

# Clinical Modulation of Oral Leukoplakia and Protease Activity by Bowman-Birk Inhibitor Concentrate in a Phase IIa Chemoprevention Trial<sup>1</sup>

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## ABSTRACT

**Bowman-Birk inhibitor is a protease inhibitor derived from soybeans that has demonstrated chemopreventive activity in a number of *in vitro* and animal systems. We conducted a 1-month phase IIa clinical trial of Bowman-Birk inhibitor concentrate (BBIC) in patients with oral leukoplakia. BBIC was administered to 32 subjects with oral leukoplakia for 1 month. We assessed toxicity and clinical and histological response of the lesions, and oral mucosal cell protease activity (PA) and serum micronutrient levels were measured. Clinical response was determined by measurement of pre- and posttreatment individual and total lesion areas and analysis of blinded clinical judgments of photographs. On the basis of prespecified response criteria, 31% of patients achieved a clinical response (two with complete and eight with partial responses). BBIC was nontoxic in doses up to 1066 chymotrypsin inhibitory units. The mean pretreatment total lesion area decreased from 615 to 438 mm<sup>2</sup> after BBIC treatment ( $P < 0.004$ ). A linear fit of the dose-response relationship between dose of BBIC and decrease in total lesion area was suggested ( $P < 0.08$ ), and analysis of blinded clinical impression from lesion photographs confirmed this relationship ( $P < 0.01$ ). Overall, at all doses tested, a 24.2% decrease in total lesion area was observed following treatment (sign rank =  $-142$ ;  $P <$**

**0.004). High pretreatment PA was associated with greater decreases in PA after BBIC administration ( $P < 0.02$ ). BBIC demonstrated clinical activity after oral administration to patients with oral leukoplakia. These results indicate that BBIC should be investigated for chemopreventive activity in a randomized clinical trial.**

## INTRODUCTION

A large body of epidemiological evidence has suggested a link between dietary habits and cancer incidence (1, 2). High levels of soybean consumption have been associated with a decreased incidence of colon, breast, and prostate cancers (3–5). Several distinct classes of compounds in soybeans (*e.g.*, isoflavones, phytic acid, saponins, and certain protease inhibitors) with chemopreventive activity have been identified (3, 4). A number of serine protease inhibitors suppress carcinogen-induced malignant transformation *in vitro*, and those with chymotrypsin inhibitory activity are the most potent chemopreventive agents in this class identified (6).

The BBI<sup>3</sup> is a soybean-derived serine protease inhibitor with both trypsin and chymotrypsin inhibitory activities (7). It is also a potential cancer chemopreventive agent with anticarcinogenic activities at concentrations well below those of other potential chemopreventive agents identified in soybeans (8, 9). *In vivo* studies have demonstrated that BBI is able to prevent development of malignancies in a large number of animal model systems (10–19). BBIC, which contains active BBI and has the same anticarcinogenic profile as purified BBI (6) has been developed for human trials. BBI and BBIC are nontoxic in several animal species, and the results of pharmacokinetic and toxicity studies for BBIC have been summarized recently (6). A phase I human chemoprevention trial in patients with oral leukoplakia demonstrated that BBIC had no acute toxicity when ingested *p.o.* as a troche (20).

In addition to the clinical response of visible premalignant lesions, phase II chemoprevention trials often rely on modulation of biomarkers to help determine potential effectiveness of the compound, and the identification and development of potential SEBMs is an important goal of phase IIa and IIb trials. We previously studied the levels of PA in cultured cells and tissues treated with ionizing radiation or chemical carcinogens as well as in tissues with higher than normal risks of cancer development (6, 21–25). We observed that exposure of cells to radiation and/or chemical carcinogens often leads to a prolonged

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<sup>3</sup> The abbreviations used are: BBI, Bowman-Birk inhibitor; BBIC, BBI concentrate; SEBM, surrogate end point biomarker; PA, protease activity; CIU, chymotrypsin inhibitory unit(s); PR, partial response; CR, complete response.

elevation of PA (6). In the hamster cheek pouch, BBI treatment suppressed the carcinogen-induced elevation of PA and inhibited development of malignant tumors (13). We have also shown that elevated levels of PA are present in patients with oral premalignant lesions, in smokers, and in ex-smokers (24). Because BBI is a serine-protease inhibitor with both trypsin and chymotrypsin activity (7), the PA level in premalignant tissues should be a good candidate SEBM for oral cancer chemoprevention trials when BBIC is used as the chemopreventive agent.

