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Authors

Armstrong, W B
Kennedy, A R
Wan, X S
et al.

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Single-Dose Administration of Bowman-Birk Inhibitor Concentrate in Patients with Oral Leukoplakia¹

William B. Armstrong, Ann R. Kennedy, X. Steven Wan, Joshua Atiba, Christine E. McLaren, and Frank L. Meyskens, Jr.²

Departments of Otolaryngology [W. B. A.] and Medicine [J. A., C. E. M., F. L. M.], Chao Family Comprehensive Cancer Center, University of California, Irvine, Orange, California 92868, and Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 [A. R. K., X. S. W.]

Abstract

The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor and a potential cancer chemopreventive agent for humans. In this Phase I clinical trial, BBI concentrate was administered as a single oral dose to 24 subjects with oral leukoplakia. Pharmacokinetics of BBI was analyzed, and subjects were monitored clinically for toxic effects. Subjects received between 25 and 800 chymotrypsin inhibitor units (CIU) of the compound in a dose escalation trial. BBI was taken up rapidly, and a metabolic product of BBI was excreted in the urine within 24–48 h. No clinical or laboratory evidence of toxicity was observed in the study. Protease activity was also measured in buccal cells to evaluate usefulness as a biomarker. Single-dose BBI concentrate administered up to 800 CIU was well tolerated and appeared to be nontoxic. Further investigation in Phase II clinical trials is being done.

Introduction

The soybean-derived protease inhibitor known as the BBI³ is a potent anticarcinogenic agent with activity demonstrated in a variety of *in vitro* and *in vivo* carcinogenesis assay systems (1). BBI was identified by Bowman in the 1940s (2) and purified by Birk in 1961 (3). The protein contains 71 amino acids and has two separate protease inhibitory sites, one each for trypsin and chymotrypsin (4). The anticarcinogenic activity of BBI has been localized to the chymotrypsin inhibitory region of the protein molecule (5, 6), but the actual mechanism producing the observed anticarcinogenic effects remains unknown (7). BBI is

resistant to digestive enzymes, and >90% of the BBI ingested reaches the colon intact. BBI is taken up by epithelial cells of the digestive tract, absorbed into the bloodstream, and distributed to all organs examined, except the brain (8, 9).

Interest in the use of soybean products as cancer-preventive agents emanated from epidemiological studies demonstrating low incidence rates of several cancers in populations with a high soy intake (10, 11). In Japan, which has a high dietary intake of soy products, the incidence rates of several cancers including breast, colon, and prostate cancer are very low (10, 12, 13). A number of compounds in soybeans have been studied, and several compounds, including phytic acid and β -sitosterol, have also demonstrated anticarcinogenic potential (11). The anticarcinogenic activity of BBI has been detected at nanomolar concentrations (5), and the ability of BBI to suppress carcinogenesis in animals far exceeds that of other potential chemopreventive agents identified in soybeans (11). Animal studies have shown that BBI is able to prevent the development of malignancies in several different animal tumor model systems (14–24). *In vitro* and animal studies using BBI and BBIC are reviewed and summarized in several recent publications (1, 7, 11, 22, 25, 26).

Soybeans are a major component of animal diets in the United States and are a human dietary staple in certain parts of the world. Early animal studies demonstrated growth inhibition when animals were fed raw soybeans (27). This was erroneously attributed to protease inhibitors (1, 22). Pancreatic changes are a potential toxicity concern because they have been associated with trypsin inhibition in rats fed very high levels of soybeans over long periods of time (28, 29). BBI contains some trypsin inhibitory activity, but BBIC has greatly reduced trypsin inhibitory activity compared with raw soybeans and many soybean products (*e.g.*, Refs. 14–22 and 24). Adverse effects on the pancreas have not been observed in animal studies using BBIC including subchronic toxicity studies in rats and dogs.⁴

The current study was designed to determine whether oral ingestion of BBIC produced any clinical or laboratory evidence of toxicity. The trial design also evaluated the pharmacokinetics of BBI and PA as a potential biomarker. Oral premalignancy was selected as the initial clinical model to study BBIC in humans because it provides an easily accessible site and has well-defined premalignant lesions that can be monitored for treatment effects. BBIC was administered as a single oral dose to 24 subjects in a dose escalation trial in which clinical and laboratory toxicity was monitored, and pharmacokinetic studies (of BBI) were performed.

