Anticarcinogenic Effects of Bowman-Birk Protease Inhibitor in vitro and in Animal Tumor Model

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Abstract: The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor with a well-characterized ability to inhibit trypsin and chymotrypsin simultaneously. BBI, both purified BBI and extract of soybeans named BBI concentrate (BBC) have been studied extensively. Purified BBI and BBC have comparable suppressive effect on the carcinogenic process in a variety of in vitro and in animal tumor model studies. BBI may be a potential cancer chemopreventive agent for humans. The article summarizes recent advances including structures, preclinical study, molecular mechanism etc. of BBI.

Key words: Bowman-Birk inhibitor; carcinogenesis; preclinical study; molecular mechanism

Bowman-Birk 蛋白酶抑制剂的体外和动物模型实验抗癌实验研究进展

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摘要：Bowman-Birk 蛋白酶抑制剂（BBI）是植物丝氨酸蛋白酶抑制剂，能同时抑制胰蛋白酶和胰凝乳蛋白酶。体外及动物实验都证实，BBI 纯品或 BBI 浓缩物均有较强的抗癌活性。本文综述了近年来有关 BBI 的一些抗癌研究进展，包括 BBI 的结构、临床前研究及可能的分子机制等。

关键词：Bowman-Birk 蛋白酶抑制剂；癌化；临床前研究；分子机理

Bowman-Birk inhibitor (BBI) is a family of serine protease inhibitors with similar structure features consisting many forms and isoforms commonly found in the seeds of legume (e.g., soybean, chickpea, and peanut)¹. The soybean-original BBI has attracted much interest due to its potent anti-carcinogenic and anti-inflammatory property in a variety of in vitro and in animal model tumor assay systems²,³.

Structure

BBI was first identified by Bowman in the 1940s⁴ and purified by Birk in 1960s⁵. The primary structure of many representatives BBI shows high homology on the conserved cysteine positions. The protein is small peptide (Mr 8~10 kDa) containing 71 amino acids and seven disulfide bridges. Disulfide bridges and (or) extensive hydrogen bond networks help to form a symmetrical structure of two tricycle domains, each containing an independent serine protease binding loop joined via a disulfide bond between flanking cysteine residues. BBI has two separate functional inhibitory domains: one domain inhibits trypsinlike serine proteases, and the other inhibits chymotrypsinlike serine proteases. BBI can form a complex with two separate proteases through the interaction between the binding loop of inhibitor and enzyme reactive center, typically allowing simultaneous inhibition of chymotrypsin and trypsin. The three-dimensional structures⁶ are now known in Molecular Modeling Database (PDB code 1BBI) and the complete Bowman-Birk type inhibitor in ternary complex with trypsin has also been reported. BBI can be cleaved to obtain two fragments using a combination of cyanogen bromide and pepsin. By testing both fragments for the ability to exert anticarcinogenesis in vitro transformation system and in animal
tumor model assay system, it could be demonstrated that the chemopreventive effect is mainly localized to the chymotrypsin inhibitory region of the molecule. The trypsin inhibitory region of BBI resulted only in a minor chemopreventive effect.

The inhibitory mechanism of BBI is well established, and the identity of the P1 residue in the binding loop is considered to define the enzyme-inhibitor specificity. The interaction of BBI with serine proteases is similar to that of enzyme-substrate. The inhibitory loop of BBI inserts deeply into the active center of the protease, taking up the reorganization sites and combination sites and forming hydrophobic bond contact between the active site residue and the P1 residue to block off the active center of protease. The protease-inhibitor complex is so tight as to result in low rates of hydrolysis\textsuperscript{11}.

