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Development of difluoromethylornithine and Bowman–Birk inhibitor as chemopreventive agents by assessment of relevant biomarker modulation: some lessons learned

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A major goal in the development of chemopreventive agents has been to develop markers that reflect the underlying process of carcinogenesis and which are modulatable by the agent under study. An important application of such markers will be to select cohorts that are at elevated risk for cancer development, which should allow use of smaller sample sizes in definitive phase III trials as well as shorter duration (and lower cost), without loss of statistical power. Susceptibility and surrogate end-point biomarkers are particularly important in this respect. Intermediate markers are probably best assessed in terms of proportionate rather than relative risk.

The systematic development of difluoromethylornithine for use in chemoprevention against human cancer has involved pilot, phase IIa and IIb trials using participants with prior colonic polyps as the study group. A unique feature of the phase IIa study was the use of a dose de-escalation design which allowed selection of the lowest effective non-toxic dose of difluoromethylornithine. The phase IIb trial now in progress is using a combination of sulindac with difluoromethylornithine; the rationale for selection of markers for this study and for a randomized phase III registration trial is discussed. We also review the findings in phase I and IIa trials of Bowman–Birk inhibitor concentrate, in which patients with measurable oral leukoplakia are the study group.

Surrogate end-point biomarkers and chemoprevention: some conceptual thoughts and applied observations

There are several discrete types of measurement that should be considered separately during the design of chemoprevention trials: susceptibility (predisposition/hereditable), exposure, intermediate marker (non-causal and causal), drug-modulatable event (related to a carcinogenesis process or not) and tumour marker (Meyskens, 1992a,b). There has been a tendency among investigators to call different types of 'marker' by the same name. The development of a relative risk profile should be the first step, rather than the last (as currently tends to be done). This strategy has been adopted only in the National Surgical Adjuvant Breast and Bowel Project breast cancer prevention trial of tamoxifen, in which an increased relative risk for breast cancer was required for study entry (Fisher *et al.*, 1998). Two different major levels of risk should be assessed: genetic and epigenetic. Genetic risk should be considered in terms of both defined molecular abnormalities (e.g., tumour-suppressor genes, oncogenes, microsatellite stability, DNA repair, metabolic polymorphisms) and familial risk by genealogical analysis only. Epigenetic assessment can be broad, but should include consideration of at least the major known risk factors in general and those specific for the organ site being studied.

The development of validated surrogate end-point biological markers is a difficult task and to date no marker for carcinogenesis equivalent to cholesterol for atherogenesis-related disease has been validated. In assessment of the value of an intermediate marker for use in chemoprevention, its predictive value as an estimator of cancer risk needs to be stated, at least qualitatively. Most investigators accept a histologically defined end-point as a surrogate end-point biomarker with a high risk and modulation of such an end-point in a favourable manner as being indicative of chemopreventive activity. Evidence of alteration in the natural history of a preneoplastic lesion such as intraepithelial neoplasia, an adenoma or metaplasia is probably *de facto* sufficient to call an agent efficacious. It is much more difficult to relate prehistological markers of cancer to risk, and even more so, those markers that may be associated or correlated with the true marker of risk, and not directly on the causal pathway.

To date, the concept of risk has been designated as relative risk. Since the maximum risk is 100%, use of the concept of proportionate risk, that is the proportion of total risk explained, could be more useful in the development of intermediate markers. An example of the principles underlying the concept of a hierarchy of markers is presented in Figure 1. In the first example, the germline absence or mutation of a tumour-suppressor gene leads to the inevitable or substantial likelihood of cancer development. Presence of the appropriate marker (M_1) would predict with high frequency the development of cancer and therefore a high proportionate risk (i.e., close to 1.0) could be assigned. Examples of this situation would include hereditary retinoblastoma and Li–Fraumeni syndrome. In such a case, an appropriate marker may be highly predictive of the development of cancer and its modulation will be predictive of a beneficial result. At the other end of the time-line of the process of carcinogenesis (M_5), a histological preneoplastic lesion also has a high chance of malignant conversion and therefore a patient with the biomarker has a high proportionate risk (i.e., close to one). There clearly is a spectrum of lesions;

