion. New treatment options using newly developed anticancer agents and molecular targeted compounds provide hope for patients with localized and advanced cancer. Therapies with high cost and small incremental improvement in survival and quality of life may find it difficult to meet the societal thresholds, providing important insights into the best use of health care expenditures. It is logical that future clinical studies should focus on examining the efficacy of dietary PIs in cancer prevention and anti-metastasis as an alternative to pharmacological agents, especially in high-risk cancer patients.

Given the limitations of proteasome-specific inhibitors and the biological evidence mentioned above, we suggest that BBI-specific target(s) other than MKP-1 should be pursued as alternative or complementary approaches to cancer prevention or anticarcinogenesis. Understanding the potential role of BBI in human cancers requires a detailed knowledge about chymotrypsin-like inhibitory activity, identification of target proteasome(s) exhibiting chymotrypsin-like activity and investigation of the mechanism by which BBI selectively enters cancer cells and inhibits the target proteasome activity. It will be determined the molecular mechanism of the BBI-mediated signaling cascade and regulation of protein kinase(s) and phosphatase(s) activity. Activation of MAP kinase requires an influx of calcium through certain calcium channels and the activation of calcium/calmodulin-dependent protein kinase (56), suggesting that BBI inhibits calcium influx-dependent signaling cascade. Furthermore, the results from the Phase IIa trial indicate that BBIC should be investigated for chemopreventive activity in a randomized large-scale Phase III clinical trial.

The most attractive hypothesis is that KTI could act as a modulator of Src/MAPK/PI3K/Akt-dependent signaling cascade, which has widespread effects on cancer and inflammation. At this time, however, signaling mechanisms linking KTI to growth factor-induced phosphorylation of Src are poorly understood. The regulation of protein phosphorylation, including Src phosphorylation, requires coordinated interaction between protein kinases and protein phosphatases. The major protein kinases involved in the activation of Src signaling may be inactivated by KTI. Another possibility is that the major phosphatases, strongly induced by KTI, may involve in the inactivation of Src signaling. In addition, the investigation of KTI should follow a structured design, incorporating parallel preclinical studies of the KTI concentrate and the isolated KTI in terms of efficacy, toxicity, biological mechanisms, and pharmacokinetics. Either the purified KTI or KTI concentrate should be selected for further development on the basis of dose-efficacy and toxicity data. Pilot clinical trials on the pharmacokinetics and mechanism-based markers of efficacy of the selected intervention should precede phase III development in suitable populations.

## 5. CONCLUSION

Soy proteins are increasingly important in the human diet. This review focused on the molecular mechanisms and possible preclinical and clinical effects of soy proteins-derived PIs, BBI and KTI. A better understanding of the factors that impact the nutrition and health-promoting aspects of soy proteins is needed. We are now entering into a second phase of human modeling that requires an emphasis on trans-disciplinary approaches to the design, analysis, and applications of human cancer models. In addition, there are a number of key questions that *in vitro* and *in vivo* animal modeling may be able to answer that cannot be addressed by analyzing human tumors. In any case BBIC is undergoing investigation in the clinical setting as a potential chemopreventive agent.

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**Key Words:** Bowman-Birk Inhibitor, Kunitz-Trypsin Inhibitor, Nutraceuticals, Soybean, Cancer, Inflammation, Review

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