**Preclinical anticarcinogenesis studies**

In nature, proteases are involved not only in assimilation and dissimilation, but also fulfill numerous other functions in the physiological processes\textsuperscript{11}. Although proteases are highly beneficial, they are very dangerous and must be strictly controlled both in time and in place. Therefore nature evolved many control mechanisms for protease, including transcriptional, translational and post-translational regulation etc. Among many different mechanisms are employed, two post-translational are direct and powerful. The first is that almost all proteases are biosynthesized as largely inactive precursors called zymogens. The activation of zymogens involves proteolysis of a peptide bond or removal of redundant peptide sequences of the enzyme, insuring the enzyme cannot function prior to the proteolysis activation. The second is that once enzymes are activated, the activity of enzyme may be controlled by the competitive inhibition of protease inhibitor. These inhibitors form completely inactive or less active complexes with their matching enzymes. It has been shown that protease activity in tissues plays an important role in cell transformation and influences greatly the development of tumor lesions and invasion and metastasis process\textsuperscript{7}. In abnormal physiological states, an imbalance of the physiological protease:protease inhibitor ratio is associated with increasing genetic instability, along with vascular, infectious, neurological diseases, and the related processes of carcinogenesis and inflammation. For example in DMBA-induced oral cancer, protease activity increased 10-fold in DMBA-exposed oral mucosa\textsuperscript{32}. 2- to 3-fold increased protease activity emerged in the case of smoking and oral leukoplakia in whole oral mucosa cells compared with protease activity in nonsmoker\textsuperscript{8}. Therefore, the inhibition of proteolytic activity represents an additional chemoprevention strategy. For example, during the carcinogenesis progression from hyperplasia, dysplasia to early invasion, several molecular changes that act as potential targets for chemoprevention\textsuperscript{13,9}, including activation of telomerase, overexpression of COX-2 and EGFR, amplification of cyclin D1, and inactivation of p16 and p53 etc, are associated with the increase of the unbalance of protease/protease inhibitor (Table 1).

Researchers are interested in the epidemiologic and dietary data that the high soybean intake might reduce incidence and mortality rates of several cancers\textsuperscript{10}. In oriental countries such as Japan, which had a high consumption of soybean, the incidence rates of several cancers including breast, colon, and prostate cancers are very low\textsuperscript{2,3}, a fact that has been attributed to the presence of the soybean derived BBI. Although several compounds in soybeans products including phytic acid, isoflavones, β-sitosterol, and saponins have also demonstrated anticarcinogenic potential in animals, the anticarcinogenic effect of BBI in animals exceed far than that of those former potential anticarcinogenic agents\textsuperscript{11}. For example, there are few literatures documenting suppression effect of the soybean-derived isoflavones and saponins studied in a pure form on animal tumor\textsuperscript{11}. As an anticarcinogenic agent, BBI has been studied as purified form as well as a concentrate of BBI (BBIC), a crude soybean-flour extract containing the classic BBI and other components. Both purified BBI and BBIC are able to prevent the development of malignancies in vitro transformation systems as well as in many animal model tumor assay systems\textsuperscript{12}. In the phase II
prevention trials of human oral cancer, a solution of BBIC (dose from 200-1066 chymotrypsin inhibitory units) was conducted to 32 patients with oral leukoplakia for one month. After the treatment of BBIC, 31% of patients achieved clinical response with reduction of oral lesion areas associated with decreasing protease activity in the oral mucosal cell. BB1 decreased this activity and oral cancer development in DMBA-induced oral cancer. The inhibitor that is similar to BBI from buckwheat was able to inhibit the cell growth in HL-60 cell line of acute myeloid leukemia and nontoxic to normal cells. These results are important to understand the mechanism of action of BBI and to improve the effect of BBI in cancer chemoprevention.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Molecular change</th>
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<tbody>
<tr>
<td>Hyperplasia</td>
<td>3p, 9p loss of heterozygosity; p16 inactivation; Telomerase activation; COX-2 overexpression; EGFR overexpression</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>8p, 17p loss of heterozygosity; p53 mutation; Cyclin D1 amplification; RAR loss</td>
</tr>
<tr>
<td>Early invasion</td>
<td>4p, 8p, 13p loss of heterozygosity</td>
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Animal studies have observed that BBI as a cancer preventive agent lies in its ability to reverse the initiation of premalignant cells and growth-inhibitory properties in several different animal tumor model systems. In animal studies, both BB1 and BBIC have been found to interfere effectively with tumors induced by chemical carcinogens or radiation, including the oral carcinogenesis in hamsters, lung carcinogenesis in mice, smoke-induced lung tumor in mice and radiation-induced lymphosarcoma in mice. In addition, BBIC even suppresses carcinogenesis in animals known to have a genetic susceptibility to cancer.

Potential molecular mechanisms

Although many theories have been brought forward to determine the mechanisms for the anticarcinogenic effects of protease inhibitors, the actual mechanisms by which BB1 suppress carcinogenesis remains unclear. The BB1 and (or) BBIC can affect several potential targets altered by carcinogen and (or) radiation exposure, including superoxide anion radical production, amounts of oncogene expression, gene amplification, immune effects, and proteolytic activity. Elevated amounts of proteolytic activities and expression of certain oncogenes are now being used as the biomarker in human cancer prevention trials with BB1. The first contributing mechanism is that BB1 may act as a selective toxic agent on the suppression of pancreatic cancer by virtue of the fact that early studies in rats and dogs demonstrated growth inhibition in case animals were fed soybeans over long periods of time.

