Cancer Chemoprevention

Cancer Drug Discovery and Development

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Volume 2: Strategies for Cancer Chemoprevention

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PREFACE

Despite significant advances in cancer treatment and early detection, overall cancer incidence has increased, cancer-associated morbidity is considerable, and overall cancer survival has remained relatively flat over the past several decades *(1,2)*. However, new technology allowing exploration of signal transduction pathways, identification of cancer-associated genes, and imaging of tissue architecture and molecular and cellular function is increasing our understanding of carcinogenesis and cancer progression. This knowledge is moving the focus of cancer therapeutics, including cancer preventive treatments, to drugs that take advantage of cellular control mechanisms to selectively suppress cancer progression.

Carcinogenesis is now visualized as a multifocal, multipath process of genetic progression occurring over a long time period and resulting in increasing loss of cellular controls. This process provides promising opportunities for chemoprevention, which involves using drugs, biologics, or nutrients to inhibit, delay, or reverse neoplastic progression at any time before the onset of invasive disease. Remarkable progress has been made in developing chemoprevention strategies, started by research on mechanisms of chemopreventive drugs and assays for evaluating these drugs in animal models *(3–6)*, and led in the clinic by early studies on prevention of head and neck carcinogenesis *(7,8)*.

Progressive disorganization provides a strong rationale for early intervention in carcinogenesis when mutations are fewer, even before tissue-level phenotypic changes are evident. However, the long latency also presents a significant challenge for prevention and treatment of early cancer *(9,10)*. That is, cancer incidence reduction studies in subjects at relatively low risk may require thousands of subjects and many years to obtain significant and definitive results. The successful trial of tamoxifen as a chemopreventive for breast cancer illustrates the vast resources required for a primary prevention study, even when the cohort is well defined and the drug effect is already well characterized. This trial was carried out in women who at a minimum had a relative risk for breast cancer equivalent to a 60-year old *(11)*. Six thousand six hundred (6600) treated women and an equivalent number of control subjects were required to achieve a significant ($p < 0.05$) treatment effect.

This inherent inefficiency in validating that chemopreventive treatment results in net clinical benefit for the patient (subject) has led to intensive research efforts to develop useful biomarkers. These biomarkers include measures of neoplastic progression, drug effect (or pharmacodynamic markers), and markers that measure prognosis as well as predict responses to specific therapy. All these biomarkers have the potential to greatly augment the development of successful chemoprevention therapies, but two specific types of biomarkers will have the most immediate impact on successful chemopreventive drug development—those that measure the risk of developing invasive life-threatening disease, and those whose modulation can "reasonably predict" clinical benefit and, therefore, serve as surrogate endpoints for later-occurring clinical disease. Thus far, the biomarker that best measures these two phenomena is intraepithelial neoplasia (IEN) because it is a near obligate precursor to cancer. As precancer, it is a very good risk marker for cancer development; and as a recognized disease that is being treated, it has been validated as a surrogate endpoint biomarker *(12–14)*. Since IEN is discussed extensively in many chapters in this volume, the three important features that characterize IEN are presented in some detail below.

IEN is a near obligate precursor to cancer. IEN occurs in most epithelial tissue as moderate to severe dysplasia, is on the causal pathway leading from normal tissue to cancer, and is close in progression to cancer (invasive neoplasia). Genetic progression with loss of cellular control functions is observed as the phenotype gradually changes from normal histology to early dysplasia then to increasingly severe IEN, superficial cancers, and finally invasive disease. For example, in the breast it is estimated that progression from atypical hyperplasia through ductal carcinoma *in situ* (DCIS) to adenocarcinoma requires 10–20 years or more *(15,16)*. Colorectal adenomas may form over a period as long as 5–20 years, and progression from adenoma to colorectal carcinoma usually requires another 5–15 years *(17–20)*. Prostatic intraepithelial neoplasia (PIN) may develop over approximately 20 years. From PIN to early latent cancer may take 10 or more years, and clinically significant carcinoma may not occur until 3–15 years later *(21)*. Progression is marked in target tissues by the appearance of specific molecular and more general genotypic damage associated with increasingly severe dysplastic histology. In many cases, critical early steps include inactivation of tumor suppressors such as APC in colon or BRCA in breast cancers, and activation of oncogenes such as *ras* in colon, lung, and pancreatic cancers. Progression is also influenced by factors specific to the host tissue's environment, such as the action of hormones and cytokines produced in stroma around

the developing epithelial tumor and changes in tissue structure. IEN shows these changes and provides a suitable target for treatment intervention because of its phenotypic and genotypic similarities and evolutionary proximity to invasive cancer.

