Prevention of Carcinogenesis by Protease Inhibitors

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Abstract

Protease inhibitors are very effective in their ability to suppress carcinogenesis in many different in vivo and in vitro assay systems. One particularly effective protease inhibitor, the soybean-derived Bowman-Birk inhibitor, has been extensively studied in our laboratory. Our results have indicated that Bowman-Birk inhibitor suppresses carcinogenesis 1) induced by several different types of carcinogens, 2) in three different species (mice, rats, and hamsters), 3) in several different tissues/organs [colon, liver, lung, esophagus, and cheek pouch (oral epithelium)], 4) when administered to animals by several different routes (including the diet), 5) involving several different types of tumors (squamous cell carcinomas, adenocarcinomas, angiosarcomas, etc.), and 6) in different cell types [epithelial cells (in the colon, liver, lung, esophagus, and cheek pouch) as well as connective tissue cells (fibroblasts, both in vitro and those in the liver which give rise to angiosarcomas). Thus, the remarkable ability of Bowman-Birk inhibitor to serve as an anticarcinogenic agent has been demonstrated in a variety of different carcinogenesis assay systems.

Although the mechanism of action of protease inhibitors as anticarcinogenic agents is unknown, many hypotheses have been presented. Our results suggest that anticarcinogenic protease inhibitors are capable of reversing the initiating event in carcinogenesis, presumably by stopping an ongoing process begun by carcinogen exposure. We have observed several effects of protease inhibitors which are thought to be related to their anticarcinogenic activity; these include 1) the ability to affect the expression of certain oncogenes (e.g., c-myc and c-fos) and 2) the ability to affect the levels of certain types of proteolytic activities (e.g., N-t-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin-hydrolyzing activity) which are elevated in carcinogen-exposed tissues.

We have also observed other effects of anticarcinogenic protease inhibitors which may be related to carcinogenesis. For example, we have reported that the inhibitors can reduce the carcinogen-induced, elevated levels of gene amplification to nearly normal levels. While all of these effects of protease inhibitors may contribute to the prevention of carcinogenesis, the mechanism by which protease inhibitors prevent cancer cannot be determined with certainty until the mechanisms involved in cancer induction are known. While the mechanism remains unclear, it is clear that protease inhibitors can reverse a number of carcinogen-induced cellular changes which may play important roles in carcinogenesis.

Introduction

The ability of protease inhibitors to serve as cancer-chemopreventive agents in both in vitro (1) and in vivo (2) systems has recently been reviewed. Although the mechanisms for the anticarcinogenic activity of protease inhibitors are unknown, many hypotheses have been discussed (1–3). Our own studies have suggested that protease inhibitors suppress both the initiation and promotion stages of carcinogenesis by stopping an ongoing process involved in carcinogenesis, as described below.

Materials, Methods, and Results

In Vitro Model Systems. Protease inhibitors have been studied as cancer-chemopreventive agents in a number of different in vitro model systems. Most of our in vitro transformation studies have been performed in C3H10T½ cells, an in vitro transformation assay system originally developed by Reznikoff et al. (4, 5). In this cell line, carcinogen treatment of cells leads to the formation of transformed foci within a background monolayer of normal-appearing cells, as shown in Figs. 1 and 2. The advantages and disadvantages of the different in vitro transformation systems commonly used in carcinogenesis research are described elsewhere (6, 7).

The ability of protease inhibitors to suppress radiation-induced transformation in vitro was first observed in C3H10T½ cells (8). It is now known that many different protease inhibitors are capable of suppressing radiation- and chemical carcinogen-induced malignant transformation in a variety of in vitro transformation systems, as recently reviewed (1). A wide variety of transformation-inducing agents have been utilized in these studies, including chemical carcinogens that do or do not need to be metabolically activated, both ionizing and nonionizing radiation, and steroid hormones. The fact that protease inhibitors suppress transformation induced by agents producing very different kinds of cellular damage suggests that carcinogenesis produced by the different agents may involve a common pathway affected by the anticarcinogenic protease inhibitors.