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² To whom requests for reprints should be addressed, at Department of Medicine, Chao Family Comprehensive Cancer Center, University of California, Irvine, 101 The City Drive South, Building 23, Suite 406, Orange, CA 92868.

³ The abbreviations used are: BBI, Bowman-Birk inhibitor; BBIC, BBI concentrate; CIU, chymotrypsin inhibitor unit(s); MCA, aminomethyl coumarin; ONG, *o*-nitrophenyl-B-D-galactopyranoside; PA, protease activity; PB, phosphate buffer; CBC, complete blood count; Hgb, hemoglobin; Hct, hematocrit; SMA, sequential multichannel autoanalyzer.

⁴ J. G. Page, personal communication.

Materials and Methods

Study Design

In this study, the effects of the administration of a single oral dose of BBIC were monitored. The highest dose used in this human trial was 800 CIU/day. This upper limit for human dosing was established from the results of BBIC toxicity testing in dogs. The highest dose of BBIC evaluated in dog toxicity tests is 1000 mg/kg, which allowed United States Food and Drug Administration approval for human trial doses of up to 800 CIU/day. Escalating doses were administered to subjects in five doses (25, 100, 200, 400, and 800 CIU). A total of 24 subjects with oral leukoplakia were studied. Subjects underwent oral examination to determine whether oral leukoplakia was present, and blood was collected for clinical laboratory assays (SMA-18 and CBC) to determine study eligibility. Each subject received the study medication in a liquid suspension, and subjects were observed for symptoms and signs of clinical toxicity or drug allergy. Timed specimens were collected after drug administration. Serum was obtained for pharmacokinetic studies at 10, 15, 20, and 40 min; 1, 3, 6, 12, and 24 h; and 2 and 4 weeks. Urine samples were also obtained at 0, 1, 3, 6, 12, 24, and 48 h and at 2 and 4 weeks. Buccal cells were collected by oral brushing before drug administration and at several subsequent staggered occasions between 6 h and 4 weeks after drug administration (6, 24, and 48 h and 2 and 4 weeks). Approximately 4 weeks after drug administration, subjects were interviewed by study personnel for symptoms of clinical toxicity. Oral examination was repeated, and blood was obtained for SMA-18 and CBC to evaluate for changes in laboratory parameters.

Sample Collection and Specimen Handling

Serum for BBI measurements was separated by centrifugation at 4°C and stored at -20°C until analyses were performed. Urine samples were also stored at -20°C until analyses were performed. Buccal cell samples were harvested by gently brushing the oral mucosa with a soft toothbrush and rinsing the mouth and toothbrush with PBS. The collected fluid was placed on ice, filtered through cheesecloth, and centrifuged at 5000 × g for 5 min at 4°C; the pellet was flash-frozen in liquid nitrogen and stored at -70°C until analyzed. The timing of brushing collections was spaced among the groups to allow reaccumulation of surface cells.

Drug Formulation and Administration

BBIC produced and provided by Central Soya Company, Inc. (Fort Wayne, IN) was prepared using methods described previously (30). Previously assayed BBIC powder containing approximately 100 CIU/g was dissolved in Roxane saliva substitute (Roxane Laboratories, Columbus, OH) containing sorbitol, carboxymethylcellulose, methylparaben, and water to yield a troche. For the 25 and 100 CIU doses, 20 ml of solution were made. At higher doses, the volume was increased up to 160 ml/dose so as not to exceed the solubility of BBIC. Doses were administered to subjects as follows: (a) 25 CIU, six subjects; (b) 100 CIU, six subjects; (c) 200 CIU, four subjects; (d) 400 CIU, five subjects; and (e) 800 CIU, three subjects. The doses administered in this study expressed in terms of CIU are in the range consumed in the Japanese diet, which contains a high intake of soy products. For comparison, the average Japanese dietary intake of soy products expressed in CIU is approximately 200 CIU/day, approximately four times that consumed in the United States diet (25).