Oral leukoplakia is an ideal model for the study of cancer chemoprevention because the lesions are readily accessible to visual examination, diagnostic sampling, and evaluation of response to treatment. In the present study, BBIC was administered daily as an oral troche for 1 month in a single-arm phase IIa trial in patients with oral leukoplakia, and the clinical and histological response, toxicity, oral mucosal cell PA, and serum micronutrient levels were evaluated.

## MATERIALS AND METHODS

### Patient Characteristics

Thirty-three participants were recruited from patients at the University of California Irvine Medical Center (Orange, California), Veterans Affairs Medical Center Long Beach, and through radio and print announcements in Orange and Los Angeles Counties. Eligibility requirements included age >18 years; presence of clinically and histologically documented oral leukoplakia and/or erythroplakia; good general health (Karnofsky performance score  $\geq 80$ ); no history of malignancy other than non-melanoma skin cancer within 5 years; presence of bidimensionally measurable lesion(s); no prior high-dose vitamin A (up to 2 multivitamin pills/day allowed), high-dose  $\beta$ -carotene (no supplements), or retinoid therapy; willingness to have periodic photographs to document findings; ability to travel to appointments; and if female, not pregnant and using an appropriate contraceptive method. A baseline oral mucosal cell PA >1.5 times normal was also required for inclusion.

Blood and urine were obtained from patients prior to enrollment. Serum was obtained for sequential multichannel AutoAnalyzer-20 (SMA-20; total protein, albumin, uric acid, blood urea nitrogen, creatinine, glucose, sodium, potassium, chloride, CO<sub>2</sub>, calcium, phosphorous, alkaline phosphatase, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, cholesterol, and triglyceride), amylase, lipase,  $\beta$ -carotene, retinol, vitamin E, and blood for a complete blood cell count. Eligibility requirements included hematocrit >35%, white blood count >4000 cells/mm<sup>3</sup>, platelet count >100,000 platelet/mm<sup>3</sup>, serum creatinine <1.5 mg/dl, bilirubin <2.0 mg/100 ml, and aspartate aminotransferase or alanine aminotransferase <2.0 times normal. In addition, urinalysis had to show less than 1+ protein, 0–3 casts, or 5 WBCs or RBCs.

### Prestudy Evaluation

All participants signed an informed consent approved by the Institutional Review Boards of the University of California Irvine or the Long Beach Veterans Affairs Medical Center. Participants had medical, tobacco, alcohol, drug use, and dietary histories taken. Urine was obtained for urinalysis. Oral exami-

nation was performed, and clinical lesions were measured, photographed, and recorded on diagrams. Oral mucosal cell brushings were analyzed for PA. Punch biopsies (3 mm) were taken from the lesion and from an uninvolved control site after infiltration with local anesthetic. Counseling and education about the carcinogenic risks of tobacco and alcohol were discussed, and subjects were offered referral to counseling and/or cessation programs.

### Patient Treatment

Eligible subjects received BBIC in doses ranging from 200 to 1066 CIU per day (Central Soya Company, Inc., Fort Wayne, IN). Drug was dispensed as a powder that was reconstituted in Roxane Saliva Substitute (Roxane Laboratories, Columbus, Ohio) immediately before use. Patients were instructed to hold the BBIC suspension in the mouth for 1 min and then swallow. BBIC was administered twice daily for 1 month. Drug administration was planned as a sequential dose escalation trial. However, because of a complex dosing calculation error discovered early in the trial, the dosage administration was as follows: subjects 1–11 received 533 CIU, subjects 12–15 received 1066 CIU, subjects 16–23 received 200 CIU, and subjects 24–32 received 800 CIU.

After completion of 1 month of drug therapy, repeat history and oral examination were performed. The oral cavity lesions were measured, photographed, and recorded on diagrams. Oral mucosal cell brushings, biopsies of lesions and control sites, and serum and urine were again obtained for the same studies performed at enrollment.

### Adherence Information and Symptom Monitoring

Patient adherence to the 28-day program was monitored by container count, self-reported compliance through a personal diary, and patient interviews. Patients receiving study medication were observed at initial ingestion for toxic or allergic reactions. Subjects were contacted by telephone after 2 weeks and underwent a personal interview at study completion to inquire about side effects, palatability, and potential toxicity through an open-ended interview process, which included specific questions about the occurrence of nausea, vomiting, diarrhea, anorexia, fatigue, oral soreness, epigastric pain, and drug palatability. Toxicity was monitored using a graded toxicity scale. Grade 1 (mild) toxicity was followed for symptomatic progression. A physician was required to evaluate grade 2 toxicity within 48 h to determine the cause and significance of the toxicity, and drug therapy was temporarily suspended until a determination was made on whether the patient would be continued on the trial. Grade 3 toxicity mandated immediate evaluation by a physician and drug stoppage; the patient was removed from the study unless a clear alternative explanation for the symptoms was promptly determined. Palatability was assessed using a four-point scale: 0 = tastes fine with good medication compliance; 1 = tastes fine with sweetener, good medication compliance; 2 = tastes bad, with good medication compliance; and 3 = tastes bad, with poor medication compliance.