Not all protease inhibitors are capable of suppressing transformation in vitro; the inhibitory profiles of those that affect transformation have been discussed elsewhere (1). For the suppression of transformation, the most effective of the protease inhibitors we have studied is chymostatin, a quite specific and potent inhibitor of chymotrypsin, as described in detail elsewhere (9). With only picomolar concentrations in the medium, chymostatin has the ability to suppress radiation-induced transformation in vitro (9). Other inhibitors of chymotrypsin, such as tosyl-phenylalanine chloromethyl ketone, are also very effective in their ability to inhibit malignant transformation (9). Inhibitors of the Bowman-Birk inhibitor family of protease inhibitors are effective at suppressing transformation in the nanomolar concentration range (10). The structure of the soybean-derived BBI1 is shown in Fig. 3. The well characterized protease inhibitory sites in BBI are shown in Fig. 3 [the structure of BBI has been described by Odani and Ikenaka (11)]; only the chymotrypsin inhibitory site is involved in the suppression of transformation by BBI (10).

The fact that BBI was so effective at suppressing transformation induced by a variety of carcinogens led us to develop this protease inhibitor into a form that could be utilized in large-scale animal and human cancer prevention trials. Because PBBI would be prohibitively costly for use in such large-scale studies, we have developed an extract of soybeans enriched in BBI, termed BBIC, which is being used for large-scale studies (2, 12). For the suppression of in vitro transformation, BBIC works as well as PBBI (10, 12).

A discussion of the potential mechanisms of action of the anticarcinogenic protease inhibitors in the suppression of transformation requires a discussion about the mechanisms thought to be involved in carcinogen-induced transformation in vitro. Results from our in vitro studies have suggested that a high-frequency initiating event is involved in transformation (13–19). The frequency of initiation is sufficiently high that it is not likely to be a mutational event. It is hypothesized that the high-frequency initiating event in transformation reflects a change in the pattern of gene expression that, like the...
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SOS system in bacteria, then determines the frequency of rare genetic events (13–19). Initiation is clearly more stable than the SOS response, however. Radiation-induced recombination in yeast, as reported by Fabre and Roman (20), is a system that remains activated for a longer period of time and represents a type of model system which could account for our observations on the kinetics of the transformation process. It is of interest that such a system can be turned off by protease inhibitors that inhibit chymotrypsin, as shown by Wintersberger (21). Although theoretically appealing for our “ongoing process” hypothesis, a system comparable to radiation-induced recombination in yeast has not yet been found in mammalian cells.

There is now much evidence that a high-frequency event is also involved in the initiating event that leads to cancer in animals. Several investigators from several different laboratories have been able to count the number of initiated cells which give rise to cancers in animals in their in vitro/in vivo systems; results from some of these studies using radiation as the carcinogenic agent are summarized in Table 1 (data from Refs. 22–24). As can be observed in the results shown in Table 1, frequencies of initiation and/or tumor formation from initiated cells are approximately 1–4% for several different systems involving cancers of breast, thyroid, and lung tissue. Similarly, high frequencies of initiation by chemical carcinogens have been discussed elsewhere (18). Such frequencies are far too high to represent single gene mutations as the initiating events in these tissues.

Thus, initiation of carcinogenesis in both in vitro and in vivo systems is likely to be brought about by a high-frequency event, as has been reviewed (18). Our data suggest that carcinogen exposure induces an ongoing cellular process (13–18); protease inhibitors appear to be able to stop this process at any point before malignant cells arise (9, 10, 19). We have observed that protease inhibitors are capable of suppressing transformation even when applied to cultures long after carcinogen exposure, provided that cells are able to proliferate at the time of exposure to protease inhibitors (6, 9, 10, 19, 25). Our results show that carcinogen-treated cells are still maximally affected by the anticarcinogenic action of protease inhibitors at 10 days and 13 cell divisions after exposure to a carcinogen (9), as demonstrated in Fig. 4. Other transformation studies of ours have suggested that protease inhibitors are capable of reversing the initiated state of carcinogen-treated cells (9). Protease inhibitors are effective even when administered to cultures for only a 1-day treatment after carcinogen exposure (8, 19, 25).