Eligibility/Exclusion Criteria

Persons at least 21 years of age with clinically observable oral leukoplakia were eligible for enrollment. Females with child-bearing potential were required to use an adequate method of contraception. Postmenopausal females not taking conjugated estrogen products were also eligible. Exclusion criteria were pregnancy, unwillingness to protect against possible pregnancy, allergy or adverse reaction (to soybeans or soybean products, Roxane saliva substitute, sorbitol, carboxymethylcellulose, or methylparaben), liver dysfunction (aspartate aminotransferase > 2× normal, alanine aminotransferase > 2× normal, total bilirubin > 2.0 mg/dl), and renal disease (creatinine > 2.0 mg/dl). After determination of eligibility and explanation of the study, all subjects signed a consent form approved by the University of California, Irvine Institutional Review Board.

Analyses Performed

Measurement of BBI Levels. Timed serum and urine samples were obtained for measurement of BBI content. We have previously produced and characterized several monoclonal antibodies that react with reduced BBI as well as metabolized BBI products in urine (31). One of the monoclonal antibodies, designated 5G2, was used in this study to measure the BBI concentrations in urine samples by an ELISA. In the early stage of the study, a solid-phase ELISA method using filter membrane-coated multiwell plates (Millipore) was used to detect BBI in urine samples from patients who received a single dose of 100, 200, or 400 CIU of BBIC. To measure BBI concentrations by this method, urine samples were heated at 95°C for 10 min in the presence of 1% β-mercaptoethanol to reduce the disulfides in the BBI molecules and then applied to Immobilon-P filter membrane-coated multiwell plates at 100 μl/well and incubated at room temperature for 1 h for the antigen to attach. The liquid was removed from each well with a vacuum manifold (Millipore) attached to the bottom of the plates, and the plates were allowed to dry in air for 2 h. 5G2 antibody was diluted 1:500 in 1% BSA in PB, applied to antigen-coated multiwell plates in quadruplicate, and incubated at room temperature for 1 h. Purified BBI (Sigma-Aldrich, St. Louis, MO) was diluted in a control urine sample at various concentrations and included in each run of the ELISA to generate a standard curve. The plates were subsequently incubated for 1 h each with β-galactosidase-conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL) and ONG substrate (Sigma-Aldrich). The plates were washed three times with PB between incubations. At the end of the incubation with the ONG substrate, the plate was read with a plate reader at a wavelength of 405 nm.

During the course of the studies, it was discovered that some urine samples contained substances that interfered with the BBI measurement by altering the hydrophobic characteristics of the filter membrane in the multiwell plates. To overcome this problem, an inhibitory ELISA method was developed and used to measure BBI concentrations in urine samples collected from patients who received a single dose of 400 or 800 CIU of BBIC. To measure BBI concentrations by the inhibitory ELISA method, 5G2 antibody was diluted 1:500 in 1% BSA and mixed with each urine sample at equal volumes and incubated at room temperature for 30 min. The antibody-urine mixtures were then applied in triplicate at 100 μl/well to polystyrene 96-well plates precoated with BBI that was reduced by a radiochemical method in the presence of etanidazole as described previously (31, 32). BBI-etanidazole was diluted in a control urine sample at various concentrations and included in each run of the ELISA

to generate a standard curve. The plates were incubated at room temperature for 90 min. After washing three times with PB, the plates were sequentially incubated with the β -galactosidase-conjugated secondary antibody for 90 min and with the ONG substrate solution for 1 h. At the end of the incubation with the ONG substrate, the solution in each well was transferred to a polystyrene 96-well plate and read with a plate reader at a wavelength of 405 nm. To compensate for the fluctuations in urine concentrations (water content), creatinine concentrations in the urine samples were determined by using Fuller's earth method (33, 34), and the concentration of BBI was normalized to the concentration of creatinine in each urine sample.

