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CANDIDATE'S THESIS

Development of the Bowman-Birk Inhibitor for Oral Cancer Chemoprevention and Analysis of Neu Immunohistochemical Staining Intensity with Bowman-Birk Inhibitor Concentrate Treatment

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Objectives/Hypothesis: **Cancer chemoprevention is a rapidly evolving approach to reverse or inhibit carcinogenesis, and there is active interest in development of effective chemopreventive agents against head and neck cancers. The retinoids are archetypal chemopreventive agents for oral premalignant lesions. They have significant clinical effect, but widespread use is limited by significant clinical toxicity. The Bowman-Birk Inhibitor is one of several nontoxic compounds exhibiting both potent anticarcinogenic activity and minimal toxicity. The purposes of the study were to summarize the preclinical and clinical development of Bowman-Birk Inhibitor and a Bowman-Birk Inhibitor concentrate against oral premalignant lesions and to evaluate Neu immunohistochemical staining intensity for lesions and simultaneously obtained biopsy specimens of normal-appearing mucosa from the Phase IIa Bowman-Birk Inhibitor concentrate oral leukoplakia chemoprevention trial.** ***Study Design:*** **Part I is a selected literature review. Part II is a retrospective analysis of pathological specimens prospectively obtained from the Phase IIa clinical trial of Bowman-Birk Inhibitor concentrate.** ***Methods:*** **Thirty-two sets of biopsy specimens from lesions and uninvolved oral mucosa before and after treatment with Bowman-Birk Inhibitor concentrate in doses ranging from 200 to 1066 chymotrypsin inhibitory units were examined in blinded fashion for Neu immunohistochemical staining intensity using the 3B-5 monoclonal antibody. Staining intensity scores among the lesion and control biopsy specimens before and after Bowman-Birk Inhibitor concentrate treatment were analyzed and compared with previously obtained values for serum Neu, oral mucosal cell Neu, protease activity, and clinical response to treatment.** ***Results:*** **Mean Neu staining score was significantly higher in lesions compared with uninvolved mucosa ($P < .001$). Pretreatment staining scores for biopsy specimens of lesions and control biopsy specimens of normal-appearing tissues were correlated (Spearman correlation coefficient [r] = 0.375, $P = .045$), but no correlation between lesion and control biopsy specimen scores was evident after treatment. The change in Neu staining score with Bowman-Birk Inhibitor concentrate treatment in control site biopsy specimens demonstrated an inverse relationship of change in lesion area with Bowman-Birk Inhibitor concentrate treatment (Spearman $r = -0.493$, $P < .007$).** ***Conclusion:*** **Bowman-Birk Inhibitor concentrate shows promise to become an effective nontoxic chemopreventive agent based on results of extensive preclinical studies, and Phase I and Phase IIa clinical trials. Bowman-Birk Inhibitor concentrate has dose-related clinical activity against oral leukoplakia and modulates levels of**

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Neu and protease activity. The current investigation identified increased Neu staining intensity in hyperplastic lesions compared with simultaneously obtained biopsy specimens of normal-appearing mucosa both before and after Bowman-Birk Inhibitor concentrate treatment. This finding supports prior observations that increased Neu expression is present in a subset of oral premalignant lesions and head and neck cancers. The trend of increased Neu staining score in control biopsy tissues of subjects exhibiting decreased lesion area following Bowman-Birk Inhibitor concentrate treatment raises questions about the mechanisms of Bowman-Birk Inhibitor concentrate action. One possible explanation is that Bowman-Birk Inhibitor stabilizes the extracellular domain of Neu, thereby preventing receptor truncation and internalization. Further study of modulation of Neu and protease activity by Bowman-Birk Inhibitor concentrate treatment may provide insights into the role of proteases and protease inhibitors in oral premalignant lesions and the mechanisms underlying Bowman-Birk Inhibitor concentrate effects. A Phase IIb randomized, placebo-controlled clinical trial to determine the clinical effectiveness of Bowman-Birk Inhibitor concentrate and further evaluate these candidate biomarkers is under way. ***Key Words:*** **Anticarcinogenic agents, oral leukoplakia, drug therapy, trypsin inhibitor, Bowman-Birk soybean, chemoprevention, receptor, erbB-2.**

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INTRODUCTION

Part I: Chemoprevention and Development of Bowman-Birk Protease Inhibitor

Chemoprevention as a therapeutic strategy. Worldwide, oral and pharyngeal tumors are the eighth most common tumors by site for cancer, with more than 500,000 new diagnoses annually.[1] Increased incidence of head and neck cancer has been noted in a number of countries, including Spain, Scandinavia, the United States, and the United Kingdom, especially among younger male patients.[2–4] A Connecticut-based study reported that, since the 1960s, male patients 30 to 39 years of age exhibited a nearly fourfold increase in oral and pharyngeal cancer incidence, which was not observed among similarly aged female patients.[5] In the United States, it was estimated that during 2002, there would be 28,900 new cases of oral and pharyngeal cancer, resulting in approximately 7400 deaths.[6] Although significant advances in surgical techniques, radiation therapy administration, and new chemotherapeutic agents have occurred, the cure rate for head and neck cancer has remained stable for at least 30 years.[7,8] Improvements in local and regional control have shifted the natural history of the disease to increased distant metastases, and a larger proportion of patients are surviving long enough to develop and, often, die of second primary tumors. The annual incidence of second primary tumors is at least 2% to 4%, and patients with stages I and II head and neck cancer are more likely to die of a second primary tumor than of recurrence of the original cancer.[9] Advances in understanding of the molecular biology of head and neck cancer are resulting in development of novel therapies to treat cancer of the head and neck region. New gene therapy protocols using viral and nonviral vectors and development of targeted antibodies against tumor cells are just two areas in which advances are being made. However, to date, these strategies appear to be evolutionary rather than revolutionary, in that they represent small, incremental advances in the fight to cure cancer as opposed to being the silver bullets that everyone hopes will greatly improve survival for the majority of patients.

Primary prevention of head and neck cancer. The most effective way to cure cancer is to prevent its occurrence. Head and neck cancer is a disease with well-defined risk factors. Tobacco and alcohol consumption are the strongest risk factors for head and neck cancer. Approximately 75% to 85% of patients with head and neck cancer have a history of significant tobacco and alcohol consumption, and together they act synergistically to markedly increase cancer risk.[10,11] Despite widespread knowledge of health risks of tobacco and alcohol, primary prevention efforts have had limited success. Overall, tobacco consumption in the United States has decreased since the surgeon general's report on smoking in 1964, but high-school aged teen smoking rates rose rapidly during the 1990s.[12] The most recent statistics demonstrate decreased prevalence, but rapid changes in the statistics emphasize the need for constant public efforts to decrease child and teenage tobacco use.[12] The trend toward decreased smoking prevalence in the United States has started to plateau, and further improvements are becoming more difficult. Nicotine is extremely addictive, and even with motivated individuals, physician support, and pharmacological intervention, long-term quit rates are well below 50%. In addition, there are strong social forces condoning smoking among children who are less concerned with mortality 40 or more years in the future compared with social acceptance in the present, which is cultivated by a tobacco industry dependent on new users to maintain sales and corporate profitability.