IEN as precancer is a risk marker for cancer. Subjects with IEN, particularly severe IEN, are at significantly higher risk than unaffected populations for developing invasive cancer in the same tissues. Among measurable risk factors, only germline mutations that occur in genetic cancer syndromes confer higher risk. For example and as reviewed previously *(14)*, very strong evidence associates the presence of colorectal adenomas with subsequent development of invasive cancer; increasing risk correlates to type of histological growth pattern (villous > tubulovillous > tubular) and increasing size and severity of dysplasia. PIN as a risk marker for prostate cancer and the characteristics of PIN progression have also been described *(21–24)*. This evidence includes similar cellular morphology and atypia in high-grade PIN (HGPIN) and prostatic adenocarcinoma (cellular atypia observed in HGPIN is virtually indistinguishable from invasive cancer, except that in HGPIN no invasion has occurred). It also includes the spatial and temporal association of HGPIN to prostate cancer, with both being found primarily in the peripheral zone, and much more infrequently in the transition zone. As PIN progresses, the likelihood of damage to the basal cell layer and basement membrane increases. Certain cytoskeletal proteins, secreted proteins, and degree of glycosylation are shared by PIN and cancer, but not by benign prostatic hyperplasia or normal prostate epithelium. The most compelling data on the temporal relationship of PIN and cancer comes from studies showing that patients with HGPIN and no detectable cancer progressed to a 40% incidence of cancer in three years and to approximately 80% incidence in ten years.

IEN is precancer and in its own right is a disease; treatment provides clinical benefit. Because IEN is a near obligate precursor to invasive cancer, it is standard clinical practice to utilize invasive surgical interventions to reduce the burden of IEN, e.g., colon adenomas, oral leukoplakia, cervical IEN (CIN) 2/3, breast DCIS. Therefore, reducing IEN burden is an important and suitable goal for medical (noninvasive) intervention to reduce invasive cancer risk and to reduce surgical morbidity *(14)*. High-risk individuals with established IEN are cohorts for clinical trials to demonstrate the effectiveness of new chemopreventive agents for IEN treatment. Moreover, treatment is needed not only for clinically apparent IEN, but also the entire epithelial sheet at risk of developing IEN ("field cancerization," e.g., ref. *25*), to ensure reduced need for surgical removal of IEN.

These features of IEN explain why it is at present the best surrogate endpoint for invasive cancer, since no serious student of cell biology or pathology questions that the morphologic changes associated with IEN are part of and predict the cancer process, and that the lesions of genetic progression manifest themselves within IEN as cytological abnormalities of neoplasia—increased nuclear size; abnormal nuclear shape; increased nuclear stain uptake; variations in cellular size, shape and stain uptake; increased mitosis; abnormal mitosis; disordered maturation (differentiation) *(26)*. In summary, because of the probability that its presence will lead to cancer, IEN is already accepted as a validated endpoint for measurement of cancer risk reduction by both surgical and drug intervention.

Other promising surrogate endpoint biomarkers—genome/proteome expression profiles. Use (validation) of surrogate endpoint biomarkers that, unlike IEN, are not obviously intrinsic to neoplastic progression mostly fail because of the complexity of neoplasia as well as the need for surrogate endpoint biomarkers to "predict patient benefit with reasonable certainty" *(27)*. The failures result because the disease of cancer is tissue-based, and surrogate endpoint biomarker development has been constrained by naive approaches to modeling the disease and its multipath, multifocal development process with isolated molecular and cellular events. Further, for biomarkers to be useful, techniques to determine them need to be robust and exhaustively validated. When using biomarkers in studies, investigators need to comply strictly with validated methods to assure confidence that what is measured is consistent across studies. Achieving this objective may require extensive efforts such as those used to establish standards for the determination of cholesterol. Criteria for biomarker measurements have been the subject of many reviews (e.g., *12,14,28*), yet much of the lack of progress derives from faulty adherence to these methodologies. Nonetheless, a sound scientific basis now exists to characterize surrogate endpoint biomarkers for developing drugs *(12)*.

The multipath, multifactorial nature of carcinogenesis is predicted by the heterogeneity that can result from processing the human genome. The 30,000 or so human genes contain as many as several hundred thousand allelic variants from single nucleotide gene polymorphisms including splicing variants *(29)*. These variations are compounded another three- to fivefold by posttranslational protein modifications leading to a multitude ($>10^6$) of protein–protein interactions *(30)*. Even if only a small fraction of the genome is critical to cancer, the number of possible molecules and interactions involved is enormous. This level of complexity highlights the uncertainties of using isolated molecular and cellular biomarkers to measure carcinogenesis. Moreover, this complexity is heightened by expected intra-/intersubject and tissue variations.

