Effects of Spermine-Conjugated Bowman-Birk Inhibitor (Spermine-BBI) on Carcinogenesis and Cholesterol Biosynthesis in Mice

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Purpose. The goals of the studies reported here were to evaluate the effects of the soybean-derived protease inhibitor known as the Bowman-Birk inhibitor (BBI) and its spermine-conjugate (spermine-BBI) on the prevention of lung tumorigenesis and the reduction of heart disease parameters.

Methods. Both spermine-BBI and purified BBI (pBBI), at a dose of 20 mg/kg body weight, were administered as intraperitoneal injections to animals treated with the chemical carcinogen 3-methylcholanthrene (MCA) to determine their effects on chemically induced lung tumorigenesis in A/J mice. In addition, the effects of spermine-BBI and pBBI on the aortic cholesterol content and the percent ester in the mice were determined.

Results. The characteristics of the animals in the various treatment groups were comparable in terms of behavioral phenomena, weight gain, and lack of deaths during the experimental period. Thus, there was no detectable toxicity in spermine-BBI–treated mice. Both spermine-BBI and pBBI had a significant suppressive effect on MCA-induced lung tumors, with spermine-BBI being more effective than pBBI. Spermine-BBI was considerably more effective than pBBI at affecting heart-disease–related parameters. The amount of esterified cholesterol present in the aortas of mice treated with spermine-BBI was 9% lower than that of the controls. Both pBBI and spermine-BBI reduced total cholesterol levels in the blood, with pBBI reducing the cholesterol level by 15.5% and spermine-BBI by 33.3%.

Conclusions. Spermine-BBI can prevent lung carcinogenesis without detectable toxic effects; therefore, it is concluded that lung targeting by the cationization of polypeptides can be achieved without apparent toxicity. The increase in retention of spermine-BBI compared to pBBI in liver tissue may make a difference for the heart disease parameters evaluated. Although spermine-BBI is capable of reducing the total cholesterol and ester levels in mice, pBBI did not have as great an effect on these parameters. Because the liver is the major site for the production of cholesterol, the localization of spermine-BBI in liver tissue may account for the greater effect of spermine-BBI on blood cholesterol levels. Spermine-BBI was administered to animals for only the first 2 months of the 4-month assay period before animal sacrifice, so the results suggest that the effects of spermine-BBI on the parameters related to heart disease are long-lasting, as are the effects of both pBBI and spermine-BBI on lung tumorigenesis.

KEY WORDS: protease inhibitor; Bowman-Birk inhibitor; conjugate; cholesterol; heart disease; malignant transformation.

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INTRODUCTION

The soybean-derived protease inhibitor Bowman-Birk inhibitor (BBI) is a potent chemopreventive agent in both *in vivo* and *in vitro* carcinogenesis assay systems, as has been reviewed elsewhere (1–3). We have prepared and performed several studies with BBI conjugates (4–8); such conjugates were produced to facilitate the absorption of BBI across cell membranes and, presumably, to increase the uptake of dietary BBI into the bloodstream, as previously reviewed (1,2). In a prior study, a BBI-poly(D-lysine) conjugate with disulfide linkage, PDL-SS-BBI, was found to be very effective in preventing lung tumor development (5); unfortunately, the PDL-SS-BBI conjugate proved to have unacceptable toxicity in animal experiments (5).

In subsequent studies, we observed that spermine-conjugated BBI (spermine-BBI) was far less cytotoxic than PDL-SS-BBI but retained an ability comparable to that of PDL-SS-BBI to localize in lung and liver tissues (6). Biodistribution studies in rats showed 14- and fivefold increases, respectively, of liver and lung localization in 3 h when BBI was conjugated to spermine (5). Thus, it appears that spermine-BBI may be a superior chemopreventive agent to PDL-SS-BBI. In the present study, both spermine-BBI and purified BBI (pBBI) were administered to animals treated with the chemical carcinogen 3-methylcholanthrene (MCA) to determine the effect on chemically induced lung tumorigenesis in A/J mice.

In addition, the effect of spermine-BBI and pBBI on heart disease parameters was also studied. Mouse aortic cholesterol content and the percentage ester were determined. It is known that the ester cholesterol content of the aorta increases with age and with developing atherosclerosis (9,10). Because liver is the major organ for the biosynthesis of cholesterol, the effect of spermine-BBI on aortic content of cholesterol is used as an indication of the liver targeting of the conjugate.