Failure of early detection results in increased mortality. Similarly, early detection efforts have had limited success. Head and neck cancer survival depends on early diagnosis and treatment. The cure rate for stage I head and neck cancer is approximately 90%, but the cure rate for stage IV disease is below 20%. Approximately two-thirds of head and neck cancers are detected with advanced local involvement and/or regional lymphatic spread.[13,14] A number of factors contribute to this situation, including delay in seeking medical or dental care, asymptomatic early disease, and a low percentage of primary care physicians and dentists practicing routine oral cancer screening.[14–16] This is unfortunate because the great majority of oral cancers are visible on careful intraoral examination, and improved oral screening examinations increases detection of early malignancies and premalignant lesions, analogous to how screening and early detection have influenced the early diagnosis and management of breast cancer, colon cancer, and cutaneous melanoma.[17–19] The 1992 National Health Interview Survey documented that only 14.3% of respondents had ever had an oral cancer screening examination.[16] Routine systematic oral examination with particular attention to the lateral tongue, floor of mouth, buccal mucosa, gingiva, and

palate by primary care physicians and dentists, especially in the "over-40" population with alcohol and/or tobacco history, can improve rates of early detection.[15,20] Although efforts are ongoing to improve knowledge among the public and health care professionals about recognition of risk factors and early symptoms and signs of oral cancer, evidence that targeted oral cancer screening is being embraced and implemented by the health care community at large is lacking.

Rationale for chemoprevention efforts. Lack of significant improvement in 5-year survival of head and neck cancer, limited success in eradication of tobacco consumption, and failure to detect cancer in its earliest stages despite efforts to promote oral cancer screening emphasize the need for alternative strategies to fight head and neck cancer. Chemoprevention provides the opportunity to decrease the risk of developing cancer by using agents that halt or reverse carcinogenic changes. Carcinogenesis is a multistep process that progresses along a continuum from normal tissue to invasive cancer over many years and results from stepwise accumulation of genetic damage.[21–25] Identification of the specific steps along the pathway to invasive cancer allows targeting of these steps to arrest or reverse carcinogenesis before it becomes clinically intractable and to prevent development of a first or subsequent primary tumor.[21,22]

Foundations of chemoprevention. *Chemoprevention* is a relatively new term, first used by Sporn et al.[26] in 1976 in a review of retinoids for prevention of carcinogenesis. It can be defined as "the use of specific natural or synthetic chemical agents to reverse, suppress, or prevent carcinogenesis before the development of invasive malignancy."[27] Strong epidemiological evidence supports the concept that dietary compounds in nature have a protective effect against a number of cancers.[28,29] In numerous studies, increased consumption of fruits and vegetables, maintenance of a low-fat diet, and increased fiber consumption were associated with a protective effect.[28,29] A number of macronutrients (eg, fiber and low-fat diet) and micronutrients (eg, β-carotene, retinoids, vitamin A, and calcium) are likely targets identified for further study. However, it is a monumental task to proceed from recognition that certain dietary habits are associated with lower cancer incidence to identification of specific compounds causing the observed effect. A number of the more than 1000 identified potential chemopreventive agents are being tested in in vitro and in vivo systems against a variety of cancers, but only a few are ready for, or have been tested in, human clinical trials.[30] A number of successful prevention trials, including several oral cancer chemoprevention trials, have demonstrated that chemoprevention is a valid strategy.[31,32] A landmark study conclusively demonstrating decreased mortality from a chemopreventive agent was the tamoxifen breast cancer reduction trial.[33] This large-scale, randomized, placebo-controlled trial of tamoxifen in women at high risk for developing breast cancer produced an impressive 49% decreased incidence of invasive breast cancer in the treatment arm. The simple idea that arresting carcinogenesis in the premalignant stage can make a meaningful impact on cancer incidence and mortality has been validated, and continued effort to find safe and effective agents are worth pursuing.

Oral premalignant lesions. Oral premalignant lesions provide a nearly ideal model for study of chemopreventive agents. White and red lesions are relatively common, but the differential diagnosis of these oral premalignant lesions is extensive and the clinical appearance alone is not a reliable predictor of malignant potential. Accurate diagnosis requires histological examination. The reported prevalence of oral leukoplakia varies extensively (from 0.2%–17%), and surveys of leukoplakia prevalence in the United States indicate a prevalence of 1% to 4%.[34–37] Reported rates of malignant transformation for oral leukoplakia range from 0.3 to 17.5% with series having longer follow-up reporting higher transformation rates.[38] A recent hospital-based study from the Netherlands of 166 patients with oral leukoplakia revealed a 2.9% annual malignant transformation rate.[39] Clinical factors shown to correlate with malignant transformation include presence of erythroplakia,[40–45] proliferative verrucous leukoplakia,[46,47] dysplastic changes,[44,45,48] and anatomical location.[45,49] No individual clinical or histological marker can accurately predict the likelihood of an individual lesion developing into cancer.[50] Oral premalignant lesions are common precursors to cancer, they are easily identified, and they are accessible for sampling and follow-up, making them nearly ideal lesions for the study of the effects of chemopreventive agents.

Head and neck cancer chemoprevention trials. The great majority of effort in oral cancer chemoprevention research has focused on the carotenoids and vitamin A and its derivatives. Carotenoids are plant-derived molecular precursors to vitamin A. They are found in high quantities in green and yellow leafy vegetables and have antioxidant activity, an immune-enhancing effect, and retinoid properties (after conversion to retinol).[51] Carotenoids are relatively nontoxic, the most common side effect being yellow discoloration of the skin following ingestion. Several randomized trials indicated that beta-carotene has chemopreventive activity.[52–54] However, promising early results in trials of β-carotene have not been confirmed in larger randomized trials, and a randomized trial with a β-carotene arm had a high rate of progression of leukoplakia to carcinoma in situ and invasive cancer.[55] In the 12-year Physicians Health Study of 22,071 male physicians randomly assigned to receive β-carotene or placebo, β-carotene failed to alter the incidence of lung cancer or the number of deaths from cancer, from cardiovascular disease, or from any other cause.[56] Of greater concern, β-carotene, thought to be an innocuous compound, is currently viewed with concern because of two studies showing an increased incidence of lung cancer in populations of smokers receiving pharmacological doses of the compound.[57–59] The reason for the procarcinogenic effect in these trials is not known, but this finding highlights the fact that "safe" dietary substances administered in pharmacological doses are potentially toxic. Early enthusiasm has also been tempered by several other negative randomized trials for cancers in other sites, including skin,[60] colon polyps,[61] and cervical intraepithelial neoplasia.[62]

Vitamin A and its derivatives have a critical role in epithelial cell differentiation, development, and growth. Because of their intimate role in epithelial cell development, they are of significant interest for chemoprevention efforts.[63,64] Vitamin A effects are mediated through a family of nuclear retinoic acid receptors belonging to the steroid receptor superfamily.[65] Retinoid binding to retinoic acid receptor ultimately leads to significant alterations of gene expression. Retinoic acid receptor expression is markedly decreased in oral premalignant lesions,[65,66] and oral administration of 13-cis retinoic acid (13-cRA) can restore retinoic acid receptor expression, which was correlated with clinical regression of lesions.[65]