Nonetheless, increasing understanding of genetic progression in cancer (e.g., *31,32*) and of signal transduction *(33)* in cancer target tissues, and observations of genotypic changes characteristic of selected cancers (e.g., *34,35*) combined with advances in technology for measuring and characterizing changes in gene and protein expression *(30)*, suggest that analyses of such patterns of gene expression have potential for development as surrogate endpoint biomarkers. For example, progress made in gene chip technology suggests that within a few years it will be trivial to measure 6–12 genes defining a genetic progression model *(36)*. As for all surrogate endpoint biomarkers, the feasibility of genome/proteome expression patterns as surrogate endpoint biomarkers will depend on careful evaluation in the context of carcinogenesis. Therefore, the characterization of molecular markers of carcinogenesis and their future development and validation as surrogate endpoint biomarkers will be most effectively done *in situ* within IEN. The further development of genetic progression models will also proceed in this context, as it has from inception. In the not-too-distant future, as understanding of the minimum number of disrupted pathways yielding malignancy grows, patterns of change representing carcinogenesis will be relatively easy to measure. This process will evolve with progress that is being made in understanding and analyzing systems biology. With this understanding will come surrogate endpoint biomarkers in predysplastic tissue (a normal morphologic phenotype); the predictive value of these data will begin to exceed the predictive value of abnormal morphology (IEN). This molecular pathology within IEN lesions, or even prior to appearance of these lesions, will also allow better identification of individuals at risk, improve study efficiency, and provide better quantitative estimation of drug efficacy than effects on IEN alone *(12,14,28)*. These advances in tissue-based biomarkers will be augmented by biomarkers that can be measured non-invasively by molecular imaging, and by functional genomic and proteomic research *(14,28)*.

Net clinical benefit is required. Drug approvals are based on clinical benefit, so the approval of drugs for chemoprevention will depend on some measure of clinical benefit—reduced morbidity, organ preservation, lower cost for surveillance—as well as efficacy against precancers. Because chemopreventive drugs will most likely be administered chronically, they will be expected to demonstrate long-term safety, duration of effect, and minimal drug resistance, or provide alternative strategies to minimize toxicity and maximize efficacy.

Promising Chemopreventive Agents, the companion volume, surveys ongoing efforts to identify drugs, natural products, and other agents that may have potential in cancer chemoprevention. The agents are grouped by pharmacological and/or mechanistic classes and vary widely in terms of stage of development as chemopreventives, ranging from extensively studied groups such as nonsteroidal antiinflammatory drugs (NSAIDs) and antiestrogens to drugs with recently identified potential based on mechanistic activity (e.g., protein kinase inhibitors, histone deacetylase inhibitors, and anti-angiogenesis agents), as well as agents yet to be evaluated in chemoprevention settings (e.g., proteasome and chaperone protein inhibitors). Attention is devoted to food-derived agents (such as tea, curcumin, and soy isoflavones), vitamins, and minerals because of their high promise for prevention in healthy populations.

Provided in this volume, *Strategies for Cancer Chemoprevention,* are guidelines for cancer chemopreventive drug development. Part I is devoted to general strategies and methods for drug discovery, preclinical efficacy, characterization of precancers, safety evaluation, clinical cohorts, and clinical trial design. Part II reviews strategies for and status of chemopreventive agent development at major cancer targets—prostate, breast, colon, lung, head and neck, esophagus, bladder, ovary, endometrium, cervix, skin, liver, and multiple myeloma. Both sections heavily document the characterization and application of reliable biomarkers in chemopreventive drug development.

The first several chapters of Part I consider discovery and preclinical evaluation of new agents. For example, an elegant approach to the challenges of identifying chemopreventive agents in natural products, particularly food plants (e.g., antioxidants, antiinflammatory compounds, and well-defined mixtures) is presented (Chapter 1); this approach addresses factors such as standardization of plant growth and extraction conditions, and considers co-development of a well-defined mixture and its likely active component. The development of preclinical models for evaluating potential chemopreventive agents is particularly important because of the potential for validating surrogate endpoints in animal models where an intermediate biomarker can be evaluated, along with subsequent effects on cancer incidence, and ultimately survival. Chapters in this volume describe well-established carcinogen-induced animal models of carcinogenesis in major cancer targets (Chapter 2), as well as newly defined transgenic and gene knock-in/knock-out mouse models of molecular targets for chemoprevention (Chapter 3), and animal models of genetically inherited cancer susceptibility (Chapter 4).