### Assessment of Clinical Response

Clinical response was assessed by recording bidimensional area of lesions as well as observing changes in color, thickness, and elevation of the lesions. Multiple lesions were recorded individually, with lesion areas summed into a total for each subject. Individual lesions were photographed (35-mm film, 100-mm macro lens and ring flash) during both the pre- and posttreatment oral examinations. Clinical response was prospectively defined as follows. Complete clinical response was defined as complete disappearance of all lesions at completion of treatment. PR was classified as at least 50% decrease in total lesion area. No response was classified as between a <50% decrease and 50% increase in total lesion area. Progression was classified as at least 50% increase in total lesion area, or development of new lesion(s).

For each lesion, the pre- and posttreatment photographs ( $4 \times 6$ -inch color prints) were mounted on the same page of an album, with relative position determined by a randomization scheme such that, within each dose, the pretreatment photo was in the superior position half the time. The order of pages in the album was randomized such that there was no relationship between dose and page number in the album. Four physicians with experience evaluating oral lesions and who had no contact with the study subjects made independent, comparative judgments of the upper and lower photographs on each page. Independent indications of clinical differences between the two photos in each pair were made. The evaluators selected from seven alternative statements that ranged from "top photo shows a complete response relative to the bottom photo," through "top and bottom photo show the same degree of disease," to "bottom photo shows a complete response relative to the top photo." The statistician later broke the code and transferred the responses to a seven-point Likert scale (26). Lesions for which photographs were unsuitable or missing were excluded from this analysis. The analysis was based on 204 judgments (55 paired photographs were reviewed by four reviewers). A total of 16 judgments were not included in the analysis because of perceived poor quality of the images by the evaluators.

### Histopathological Analysis

Specimens were processed in a standard manner with fixation in 10% neutral buffered formalin. Each biopsy was step-sectioned and stained with H&E. The biopsies were evaluated for the presence of hyperkeratosis, parakeratosis, acanthosis, inflammation, dysplasia, and malignancy. For purposes of this study, dysplasia was defined as neoplastic epithelium confined within the basement membrane of the stratified squamous epithelium within which it arose. The degree of dysplasia was graded using the terminology of low-grade dysplasia, high-grade dysplasia, and atypia. The criteria for grading dysplasia were as follows:

- Low-grade dysplasia—increased nuclear:cytoplasmic ratio, nuclear hyperchromatism, nuclear pleomorphism, and increased mitotic activity with or without atypical mitotic figures, maturational pattern disturbances with or without dyskeratosis. These changes involve 50% or less of the epithelial thickness.

- High-grade dysplasia—findings of low-grade dysplasia involving >50% of the epithelial thickness.
- Atypia—term used when there was uncertainty as to whether the observed histological abnormalities reflected reactive change or actual dysplasia.

### Laboratory Procedures

**Collection of Oral Mucosal Cells and Serum.** Oral mucosal cell collection was performed atraumatically using techniques developed in our earlier studies (27). Subjects were instructed not to brush their teeth the morning of oral mucosal cell collection. After patients rinsed their mouths twice with tap water, they were instructed to place ~15 ml of sterile PBS in their mouths and gently brush the entire inside surface of the mouth with a cytology brush. The cells brushed into the saline solution were collected into a 50-ml conical tube. The mouth and cytology brush were rinsed with 30 ml of PBS, the rinse was collected into a 50-ml centrifuge tube, and the cells were centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was removed, and the cell pellet was stored at -70°C until the assays were performed. Approximately 1 to 6 million cells could be easily and comfortably collected in this manner. Collected blood samples were allowed to clot and centrifuged at 1500 rpm for 10 min to separate the blood clot from the serum. The serum samples were saved and stored at -70°C before analysis.

**Oral Mucosal Cell PA Measurements.** Oral mucosal cell PA was determined as described previously (28). PA was measured by the Boc-Val-Pro-Arg-MCA substrate hydrolysis method, and the results were expressed as number of  $\mu\text{M}$  of substrate hydrolyzed per hour per  $\mu\text{g}$  of sample protein ( $\mu\text{M}/\text{h}/\mu\text{g}$ ).