The agent 13-cRA is the most extensively studied chemopreventive agent for oral premalignant lesions, and randomized, placebo-controlled clinical trials had encouraging results.[67,68] Hong et al.[68] found a 67% response rate (vs. 10% placebo response) with 13-cRA treatment of oral leukoplakia for 3 months. However, drug toxicity limited subject tolerance of medication, and lesion recurrence in half of the subjects in the treatment arm was observed within 3 months after stopping medication. A follow-up study compared high-dose induction therapy with 13-cRA followed by maintenance low-dose treatment of responders with either 13-cRA or β-carotene for an additional 9 months. Fifty-five percent of subjects responded to induction 13-cRA, and this was maintained in 90% of subjects randomly assigned to low-dose 13-cRA versus only 45% in the β-carotene maintenance arm. Five subjects had progression to invasive cancer, and one patient to carcinoma in situ in the β-carotene arm, but only one subject developed carcinoma in situ in the 13cRA maintenance arm.[55] The agent 13-cRA also decreased the incidence of second primary tumors from 24% to 4% following treatment with 50 to 100 mg/d for 12 months in a randomized, placebo-controlled trial, and the effect persisted at 55-month follow-up.[69,70] Drug toxicity was significant, and no survival advantage was seen, most likely because recurrences among the large percentage of stages III and IV tumors in both groups decreased the power of the study to evaluate any differences in survival attributable to prevention of second primary tumors. A long-term (3-year), low-dose (30 mg/d) study of 13-cRA for prevention of second primary tumors in persons with stages I and II head and neck cancer has completed accrual, and release of the results is anticipated. Combining retinoids with other chemopreventive agents has been attempted in an effort to boost retinoid effectiveness and limit toxicity. A prospective nonrandomized biochemoprevention trial of 13-cRA, vitamin E, and α-interferon administered to 36 subjects with high-risk oral premalignant lesions produced complete lesion response in one-third of evaluable subjects at 6 and 12 months with acceptable toxicity.[71]

Although 13-cRA is clinically active, significant toxicity and relapse after discontinuation of treatment limit its clinical utility.[64,68] The retinoids are the most studied chemopreventive agents to date for aerodigestive malignancies and are the current standard against which other agents are compared. Nevertheless, there is active interest in identifying and developing alternative agents that are both effective and have fewer side effects than currently available retinoids. A number of compounds are under active study, some of which are in the preclinical testing stage, and a few, including epigallocatechin from green tea, nonsteroidal anti-inflammatory agents, and the Bowman-Birk Inhibitor (BBI) are ready for, or are already in, human clinical trials.[72] Bowman-Birk Inhibitor, a plant chymotrypsin-like protease inhibitor, is of interest because of its potent anticarcinogenic properties and lack of toxicity.

Protease inhibitors as chemopreventive agents. Proteases are a diverse family of proteins that catalyze the hydrolysis of peptide bonds. They are broadly subdivided into exopeptidases, which cleave amino or carboxy terminal amino acids, and the endopeptidases, which cleave proteins at specific points within their sequence. The endopeptidases are further subclassified into serine, cysteine, aspartate, and metalloproteases. Proteases from the serine protease and metalloprotease families are involved in a number of cellular regulatory pathways and have been implicated as promoters of cancer cell growth, invasion, and metastases.[73] Protease inhibitors are also a diverse group of proteins that are widely distributed throughout the plant and animal kingdoms. They counteract the effects of proteases, prevent cellular destruction, and act as important regulators in a wide variety of cellular biomolecular pathways. The serine protease inhibitors (serpins) are a superfamily of protease inhibitors of 350 to 500 amino acids that inhibit proteases by a unique suicide substrate–like inhibitory mechanism.[74] They play an important role in controlling cellular activity, and several serpins are known to be downregulated in cancer cell lines and tumors.[75–77] A number of serpins play regulatory roles in cancer development, and there are indications that some may act as tumor suppressors. Mammary serine protease inhibitor (maspin) has tumor suppressor function in breast and prostate cancer,[78] and high tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma (SCCA).[79] SCCA1 and SCCA2 are serpins isolated from the SCCA antigen, a serological marker for squamous cell tumors of the cervix, lung, and oropharynx.[80] SCCA2 is a chymotrypsin-like serine protease inhibitor with activity against a number of proteins including mast cell chymase and cathepsin G. A novel serpin (headpin) has recently been discovered and is expressed in normal epithelium of the oral mucosa, skin, and cervix, but is downregulated in oral cavity SCCA and head and neck SCCA cell lines.[75,76] Like maspin, SCCA1, and SCCA2, headpin also appears to have tumor suppressor activity.

Plant protease inhibitors are generally small (8–10 kDa) proteins widely distributed throughout the plant kingdom and are present in many food products. They are most concentrated in plant seeds but are also localized in the leaves and tubers.[81] These proteins generally inhibit trypsin and/or chymotrypsin. The first plant protease inhibitor identified was a *t*rypsin *i*nhibitor isolated from soy*b*eans (SBTI).[82] In legume seeds the predominant protease inhibitor is BBI, which has inhibitory activity against chymotrypsin and possesses a second trypsin inhibitory domain.[81] Since the initial identification of BBI, a number of related protease inhibitors making up a BBI

family have been isolated from soybeans and other plants. Protease inhibitors from the BBI family and the soybean trypsin inhibitors make up the great majority of protease inhibitor activities in plants. Bowman-Birk Inhibitor is the only protease inhibitor in soybeans that inhibits chymotrypsin. The physiological role of protease inhibitors in plants is a subject of debate. To date, no chymotrypsin-like serine proteases have been isolated from plants, which raises the question of whether BBI has a natural target in plants.[83] It is likely that these proteins function primarily as antidigestive enzymes designed to protect vital plant components from destruction by insects.[81]

Preclinical data demonstrate anticarcinogenic activity of protease inhibitors. The notion that some dietary protease inhibitors are anticarcinogenic evolved from a number of epidemiological studies which suggested that some components of vegetables, and legumes in particular, might be partially responsible for differences in cancer incidence between populations.[84] Legumes and cereals have high concentrations of protease inhibitors, and several studies have associated high intakes of these products with decreased cancer incidence at a variety of sites.[85] Although epidemiological studies provide clues to the mechanisms of cancer development by demonstrating differences in environmental exposures, these associations require independent confirmation by experimental studies. Over-reliance on epidemiological data can lead to initiation of expensive large-scale trials that fail to demonstrate clinical benefit of the agent tested.[32,86,87]

Epidemiological associations of protease inhibitors and decreased cancer incidence are supported by experimental data showing a protective effect of these compounds. A number of protease inhibitors have the ability to suppress carcinogenesis in vitro, and there is considerable animal data indicating that protease inhibitors have anticarcinogenic activity.[88–90] The most potent protease inhibitors, including chymostatin, antipain, leupeptin, and BBI, all have strong chymotrypsin inhibitory activity.[91] Finding anticarcinogenic activity with a variety of chymotrypsin specific protease inhibitors in multiple in vitro and in vivo systems stimulated a search for an effective, nontoxic protease inhibitor that could be produced in an economical fashion. Soybeans are a particularly rich source of protease inhibitors, which make up as much as 6% of total soybean protein. The two most abundant and best-characterized protease inhibitors in soybeans are SBTI, which has only weak anticarcinogenic activity,[92–94] and BBI, a potent anticarcinogen described in detail later in the present study.[95,96] There is also a large body of epidemiological evidence specifically linking soybean intake with decreased incidence of several cancer types. Soybeans contain several compounds including phytoestrogens that have anticarcinogenic activity but, unlike the phytoestrogens and other components with anticarcinogenic action in the soybean, the anticarcinogenic activity of protease inhibitors occurs at physiological levels roughly equivalent to those ingested in Asian diets. It is likely that a large proportion if not most of the anticarcinogenic effect against nonhormone-dependent tumors results from protease inhibitor actions.[91,97,98]

St. Clair et al.[99] determined that as little as 0.1% dietary protease inhibitor could decrease dimethylhydrazine-induced mouse gastrointestinal tract and liver carcinogenesis. Assuming extrapolation of the mouse data to humans provides a reasonable estimation of the amount of protease inhibitor required in the diet to achieve anticarcinogenic effect: 1600 mg per day of dietary protease inhibitor would be necessary. The average Western diet contains approximately 330 mg per day of protease inhibitor. To make up the remaining 1300 mg per day, between 8 and 9 cups of tofu (150 mg/cup) or 2 quarts of commercial soy drink (600 mg/quart) would be required.[90] Although protease inhibitors are dietary components, supplementation using commercially available products (eg, tofu and soy drinks) would be impractical because the extreme volume required to be ingested is prohibitive. The use of pure isolates is also impractical because of the extremely high costs required to isolate the pure compound. The only practical solution for production of a cost-effective product that would not require major changes in the diet is to produce a concentrated extract containing high levels of the desired protease inhibitor that can be ingested in pill or liquid form.