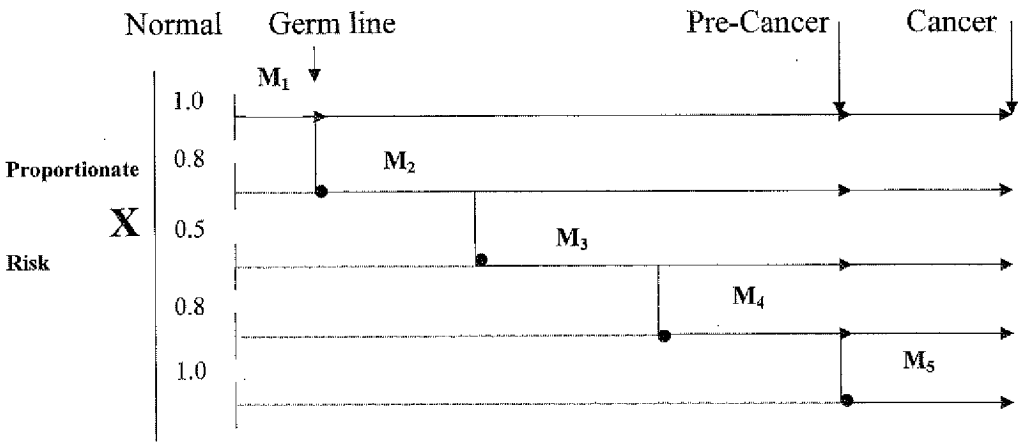

Figure 1. Development of a proportionate risk hierarchy of markers

The maximum likelihood of developing a cancer is 100% (1.0). Therefore, the risk assigned to any marker should be a proportion of this risk rather than portrayed as a relative risk. If the predictive properties of all markers were known, then choosing a few would likely result in a high prediction rate. Time to event would influence this selection process as well. A scoring system for markers related to risk of development of cancer based on this principle would be:

For x=0 to 1; $y=x_1M_1+x_2M_2+x_3M_3+x_4M_4+x_5M_{5=}$ 1; $M_{1,2,3,4,5}$= degrees of certainty

for example, oral leukoplakia has a relatively low conversion rate, while erythroplakia with dysplasia has a high rate of malignant transformation.

However, in most cases the extent to which an intermediate marker predicts the development of cancer is not as great (e.g., M_2, M_3). Assessing the proportionate risk associated with a marker that falls somewhere between a germline mutation and a histological change has been considerably more difficult. At some point late in the carcinogenesis cascade, the value of a marker may again rise as the state of histological preneoplasia is approached; an example might be optically-measured nuclear morphometry (e.g., M_4).

Stringent criteria have previously been proposed to validate an intermediate marker as a surrogate end-point biomarker. These correspond to several steps of an algorithm:

- The identified marker must represent a step on the causal pathway to carcinogenesis
- The marker must be modulated by a chemoprevention agent
- Modulation of the marker must correlate with reduction of cancer incidence

Fulfilling these criteria requires a lengthy and expensive process that in the worst case might be marker- and/or chemoprevention agent-specific.

In the rational development of surrogate end-point biomarkers, it might be more productive to link biomarkers to predisposition for risk rather than to attempt to assign a relative risk based on biological plausibility. An attempt to link these various features is presented in Table 1, in which the class of risk, relative risk and attributable risk in a population are assessed. Quantification (or even intelligent qualification) of these factors may allow identification of cohorts for chemoprevention trials that provide individuals that are at relatively high relative risk, but nevertheless, fairly representative of the more general population. For example, individuals with a strong family history for a cancer in which there are organ-specific polymorphisms predictive of risk might constitute a particularly useful cohort to study. Hopefully, identification of such cohorts will lead to more efficient trials that are of shorter duration and smaller size and hence less costly and of greater feasibility.

Development of difluoromethylornithine as a chemoprevention agent

Experimental data

Difluoromethylornithine (DFMO) is an irreversible enzyme-activated inhibitor of ornithine decarboxylase (Meyskens & Gerner, 1999). This enzyme catalyses the first step in the synthesis of polyamines in eukaryotes, the decarboxylation of

Table 1. Identification of high-risk cohorts that are generalizable

Class	Relative risk	Attributable risk
Predisposition		
Hereditary	High	Low
Familial	Moderately high	Higher
Polymorphism	Low	Highest
Epi/classical	Low	Higher
Intermediate end-point biomarker		
Preneoplasia	High	Moderate
Intermediate marker	Low	High

Optimal cohort = Relative risk (familial/metabolic polymorphism/epi) + proportionate risk (intermediate end-point marker)

ornithine to putrescine. In sensitive tissues, putrescine levels fall rapidly after administration of DFMO. Levels of the derived polyamine product spermidine also fall with time as does that of the terminal polyamine spermine, although to a much lower degree. DFMO was originally developed for therapeutic purposes, but was found to be ineffective against established malignancies (Abeloff *et al.*, 1986). A number of workers demonstrated that an increase in ornithine decarboxylase follows carcinogen exposure and inhibition of this rise with DFMO blocks cancer formation in essentially all in-vitro and animal models studied, including colon polyps and cancer (Verma, 1990; Halline *et al.*, 1990). These findings led to a renewed interest in DFMO as a potential chemopreventive agent.