Many protease inhibitors are strongly antagonistic to the enhancing effects of promoting agents in the induction of transformation in vitro (25, 26). Protease inhibitors have been thought to be highly “antipromotional” in many of the in vivo two-stage carcinogenesis experiments which have been performed (27, 28). It has recently been proposed that Bloom’s syndrome is a human disease representing a defect in the area of promotion (discussed in Ref. 29). We have observed that protease inhibitors, including BBI and BBIC, greatly reduce the levels of spontaneously occurring chromosome aberrations and sister chromatid exchanges in cells of patients with Bloom’s syndrome (29). Bloom’s syndrome is an autosomal recessive genetic disease in which high levels of chromosomal abnormalities are thought to be related to the high risk of cancer in people with the disease (30). We have hypothesized that radiation and chemical carcinogens may induce in normal cells a state or ongoing process resembling that which always occurs in the Bloom’s syndrome cells, with protease inhibitors being capable of stopping that process (29).
Recently, evidence has been presented that such an ongoing process can be induced by carcinogens; it has been reported that radiation can produce chromosomal aberrations via an indirect process operating long after exposure (31). Our hypothesized carcinogen-induced process could be characterized by a state of genomic instability. It is a well known phenomenon that a state of genomic instability exists in malignant cells (32, 33). The presence of a variety of chromosomal abnormalities in preneoplastic cells (33) suggests that a state of genomic instability precedes malignancy. Many premalignant cells have also acquired the ability to amplify genes, another measure of genomic instability (34). From the data cited above, it seems reasonable to suggest that the state of genomic instability can occur very early in carcinogenesis and that this state represents an ongoing process which could serve as a target for cancer-chemopreventive agents such as protease inhibitors.

With the availability of relatively new molecular biology techniques, an overwhelming number of specific mutational changes in genes have been reported. However, what may be far more important to the field of cancer chemoprevention than the generation of any specific mutational change is the process driving initiated/premalignant cells to continuously produce the specific mutational changes. If the system can be turned off before a critical mutation is produced, cancer development could be prevented.

Protease inhibitors have been shown to affect a number of different endpoints which could be related to their ability to suppress transformation in vitro. The major lines of investigation regarding the mechanism of the protease inhibitor suppression of transformation relate to the ability of anticarcinogenic protease inhibitors to affect 1) the levels of certain types of proteolytic activities, 2) the expression of certain oncogenes, and 3) gene amplification. The most direct approach to determining the mechanism of action of the protease inhibitors is to identify and characterize the protease or proteases with which they are interacting; such proteases have been identified by substrate hydrolysis and affinity chromatography (35-43). Utilizing substrate hydrolysis, we have examined the ability of cell homogenates to cleave specific substrates and have then determined the ability of various protease inhibitors to affect that hydrolyzing activity. With affinity chromatography, specific proteases directly interacting with the anticarcinogenic protease inhibitors have been identified. Utilizing in vitro systems, the Boc-Val-Pro-Arg-MCA-hydrolyzing activity and the succinyl-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin-hydrolyzing activity were identified by substrate hydrolysis (44), and a M, 43,000 protease has been identified by affinity chromatography (38, 40). Although the functions of these proteases are not known, the Boc-Val-Pro-Arg-MCA-hydrolyzing activity has characteristics similar to those of specific proteases known to be involved in growth factor processing, which has led to speculation that it has a similar function (36).

Our studies on the effects of protease inhibitors on the expression of oncogenes have focused mainly on c-myc, c-fos, c-erb-B, and c-H-ras (44–51). Our initial studies in this area of research were with C3H10T½ cells. In these studies, total RNA was extracted from logarhythmically growing irradiated and control cells maintained with or without protease inhibitors. In these studies, we observed that c-myc transcripts are reduced in irradiated C3H10T½ cells treated with protease inhibitors, as discussed in detail elsewhere (44). Under conditions in which protease inhibitor treatment results in a reduction in c-myc expression, there is no effect on proliferation rates, as shown in Fig. 5. Further studies in this area of research demonstrated that c-fos expression was similarly affected by the anticarcinogenic protease inhibitors (47). The effect of protease inhibitors on c-myc expression is thought to be related to the ability to suppress transformation in vitro, for the following reasons. 1) The protease inhibitor suppressing effect on transformation is correlated with the ability to down-regulate c-myc expression (49). 2) Protease inhibitors can down-regulate c-myc expression in normal, but not transformed, C3H10T½ cells (49). This phenomenon parallels the effect of protease inhibitors on transformation; protease inhibitors suppress the