Bowman-Birk Inhibitor. The BBI is an abundant protease inhibitor in soybeans. It was identified by Bowman in the 1940s and purified by Birk[100] in the early 1960s. Bowman-Birk Inhibitor had particularly strong anticarcinogenic properties when tested in C3H/10T1/2 cells.[101] Subsequent work demonstrated that BBI had anticarcinogenic effect at nanomolar concentrations (0.125 nmol/L), several orders of magnitude lower than other potential chemopreventive agents in soybeans had.[84,93,102] Bowman-Birk Inhibitor is a 71-amino acid protein with a molecular weight of approximately 8000 d and has seven disulfide bonds, which stabilize the protein, making it resistant to heating (not autoclaving) and digestive enzymes (Fig. 1). The protein has a double-headed structure with a trypsin inhibitory domain on one head and a chymotrypsin inhibitory domain on the other. The protein has been sequenced, and X-ray crystallographic structure of BBI has revealed the three-dimensional protein structure.[103,104]

Pure BBI is prepared from acetone-defatted soybean flower subjected to diethylaminoethyl-cellulose ion ex-

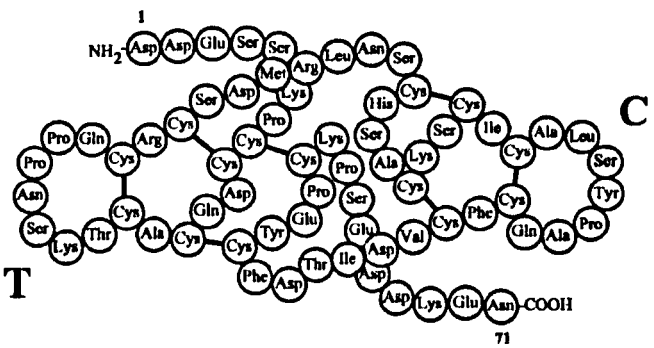

Fig. 1. Schematic representation of the structure of Bowman-Birk Inhibitor. The shaded areas represent the trypsin (T) inhibitory and chymotrypsin (C) inhibitory domains. The seven disulfide bonds are represented by dark lines. (Adapted from Odani and Ikenaka[110]).

change chromatography.[93] Purified BBI (Sigma Chemical Company) is exceedingly expensive, costing approximately $500,000 per kilogram. To make clinical evaluation of BBI possible, a concentrate extract containing BBI was developed. Bowman-Birk Inhibitor concentrate (BBIC) contains BBI and four other distinct protease inhibitors, but no SBTI. Of the protease inhibitors present in BBIC, all have trypsin inhibitory activity, but only BBI has chymotrypsin inhibitory activity.[96,105] The production and detailed analysis of the composition and properties of BBIC have been described in detail elsewhere.[106] In vitro and animal models studied have indicated that BBI and BBIC have nearly identical clinical activity.[88]

Bowman-Birk Inhibitor and BBIC have a broad spectrum of anticarcinogenic activity. In vitro studies in both radiation-induced and chemically induced carcinogenesis models have demonstrated inhibition of carcinogen-induced transformation with BBI and BBIC.[93,101,107,108] In animal models studied, BBI and BBIC suppressed carcinogenesis in studies involving mice, rats, and hamsters. Tissues evaluated included colon, esophagus, oral cavity, lung, and liver. In addition to epithelial tissue, transformation is suppressed in fibroblasts and connective tissues giving rise to hepatic angiosarcomas.[88,90] Furthermore, the drug is effective when administered by multiple routes (by mouth, intravenously, intraperitoneally, and by direct application).[90,109] Of interest for head and neck chemoprevention, Messadi et al.[94] evaluated the effect of BBI on development of cheek pouch cancers induced by 7,12-dimethylbenz[a]anthracene (DMBA) treatment over a 20-week period. Bowman-Birk Inhibitor, but not SBTI or autoclaved BBI, produced a greater than 50% decrease in the number of invasive carcinomas. These results suggest that BBI may be useful as a chemopreventive agent against oral cancer.

Possible mechanism of anticarcinogenic effect of Bowman-Birk Inhibitor. The mechanism(s) by which BBI exerts its anticarcinogenic effect remain unknown. A number of biochemical effects result from BBI activity, but which of these are directly responsible for anticarcinogenic activity and which are bystander effects is not known. The chymotrypsin-inhibiting fragment of the protein is the portion associated with anticarcinogenic effect.[110] Proteases and their inhibitors are intimately involved in every aspect of cellular function, and the proteases make up one of the largest and most diverse enzyme families.[73] A number of proteases are involved in carcinogenesis, and several serpins act as tumor suppressors.[75,76,78–80] It is possible that BBI may be acting in a similar fashion to one or more endogenous tumor suppressor proteins possessing protease inhibitory activity. It is also possible that BBI acts on targets of endogenous serpins or could regulate the activity of type II transmembrane serine proteases, a class of proteases receiving intense study for their possible role in regulation of cell function and oncogenesis.[111]

Although BBI acts to decrease cellular protease action and it is hypothesized that BBI may act directly to affect the activity of one or more proteases, specific protease targets have not been sequenced. However, a neutral serine protease has been identified as a potential substrate for BBI in mouse fibroblast cells,[112,113] and other potential protein targets have also been identified.[114–116] Yavelow et al.[117] have identified two membrane bound proteases that are inhibited by BBI as well. It is possible that one or more of these proteases could be cellular targets for BBI.

Bowman-Birk Inhibitor also has anti-inflammatory properties and inhibits free radical production. The protein inhibits proteases released from inflammatory cells, including neutrophil elastase, mast cell chymase, and cathepsin G.[118–121] In addition, BBI inhibits superoxide anion free radical production in purified human polymorphonuclear lymphocytes[122] and HL-60 cell lines.[123] These properties are associated with other potential chemopreventive agents and may partially account for the chemopreventive effect of BBI.

Bowman-Birk Inhibitor alters the levels of several oncogenes, but it is not known which, if any, are direct effects of BBI.[124–126] Expression of *c-myc* is decreased in normal and proliferating C3H/10T1/2 cells grown in medium containing BBI, which was also observed with other protease inhibitors (leupeptin and antipain).[127] Similarly, *c-fos* expression is decreased in BALB/c/3T3 cells in the presence of BBI as well as antipain.[124]

Although proteases and their inhibitors are intimately involved in oncogenesis, Whether the anticarcinogenic effect of BBI is a direct or an indirect effect of the chymotrypsin inhibitory activity of BBI is unknown. Anticarcinogenic activity has been linked to the chymotrypsin inhibitory domain of BBI, but whether direct inhibition of chymotrypsin or some other activity on this portion of the protein is responsible for its anticarcinogen effect is unknown. In addition to anticarcinogenic activity, BBI exerts a radioprotective effect on tissues. This property is localized to the portion of the protein containing the chymotrypsin inhibitory site. Experiments using linearized BBI protein fragments devoid of chymotrypsin inhibitory enzymatic activity revealed that the fragments maintained radioprotective ability independent of chymotrypsin inhibitory activity.[128] The possibility exists that the structural factors responsible for radioprotection, which are independent of chymotrypsin inhibitory activity of the molecule, may also be responsible for the anticarcinogenic activity as well.

