The Biology Behind

Protease Inhibitors in Oral Carcinogenesis and Chemoprevention

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We are honored to write the commentary for the inaugural report of “The Biology Behind...” series in Clinical Cancer Research. We confess at the outset, however, that the reader conducting the most thorough survey of the cancer literature would not turn up any publications on BBI with either of our names in the author list (before now). Nevertheless, the scientific roots of “Clinical Modulation of Oral Leukoplakia and Protease Activity by Bowman-Birk Inhibitor Concentrate in a Phase Ia Chemoprevention Trial,” by Armstrong et al. (1), grow within two respective fields of our specialized research: translational study of oral carcinogenesis and chemoprevention (Lippman) and the contribution of proteolysis to tumor progression (Matrisian). The three major avenues of biology behind BBI study in oral carcinogenesis are (a) the long-term development of OPLs as a clinical and translational model for assessing drug activity; (b) preclinical (and early clinical) study of BBI itself; and (c) broader basic study of PIs. Our charge is to “translate” the contribution of the background biology to the currently reported findings on clinical BBIC activity. This background biology and clinical BBIC study together raise important issues concerning the larger picture of drug development for cancer chemoprevention, which we also discuss below.

The study of PIs in preclinical cancer chemoprevention began about 30 years ago (2). The preclinical study of BBI began about 15 years ago in colon and oral-cavity carcinogenesis and now involves many sites that could be studied clinically (3). The authors’ choice of OPLs for the early clinical study of BBIC, however, is commendable. Work in the OPL model has been a driving force behind the entire field of chemoprevention, which matured recently with the hard-earned FDA approvals of tamoxifen (for reducing breast cancer risk) and celecoxib (for controlling familial adenomatous polyposis; Ref. 4). The OPL model offers several important research advantages, including lesions that are easily monitored and sampled; an association with clonal expansion and the risk of second primary tumors; and statistical models for analyzing biomarker modulations and correlations. These methodological issues have been worked out in the more than 20 years of clinical and translational study of retinoids in OPLs (phenotype-genotype reversal/suppression of OPLs and extension to the prevention of second primary tumors associated with head-and-neck cancer), which have been documented extensively elsewhere (5–7). The current clinical/translational study of BBIC is approximately where the study of retinoids was 20 years ago but likely will benefit from the earlier work. OPL study has produced major translational findings involving retinoic acid receptor-β loss and retinoid regulation (8), clonal expansion indicated by 3p14 and 9p21 loss of heterozygosity (9), p53, and genetic instability (10), among others; markers associated with carcinogenic progression and/or pharmacological retinoid activity (Ref. 4; Fig. 1), and the first systematic statistical model for analyzing biomarkers within chemoprevention trials (11). The OPL model also offers a specific advantage for BBIC study, which is the ease of topical delivery. In the current Phase Ia study, a solution of BBIC was held in the mouth (before swallowing; Ref. 1). Protease expression and activation patterns beginning to be worked out in head and neck cancer will be extended to OPLs [adding to the recent findings of increased protease activity in OPLs and “normal” oral mucosa of smokers (Refs. 2 and 12; see Fig. 1)]. Because BBIC has preclinical activity in several sites besides the oral cavity, the early clinical development of BBIC within the well-developed OPL model may be relevant to clinical BBIC study elsewhere.

The BBI family consists of many forms and isoforms of natural polypeptide serine PIs and is found in the seeds of legumes (e.g., Soybean, chickpea, and peanut) and other plants (e.g., barley). Plant serine PIs function as natural insecticides.

BBIs are one of the two main families of serine plant PIs (the other is the Kunitz-type PIs). BBIs come from dicotyledonous or monocotyledonous seeds and beans, and the amino acid sequences of about 100 BBIs have been worked out, as have the three-dimensional structures of a few (13). The classic and first BBI, the one that is most commonly referred to in the literature, is a protein that was identified in soybeans by Bowman in the 1940s and purified by Birk in the early 1960s (14). This molecule is a typical dicotyledonous BBI and has a M, 8,000, 71 amino acids, and two separate PI sites: subdomain 1 (NH2-terminal) and subdomain 2 (COOH-terminal) for trypsin- and chymotrypsin-like serine proteases, respectively. The more important PI site for chemoprevention is subdomain 2, suggesting the importance of chymotrypsin inhibition for BBIC chemoprevention activity (Ref. 3; recent findings on subdomain specificity for protease binding are discussed later).