Clinical studies

Two major obstacles needed to be overcome to demonstrate that DFMO was worth studying as a chemoprevention agent in humans. First, although the results in animals with DFMO were impressive, its potential as a chemopreventive compound in humans was unknown. It was soon found, however, that many human preneoplastic tissues had elevated basal ornithine decarboxylase activity compared with control tissues (Hixson *et al.*, 1993), thereby providing a rational basis for the use of DFMO as a chemoprevention agent for patients at risk in clinical settings. Second, at the high doses used in therapeutic trials, hearing loss, although reversible upon drug discontinuation, was substantial (Croghan, 1991). It was therefore necessary to establish whether a dose of DFMO could be found that would deplete polyamines in the organ of interest, but was below the threshold for producing hearing changes and other side-effects.

The colon was selected as the target organ for our studies, as animal experiments had demonstrated substantial anticarcinogenic activity of DFMO in this tissue (Nigro *et al.*, 1986; Verma, 1990) and the flat mucosa of patients with colon polyps and cancer has elevated levels of ornithine decarboxylase (Rozhin *et al.*, 1984; Hixson *et al.*, 1993). In a pilot study, we demonstrated that a modest dose of DFMO could lower polyamine levels in rectal mucosal biopsies, but not in shed oral mucosal cells (Boyle *et al.*, 1992). This was disappointing, in that these results indicated that oral mucosal cells were not a satisfactory surrogate for rectal biopsies, thereby limiting the number of studies that could be carried out in this target patient population. However, clear evidence of suppression of polyamine content in colonic mucosa by DFMO was demonstrated.

Our subsequent phase IIa trial used a unique design to determine the lowest dose at which DFMO was effective in lowering polyamines in the rectal mucosa without producing side-effects (Meyskens *et al.*, 1994). From the results of the prior therapeutic trials and pilot study in humans, we selected a moderate dose of DFMO as the first and highest dose of drug to be studied in a cohort of individuals who had prior colonic polyps removed. The flat mucosa was biopsied, before and after one month of DFMO treatment, and polyamines were measured. The dose of DFMO was progressively reduced until no effect on the polyamine content was seen. On the basis of the results from this detailed investigation, a range of doses (75–400 mg/m^2 per day) was selected for subsequent longer-term studies. Analysis of the results also indicated that the age of the participant affected baseline and changes in polyamine values in response to DFMO, parameters which were important in evaluating the overall results of the phase IIa trial and subsequent studies.

A subsequent placebo-controlled phase IIb trial of 12 months' duration measured the effect of a range of low doses of DFMO on polyamine content in rectal mucosa over time, and general side-effects (Meyskens *et al.*, 1998) and specific hearing changes, as determined by pure tone audiometry and otoacoustic emission analysis (M.J. Doyle, unpublished) were carefully assessed. The critical parameters putrescine content and spermidine/spermine ratio in the rectal biopsies were decreased by doses as low as 200 mg/m^2 per day. Although well tolerated at all doses tested, DFMO at the highest dose of 400 mg/m^2 per day produced more side-effects than the placebo arm and subtle changes in the lowest frequencies of the pure tone audiogram were evident. In contrast, doses of DFMO of 200 mg/m^2 per day produced no side-effects or audiometric changes greater than placebo and produced effects on polyamine content nearly equivalent to the higher doses of DFMO. Therefore in all subsequent chemoprevention trials, a dose of DFMO of 200 mg/m^2 per day is being used.

We next had to consider whether to undertake a phase III trial of polyp risk reduction using DFMO alone or to develop DFMO in combination with other agents. We were significantly influenced in our decision to develop combination trials by the results from animal studies, in which marked inhibition of tumour formation was achieved by using doses of individual drugs in combination that were considerably lower than the doses of single agents alone (Kelloff, 1996). Since absence of side-effects is at least as important as efficacy in developing chemoprevention agents for human beings, we accordingly elected to develop combination clinical chemoprevention regimens.