Toxicity and safety of Bowman-Birk Inhibitor and Bowman-Birk Inhibitor concentrate. A number of studies have addressed clinical toxicity of BBI and BBIC in a variety of animal models.[88,90,129,130] Subchronic and chronic preclinical toxicology studies sponsored by the National Cancer Institute have been completed in rats and dogs. Animal toxicology studies were coordinated by John R. Page at the Southern Research Institute (Birmingham, AL). In rats, no toxicity was identified at daily doses up to 1000 mg/kg-body weight per day. In dogs, BBIC produced sporadic diarrhea at daily doses of 500 to 1000 mg/kg-bw, approximately 100 times the maximum doses planned for human studies. Human clinical trials at several organ sites have been completed or are in progress, and toxicity data are being accumulated in these studies.

Clinical studies of Bowman-Birk Inhibitor and Bowman-Birk Inhibitor concentrate for oral leukoplakia. Bowman-Birk Inhibitor concentrate has been tested in two chemoprevention trials against oral prema-

lignant lesions. A Phase I trial of BBIC for oral leukoplakia is the first reported human clinical trial of BBIC.[131] Bowman-Birk Inhibitor concentrate was administered orally as a troche to 24 volunteers with oral leukoplakia and was well tolerated by all subjects, with no clinical or laboratory evidence of toxicity identified at doses ranging from 25 to 800 chymotrypsin inhibitory units (CIU). Orally administered BBIC was rapidly absorbed following ingestion and excreted in the urine in a manner consistent with findings in animal studies.[132]

A Phase IIa study of BBIC has been completed, and results recently published.[133] Bowman-Birk Inhibitor concentrate was administered twice daily as an oral troche to 32 subjects with oral leukoplakia (dose range, 200–1066 CIU) for 1 month to assess toxicity and measure lesion clinical response, histological response, and mucosal cellular protease activity (PA). Clinical response was assessed by measurement of total lesion areas before and after treatment and by analysis of clinical judgments of lesion photographs.

Bowman-Birk Inhibitor concentrate was nontoxic in doses up to 1066 CIU and was well tolerated by the patients, with an overall compliance rate greater than 90%. Bowman-Birk Inhibitor concentrate has clinical activity following oral administration to patients with oral leukoplakia. Clinical response (partial or complete response) to BBIC administration was seen in 31% of subjects (10 of 32). The mean pretreatment total lesion area decreased 24.2% from 615 to 438 mm^2 after BBIC treatment ($P < .004$). A possible linear relationship between dose of BBIC and decrease in total lesion area was also evident ($P < .08$) but did not reach statistical significance. Independent analysis of blinded clinical impression of clinical response from lesion photographs confirmed a dose-response relationship ($P < .01$).[131] Pathological review of the lesion biopsy specimens before and after BBIC treatment revealed neither histological evidence of progression nor resolution of dysplastic or hyperplastic lesions, which was not be expected in the short-term study. The results of the Phase I and Phase IIa trials are encouraging but require confirmation. A larger scale Phase IIb randomized, placebo-controlled trial is currently under way.

Biomarker modulation following Bowman-Birk Inhibitor concentrate administration. The use of surrogate end points for the development of cancer in prevention studies is necessary to allow more rapid and efficient screening of candidate chemopreventive agents. The time and cost required to accrue subjects, treat them for a number of years, and follow them until cancer develops make assessing more than a handful of the large number of potential agents impossible if intermediate end points are not used. Intermediate markers encompass a broad variety of changes in cells and tissues thought to correlate with the development of cancer. Examples of surrogate end points include clinical and histological regression of premalignant lesions, nonspecific genomic markers such as the presence of micronuclei in cells, an alteration or change of specific genetic markers such as oncogenes and tumor suppressor gene products, the presence of markers of cellular differentiation, and markers of apoptosis. Measurement of these biomarkers, as well as changes in their levels, is useful to screen for effective compounds.[134,135] Although the relationship of these intermediate markers to cancer has not been proved conclusively, these are currently the best methods available to screen potential agents.[67] Two intermediate markers, PA and *neu* expression, are under investigation in oral cancer chemoprevention trials of BBIC.

Protease activity has been developed as a potential biomarker for activity of BBI. The PA measurement is a substrate hydrolysis technique measuring hydrolysis of the synthetic tripeptide fluorescence substrate Butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (Boc-Val-Pro-Arg-MCA). In mouse C3H/10T1/2 cells, this hydrolysis has been linked to a 70-kd neutral serine endopeptidase that is inhibitable by anticarcinogenic serine protease inhibitors including soybean-derived BBI, chymostatin, L-tosylamido2-phenylethyl chloromethyl ketone, and antipain. DMBA treatment of hamster cheek pouches resulted in a 10-fold elevation of PA, which was lowered to normal range after treatment with BBI, but not after treatment with SBTI or autoclaved BBI. Both smokers and persons with oral leukoplakia had twofold to threefold elevations of levels of PA compared with normal oral epithelium.[136]

In the Phase IIa trial of BBIC there did not appear to be a pattern of change in PA levels following BBIC administration for the study population. However, the initial oral mucosal cell PA level negatively correlated with the relative percentage of change in oral mucosal cell PA level after BBIC treatment (correlation coefficient [r] = −0.44, $P < .02$ [n = 30]), which suggests that BBIC may reduce elevated levels of PA but does not affect PA levels when they are within a normal range.[133] This finding is consistent with previous observations that BBI or BBIC can lower abnormally elevated levels of other biomarkers such as *c-fos*[124,126] and *c-myc*,[125,126] while not significantly affecting the normal levels of expression of these biomarkers. There was no statistically significant correlation between changes in PA and clinical response. The power of the analysis was low, but there are several possible reasons for the lack of association. The most likely reason is the short duration of the Phase IIa trial. Another confounding factor may be that significant responses in lesion epithelial cells were masked by contamination with a preponderance of normal sloughed mucosal cells during collection of oral mucosal cells.

Recent work has focused on possible activity of the *neu proto-oncogene* and how BBIC administration affects Neu expression in serum and oral mucosal cells. The proto-oncogene (also known as c-erbB-2 or Her-2/*neu*) encodes a 185-kd transmembrane glycoprotein with tyrosine kinase activity (*neu* protein or Neu). Neu has approximately 40% sequence homology to the epidermal growth factor receptor (EGFR), and it is likely that Neu functions as a growth factor receptor.[137] Oncogenic activity of *neu* is generally associated with gene amplification, resulting in receptor overexpression.[138] Overexpression of Neu is seen in a proportion of breast, ovarian, colon, and head and neck cancers and is associated with decreased survival.[139,140] Overexpression of Neu is also seen in oral premalignant lesions, and the level of expression increases

with severity of dysplasia.[141–143] Cleavage of the extracellular domain of the protein is associated with constitutive tyrosine kinase activity and loss of regulatory control.[144,145] This cleavage is mediated by cellular proteases, and although the target protease for Neu has not been identified, extracellular domain cleavage following epidermal growth factor binding has been demonstrated with the EGFR.[144,146,147] Correlation of Neu levels between serum and the surface of breast and other cancer cells has also been found, and serum Neu levels are undergoing evaluation as a prognostic marker for treatment response of breast cancer.[148–151]

Expression of Neu in serum and oral mucosal cells was assessed by an enzyme-linked immunosorbent assay (ELISA) using antibody specific for the N-terminal portion of Neu.[152] Correlations between cellular Neu and serum Neu levels were identified, and relationships between oral mucosal cell PA, serum Neu, and oral mucosal cell Neu were also discovered.[152] Before BBIC administration, correlation between serum and oral mucosal cell Neu levels was seen ($r^2 = 0.416$, $P < .001$). Following BBIC administration for 4 weeks, changes in oral mucosal cell Neu level correlated with changes in serum Neu level ($r^2 = 0.428$, $P = .001$). However, the absolute levels of Neu protein in serum and oral mucosal cells were not correlated ($P > .15$). Following BBIC treatment there was no correlation between Neu in either serum or oral mucosal cells and clinical response. Relationships between Neu levels and PA were identified. Changes in serum and oral mucosal cell Neu correlated to changes in mucosal cell PA (P values $< .001$). In addition, no correlation between mucosal Neu protein level and mucosal PA level was identified before BBIC treatment, but post-treatment levels were correlated. The significance and meaning of modulation of PA and relationships between PA and Neu protein remain unclear. It has been previously established that the extracellular domain of Neu measured in this assay is released by proteolytic cleavage.[144] These findings suggest the possibility that anticarcinogenic activity of BBI may be due to inhibition of proteolytic cleavage of the extracellular domain of Neu. Therefore, BBI may act to stabilize Neu and prevent conversion of the protein into a constitutively active conformation by blocking cleavage of the extracellular domain.