Table 1 Frequency of initiation by radiation in in vivo and in vitro experiments

<table>
<thead>
<tr>
<th>Cell system</th>
<th>No. of cells (surviving) exposed to carcinogen</th>
<th>Type of radiation (dose)</th>
<th>Frequency of initiation</th>
<th>Authors (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo rat tracheas exposed to carcinogen; cell suspensions obtained from individual tracheas and cultured in vitro</td>
<td>~150 colony-forming cells/dish</td>
<td>X-rays (600 cGy)</td>
<td>Frequency of altered cells (number of altered cells/number of colony-forming cells): X-rays, 2 x 10⁻²; neutrons, 2.8 x 10⁻²</td>
<td>Terzaghi-Howe (24)</td>
</tr>
<tr>
<td>In vitro carcinogen exposure; in vivo growth of tumors (thyroid clonogenic cell transplantation system)</td>
<td>3.3 surviving ‘clonogens’/animal graft site</td>
<td>X-rays (500 cGy)</td>
<td>Incidence of gross tumors: 42/10³ clonogens, 4.2 x 10⁻⁴ clonogen</td>
<td>Clifton et al. (22)</td>
</tr>
<tr>
<td>In vivo carcinogen exposure of mammary epithelial cells; mammary outgrowths derived by injecting cells into mammary fat pads of mice</td>
<td>~3 surviving stem cells/fat pad</td>
<td>γ-rays (100 cGy)</td>
<td>100% of carcinogen-exposed animals had initiated cells; initiation frequency, 1.5 x 10⁻²</td>
<td>Ethington and Ullrich (23)</td>
</tr>
</tbody>
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* 20% of the altered cells maintained in culture developed tumorigenic potential (M. Terzaghi-Howe, personal communication).

b R. Ullrich, personal communication.
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Fig. 4. In the experiments shown, cultures of C3H10T½ cells were irradiated and allowed to proliferate until they were nearly confluent, at which time they were subcultured to a lower density and seeded into dishes so that there would be approximately 300 viable cells/dish. At that point, protease inhibitors were added to cultures, which were then maintained as for a routine transformation assay. The results showed that protease inhibitors are capable of suppressing radiation-induced transformation in vitro even when applied to cultures as long as 10 days and 13 cellular generations after cells were exposed to the carcinogenic agent. Data are from Ref. 9.

process involved in the induction of transformation but have no effect on cells that are already transformed (25). 3) Protease inhibitors only affect transformation when applied to proliferating cells; similarly, protease inhibitors only affect c-myc expression when applied to proliferating cells (45, 46, 49, 51). Another promising area for mechanistic studies involving protease inhibitors is gene amplification, because that is one type of genetic event which can be induced in a widespread fashion in a population of mammalian cells by a number of different carcinogens (52, 53). We have observed that radiation-induced gene amplification can be affected by the anticarcinogenic protease inhibitors in a manner that corresponds to their ability to suppress radiation-induced transformation (54), suggesting that the protease inhibitor suppression of gene amplification may be related to the anticarcinogenic activity of the inhibitors.