**Micronutrient Analysis.** Approximately 4 ml of fasting blood per subject was collected by venipuncture into a foil-wrapped, green-top (heparinized) Vacutainer and immediately placed on ice. The blood was centrifuged at  $1200 \times g$  for 10 min at 4°C, and the plasma was transferred into two microcentrifuge tubes (Intermountain Scientific Corp., Bountiful, UT), at 1 ml/tube, and stored at -70°C. The samples were shipped to The Arizona Cancer Center periodically for micronutrient (carotenoids, retinoids, and tocopherols) analysis using previously published methods (29, 30).

### Statistical Methods

Data were entered into a computer database by staff of the Biostatistics Shared Resource of the University of California Irvine Chao Family Comprehensive Cancer Center and were subject to 100% verification by the statistician. Except for lesion area and dose, all variables had small but varying amounts of missing data because of inadequate or unavailable samples. Given the small number of patients, no attempt was made to impute missing values. The analyses presented here are intent-to-treat. Data analyses were performed using SAS statistical software (31). Because the study was hypothesis generating in nature, no attempt was made to preserve a study-wise type one error rate.

Bivariate relationships were summarized by the Pearson correlation coefficient, and trend lines were fitted by the least-squares criterion (32). Judgments of photographs were analyzed

Table 1 Clinical response of BBIC with respect to dose administered

Dose	Prog <sup>a</sup>	NR <sup>b</sup>	PR <sup>c</sup>	CR <sup>d</sup>	n	Response
200	0	7	1	0	8	12.50
533	0	7	3	1	11	36.36
800	2	5	2	0	9	22.22
1066	0	1	2	1	4	75.00
Total	2	20	8	2	32	31.25

<sup>a</sup> Prog (progression), appearance of new lesions, or >50% increase in total lesion area.

<sup>b</sup> NR (no response), <50% decrease in size to 50% increase in size.

<sup>c</sup> PR, at least 50% reduction in total area of all lesions.

<sup>d</sup> CR, complete resolution of all lesions at completion of 1 month BBIC.

Table 2 Mean judgments of clinical response of photographic images of clinical lesions

BBIC dose (CIU/day)	Mean clinical response score <sup>a</sup>
200	4.15
533	4.40
800	4.45
1066	5.26

<sup>a</sup> Clinical responses were assessed in a blinded fashion described in "Materials and Methods" using a Likert scale. The analysis was based on 204 judgments. The  $\chi^2$  approximation of Kruskal-Wallis test is 12.4; *df*, 3; *P* < 0.01.

by one-way (BBIC dose), nonparametric ANOVA using the Kruskal-Wallis test (33). Ordinary, simultaneous multiple regression was used to estimate the residual association of BBIC and total lesion area, adjusting for pretreatment PA (32).

## RESULTS

**Clinical Effects.** Of the 33 patients enrolled, 1 dropped out for reasons unrelated to the trial and contributed no information to the study. The remaining 32 patients (24 males and 8 females) completed the full 1 month of BBIC treatment. The mean age at enrollment was 59 years (range, 28–82 years). Tobacco and alcohol use were analyzed before and after BBIC treatment. No significant changes were reported for the groups.

Clinical response (PR + CR) was demonstrated in 10 of 32 subjects (31%; 95% confidence interval, 18–49%). There were two CRs and eight PRs. Progression, defined as a >50% increase in total lesion area or appearance of new lesions, was found in two subjects (outliers, described below). The clinical results are tabulated in Table 1. Blinded judgments of lesion photographs by four physicians showed a dose-dependent (*P* < 0.01) increase in mean clinical response scores after BBIC treatment (Table 2).

The lesion sizes before and after BBIC treatment are depicted graphically in Fig. 1. The mean pretreatment total lesion area was of 615 mm<sup>2</sup> (range, 40–5270 mm<sup>2</sup>) and decreased to 438 mm<sup>2</sup> (range, 0–5030 mm<sup>2</sup>) after BBIC treatment. This represents a 24.2% decrease in total lesion area across all doses administered (sign rank = -142; *P* < 0.004). For the 75 individual lesions in the data set, 44 (58.7%) showed an arith-

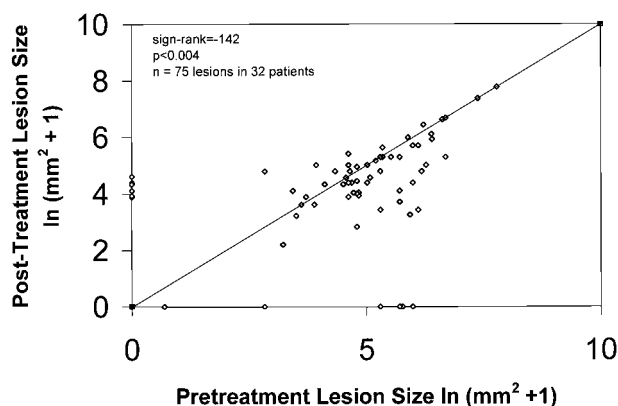


Fig. 1 Clinical response of individual oral leukoplakia lesions to BBIC. The area of oral leukoplakia lesions was measured for each patient before and after a 1-month treatment with BBIC. Shown is a natural logarithmic plot of pretreatment bidimensional lesion area against posttreatment lesion area after 1 month of BBIC treatment. The data are on a log scale only to achieve a compact graph: the statistical results are not materially different in the raw data. The line represents no change. The points on the X axis represent CRs, and the points on the Y axis represent new lesions that developed during the treatment period.