Measurement of PA. PA was analyzed using the synthetic substrate Boc-Val-Pro-Arg-MCA (35, 36). Buccal cell pellets were thawed on ice and homogenized in 600 μ l of PBS. Samples were analyzed using 50- μ l aliquots of sample in 0.1 M Tris (pH 7.5)-5 mM CaCl₂ with the synthetic substrate. Samples were incubated for 2 h at 37°C, and the reaction was terminated by dilution with 1.8 ml of distilled H₂O. Fluorescence from released aminomethyl coumarin was determined spectrophotometrically at excitation and emission wavelengths of 380 and 460 nm, respectively, in a Perkin-Elmer fluorescence spectrophotometer. Protein content was determined by the Bradford method (37), with BSA used as a standard. The spectrofluorometer was standardized such that 10⁻⁷ M aminomethyl coumarin = 700 relative fluorescence units. Results were expressed as relative fluorescence units/h/ μ g protein.

Statistical Analysis. Mean values before and after BBIC administration were compared for serum and urine laboratory values using the paired *t* test. For each patient, the blood and serum CBC and SMA-18 test results were classified as normal (within the laboratory reference range) or abnormal (outside the laboratory reference range). McNemar's statistical test (38) was then used to examine whether or not the proportion of laboratory test values falling within or outside the reference range remained the same after BBIC administration. For each statistical test, the exact two-tailed *P* was computed. Pharmacokinetic data from the urine analyses were analyzed by first calculating the percentage increase in urine BBIC concentration from baseline for each sample. A Wilcoxon signed rank test was performed to examine changes from baseline. Buccal cell PA was analyzed similarly.

Results

Twenty-four subjects were enrolled in the study, consisting of 16 males and 8 females ranging in age from 48–73 years. Median age was 57.5 years. Twenty Caucasians, two Hispanics, one Asian, and one African American participated in the trial. Administration of BBIC as a single-dose troche in doses ranging from 25–800 CIU in a total of 24 subjects resulted in no clinically observed toxicities. All subjects completed follow-up visits to assess for clinical toxicity. No subjects reported adverse effects after ingestion of BBIC. No allergic reactions, gastrointestinal side effects, or constitutional symptoms were elicited. There was no change in the leukoplakic oral lesions in any of the subjects.

Blood (serum) was obtained before and approximately 4 weeks after BBIC administration. Complete pre- and post-SMA-18 values were available for 21 of 24 subjects, and complete pre- and post-CBC values were available for 14 of 24 subjects (incomplete data collection for CBC was due to a systematic technical error in obtaining and handling specimens during the collection process). The majority of laboratory values were within the reference range for each test. Mean test

values were calculated before and after drug administration and compared with a two-tailed *t* test. Statistically significant (*P* < 0.05) changes were observed for RBC, Hgb, total protein, and globulin. Hct was also shown to have a nearly significant (*P* = 0.06) change. RBC, Hgb, and Hct means all decreased slightly. However, the absolute magnitude of the shifts in the means was small, and all of the means were within the normal range for each parameter. RBC count decreased from 4.79 to 4.63 million cells/mcl, Hgb decreased from 15.28 to 14.81 g/dl, and Hct decreased from 44.88% to 43.79%. Closer examination of changes in the mean CBC values demonstrated that three subjects accounted for most of the change. Two males had decreases in Hct of -4.5% and -3.1%, and one female had a decrease in Hct of -2.6%. All three had starting Hcts over 40%, and the lowest Hct encountered in the study was 39%. Mean total protein decreased from 7.35 to 7.08 g/dl, and globulin decreased from 3.08 to 2.83 g/dl. Mean lactate dehydrogenase values were not calculated because approximately half way through the study, the clinical laboratory changed instrumentation for this test, and the reference range decreased from 313–618 IU/liter to 91–180 IU/liter, rendering calculation of the mean values of little use.