MATERIALS AND METHODS

Spermine-BBI was prepared by coupling BBI with spermine in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, as described in our previous report (5). Seventy male strain A/J mice were received from Jackson Laboratories (Bar Harbor, ME). This particular strain of mice has a high incidence of spontaneous lung adenomas, and lung tumors readily develop in response to carcinogens (11). On arrival in the laboratory, the mice were randomly sorted into three treatment groups. They were housed four mice per cage on hardwood bedding in plastic cages and had free access to conventional laboratory food and water. [Conventional lab chow was used for this study so that the results obtained for this study of BBI and a conjugate could be easily compared to previous results obtained from similar studies in which conventional lab chow was used as the food source (8).] The animals were kept in a temperature- and humidity-controlled environment for a 7-day acclimatization period. At this time, all mice received a single intraperitoneal (i.p.) injection of 3-methylcholanthrene (MCA, Sigma Chemical Co., St. Louis, MO). MCA was initially dissolved in corn oil and was then administered to the animals at a dose of 20 mg/kg body weight

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(injection volume = 0.1 ml/10 g body weight). Because both BBI and spermine-BBI are water soluble, they are administered to animals in an aqueous solution. Beginning 3 days after the single MCA injection, the three different groups of animals received the following treatments: group 1 (controls), 0.9% NaCl solution only at a dose of 0.1 ml/10 g body weight; group 2, 0.9% NaCl plus pBBI at a dose of 20 mg/kg body weight; and group 3, 0.9% NaCl plus spermine-BBI conjugate at a dose of 20 mg/kg body weight. The dose of spermine-BBI was based on the weight of the conjugate. Because the molecular weight of spermine is only about 2.5% of that of BBI, the amount of this polyamine in the conjugate is negligible.

All of the treatments were administered as i.p. injections into the right lower abdominal quadrant three times per week over an 8-week period. The mice were sacrificed by CO_2 asphyxiation 16 weeks after the MCA injection. For each mouse, 1 ml of Telleysnicki's fluid was injected through the trachea to inflate and fix the lungs. [The use of Telleysnicki's fluid in carcinogenesis studies of the sort performed here has been established by Shimkin and Stoner (11); it is used because the acetic acid in Telleysnicki's fluid shrinks the normal lung, which makes the nodules more evident during analysis.] The aorta was removed from each animal for measurement of the total cholesterol (mg/100 g) and percentage ester. The aorta's point of origin from the heart was located and followed to the end of the aortic arch. This segment was cut, removed, and flash frozen in liquid nitrogen. Because of the sensitivity of subsequent measurements, aortas with the same size from each group were pooled together and stored at –80°C. The pooled aortas were weighed and minced, and the lipid extracted with chloroform-methanol (2:1) (12). The ester cholesterol content of the lipid extracted was determined using the method of Sperry and Webb (9).

To study lung tumorigenesis, the lungs, heart, and mediastinum were excised *en bloc* and stored in Telleysnicki's fluid. At the time of sacrifice, each animal was examined carefully for abnormalities. If abnormalities were observed in organs other than the heart and lungs, these organs would also be removed, fixed, and processed for histopathologic observation. The number of tumors visible on the surface of each animal's lungs was determined under a dissecting microscope; the data for tumor multiplicity (average number of tumors per lung) were tabulated and calculated as described previously (8).

RESULTS

There was no detectable toxicity in spermine-BBI-treated mice. No animals died during the 16 week assay period, and animal weights and behavioral characteristics were comparable for all three treatment groups throughout the study. The weights (grams ± SD) of the animals in the treatment groups at the beginning of the assay period were as follows: MCA + saline, 19.5 ± 3.9; MCA + pBBI, 20.1 ± 1.8; MCA + spermine-BBI, 19.8 ± 4.5; the weights at the end of the assay period were as follows: MCA + saline, 24.7 ± 1.9; MCA + pBBI, 24.2 ± 2.0; and MCA + spermine-BBI, 24.9 ± 1.5. At the time of the animal autopsies, all of the organs except lungs from the animals in each of the different treatment groups were normal in appearance; thus, no tissues were examined for histopathologic alterations. The average number of tumors per lung for the animals in each of the three treatment groups is shown in Table I. Spermine-BBI was slightly more effective than pBBI ($p < 0.01$ vs. $p < 0.02$) at suppressing MCA-induced lung tumorigenesis, but both treatment groups had a significant suppressive effect on MCA-induced lung tumors.