Many of the theories on the anticarcinogenic mechanism are related to the fact that BB1 prevents the release of the superoxide anion radical and hydrogen peroxide from polymorphonuclear leukocytes and HL-60 cells stimulated by carcinogens, which may reduce the likelihood for free radical DNA damage and cell membrane damage. Although BB1 do not function as free radical-scavenging agents, they can achieve the same final suppressant effect that they can keep free radicals from being produced in cells and thereby decrease the level of oxidative damage. The correlation between the ability of a protease inhibitor to prevent the release of oxygen free radicals from cells and its ability to inhibit carcinogenesis, are associated with chymotrypsin inhibitor activity-such as BB1-having the greatest potency. It is assumed that the ability to prevent the release of oxygen free radicals is also related to the potent anti-inflammatory activity of BB1.

The third potential mechanism contributing to the anticarcinogenic activity of BB1 is that BB1 may inhibit inflammation-associated proteases such as cathepin G, elastase and chymase secret by inflammation associating cells. It is well known that inflammation is a predisposing factor for carcinogenesis and a number of anti-inflammation agents have been shown to have a suppressive effect on the carcinogenic process. BB1 can suppress damage of oxygen radicals.
released by many inflammation-mediated immunocytes and inhibit the activities of the above three matrix-degrading proteases[21]. There is also a report that BBI may be able to inhibit histamine release from rat mast cells[21]. Therefore, BBI may be able to inhibit several inflammatory mechanisms, making it a highly effective anti-inflammatory agent.

The fourth mechanism is that BBI may suppress carcinogenesis by affecting the expression of certain oncogenes. One such oncogene is the HER2/neu oncogene, which codes for a 185 kD protein commonly referred to as p185. The neu protein that is derived from the extracellular domain of the p185 by serine protease proteolytic cleavage is a tumor-associated antigen, which can be useful biomarker for human cancers studies. Although the overexpression of neu antigen on the surface of cancer and premalignant cells could make the cells vulnerable to be attacked by the host immune surveillance mechanisms, those cells may avoid being detected and targeted by antibodies and T lymphocytes against neu antigen by shedding off the neu protein from the cell surface[19]. The released neu protein binds to anti-neu antibodies and T lymphocytes and thereby prevents them from attacking the neu-expressing cells. The overexpression levels of p185 on tumor cells have been shown to correlate with the elevated concentration of neu protein in serum. Wan observed that the BBIC would decrease the concentration of neu protein in the serum and the correlation between the p185 and neu protein disappear after the treatment by BBIC, thereby preventing the neu-expressing cancer cells from escaping host immunological control system[19]. It is likely that BBI inhibits the proteases involved in the proteolytic cleavage of the neu protein for its ability to inhibit both trypsin and chymotrypsin-like serine proteases.

BBI may also affect other proteolytic events that are related to cancer development. An interesting example is that the natural serine protease inhibitor in saliva can suppress MMPs activity by keeping MMPs precursor from mature MMPs, which may act as extracellular matrix degrading agents in the process of inflammation and carcinogenesis[22]. BBI may stabilize specific tyrosine-phosphatases that interfere with EGFR activation after radiation exposure[23]. BBI also inhibits the proteolytic processing of gastrin-releasing peptide, a growth factor derived from small cell lung carcinoma cells[24].

Perspect

Chemoprevention is an important and viable strategy to decrease cancer incidence and mortality. In vitro and animal studies support the preventive effects of BBI, which are reported to be broad spectrum (many sites) and irreversible. Currently BBIC has undergone preliminary pharmacokinetic evaluation and achieved to INDs (investigational new drug status) for prostatic disease[25].

Further studies are needed to investigate whether BBI acts as an inhibitor of unknown proteases or whether it acts indirectly on non-proteases molecules, which are involved in the regulation of cellular carcinogenesis. For example, BBI anti-carcinogenic function may not independent on its protease inhibition property, just as the anti COX-2 drugs are to activate “COX-2 inhibitors”, not to work via COX-2[26]. Further anticarcinogenic studies of BBI are relied on the combination with preclinical (and early clinical) study and broader basic structural and functional study of BBI itself[3]. Investigation of synthesized BBI- (PEO-PPO) showed that the modification increased the retention time in the blood and higher concentrations in tumor organs comparison with native BBI[7]. Koepke observed the putative key PI site for cancer prevention binds specifically to chymotrypsin as well as trypsin by means of high-resolution X-ray crystal graph studies[27]. This work will help to find the BBI target enzyme(s) and also synthesize small-molecule BBI analogues. Recently we constructed a fast active-staining gelatin-PAGE method, which would help to find and detect new serine protease inhibitor rapidly[28].

Reference

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