Epidemiological and experimental as well as a few clinical studies have indicated nonsteroidal anti-inflammatory agents (NSAIDs) to be compounds likely to be effective for chemoprevention (IARC, 1997). After a careful review of the mechanism of action of the NSAIDs (COX-1, COX-2, and other), the profile of putative side-effects and the experimental (as well as clinical) activity, we chose to study sulindac for use in combination with DFMO. We have recently initiated a 36-month phase IIb trial in which half of the participants receive placebo and half a combination of low doses of DFMO and sulindac. For this trial, 250 individuals with prior colonic polyps are being recruited. A number of surrogate end-point biomarkers will be measured in the flat rectal mucosa before and after 12 and 36 months of therapy. These include nuclear morphometry, uninduced apoptosis, polyamine and prostaglandin content, Ki67, and a number of preneoplastic antigens (CEA, sialy TN, p53, Bcl-2) measured by immunostaining in flat mucosa. Changes in these markers are being correlated with appearance of new incident adenomatous polyps. The presence of K-*ras* mutations in incident polyps is also being studied. The study has sufficient power to ensure that significant correlations between the appearance of colonic polyps (the primary biomarker) and changes in the spermidine/spermine ratio and occurrence of secondary biomarkers will be detectable. However, the study was not designed to have power to detect a modest difference (25%) in new incident adenomas between the two arms, although a large (75–90%) difference would be detectable.

We have also planned a large placebo-controlled chemoprevention trial of DFMO and sulindac using a 2×2 factorial design in which the rate of incident polyps after three years of therapy will be the primary end-point. Surrogate end-point biomarkers will also be measured 12 and 36 months after therapy and include those listed above. By using a Cochrane–Armitage analysis, we have been able to reduce the projected sample size from 1800 to 1000 participants without losing power; however, the design assumes that the combination is more effective in reducing the appearance of new colonic adenomas than either compound alone and that single agents will be more effective than the placebo.

Development of Bowman–Birk inhibitor as a chemoprevention agent

Experimental data

In epidemiological studies, high levels of soybean consumption have been associated with a decreased incidence of epithelial cancers (Kennedy, 1998). Four major classes of candidate chemopreventive compounds have been identified in soybeans: isoflavones, phytic acid, saponins and certain protease inhibitors. We have focused our studies on the Bowman–Birk inhibitor, a soybean-derived serine protease inhibitor with anticarcinogenic activity at doses well below those of other chemopreventive agents identified in soybeans. A series of studies in animals have demonstrated that a Bowman–Birk inhibitor concentrate is an effective inhibitor of protease activity and oral carcinogenesis (Messadi *et al.*, 1986; Kennedy *et al.*, 1993).

Clinical trials

Bowman–Birk inhibitor concentrate, which contains active Bowman–Birk inhibitor and has the same anticarcinogenic profile as the purified substance, has been developed for human trials. Proteolytic activities are elevated in the oral mucosa cells of patients with oral leukoplakia, and markedly so in those who are actively smoking (Manzone *et al.*, 1995), providing a direct rationale for the use of this agent in clinical trials at this tissue site. Overexpression of c-erbB-2 (neu) is important in human oral carcinogenesis and progression (Craven *et al.*, 1992; Hou *et al.*, 1992), and the relationship between protease activity and *neu* oncogene expression in patients with oral leukoplakia treated with the Bowman–Birk inhibitor has been established (Wan *et al.*, 1999). A phase I trial of

Bowman–Birk inhibitor concentrate showed that the compound was non-toxic up to the maximum doses that were allowed by regulatory agreement (Armstrong *et al.*, 2000).

We have recently completed a one-month phase IIa dose-escalation chemoprevention trial of Bowman–Birk inhibitor concentrate in patients with oral leukoplakia lesions (Wan *et al.*, 1999). There was a linear fit of the relationship between the dose of the agent and the decrease in total lesion area, which was confirmed by a blinded analysis of clinical impression of lesion photographs (W. Armstrong & F. Meyskens, unpublished). Pretreatment levels of cellular protease activity affected the clinical response, with lesions having lower initial levels of protease activity responding better. There were a number of important relationships confirmed in relation to administration of Bowman–Birk inhibitor concentrate:

- High pretreatment levels of protease activity were associated with greater decreases in protease activity.
- A dose-dependent increase in serum neu protein was observed.
- Higher pretreatment serum neu protein levels were associated with greater relative decreases in serum and cellular neu protein and cellular protease activity.
- Pretreatment levels of cellular neu protein correlated inversely with changes in cellular neu protein.

Overall, these results were quite encouraging, as they suggested that Bowman–Birk inhibitor concentrate had clinical activity against oral leukoplakia that was associated with a change in predefined surrogate end-point biomarkers. We have now begun a randomized phase IIb trial in which patients with oral leukoplakia receive either Bowman–Birk inhibitor concentrate or placebo for six months. Serial shed oral mucosal cells will be collected for analysis of surrogate end-point biomarkers and clinical lesions will be monitored. Patients achieving a partial or complete clinical response will continue treatment for up to 18 months.

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