In Vivo Studies. Many different types of in vivo carcinogenesis studies have been performed with protease inhibitors, as recently reviewed (2). It is clear from the results of these studies that certain protease inhibitors are powerful anticarcinogenic agents, with the ability to completely prevent cancer in many different animal model systems. Our own studies have focused on BBI administered to animals as PBBI or BBIC (reviewed in Ref. 2). In our studies, BBI has been shown to suppress carcinogenesis 1) in three different species (mice, rats, and hamsters), 2) in several organ systems/tissue types [colon, liver, lung, esophagus, and cheek pouch (oral epithelium)], 3) in cells of both epithelial and connective tissue origin, 4) when it was given to animals by several different routes of administration (including i.p. or i.v. injection, topical administration, and dietary administration), 5) leading to different types of cancer (e.g., squamous cell carcinomas, adenocarcinomas, angiosarcomas, etc.), and 6) induced by a wide variety of chemical and physical carcinogens. We originally identified BBI as an anticarcinogenic agent in the C3H10T½ in vitro transformation assay system (55), as described above. It was then shown to work in several different animal model carcinogenesis systems (reviewed in Ref. 2). The anticarcinogenic effects of BBI in vivo are similar to those observed in our in vitro studies, as summarized here. 1) Like the irreversible effect on in vitro transformation, BBI has an irreversible suppressive effect on carcinogenesis in vivo, as shown in the hamster oral carcinogenesis assay system (demonstrated in Fig. 6; data are from Ref. 56). 2) Like the effect of protease inhibitors on in vitro transformation, BBI treatment can be delayed for a long time after carcinogen treatment and still have a suppressive effect on in vivo carcinogenesis, as shown in Fig. 6. 3) The dose-response relationship for the BBIC and PBBI suppression of oral (56) and colon (57) carcinogenesis is like that observed in vitro (9, 10), in that the same suppressive effect is observed for differences in dose/concentration of BBI which vary over orders of magnitude. 4) Like the in vitro studies showing that protease inhibitors suppress transformation without toxicity (8, 25), BBI is able to suppress carcinogenesis in animals without toxic side effects (2). In fact, anticarcinogenic levels of BBIC have a significant life-lengthening effect in mice (12), as illustrated in Fig. 7. BBI has recently advanced to the human trial stage. It is planned that the IME to be studied in people will be those that were originally identified in in vitro systems and that also
assay indicate that BBIC treatment can be delayed for a relatively long time after there is an irreversible suppressive effect on carcinogenesis. The results of studies on the effects of BBIC when treatments began during the later periods of the transformation suppressive effect on carcinogenesis. The results showing a suppressive effect on carcinogenesis when BBIC was present for the first third of the assay period demonstrate that have to be continuously present during the carcinogenesis assay period to have a as effective as BBIC in the suppression of oral carcinogenesis. In addition, BBIC does not appear to play a role in in vivo carcinogenesis systems. As shown in Fig. 8, both the expression of specific oncogenes (e.g., c-myc) and specific proteolytic activities (e.g., Boc-Val-Pro-Arg-MCA-hydrolyzing activity) are elevated above normal levels in carcinogen-treated epithelial tissue in vivo. For both of these IME, PBBI/BBIC treatment results in normal levels of expression in the in vivo tissues studied, and this effect on the IME is correlated with a reduction in tumor yields. Our hypothesis is that human premalignant tissue or tissue at a higher than normal risk of developing into cancer will be analogous to carcinogen-treated tissue in the animal model carcinogenesis assay systems we have studied. Thus, it is expected that we will observe elevated levels of these IME in the human tissues at elevated risk for cancer development. As discussed above, in our previous animal studies we have observed that BBI is capable of bringing such elevated levels of IME back to normal levels. In the in vivo studies performed, BBI treatment did not affect normal levels of either c-myc expression (48) or proteolytic activity (40) but was able to reduce the elevated levels of each of these IME to normal levels in the carcinogen-treated tissues.

Discussion

While the mechanism of action of protease inhibitors in the prevention of cancer is not yet clear, it is clear that the inhibitors are powerful anticarcinogenic agents. Those protease inhibitors with the ability to suppress transformation are capable of reversing several carcinogen-induced changes to a normal state. While it is possible that we have not yet studied the critical carcinogenic-induced change that is involved in the conversion of a cell to a malignant state, it is highly likely that the anticarcinogenic protease inhibitors are able to reverse other important carcinogen-induced changes related to cancer development in the same manner that we have already observed for the IME discussed above. The mechanism(s) involved in cancer causation must be known with certainty before we can feel confident about the mechanism by which protease inhibitors prevent cancer.

Protease inhibitors are unlike most of the other potential classes of cancer-chemopreventive agents that have been studied, in a number of different ways. The most dramatic difference we have discovered is their ability to affect the carcinogenic process in an irreversible manner. When protease inhibitor administration is stopped in either in vivo (56, 58) or in vitro (9, 10) experiments, malignant cells or tumors do not arise in the assay systems we have utilized. Other cancer-preventive compounds we have studied, including vitamin E, dimethylsulfoxide, β-carotene, retinoids, etc., have a reversible effect on in vitro transformation (e.g., see Ref. 59). When most cancer-chemopreventive agents are removed from carcinogen-treated cell cultures, transformed cells do arise (59). While there are other compounds which appear to have the ability to reverse the initiated state of cells in a manner similar to that which we have observed for the protease inhibitors, they are not commonly used as cancer-chemopreventive agents, because the agents which have been described are potent carcinogens in other organ systems. For example, sulfur mustard appears to inactivate initiated cells in the skin (60) but serves as a carcinogen in the respiratory tract (61). Similarly, aflatoxin B1 and actinomycin D are thought to inactivate initiated cells in the skin but are potent carcinogens for other tissues (60).