The agent in the study reported in this issue of Clinical Cancer Research (p. 4684) by Armstrong et al. was not pure BBI, but BBIC, a crude acetone-defatted soybean-flour extract containing the classic BBI, four other soy PIs (with trypsin, but

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3 The abbreviations used are: BBI, Bowman-Birk inhibitor; BBIC, Bowman-Birk inhibitor concentrate; OPL, oral premalignant lesion; PI, protease inhibitor; FDA, United States Food and Drug Administration; COX, cyclooxygenase; MMP, matrix metalloproteinase; MMPI, matrix metalloproteinase inhibitor.
not chymotrypsin, inhibitory activity and not yet fully characterized), and other components. BBIC was chosen over purified BBI because the latter would be prohibitively expensive for large-scale clinical trials. One g of BBIC has 100 chymotrypsin inhibitory units and 40 trypsin inhibitory units of activity. Preclinical studies suggest that BBI and BBIC have similar effects (3). Nevertheless, the use of BBIC raises questions about the specific activities and long-term toxicities of the individual constituents. In regard to cancer chemoprevention, no other BBI or BBI-containing plant extract has received as much attention as has soy BBI or BBIC in preclinical study or is near to entering clinical trials.

Currently, the focus of cancer chemopreventive drug development is the identification of molecular targets (15–17). BBIC, like many other natural compounds, however, has developed through epidemiology and in vitro and in vivo studies of overall activity. The ecological and dietary epidemiological data supporting BBI for cancer chemoprevention show that soybeans, breads, and cereals may reduce the risk of oral and other cancers (3). The epidemiological associations between the intake of these foods and reduced cancer risk/mortality are difficult to attribute specifically to BBI, however. For example, the study of soy (assessed in Japanese and other dietary studies) is confounded by soy’s content of BBI and several other potential chemopreventive agents, including isoflavones (e.g., genistein), saponins, and other PIs (18).

More direct support for the protective effects of BBI comes from preclinical findings of suppression of transformation in vitro and of carcinogenesis in vivo (3). BBI chemopreventive activity is reported to be broad spectrum (many sites) and irreversible. The mechanism of BBI activity is unclear and may be independent of PI effects. In more than 15 years of investigation, the specific target protease(s) inhibited by BBI or BBIC has not been identified, although several potential protease targets of Mr 43,000–125,000 have been reported (3, 19, 20). Other reported effects of BBI include indirect effects on other proteases and the modulation of superoxide anion radical production, oncogene levels, DNA repair, immune effects, and arachidonic acid metabolism among other effects. In DMBA-induced oral cancer, protease activity increased 10-fold in cancer tissue and normal-appearing DMBA-exposed oral mucosa; topical BBI (and BBIC) decreased this activity and oral cancer development; and soybean trypsin inhibitor was not active, nor was a potato chymotrypsin inhibitor, which is of interest be-

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**Fig. 1** Potential chemoprevention molecular targets in oral carcinogenesis. The top of the figure depicts the progression of a squamous lesion of the oral cavity from normal cells to early invasion. The boxes (yellow) summarize molecular changes associated with carcinogenesis in this system that represent potential targets for chemoprevention. The upper triangle (orange) represents increasing genetic instability with advancing steps of oral carcinogenesis. The lower triangle (lavender) indicates that there is an imbalance in the natural protease:PI ratio in favor of proteolysis that occurs as early as in carcinogen-exposed (e.g., to tobacco), normal-appearing tissue and can increase with carcinogenic progression. Therefore, the inhibition of proteolytic activity represents an additional chemoprevention strategy.
cause of the suggested importance of chymotrypsin inhibitory activity in BBI chemoprevention (3, 21).

Of course, studying BBIC in humans is more complex. Smoking and OPLs are associated with highly variable but generally 2- to 3-fold increased protease activity in whole-mouth brushings (compared with protease activity in nonsmoker normal controls; Ref. 12). Subsequent to animal pharmacokinetic studies, the first clinical BBIC study was a Phase I pharmacokinetic study (in OPL patients) that assessed one-time-only BBIC doses of from 25–800 chymotrypsin inhibitory units (22). There was no toxicity. BBIC was not detected in plasma by ELISA, but a metabolic product of BBIC was detected in urine and cleared within 1–2 days after the dose of BBIC. In the current Phase Ia study, BBIC (doses from 200-1066 chymotrypsin inhibitory units for 1 month) appeared to decrease protease activity in the oral cells of OPL patients with high baseline protease activity (consistent with preclinical studies), but was most clinically active in patients with lower baseline protease activity (1). There was no correlation between clinical-histological outcome and a change in protease activity.