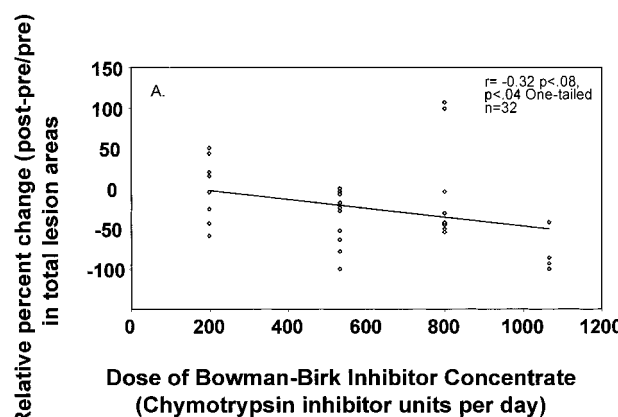


Fig. 2 Clinical response in relation to BBIC dose. The area of oral leukoplakia lesions was measured for each patient before and after a 1-month treatment with BBIC. The percentage of change in total lesion area for all recorded lesions for each subject after BBIC treatment was plotted against BBIC dose. The line shows the estimated linear trend fit by least squares.

metic decrease in size, 11 (14.7%) remained arithmetically the same, and 20 (26.7%) showed an arithmetic increase in size. The null hypothesis (no change in lesion size after BBIC treatment) was rejected by the sign-rank test (sign rank = -142; *P* < 0.004). The relative percentage of change in total lesion area for each subject versus dose of BBIC is shown in Fig. 2, with a best-fitting (least-squares) trend line superimposed on the data. An increased dose of BBIC was associated with a decrease in lesion area (*P* < 0.08; Pearson two-tailed *r* = -0.32). Two outliers in the 800-CIU dose group who registered increased total lesion areas after drug treatment greatly influenced these data. One of the two patients had a very subtle lesion observed at completion of treatment that had not been identified as



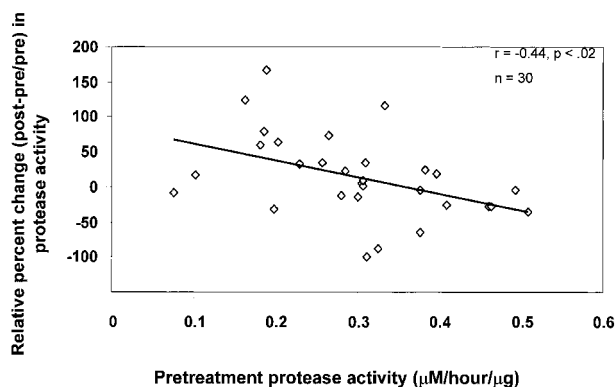


Fig. 3 Relationship between pretreatment oral mucosal cell PA and relative percentage of change in PA after BBIC treatment. The oral mucosal cell PA was quantified by the Boc-Val-Pro-Arg-MCA substrate hydrolysis method. Relative percentage of change [(post-pre/pre)  $\times$  100] was plotted against the cellular PA levels measured in the patients before BBIC treatment.

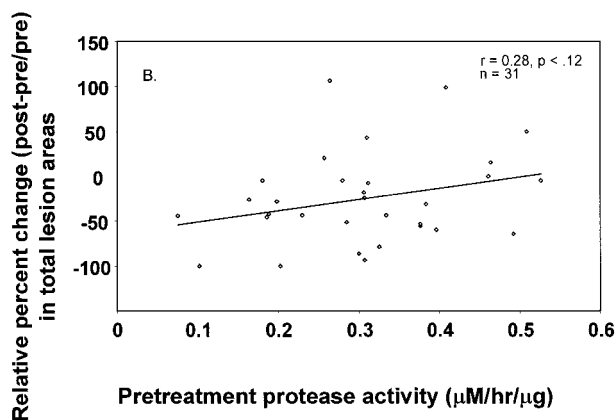


Fig. 4 Clinical response in relation to pretreatment oral mucosal cell PA level. The PA levels in oral mucosal cells were determined as described in the legend for Fig. 3. The percentage of change in total lesion area for all recorded lesions for each subject after BBIC treatment was plotted against initial PA level. The line shows the estimated linear trend fit by least squares.

abnormal at initial examination. The other patient developed a new lesion at the site of the prior control biopsy of normal-appearing mucosa, which accounted for the entire increase in total lesion area observed in this patient. Exclusion of both outliers produced a stronger correlation ( $P < 0.001$ ; Pearson two-tailed  $r = -0.60$ ).