The importance of precancerous histopathology, particularly IEN, in chemoprevention has been stated. Characteristics and progression of this pathology in most cancer targets are comprehensively reviewed in Chapter 5. The use of computer-assisted image analysis to analyze precancerous tissue in the prostate is described as

an example of the potential application of new quantitative imaging techniques to evaluate chemopreventive efficacy in IEN (Chapter 6). As noted before, genome/proteome expression profiles have high potential as surrogate endpoints for carcinogenesis because they correlate with the clinical progression of carcinogenesis. Several chapters in the book assess potential applications of genomics and proteomics to chemoprevention. Uses of genomics databases in discovery of chemopreventive agents and in designing chemopreventive strategies are surveyed (Chapter 7). Surrogate endpoint biomarkers for breast cancer based on functional genomics are described (Chapter 8), as are applications of proteomics in clinical cancer settings (Chapter 9) and interpretation of genome-based data (Chapter 10).

Determining which populations will likely benefit from chemopreventive intervention, particularly those who are asymptomatic, is a significant challenge and an opportunity for chemoprevention. Two approaches are laid out in this volume. One is the construction of multifactorial models of absolute risk, based primarily on epidemiological statistics (Chapter 11). The second explores the correlation of genetic polymorphisms to cancer susceptibility (Chapter 12). The remaining two chapters in Part I examine some practical aspects of clinical evaluation of chemopreventive agents—i.e., clinical trial design issues (Chapter 13) and subject recruitment (Chapter 14).

For each cancer target organ covered in Part II, one chapter provides an overview of carcinogenesis in the target organ, including cancer and precancer incidences, genetic progression, and risk factors, along with potential opportunities for chemoprevention. Known and promising chemopreventive agents, surrogate endpoints, and clinical trial designs are summarized. For a number of these targets, additional chapters address specific topics that contribute to chemoprevention strategies.

In addition to an overview of strategies for prostate cancer chemoprevention (Chapter 15), a second chapter addresses the controversial topic of using prostate-specific antigen for determining risk and monitoring the progression of prostate cancer (Chapter 16). The overview of breast cancer chemoprevention focuses on defining populations at risk based on evidence of early genetic progression (Chapter 17) and is accompanied by two supplemental chapters. One describes development of ductal lavage as a technique for sampling breast cells in assessment of early neoplasia (Chapter 18), and the second addresses the well-recognized need to control estrogenic activity in suppressing breast carcinogenesis (Chapter 19). Quite possibly, the most significant advances in clinical chemoprevention have been made against colorectal carcinogenesis (Chapter 20) where genetic and histopathological progression of early dysplasia to adenoma to cancer has been well-studied. An additional important preventive strategy in colon is screening for and excision of adenomas (Chapter 21). Chapter 22 provides an overview of lung cancer chemoprevention accompanied by an article on topical delivery as a strategy to allow administration of drugs to lung that may be too toxic for systemic administration (Chapter 23); topical administration is also a promising strategy in other accessible targets such as skin, oral cavity, colon and cervix.

The review of bladder cancer chemoprevention (Chapter 24) focuses on the potential use of chemopreventive drugs to stop the recurrence of superficial bladder cancers; this cohort is at very high risk for recurrence and progression, and a successful chemopreventive intervention could be expected to provide clinical benefit from organ preservation (by delaying or reducing the need for cystectomy). Chapters on the esophagus discuss prevention and delay of progression of Barrett's esophagus, a precursor to adenocarcinoma and an increasing risk factor for esophageal cancer in western populations (Chapter 25), as well as prevention of squamous cell carcinoma (Chapter 27). A third chapter looks at sophisticated new techniques for imaging esophageal dysplasia (Chapter 26). The high rate of second primary tumor formation has been well-documented for the head and neck, which have been studied for more than 20 years as a site for chemoprevention (Chapter 28). Recently, aneuploidy and other biomarkers of genetic progression have been carefully documented as risk and prognostic indicators of head and neck carcinogenesis and potential endpoints for chemoprevention studies (Chapter 29). Incidences of non-melanoma skin cancer are higher by far than any other cancer, and melanoma incidence is increasing (Chapter 30). In addition to oral and topical small molecule drug treatments for prevention and treatment of non-melanoma precursor lesions (actinic keratoses, basal cell nevus syndrome), opportunities exist for novel immunotherapies in skin carcinogenesis and vaccination against melanoma (Chapter 31).