Protease inhibitors are also unlike many of the other agents we have used as cancer-chemopreventive agents, in that they are effective at extremely low levels while most of the other agents we have studied
are effective only at very high levels. For example, vitamin E is effective only at levels near the toxic level in our in vitro studies (62). Protease inhibitors such as BBI are effective in the nanomolar range (10) and have shown no toxicity at any concentration evaluated in our in vitro studies (reviewed in Ref. 1). The dose-response curve for the BBI suppression of carcinogenesis in both in vivo (56, 57) and in vitro (9, 10) systems is unusual, in that concentrations/dose levels of BBI varying over orders of magnitude have the same suppressive effect. This phenomenon may be due to the characteristics of the target protease involved in carcinogenesis. Studies on the M, 44,000 protease described above have shown that this protease must be activated by trypsin to be active (40, 41). It is thought that proteases, such as this, requiring trypsin activation are carefully controlled by cells and only very small amounts of these enzymes are active at a given time; thus, very low concentrations of protease inhibitors would be effective at inhibiting such a protease (40).

Perhaps the most important difference between the anticarcinogenic protease inhibitors and other cancer-preventive agents is their ability to affect so many different kinds of carcinogenesis. As mentioned above, their anticarcinogenic effects are not restricted to specific organs or carcinogens. The effects of most cancer-chemopreventive agents are far more limited than are the effects of the anticarcinogenic protease inhibitors. Protease inhibitors can almost be considered "universal" anticarcinogenic agents in their wide-ranging ability to affect the carcinogenic process.

The one type of carcinogenesis that the protease inhibitors have not been expected to be able to affect is gastric carcinogenesis. Because the inhibitory activity of protease inhibitors is pH dependent, they would not be expected to have activity at the low pH of the stomach environment or to function as cancer-chemopreventive agents there. There are epidemiological data, however, demonstrating that high levels of soybean in the diet are associated with low gastric cancer rates (63). While this effect could be due to other anticarcinogenic agents in soybeans (reviewed in Ref. 64), it could also be due to soybean protease inhibitors such as BBI. [In our studies, BBI was the only compound in soybeans with the ability to suppress in vitro transformation (10)]. Protease inhibitors could serve as cancer-chemopreventive agents for gastric carcinogenesis by being internalized by the cells lining the stomach. Once internalized, protease inhibitors such as BBI would have activity at the normal pH of the stomach epithelial cells.

High levels of protease inhibitors in the diet are associated with low incidence rates for breast, colon, prostate, oral, and pharyngeal cancers, as recently reviewed (2). The particular kind of protease inhibitor most closely associated with the ability to prevent cancer is chymotrypsin inhibitory activity (reviewed in Ref. 12). Soybeans have a particularly high content of chymotrypsin inhibitory activity (65). Both the Japanese (66) and members of the Seventh Day Adventists (67, 68) have high dietary levels of soybean products and extremely low cancer rates for the common Western cancers. The Japanese do have high rates of stomach cancer (69, 70); however, there is no evidence to suggest that high levels of soybean products in the diet contribute to the high stomach cancer rates in Japan. If there were such an association, one would expect stomach cancer rates to be higher in the vegetarian Seventh Day Adventist population too, and they are not. Further evidence against such an association includes the data cited above, which indicate that diets high in soybean content are associated with a decreased stomach cancer risk (65).

While there are no doubt many factors contributing to the cancer incidence and mortality data utilized for epidemiological studies, the data showing the association between high dietary levels of soybean products and low cancer mortality rates suggest that soybeans may contain compounds capable of serving as anticarcinogenic agents in human populations. As discussed here, at least one compound in soybeans, BBI, has clear anticarcinogenic activity in both in vivo and in vitro carcinogenesis assay systems.

**References**


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