Despite the host of biological leads and possibilities listed above, the understanding of the specific biological activity of BBIC or BBIC is virtually nonexistent. The molecular or cellular mechanisms and effects of BBIC are not nearly as well studied or understood as, for example, the biology of selective estrogen-receptor modulators or COX-2 inhibitors (4, 15–17). The clinical and preclinical evidence of protease inhibition by BBICs is indirect, and it is not clear what proteases are inhibited. The measure of protease activity in the present clinical trial is a substrate hydrolysis method and not a specific enzyme assay. As with nonsteroidal anti-inflammatory drugs to inhibit COX-2, the use of PIs to down-regulate elevated protease activity in OPLs is logical. But just as clinically active “COX-2 inhibitors” (4) may not work clinically via COX-2 (23, 24), BBIC may not work via protease inhibition. The apparent BBIC clinical activity in the current study is not correlated with measurable protease inhibition. Armstrong et al. suggest that this lack of correlation is primarily attributable to the short-term (1-month) BBIC treatment (1). This is not, however, likely. If the BBIC clinical activity (OPL regression) resulted from protease inhibition [i.e., if protease activity were an early surrogate end point biomarker (25), as the authors suggest (1, 12)], suppression of protease activity should precede the clinical activity. These findings do not clarify the mechanism of BBIC activity in the clinical setting. Notwithstanding the many caveats concerning BBIC, the more general concept that protease inhibition could be an effective chemoprevention strategy is worth investigating.

There is a diverse array of natural proteases with a corresponding array of natural PIs. An imbalance of the physiological protease/PI equilibrium is associated with vascular, infectious, and neurological diseases, and the related processes of carcinogenesis and inflammation (Refs. 26 and 27; Fig. 1). Selective synthetic PIs are being developed to treat these diseases. Protease inhibition is considered a means of reducing tumor invasion and metastasis. Extracellular proteases, such as the MMPs, urokinase, and cathepsins B and D, have been implicated in the matrix degradation viewed as being essential to tumor cell invasion, extravasation, and intravasation. On the basis of this concept and supported by a substantial number of preclinical studies, synthetic MMPIs were developed and advanced to the clinic (28).

The initial Phase III MMPI clinical trials targeted advanced stage cancers with MMPIs either as monotherapy or in combination with cytotoxic agents. The results of these trials (seven in all) have been disappointing. For example, treatment with the MMPI Marimastat (British Biotech) provided no benefit to patients with advanced pancreatic cancer (28). Patients with advanced small cell lung cancer treated with Tanomastat (Bayer Corp.) fared worse than placebo controls, resulting in the cessation of all clinical trials with this agent.4 However, although a clinical trial with Marimastat in gastric cancer patients did not meet its primary end point, there was a significant benefit of Marimastat treatment in a subset of patients with node-negative disease (29). These results are consistent with the more recent appreciation for the role MMPs play in tumor establishment, growth, and angiogenesis (30). This concept has extended to a role for protease inhibition in preventing the conversion of a premalignant cell into a malignant one. For example, inhibition of MMP activity with the synthetic MMPI Batimastat (British Biotech) reduces the number of intestinal adenomas in the Min mouse, a model of human familial adenomatous polyposis (31). A specific MMP, matrilysin (MMP-7) has been implicated in this effect, and Min mice in which matrilysin has been genetically ablated demonstrate a significant reduction in adenoma number and size (32). [BBIC also has activity in the Min mouse model, but, in contrast to MMPIs, has no known molecular target (33).] In addition, treatment of hyperplastic pancreatic islets in transgenic mice expressing SV40 T antigen under the control of a β cell-specific promoter with Batimastat reduces the frequency of conversion of these islets to malignancy (34). This effect has been attributed to the MMP gelatinase B (MMP-9) in that mice genetically deficient in MMP-9 show a similar reduction in the number of angiogenic islets (35). Thus, in both cases, protease inhibition was effective in a chemoprevention strategy, and specific molecular targets responsible for this activity have been identified.

It would be fitting, perhaps, in this commentary on the “biology behind” BBIC, to add a few comments on the biology ahead as BBIC proceeds toward potential FDA approval for chemoprevention. Although the present BBIC study had only a 30% response rate in early (hyperplastic) premalignant lesions, the short treatment duration and dose-response trends support proceeding to a Phase Iib, or randomized, placebo-controlled, trial to confirm activity. This is the next step in chemoprevention study (36), which is critical to control for many factors, especially the possibility of spontaneous regression. Previous chemoprevention studies in tobacco-related early carcinogenesis illustrate the importance of this step (37).