**PA.** The initial oral mucosal cell PA level negatively correlated with the relative percentage of change in oral mucosal cell PA level after BBIC treatment, and the correlation was statistically significant (Fig. 3;  $r = -0.44$ ;  $P < 0.02$ ;  $n = 30$ ). A possible relationship between initial PA and relative change in total lesion area after BBIC treatment was determined (Fig. 4;  $r = 0.28$ ;  $P < 0.12$ ;  $n = 31$ ), but this did not reach statistical significance. There was no evident relationship between pre- and posttreatment mucosal cell PA levels (Fig. 5; sign rank = 34.5;  $P < 0.49$ ;  $n = 30$ ). The distribution of pretreatment PA

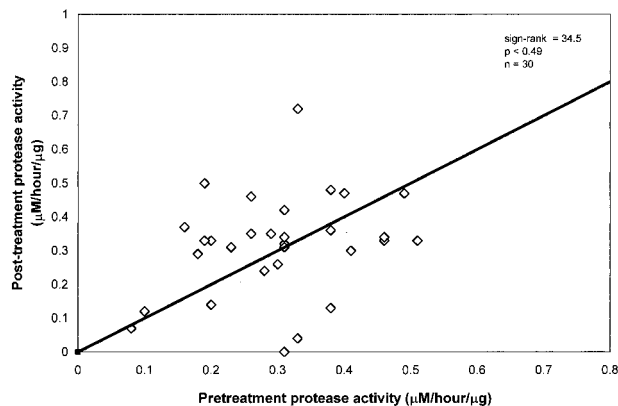


Fig. 5 Relationship between pretreatment oral mucosal cell PA and protease activity after BBIC treatment. The PA levels in oral mucosal cells were determined as described in the legend for Fig. 3. Oral mucosal cell PA levels observed after 1 month of BBIC treatment were plotted against the cellular PA levels measured in the patients before BBIC treatment. The line represents no change in PA.

values among subjects receiving different doses of BBIC was comparable between dosing groups ( $P < 0.27$ ;  $n = 31$ ; ANOVA, data not shown).

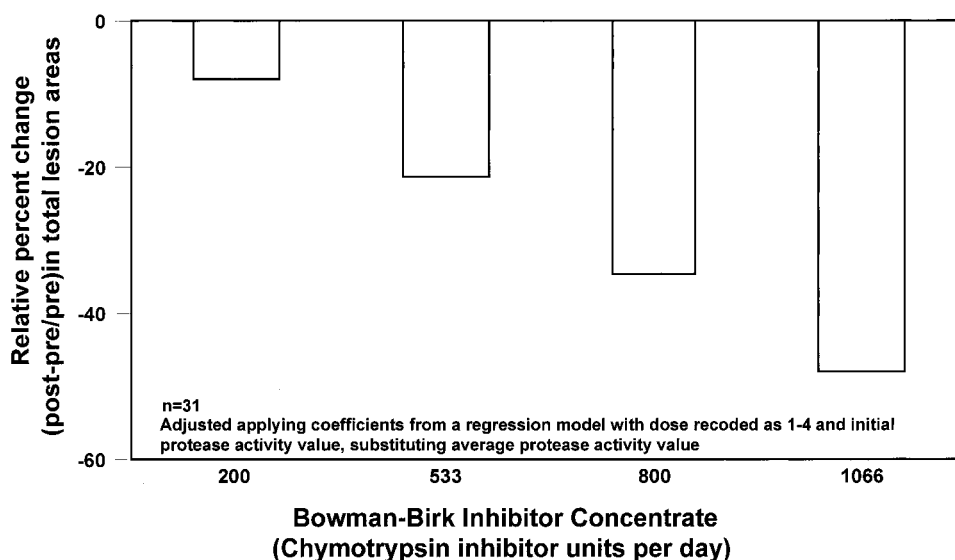
**Histological Response.** Histological analysis was performed to document microscopic tissue characteristics before and after BBIC treatment. The majority of patients in this series demonstrated lesions with hyperplastic characteristics (30 of 32, 93.75%), and 2 of 32 (6.25%) had evidence of low-grade dysplasia before treatment. Pathological review of the biopsies of lesion sites before and after BBIC treatment revealed no histological evidence of resolution or progression of dysplastic or hyperplastic lesions.