Part II: Evaluation of Neu Immunohistochemistry in Oral Premalignant Lesions Treated With Bowman-Birk Inhibitor Concentrate

The identification of interactions between PA and serum and oral mucosal cell Neu provide insight into possible mechanisms of action of BBI. However, no correlation between levels of either PA or Neu and clinical response to BBIC treatment was identified in the Phase IIa BBIC oral leukoplakia trial. One possible reason for lack of association was that a meaningful relationship was obscured by the technique of harvesting oral mucosal cells. Because the oral mucosal cell brushings represent cells obtained throughout the oral cavity, it is possible that the changes of surrogate endpoint biomarkers in cells collected from the lesions were masked by a lack of change in the same SEBMs in uninvolved epithelial cells. Consideration has been made of using oral lesion scrapings to more directly assay lesions as performed by other authors,[52,153] but the presence of even small amounts of blood markedly affects PA measurements and the number of cells acquired is not adequate for measurement of PA or Neu levels. The heterogeneity of clinically observed lesions may also contribute to the apparent lack of correlation between cellular PA, Neu expression, and clinical response.

In addition to serum and oral mucosal cells collected during the Phase IIa trial of BBIC, biopsy specimens were obtained from lesions and normal-appearing mucosa both before and after treatment with BBIC. These formalin-fixed specimens could provide a more direct and representative assessment of Neu expression in the lesions themselves and provide a comparison to the status of clinically uninvolved tissues in the same subject. Additional information about Neu expression could help answer a number of questions raised in the Phase IIa trial. For example, is there a difference in Neu expression between the biopsy specimens of normal-appearing mucosa and biopsy specimens of the lesions? Are there any effects of BBIC on Neu expression in the tissues? Is there any correlation between Neu expression in the tissues and clinical response? Are there any interactions between Neu expression and PA? Are there any correlations between serum Neu, oral mucosal cell Neu, and Neu measured by immunohistochemical staining techniques from the biopsy specimens? Will measurement of Neu expression in tissues be a useful biomarker in subsequent studies of BBIC?

As an extension of previously reported Phase I and Phase IIa trials of BBIC treatment for oral leukoplakia, the purpose of the current investigation was to describe the expression of Neu oncoprotein in subjects treated with BBIC in the Phase IIa chemoprevention trial and determine the potential utility of Neu immunohistochemical staining intensity as a biomarker for BBIC treatment of oral premalignant lesions. Neu expression in biopsy specimens measured by immunohistochemical staining of formalin-fixed tissues was analyzed and compared with previously measured Neu levels from simultaneously collected serum and from oral mucosal epithelial cells. Relationships to PA in oral mucosal cells and clinical response to treatment with BBIC were also be assessed.

MATERIALS AND METHODS

Specimens

Paraffin-embedded tissue blocks from subjects enrolled in a Phase IIa trial of BBIC for oral leukoplakia were collected. Specimens consisted of pretreatment and post-treatment biopsy specimens of lesions and normal-appearing mucosa from 32 subjects. Six-micrometer-thick sections were prepared and visually inspected to ensure that adequate tissue was obtained from the tissue blocks. Protease activity was measured by the Boc-Val-Pro-Arg-MCA substrate hydrolysis method, as described previously.[152] Neu protein levels in oral mucosal cell homogenates and serum samples were measured using a dual-antibody ELISA kit (Calbiochem, San Diego, CA) with specificity for the extracellular domain of Neu as previously described.[152] Clinical response was

measured by summation of bidimensional recording of lesion areas for all visible lesions.

Immunohistochemical Staining

Samples were deparaffinized and hydrated by immersion in xylene followed by an ethanol gradient rinse. After washing in phosphate-buffered saline (PBS) for 10 minutes, then 10 mmol/L citrate buffer (pH 6.0), samples were microwaved at high power for 3 minutes and at 50% power for 10 minutes and allowed to cool to room temperature to retrieve antigen. Endogenous peroxidase activity was quenched with 30% hydrogen peroxide immersion for 15 minutes. Nonspecific binding was blocked with 5% bovine serum antigen (BSA)–PBS incubation for 1 hour. Mouse antihuman Neu antibody (3B-5 clone) (Oncogene Research Products, San Diego, CA) diluted in BSA-PBS was applied to sections and incubated overnight, followed by PBS rinse. Specimens were next treated with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (Southern Biotechnology Associates, Birmingham AL) 1:1000 diluted in BSA-PBS, incubated for 1 hour, then rinsed with PBS. Three hundred microliters of 3,3'-diaminobenzidine (DAB) substrate solution was applied to each section for 10 minutes, then rinsed with distilled water, and sections were covered with mounting solution and coverslips. SK-BR-3 breast tumor cells served as positive control, and oral biopsy specimens and SK-BR-3 prepared in identical fashion but not stained with primary or secondary antibody were used as negative controls. An observer experienced in preparation and interpretation of immunohistochemical stains, who was blinded to clinical data, reviewed all slides. Slides were graded on a five-point rating scale as follows: no staining, 0; faint focal staining, 1+; moderate staining, 2+; and heavy to intense homogeneous staining of the epithelial component of the samples, 3+ to 4+.

Statistical Methods

Data were tabulated in a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA) and analyzed using SigmaStat statistical software, version 2.03 (SPSS Corporation, San Rafael, CA). Spearman rank order correlations were calculated to identify relationships between Neu staining score and other biomarkers tested in the Phase IIa trial (serum Neu, oral mucosal cell Neu, PA, and change in lesion area). Differences in Neu staining score with BBIC treatment and differences in Neu staining score between lesion biopsy specimens and simultaneous biopsy specimens of normal-appearing mucosa were analyzed by performing two-way repeated-measures ANOVA to account for possible interactions between treatment effect and biopsy site with repeated measurements.

RESULTS

The staining intensities from biopsy specimens of lesions and normal-appearing mucosa (control biopsy specimens) before and after treatment are displayed in Table I. Complete immunohistochemical staining results were available for all four biopsy specimens in 27 of 32 cases. In several specimens there was insufficient tissue remaining on the tissue blocks, and one subject refused to allow post-treatment biopsy specimens. Using a score of 3 or 4 to indicate intense staining, the percentage of biopsy specimens of normal-appearing mucosa showing intense staining was low (16%–17%), whereas among biopsy specimens of lesions, intense staining was recorded in 40% of specimens before treatment and in 30% after treatment with BBIC (Table I).