The results of total cholesterol and percent ester measured for the heart disease parameters are shown in Table II. These findings show that spermine-BBI was considerably more effective than pBBI at affecting these heart-disease–related parameters. The amount of esterified cholesterol present in the aortas of the mice treated with spermine-BBI was 9% lower than that of the controls. Both pBBI and spermine-BBI reduced total cholesterol levels in the blood, with pBBI reducing the cholesterol level by 15.5% and spermine-BBI by 33.3%. As described in Materials and Methods, the aortas isolated from the mice in each group were pooled, resulting in one total value representing each group for the studies reported here. In experiments for rats maintained on diets with and without pBBI for 6 months, we found that pBBI treatment resulted in a reduction in total cholesterol levels comparable to the results reported here (Kennedy, A.R. and Kritschevsky, D., unpublished data).

DISCUSSION

The studies reported here indicate that spermine-BBI is considerably less toxic to animals than PDL-SS-BBI, a BBI conjugate that has been previously used in lung targeting studies (4–6). In this study, there was no detectable toxicity observed for spermine-BBI in terms of behavioral characteristics, weight gain, or animal deaths. We observed that both spermine-BBI and pBBI have the ability to suppress lung tumorigenesis in strain A/J mice, with spermine-BBI being slightly more effective than pBBI (spermine-BBI, $p < 0.01$ vs. pBBI, $p < 0.02$), when compared to the treatment group receiving MCA and saline only. The finding that spermine-BBI is only slightly more effective than pBBI in its ability to affect lung tumor development is consistent with the extent of targeting of this conjugate to the lungs, i.e., with an approximately fivefold increase of lung localization but a 50% decrease of the *in vitro* chymotrypsin inhibitory activity (5). We have reported that the increase in lung-localization of PDL-SS-BBI was at least 2-fold higher than that of spermine-BBI (6). Even though the effect was moderate for the improvement of the chemopreventive effect in lung cancer, results in this report suggest that lung targeting by the cationization of polypeptides can be achieved without any serious toxicity.

Although spermine-BBI has relatively low localization in the lungs when compared to PDL-SS-BBI, these two conjugates are equally effective in liver targeting, and both are

Table I. Tumor Multiplicity in Mice Treated with MCA and Purified BBI, Spermine-BBI, or Saline

Treatment group	No. of mice	No. of tumors	Mean no. of tumors/lung (±SE)[a]
1. MCA Saline	23	58	2.6 ± 0.6
2. MCA pBBI	24	21	0.9 ± 0.3
3. MCA spermine-BBI	23	17	0.7 ± 0.2

[a] Statistical analysis (Student's *t*-test): group 1 vs. 2, $p < 0.02$; group 1 vs. 3, $p < 0.01$; Groups 2 vs. 3, $p > 0.05$.

Table II. Effects of pBBI and Spermine-BBI on Total Cholesterol and Percentage Ester

Treatment group	Total cholesterol (mg/100 g)	Percentage ester	Cholesterol: % reduction (compared to control)
1. MCA + Saline	181	37.3	—
2. MCA + pBBI	153	39.2	15.5%
3. MCA + spermine-BBI	121	33.9	33.3%

significantly higher than native BBI (6). The increase in retention of spermine-BBI compared to pBBI in liver tissue may make a difference for the heart disease parameters evaluated in these studies. Although spermine-BBI is capable of reducing the total cholesterol and percentage ester in mice, pBBI did not have as great an effect on these parameters. Because the liver is the major site for the production of cholesterol, the localization of spermine-BBI in liver tissue may account for the greater effect of spermine-BBI on blood cholesterol levels. Although the mechanism for the effect of pBBI and spermine-BBI on cholesterol levels is still unclear, proteolytic activity is known to control the activities of sterol-regulatory element binding proteins (13,14), which control cholesterol homeostasis (14–16). It is conceivable that BBI is capable of inhibiting this proteolytic activity involved in cholesterol production. The animals were exposed to spermine-BBI for only the first 2 months of the 4-month assay period, suggesting that the effects of spermine-BBI on the parameters related to heart disease are long-lasting, as are the effects of both pBBI and spermine-BBI on lung tumorigenesis.