Phase Iib trials eventually may support taking BBIC to the next step, Phase III testing. However, they cannot completely address one complex and important issue with BBIC and many other relatively nontoxic compounds: the optimal dose and formulation. Precisely what dose will have the best chance of activity in Phase III trials? Precisely what dose will have the
least chance of producing infrequent or unexpected long-term side effects? The effect of β-carotene on lung cancer incidence in smokers illustrates the latter concern (16). The Phase I and IIa BBIC data were not helpful in this regard because there was no dose-limiting toxicity. Besides teratogenic effects, for which safety measures can be taken, the major potential long-term safety issue with soy extracts is potential pancreatic toxicity and cancer thought to be related to trypsin inhibitory activity (which is reduced in BBIC) and soy lectins (3). Future Phase IIb or III trials must ensure that the BBIC formulation has batch-to-batch consistency and can be reproduced, especially with respect to the percentages of the potentially active constituent PI (e.g., cysteine PI has been reported to appear in BBIC preparations; Ref. 38). Any prospective Phase III testing must use the formulation supported by Phase IIb testing and, if conclusively beneficial, must supply the FDA with a specific formulation to be approved for reducing cancer risk. Many other food-derived extracts (e.g., the soy phytochemical concentrate that includes isoflavones and saponins) also are being investigated for chemopreventive activity and confront the issues just described (18, 39).

Future translational study of BBIC will be aided by very recent and developing research. For example, high-resolution X-ray crystallographic studies have found that subdomain 2, the putative key PI site for cancer prevention, is not specific only for chymotrypsin (discussed earlier) but binds trypsin as well (40). This work will help find the BBI target enzyme(s) and will allow structure-based development of synthetic small-molecule BBI analogues.

The present translational report offers encouragement for the clinical application of PIs as chemopreventive agents. Current work on the potential molecular targets for PIs involves primarily proteases in the proteinase (endopeptidase) group, which have five known classes: serine, cysteine, metallo, aspartyl, and threonine proteases. There are substantial complexities involved with these potential targets, including functional overlaps in substrate specificities of different proteolytic pathways, stromal and extracellular matrix interactions, transcriptional regulation of the synthesis of proteases and PIs, and interactions between protease/PI classes (26, 27). An interesting example of this last complexity is that the natural serine PI found in saliva can suppress MMP activity (41), which may play a role in the control of oral inflammation and carcinogenesis. Refinement and additional progress in this area is likely to come from an identification of the specific enzymes involved in the preinvasive clonal spread (42) and progression (4, 9) of OPLs and other premalignant conditions and the design of specific molecular inhibitors of the appropriate enzymatic activities. For example, although certain specific proteases are thought to be involved in advanced head and neck cancer (e.g., MMP-1, -2, -3, -9, -10, and -11), none as yet have been identified in premalignant oral carcinogenesis. This line of investigation requires a translational approach in addition to a concerted effort to develop appropriate animal models for proof-of-concept experimentation. Proteomic methods are being developed for identifying new protease targets that will allow the development of novel synthetic PIs via traditional medicinal, computational, and combinatorial chemistry (16).

Initial steps have been taken to form a consortium of investigators interested in the application of PI as therapeutic and preventive agents (43). By selecting and refining specific mouse model systems for the study of pharmacological and genetic modulation of proteolytic activities, a database of comparable information can be generated to identify the most appropriate targets for intervention. The consortium approach offers the opportunity to more rapidly and rationally advance the addition of PIs to the cancer prevention arsenal. Highlighted by MMPIs, the development of selective versus broad-spectrum inhibitors (different MMPIs can be either) is a major issue of chemopreventive drug development. The highly selective agents may have less toxicity, but less activity as well (16).

BBI study appears to stand just this side of a biological watershed that was crossed by retinoids nearly 15 years ago: the discovery of specific molecular targets of activity. The curtain dropped on an era of now-obscure biology behind retinoids (well documented elsewhere; Ref. 44 with the discovery of the nuclear retinoic acid receptors (45, 46). Simultaneously, the clinical and mechanistic retinoid biology ahead of these receptor discoveries (including receptor-dependent modulation of proteolytic pathways; Ref. 47) took off at a lightning pace that has yet to slacken (4, 8, 15–17, 36). The coming discovery of the primary molecular target(s) of BBIs promises to trigger a similar explosion of biological study.

References

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5 Internet address: www.vicc.org/protease.


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