The mean staining score was calculated for each group of biopsy specimens performed. A higher staining intensity was recorded in the lesion biopsy specimens compared with the control site biopsy specimens both before and after treatment with BBIC. Mean staining scores for the control site biopsy specimens were 1.22 (95% confidence interval [CI], 0.81–1.63) and 1.43 (95% CI, 1.07–1.79) before and after treatment, respectively. Mean staining scores for the lesion biopsy specimens were 1.87 (95% CI, 1.40–2.34) and 2.00 (95% CI, 1.55–2.45) before and after treatment, respectively. Differences in staining related to biopsy location (lesion vs. control specimen) and BBIC treatment (before vs. after treatment) were assessed by performing a two-way repeated-measures ANOVA. A statistically significant difference in median staining score between control biopsy specimens of normal-appearing mucosa and lesion biopsy specimens was identified ($F = 13.8$, $P < .001$). There was no effect of BBIC treatment ($F = 0.168$, $P = .685$) on lesion staining score, and no treatment and biopsy site (lesion/control) interaction ($F = 0.119$, $P = .733$) was observed. Staining intensity scores of lesion and normal-appearing mucosal biopsy specimens were correlated before BBIC treatment (Spearman $r = 0.375$, $P = .045$) (Fig. 2) but not after BBIC

TABLE I.
Neu Immunohistochemical Staining Scores of Lesion and Control Biopsy Specimens.

	Pretreatment		Post-Treatment	
Score	Control	Lesion	Control	Lesion
0	9	5	5	4
1	12	8	12	5
2	5	5	8	12
3	4	10	5	5
4	1	2	0	4
# Sampled	31	30	30	30
Scores 3 and 4 (%)	16.1	40	16.7	30
Scores 0–2 (%)	83.9	60	83.3	70
Mean score	1.22	1.87	1.43	2.00
Median score	1	2	1	2

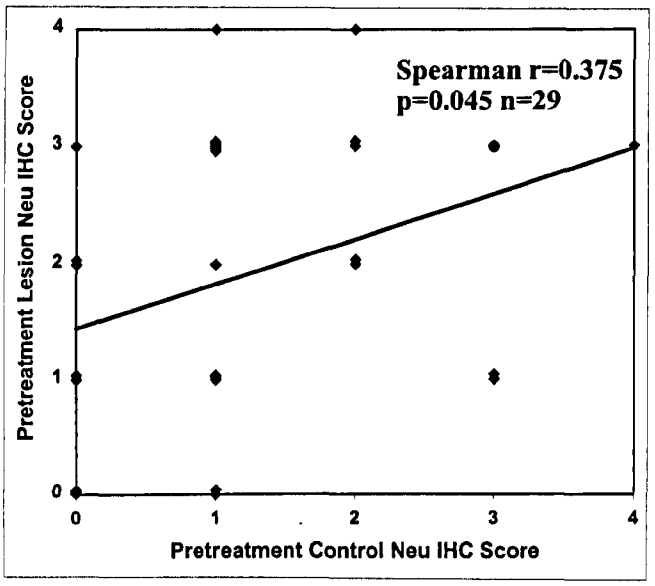

Fig. 2. Pretreatment Neu immunohistochemical staining scores for lesion and control biopsy specimens. The pretreatment Neu staining intensity scores for control site biopsy specimens was plotted against the pretreatment Neu staining intensity scores for lesion biopsy specimens. The line shows the estimated linear trend fit by least-squares analysis.

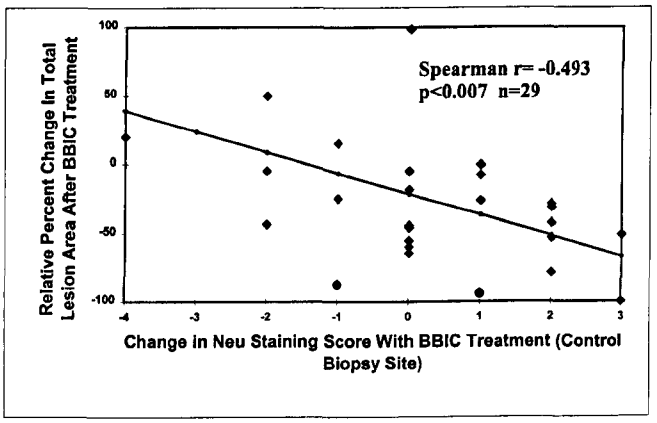

Fig. 3. Clinical response in relation to change in Neu immunohistochemical staining score with Bowman-Birk Inhibitor concentrate (BBIC) treatment. The total area of oral leukoplakia lesions was measured for each subject before and after a 1-month treatment with BBIC. The percentage of change in total lesion area for all recorded lesions for each subject after BBIC treatment was plotted against the change in Neu staining score from biopsy specimens of normal-appearing mucosa. The line shows the estimated linear trend fit by least-squares analysis.

treatment (Spearman $r = 0.226, P = .236$). No correlation between pretreatment and post-treatment biopsy scores for either lesions or normal-appearing mucosa was identified.

Analysis for possible relationships between Neu staining intensity and serum Neu, oral mucosal cellular Neu, PA, and change in lesion area was performed. An inverse relationship between changes in Neu staining score with BBIC treatment for the control site biopsy specimens and relative percentage of change in total lesion areas was identified (Spearman $r = -0.493, P < .007$) (Fig. 3). However, there was no corresponding relationship between change in lesion staining score and relative change in lesion area (Spearman $r = 0.0156, P = .935$). No other statistically significant relationships were identified for before treatment or after treatment or for change in Neu staining score with serum Neu level, oral mucosal cell Neu level, PA, or change in lesion area following BBIC treatment.

DISCUSSION

Chemoprevention has a strong theoretical foundation and is gaining wider acceptance as a therapeutic intervention for head and neck premalignant lesions and prevention of second primary tumors. Retinoids have significant clinical effect against oral premalignant lesions and are the most studied and best understood oral cancer chemopreventive agents. However, this effect comes with a price, namely, significant toxicity and rapid relapse after termination of therapy. There is clearly a need for identification of effective but also nontoxic compounds. Although ingestion of drugs with significant toxicities to prevent malignant transformation in persons with carcinoma in situ or severe dysplasia is justified, ingestion of these same compounds in the majority of persons who have lesions displaying hyperplastic changes or mild atypia is not acceptable. Use of more toxic compounds is justified when the risk of malignant degeneration is known to be high but, in many cases, selecting the subset of persons without histological evidence of dysplasia at high risk for progression is not possible. Several groups have attempted to model the likelihood of lesions developing into cancer with varying success.[50,154,155] Most analyses were retrospective, and the one prospective study, by Sudbø et al.,[154] examined only dysplastic lesions, which already have a high baseline rate of malignant transformation.

The subsequent clinical course of the subjects enrolled in the Phase IIa BBIC trial illustrates the need for identification of effective and nontoxic chemopreventive agents. Three of the 32 subjects treated in the Phase IIa trial have subsequently developed head and neck squamous cell cancers. None had evidence of dysplasia on any of his or her biopsy specimens before or after treatment with BBIC, and the study cohort was composed almost exclusively of hyperplastic lesions. This finding underscores the observation that histological findings are a poor predictor of long-term clinical behavior of oral premalignant lesions. For effective new chemopreventive agents to make a significant impact on cancer incidence, nontoxic drugs that can be taken by practically everyone at risk for developing the targeted cancer, not just those with clinically advanced premalignant lesions, must be developed and made widely available.

Bowman-Birk Inhibitor concentrate has potent anticarcinogenic effect both in vitro and in animal models and has shown clinical activity, favorable biomarker modulation, and lack of clinical toxicity in early Phase I and Phase IIa clinical trials.[131,133] Along with clinical response, modulation of Neu and PA were observed following BBIC administration. Relationships between PA and

Neu levels after BBIC treatment were identified, but no correlations between PA or Neu protein and clinical response were seen. To help answer questions raised by the complex biomarker findings in the Phase IIa clinical trial of BBIC, immunohistochemical staining of the tissue samples from the trial to assess Neu expression in lesions and normal-appearing mucosa in the oral cavity was performed.