Serum data were also analyzed to determine the proportion of values outside the normal reference range for each test. Overall, the majority of laboratory values were normal both before and after treatment. Of the subjects who had an abnormal laboratory value, most were aberrant both before and after drug administration (15 of 126 CBC values and 18 of 418 SMA-18 values). The proportion of values that normalized after drug administration and the proportion of normal values that became abnormal after drug administration were approximately equal. Four of 126 abnormal CBC values were within the reference range after BBIC, whereas 5 of 126 normal values became abnormal. For serum SMA-18, 20 of 418 values normalized, and 22 of 418 values became abnormal. The proportion of normal and abnormal test values did not differ significantly before and after BBIC administration [*P* > 0.12 for all laboratory tests (McNemar's test)].

Serum and Urine Pharmacokinetic Results. A solid-phase ELISA method was used on the initial urine and serum samples from the patients who received a dose of 100, 200, or 400 CIU of BBIC. BBI was not detected in the serum samples tested using this assay. Because of this finding, further serum BBI assays were not performed in this trial. Using the initial solid-phase ELISA method for the detection of BBI, identifiable peaks in urine BBI were seen in 8 of 11 subjects, occurring between 2 and 9 h after drug administration. The relative percentage increase of BBI in those subjects exhibiting increased urine BBI over baseline ranged from 5.9–118%. Urine BBI levels, measured by modification of the initial protocol to an inhibitory ELISA, demonstrated peak excretion 3–10 h after drug administration in the four subjects studied with this improved method. The range of peak percentage increase was 154–895% (findings are displayed in Table 1). There was not enough urine remaining from the first 11 subjects to repeat the assays using the improved methods.

PA. Changes in PA measured in buccal cells after BBIC ingestion were highly variable. There were marked changes in levels of PA at 6 h for six of the seven subjects measured at this time point, with a marked increase in PA for three patients (with the levels increasing by 90.9 \pm 34.7%) and a marked decrease in PA for three patients (with the levels being reduced by 55.3 \pm 51.1%). The mean PA values for each time period analyzed are shown in Table 2. The number of samples ob-

Table 1 Urinary excretion of BBI^a

Subject no.	Dose (CIU)	Baseline BBI (ng/mg)	Time to peak excretion (h)	Peak BBI (ng/mg)	Increase (ng/mg)	Percentage increase
20	400	125	10	621	497	399
21	400	24.3	6	61.6	37.3	154
22	800	10.6	3	79	68.4	642
23	800	1.85	4	18.4	16.6	895

^a BBI measurements were recorded as nanograms of BBI per milligram of creatinine using the inhibitory ELISA technique described in "Materials and Methods." Normalization to milligrams of creatinine was performed to account for variable urine concentrations.

Table 2 Buccal cell PA as measured with the Boc-Val-Pro-Arg-MCA substrate

	Time after BBIC administration			
	6 h	24–48 h	2 wks	4 wks
No. of patients	7	10	10	12
Mean percentage increase from baseline	25%	15%	32%	19%
95% confidence interval	–37% to 86%	–36% to 66%	–16% to 79%	–28% to 66%
<i>P</i>	0.69	1.0	0.21	0.57

tained at each time interval was small because buccal cells were not harvested from each subject at each time period. For each individual patient, a sufficient time between buccal cell harvests had to occur to allow for epithelial cell maturation and sloughing. There was a trend toward a slight increase in PA after BBIC administration, but due to the wide variability in PA response after BBIC administration, no statistically significant change in mean PA from baseline was detected.

Discussion

BBIC was well tolerated when administered p.o. in this trial. No toxic or allergic reactions were recorded during the study. The doses administered ranged from levels near those obtained in the Western diet (25 CIU) to approximately four times those ingested in the Japanese diet (800 CIU).