**Symptom Monitoring, Side Effects, and Compliance.** No acute toxic or allergic reactions were documented. Grade 1 nausea, diarrhea, and oral soreness were recorded in one patient each at 2 weeks. After 4 weeks of BBIC treatment, grade 1 nausea was observed in two subjects, and grade 1 diarrhea, oral soreness, and epigastric pain were each observed in one patient. No grade 2 or higher side effects were observed in any subject during the study.

At 2 weeks, 24 of 32 patients rated the palatability as grade 0, 2 as grade 1, 6 as grade 2, and 0 as grade 3. At 4 weeks, palatability decreased. Only 14 of 32 patients rated palatability as grade 0, 4 rated palatability as grade 1, 14 as grade 2, and 0 as grade 3. Despite the high percentage of patients describing poor palatability, compliance in this trial was very high. Overall doses, the average number of bottles not returned and presumed used, exceeded 92% of expected. More than 80% compliance as measured by bottle count was obtained in 29 of 32 subjects (90.6%).

**Standard Laboratory Test Changes.** No evidence of toxicity was identified in the serum tests performed after BBIC administration. The majority of laboratory values (SMA-20 and complete blood count) were within the test reference ranges both before and after treatment, and there were no patterns of major shifts in normal pretreatment serum values to abnormal values after BBIC treatment. Compared with the respective

Fig. 6 Modeled BBIC dose-clinical response relationship following multiple regression analysis. Simultaneous least-squares multiple regression was performed. Clinical response was modeled by an intercept, baseline PA, and dose to correct for initial PA level. This histogram shows the estimated clinical response to BBIC at each dose, assuming patients have the average initial value of PA (estimated from the data).



pretreatment values, the mean serum sodium increased from 137.8 to 139.0 meq/l ( $n = 24$ ;  $P < 0.04$ ), mean serum glucose decreased from 119.5 to 92.4 mg/dl ( $n = 24$ ;  $P < 0.03$ ), and mean serum lipase decreased from 13.3 to 9.6 units/dl ( $n = 25$ ;  $P < 0.04$ ). One subject had a marked decrease of an elevated serum glucose level from above the reference range before treatment (327 mg/dl) to below reference range after treatment (68 mg/dl) that accounted for the majority of the shift in the mean value of the group seen for this test.

**Micronutrients.** The serum levels of  $\beta$ -carotene, vitamin E, and retinol did not change significantly after 1 month of treatment with BBIC either as a function of dose or across all doses combined ( $P > 0.23$  for all three). No relationship was observed between the percentage of reduction in lesion area and change in or initial value of  $\beta$ -carotene, vitamin E, or retinol ( $P > 0.17$  for all).

## DISCUSSION

BBIC treatment for 1 month in patients with oral leukoplakia produced a partial or complete response in 31% of patients. Overall, there was a 24.2% mean decrease in total lesion area for the study group. Because leukoplakia can have a waxing and waning course, the identification of a probable dose-related clinical response relationship is remarkable. To further establish this finding, an independent confirmation of clinical improvement based on blinded analysis of clinical photographs was done, which strongly suggested that BBIC had a therapeutic effect on oral leukoplakia. The measurement of change in individual lesion areas and blinded evaluation of pre- and posttreatment lesion photographs represent independent tests of the same hypothesis. The probability that these two independent tests of the same hypothesis (*i.e.*, changes in lesion size are attributable to BBIC) represent a random occurrence is very low. Using the "combining of  $P$  value method" (32),  $P$  is  $< 0.0004$ . A second more conservative test statistic is the Bonferroni method, which estimates the chances that either or both

of the statistical tests is a type 1 error (32). The Bonferroni test produced a combined  $P$  of 0.01.

It has been reported that leukoplakia can respond to treatment with retinol or other retinoids, and/or vitamin E (34–36). In the present trial, the decrease in oral lesion areas observed after BBIC treatment was not related to alterations in levels of these three compounds because no relationship between changes in these micronutrients and clinical response was observed.

There appeared to be a possible direct interaction between the pretreatment oral mucosal cell PA level and clinical response (Fig. 4). Patients with lower initial PA levels tended to have an overall greater clinical response than patients with elevated PA (Fig. 4). Because these data suggest that the baseline PA may affect clinical response to BBIC, an ordinary, simultaneous least-squares multiple regression was performed on the data, where clinical response was modeled by an intercept, baseline PA, and dose to adjust for initial PA level (37). The model estimates coefficients for dose and for baseline PA, each adjusted for the effects of the other. The model was used to generate predicted values of clinical response as a function of dose, fixing the baseline PA at the average value for all patients in the study for whom we have data, regardless of dose. The predicted results, shown in Fig. 6, demonstrate that after accounting for the effect of initial PA level, the dose-response relationship remained intact.