Mean Neu staining intensity scores from lesion biopsy specimens were increased compared with normal-appearing mucosa biopsy specimens both before (+0.65) and after (+0.57) after BBIC treatment. Before BBIC treatment, 40% of lesion biopsy specimens showed heavy staining for Neu, whereas only 16% of biopsy specimens from normal-appearing mucosa showed intense Neu staining. The lesions treated in the present study were predominantly early, nondysplastic lesions; 30 of the 32 lesions showed only hyperplastic changes.

The prevalence of Neu overexpression in head and neck squamous cell carcinomas has been variously reported to be between 0% and 47%.[156] The proportion of head and neck cancers having aberrations of Neu expression is debatable, but there are compelling data linking overexpression of Neu to, at least, a significant minority of head and neck malignancies, analogous to findings in breast and other cancer sites.[137,156,157] Elevated Neu expression is also seen in premalignant lesions, and progressively increased Neu expression is associated with histological progression towards malignancy. Hou et al.[141] described progressive increase in expression of Neu with increasing dysplasia. That series consisted of 86 specimens, including 7 normal specimens, 9 specimens with simple hyperplasia, and 15 with mild dysplasia. There was no quantitative grading reported; only the presence or absence of staining was reported. Eleven percent of simple hyperplastic lesions demonstrated positive staining. Wilkman et al.[143] also found progressive increase in staining intensity with progressive dysplasia in 37 samples, including 6 demonstrating hyperplastic changes and 7 with slight dysplasia. Using a five-point scale (range, 0–4) similar to the rating scale used in the present study, mean score for hyperplastic lesions was 1.3; for dysplastic lesions, 1.5; and for SCCA, 2.7. In the present study, scores for normal-appearing tissues and hyperplastic lesions were 1.22 and 1.87, respectively. In contrast, Werkmeister et al.[142] reported that 2 of 13 nondysplastic leukoplakias had aberrations in *neu* proto-oncogene, both of which were deletions.[142] Neu staining intensity scores for lesions and normal-appearing mucosa were correlated before treatment but not after treatment. The intensity of staining in "normal"-appearing mucosa in the series, although low (mean score, 1.22), was also slightly higher than staining intensities for normal mucosa in other series.[141,143] The correlation between pretreatment Neu staining scores and slight elevation in staining may be a reflection of field effects on the tissues.

No statistically significant effect of BBIC treatment on Neu staining scores was identified. In addition, there was no relationship identified between Neu staining score from histological preparations and serum Neu or oral mucosal cell Neu levels. The lack of change parallels findings for serum and oral mucosal cell Neu measurements in the BBIC Phase IIa trial.[152]

Assessment of relationships between change in Neu staining score with BBIC treatment and clinical response demonstrated an inverse relationship between change in Neu staining in biopsy specimens from normal-appearing mucosa and change in lesion area ($r = -0.493$, $P < .007$). Paradoxically, decreased staining intensity was associated with an increase in relative total lesion area. The same analysis for the lesion biopsy staining scores failed to reveal any relationship ($P = .935$). The slope of the estimated linear fit to the data was a 15.7% increase in lesion area for every unit of decrease in Neu staining score. The observation of decreased lesion area associated with an increase in Neu staining score contrasts with observed patterns of staining in oral mucosal lesions. Increased staining is associated with more severe histological atypia or dysplasia in studies comparing staining intensity with lesion histological appearance.[141,143] Although the trend is statistically significant with a less than 0.7% likelihood that the findings were due to chance, the slope was small and may not be biologically significant. One possible explanation is that BBIC inhibits proteolytic cleavage of the extracellular domain of Neu, which in turn blocks turnover of the intact receptor. In the short term, BBIC inhibition of proteolytic cleavage of the extracellular domain could stabilize the receptor and slow receptor turnover in normally functioning epithelial cells, resulting in, at least, a temporary increase in receptor concentration in the cells. Intact Neu and EGFR are stable on the cellular membrane, but activation by ligand or binding by monoclonal antibody results in receptor-mediated endocytosis.[158–161] Truncation of Neu has not yet been proved to also cause endocytosis, but the closely related and more extensively studied EGFR is truncated after EGF binding, and subsequent endocytosis occurs.[146] If this process is occurring with the Neu receptor in the control biopsy specimens, it is likely to be operative for the lesions as well, but the heterogeneity of tissue responses could be masking any effect. As an alternative, the lesions could be reacting to BBIC in a manner different from control biopsy tissues, but the distribution of the data points either suggests heterogeneous response in the lesions or reflects imprecision inherent with immunohistochemical staining techniques. Confirmation of these findings is necessary to verify these results.

A number of factors may be responsible for the lack of correlation of Neu staining score with other clinical variables or change in Neu staining score with clinical response, PA, or serum and oral mucosal cellular Neu levels. As discussed earlier, the short duration of the study may have accounted for lack of relationship with clinical response. In addition to the short treatment time, it is possible the precision of the assay techniques for ELISA for Neu on serum and mucosal cells, PA measurements, and the immunohistochemical techniques for Neu from biopsy specimens were not great enough to reveal trends for the number of samples tested. In particular, immunohistochemical staining techniques are affected by a number of variables, including antibody selected, age of histological preparations, methodology for preparation of specimens,

criteria for assessing positive staining, and subjective variability of the examiner rating the staining intensity.[162] Further, disruption of Neu expression is only present in a proportion of oral premalignant lesions and cancers. For this reason, changes in Neu in a minority of specimens may not be readily apparent with the number of samples tested. Because of the sample sizes in the trial, the power of these analyses is low and type II errors are possible. The findings identified in these experiments are to be considered exploratory and will require confirmation in a larger study. This is an inherent limitation in the design of Phase IIa chemoprevention trials, which are designed to demonstrate clinical modulation of biomarkers and identify clinical toxicity. Encouraging preliminary results justify the expense of performing a larger scale trial, but identification of toxicity or lack of clinical effect or favorable biomarker modulation can prevent devotion of extensive resources to study an ineffective or unsafe compound.

CONCLUSION

Bowman-Birk Inhibitor concentrate is a novel chemopreventive agent that has dose-dependent clinical effect and favorable modulation of Neu and PA on oral leukoplakia, with no evidence of significant (grade 2 or higher) toxicity observed in the Phase I and Phase IIa oral leukoplakia trials.[131,133] Neu immunohistochemical staining of lesion and control biopsy specimens before and after BBIC treatment for 1 month showed a significant difference in staining intensity between lesions and control specimens but did not produce an overall change with treatment. Prior studies of Neu staining in premalignant lesions had small numbers of normal and hyperplastic lesions.[141–143] The large number of hyperplastic lesions having increased staining intensity compared with control biopsy specimens of clinically normal tissues further supports prior observations that changes in Neu expression are involved in a proportion of premalignant lesions. The observation of an inverse relationship between change in Neu staining intensity in the control site biopsy specimens and clinical response of lesions raises interesting questions about the possible mechanisms of action of BBI, which will require further evaluation and confirmation. Neu immunohistochemical staining provides information about receptor expression in tissues, but longer-term study will be required to demonstrate whether BBIC treatment modulates levels of Neu staining in premalignant lesions in such a way that it can be used as a reliable biomarker. Bowman-Birk Inhibitor concentrate is currently being tested in a Phase IIb randomized, placebo-controlled trial, and accrual is under way.

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