The results presented here suggest that spermine-BBI is comparable to, or slightly better than, pBBI in the ability to affect lung tumorigenesis and more effective than pBBI in the ability to affect parameters related to heart disease. These effects correlate with the targeting of spermine-BBI to the lungs and liver, suggesting that BBI is directly involved in cancer chemoprevention in the lungs and the reduction of cholesterol biosynthesis in the liver. These results suggest that spermine-BBI could be more effective than BBI for a number of different therapeutic applications. At present, BBI is being evaluated for its therapeutic effects (primarily anticarcinogenic or antiinflammatory) in a number of human trials utilizing patients with: (a) higher than normal risks for oral or prostate cancer, (b) cancer (lung or prostate), (c) ulcerative colitis, (d) benign prostatic hyperplasia, and (e) gingivitis. In addition, several other areas of clinical investigation are in various stages of development. It is conceivable that spermine-BBI could be more effective than BBI for a number of different potential therapeutic applications for compounds with characteristics similar to those of BBI.

Diets with high soybean content have been well established as having beneficial effects on heart disease parameters. It is noteworthy that BBI was the sole soybean-derived compound evaluated in these studies. Therefore, our studies suggest the possibility that, as well as its anticarcinogenic activities (1,2), BBI could contribute to the beneficial effect of high-soybean diets in the prevention of heart disease.

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REFERENCES

1. A. R. Kennedy. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **78**:167–209 (1998).
2. A. R. Kennedy. Overview: anticarcinogenic activity of protease inhibitors. In W. Troll and A.R. Kennedy (eds.), *Protease Inhibitors as Cancer Chemopreventive Agents*, Plenum, New York, 1993, pp. 9–64.
3. A. R. Kennedy. *In vitro* studies of anticarcinogenic protease inhibitors. In W. Troll and A.R. Kennedy (eds.), *Protease Inhibitors as Cancer Chemopreventive Agents*, Plenum, New York, 1993, pp. 65–91.
4. S. Persiani, A. Yeung, W. C. Shen, and A. R. Kennedy. Polylysine conjugates of Bowman-Birk protease inhibitor as targeted anticarcinogenic agents. *Carcinogenesis* **12**:1149–1152 (1991).
5. H. Ekrami, A. R. Kennedy, H. Witschi, and W. C. Shen. Cationized Bowman-Birk protease inhibitor as a targeted cancer chemopreventive agent. *J. Drug Targeting* **1**:41–49 (1993).
6. H. Ekrami, A. R. Kennedy, and W. C. Shen. Disposition of positively changed Bowman-Birk protease inhibitor conjugates in mice: Influence of protein conjugate charge density and size on lung targeting. *J. Pharm. Sci.* **84**:456–461 (1995).
7. H. M. Ekrami, A. R. Kennedy, and W. C. Shen. Water soluble fatty acid derivatives as acylating agents for reversible lipidization of polypeptides. *FEBS Lett.* **371**:283–286 (1995).
8. H. Witschi and A. R. Kennedy. Modulation of lung tumor development in mice with the soybean-derived Bowman-Birk protease inhibitor. *Carcinogenesis* **10**:2275–2277 (1989).
9. W. M. Sperry and M. Webb. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* **187**: 97–106 (1950).
10. H. A. I. Newman and D. B. J. Zilversmit. Accumulation of lipid and nonlipid constituents in rabbit atheroma. *Atherosclerosis Res.* **4**:261–276 (1964).
11. M. B. Shimkin and G. D. Stoner. Lung tumors in mice: application to carcinogenesis bioassay. *Adv. Cancer Res.* **21**:1–28 (1975).
12. J. Folch, M. Lees, and G. H. Sloanne-Stanley. A simple method for the isolations and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:487–509 (1957).
13. R. Sato, X. Yang, M. J. Wang, Y. K. Evans, J. L. Ho, J. L. Goldstein, and M. S. Brown. Assignment of the membrane attachment, DNA binding, and transcriptional activation domains of sterol regulatory element binding protein-1 (SREBP-1). *J. Biol. Chem.* **25**:17267–17273 (1994).
14. X. Wang, M. S. Sato, M. S. Brown, X. Hua, and J. L. Goldstein. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* **77**:53–62 (1994).
15. X. Hua, C. Yokoyama, J. Wu, M. R. Briggs, M. S. Brown, J. L. Goldstein, and X. Wang. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. USA* **90**:11603–11607 (1993).
16. C. Yokoyama, X. Wang, M. R. Briggs, A. Admon, J. Wu, X. Hua, J. L. Goldstein, and M. S. Brown. SREBP-1, a basic helix-loop-helix leucine zipper protein that controls transcription of the LDL receptor gene. *Cell* **75**:187–197 (1993).