A negative correlation between the initial levels of oral mucosal cell PA and relative change in oral mucosal cell PA after BBIC treatment (Fig. 3) was identified. This finding suggests that BBIC may reduce elevated levels of PA but does not affect PA levels when they are within a normal range. This is consistent with previous observations that BBI or BBIC can lower elevated levels of other biomarkers such as *c-fos* (38, 39) and *c-myc* (39, 40) when they are abnormally elevated, while not affecting to a significant degree the normal levels of expression of these biomarkers (see Ref. 6 for a review).

The mechanisms of BBI chemopreventive activity remain

unknown. PA is a potentially useful biomarker for chemoprevention trials using BBIC. Although a possible relationship between lowered initial PA and relative decrease in total lesion area was seen (Fig. 4), no correlation between change in PA and clinical response was found. There are several possible reasons for this. One likely reason is the short duration of this trial, which is a standard length for these types of studies. The tissues undergo dynamic changes secondary to drug administration and may not have reached a steady state by the end of the study. We would anticipate that the effects on biomarkers would be more pronounced with more prolonged drug administration. This problem is inherent with the phase IIa study design, which has the dual purpose of identifying toxicities and determining whether there is sufficient clinical response or change in other biomarkers to warrant a longer term randomized placebo controlled trial.

Another confounding factor may be that the oral mucosal cell harvest technique did not collect a high proportion of dislodged epithelial cells from the clinically observed lesions. Because the oral mucosal cell brushings represent cells obtained throughout the oral cavity, it is possible that the changes of SEBMs in cells collected from the lesions were masked by a lack of change in the same SEBMs in uninvolved epithelial cells. In future trials, this issue could be addressed by collecting oral scrapings (35, 41) directly from lesion sites as well, but we have found that this is surprisingly difficult to do for a number of technical reasons and may not be reliable. The biological heterogeneity of clinically observed lesions may also contribute to the apparent lack of correlation between cellular PA levels and clinical response.

No significant histological changes were identified in this trial. This is not surprising given the short duration of the trial. The population had a very low incidence of dysplasia, present in only 2 of 32 subjects. However, the absence of dysplasia does not adequately predict that patients will have a low risk of eventually developing malignancy (42, 43). After the BBIC trial, two patients were known to have subsequently developed cancers. One subject developed a T<sub>1</sub>N<sub>2c</sub>M<sub>0</sub> squamous cell carcinoma of the soft palate 4 months after study completion, and a second subject developed a T<sub>1</sub>N<sub>0</sub>M<sub>0</sub> squamous cell carcinoma of the oral tongue 16 months after study completion. Both tumors developed at the sites of documented lesions. Review of biopsy specimens from both cases confirmed absence of dysplasia in all pre- and posttreatment biopsy specimens. The development of two malignant transformations is within the range observed in "studies of oral premalignancy." Published malignant transformation rates are variable for oral leukoplakia, and range from 0.13% in an Indian population to >6% in a series in the United States (42, 44). Transformation rates are also dependent on the location in the oral cavity as well (42, 43). Both subjects that developed squamous cell carcinoma had lesions at high-risk sites for malignant transformation. Given the fact that BBI is an extract of a food product that was administered in doses near that ingested in the Japanese diet, it is extremely unlikely that BBI acted as a procarcinogen.

BBIC was well tolerated by the patients, with an over 90% overall compliance rate. The palatability of BBIC suspension did not affect compliance, and no clinical or laboratory evidence of toxicity or drug allergy was observed other than infrequent

reporting of nausea, diarrhea, or epigastric discomfort, which did not seem related to drug ingestion. The population in this trial was extremely motivated, and it is expected that the compliance over a longer term of administration in future trials will not change significantly. Although statistically significant changes in mean sodium, glucose, and lipase values were seen for the group as a whole, the changes were not clinically significant and do not appear to represent clinical toxicity to BBIC. These results suggest that BBIC is safe to be used as a cancer chemopreventive agent at the doses tested in this trial.

Chemoprevention is an important and viable strategy to decrease cancer incidence and mortality. In this short-term clinical trial, BBIC demonstrated clinical activity on oral leukoplakia lesions with no dose-limiting toxicity. The analysis of SEBMs tested in this trial support our hypothesis that BBIC acts through modulation of PA in premalignant cells. Subsequent trials are planned to better define the utility of PA as a candidate biomarker. The dose-dependent decrease in oral leukoplakia lesion area and the lack of clinical toxicity observed after BBIC treatment indicate that this compound should be assessed in a randomized clinical trial.

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