Statistically significant decreases in mean RBC, Hgb, total protein, and globulin were observed for the group as a whole. The absolute magnitudes of the changes were small, and for these tests, almost every laboratory value remained within the normal range for the test, and all mean values were well within the normal range for the test. The magnitude of changes in the mean values, although statistically significant, is not biologically indicative of toxicity from BBIC. This is supported by analysis of shifts in the proportion of values falling within or outside the normal range after BBI administration. For each of the four tests showing a statistically significant shift in the mean values, McNemar's test *P* was either 1.0 or was not calculable because no values were outside the reference range for the test. Further study with a larger sample size is planned to determine whether these subtle observations of concern are due to BBI or other factors or represent random findings.

Analysis of urine pharmacokinetic data for BBIC was complicated by discovery in the early studies that the measurement of BBI by the solid-phase ELISA using filter membrane-coated multiwell plates can be compromised by the presence of unidentified urine substances and by the great fluctuations in the water content of the urine samples. To overcome these problems, urinary BBI concentrations in the later studies were determined by the inhibitory ELISA method and normalized to the urinary concentration of creatinine. Urine samples from four patients who received a single dose of 400 or 800 CIU of BBIC were measured by this method (urine samples from other

patients were not measured by this method because the samples were exhausted before this improved method was developed). The results from both assays showed BBIC to be rapidly excreted in the urine between 3 and 12 h after administration, decreasing markedly by 48 h. This is consistent with observations made in animal pharmacokinetic studies that demonstrate that approximately half of p.o. ingested BBI is taken up in the bloodstream and distributed through the body and that BBI has a serum half life of 10 h in rats and hamsters and is excreted in both the urine and feces (1, 8, 9). Animal pharmacokinetic studies of BBI have been summarized by Kennedy (1). The origin of the late peak seen in one subject (subject 22) is unclear and inconsistent with observations in animal studies but could have resulted from consumption of food products containing BBI in the diet.

PA in the buccal mucosal cells was highly variable in this trial, with both increases and decreases in PA observed in the patients. The mean values of Boc-Val-Pro-Arg-MCA hydrolyzing activity increased between 19% and 32% from baseline at all time periods sampled for each dosage group, but the responses were very variable as shown by the confidence intervals, and the changes were not found to be statistically significant. Several factors may account for the variable response observed. First, the population studied was heterogeneous, and no control for diet was made. Significant ingestion of products containing high levels of dietary protease inhibitors before or during the study could alter the assay results and camouflage the biological effects of BBI. Additionally, it is possible that there is a differential response to BBI administration among different subjects. No control was made for smoking status, which elevates PA (36). Histopathological analysis of the oral lesions was not performed. It is possible that PA may respond differently with severely dysplastic lesions than with mildly hyperplastic lesions. Both sustained increases and decreases in PA were observed in several subjects after BBIC administration, which could represent differential tissue responses to BBIC treatment.

Although no statistically significant changes or patterns of change in PA were observed in this single-dose study, more work needs to be completed to better characterize the effects of BBIC on PA in exfoliated mucosal cells and to define its utility as a potential intermediate marker end point. Animal data

clearly demonstrate reduced PA in normal cells exposed to carcinogenic agents and subsequently treated with BBIC (17, 35, 39). We are currently examining PA in detail in a follow-up Phase II clinical trial of BBIC (40, 41). We have demonstrated interrelationships between neu protein levels and PA after BBIC administration, providing insights on possible mechanisms of BBI activity (40).

BBIC was found to be nontoxic when administered as a single oral dose up to 800 CIU to human volunteers with oral leukoplakia. p.o. administered BBI was absorbed and rapidly excreted in the urine. Based on the lack of toxicity and the demonstrated *in vitro* and animal model anticarcinogenic effects, we have recently completed a short-term (1-month) Phase IIa study of BBIC and demonstrated a substantial clinical effect against oral leukoplakia and effects on potential biomarkers. A longer term (12-month) randomized Phase IIb study is planned.

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