Screening for and surgical removal of suspect CIN is well established, and drugs have shown activity in reducing CIN severity (Chapter 32). Moreover, human papillomavirus infection is strongly associated with onset of cervical cancer, and immunoprevention strategies (both treatment and prophylactic) are under development for populations at risk (Chapter 33). Thus far, chemoprevention strategies in endometrium have not been established; however, remarkable advances have been made in documenting and quantifying carcinogenesis-associated changes in endometrial tissue that provide opportunities for preventive intervention (Chapter 34). In ovary (Chapter 35), pancreas (Chapter 36), liver (Chapter 37), and multiple myeloma (Chapter 38), precancerous lesions that may be targets for chemoprevention

are suggested, along with potentially effective chemopreventive drugs.

The two volumes of *Cancer Chemoprevention* demonstrate that the science of chemoprevention research is solidly established, very active, and offers great promise for lessening the burden of human cancer. Progress in building and understanding genetic/molecular progression models of many human cancers based on seminal work described by Vogelstein and colleagues for the adenoma-carcinoma sequence in colon cancer *(37)* has been substantial and is being enhanced by newer and better animal models. Understanding molecular progression leads to synthesis and discovery of new molecularly targeted agents with high promise of efficacy that, once evaluated for safety, will have an impact on cancer incidence and mortality. The evaluation of drug effect and drug efficacy biomarkers along with better technologies for their measurement is progressing, and the science and utility of surrogate endpoint biomarkers in developing cancer chemopreventive agents against sporadic cancers are solidly established. The issue of validation is a relative one, and IEN is validated for most target organs sufficiently to establish that its prevention/removal provides clinical benefit. With rigorous attention to methodology and to emerging scientific data and new technologies, there is every expectation that new surrogate endpoint biomarkers will now be developed in the context of IEN. These new surrogate endpoint biomarkers will improve the efficiency of clinical chemopreventive agent development, better identify those patients (subjects) who are likely to benefit (or not to benefit), while also opening the door to even earlier identification of individuals at risk (e.g., those with predysplastic molecular lesions that occur prior to IEN). The rapid pace at which systems biology and new technologies are evolving will make surrogate endpoint biomarker science a very productive and exciting area, but will also evoke the need for careful validation of such markers in the context of clinical trials.

Prospects are bright that surrogate endpoint biomarkers will make cancer chemoprevention studies more efficient and informative; however, hard work and exceptional dedication to sound, standardized methods will be required to assure that the application of these efforts in developing chemopreventive drugs is fruitful. The eventual acceptance of surrogate endpoint biomarkers may entail more than scientific rationale. Scientific and regulatory policy changes may also be required (e.g., *38*). It is often observed that candidate surrogate endpoint biomarkers are expressed at higher incidences than the symptomatic clinical disease that they approximate. Such will always be the case when completely unrelated endpoints (other causes of death) do not allow carcinogenesis to go to completion. Based on existing disease models, it is likely that all high-grade IEN-carrying confirmed genetic lesions would end in cancer if the host lived long enough *(12,32)*. Validation, like causality, is a relative term that only becomes absolute when all variables and elements of a process are known and can be studied quantitatively. It is undesirable and short-sighted to require any data more rigorous than validation based on probabilistic estimates that are consistent with current medical and regulatory practice. Based on existing knowledge, we can assume that IEN and the earlier biomarkers within IEN are on one or more of the possible causal pathways to carcinogenesis. Because they sometimes precede the cancer endpoint by several years, there is potential for interference, diversion, role in other biological processes, etc., that would keep these events from being ideal surrogate endpoint biomarkers. However, intervention to treat or prevent could be shown to provide clinical benefit, much like lipid-lowering in cardiovascular disease and viral load reduction in AIDS *(27,39)*. If interventions show compelling efficacy against surrogate endpoint biomarkers and can be administered safely to populations at risk, it would seem prudent to formulate scientific and regulatory policy changes allowing the use of these biomarkers in evaluating interventions that would lead to more efficient drug approvals to prevent this dread disease.

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VALUE-ADDED EBOOK/PDA

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I CHEMOPREVENTIVE AGENT DEVELOPMENT SCIENCE

1 Characterization of Natural Product Chemopreventive Agents

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1. INTRODUCTION

Cancer is a complicated group of diseases characterized by the uncontrolled growth and spread of abnormal cells *(1)*. In 2002, 1,284,900 new cases of cancer were estimated to be diagnosed in the United States (US), and about 555,500 persons were expected to die of cancer, i.e., more than 1500 every day *(2)*. Despite small decreases in overall cancer incidence and mortality rates in the US since the early 1990s, the total number of recorded cancer deaths continues to increase due to an aging and expanding population *(2)*. Furthermore, deaths from certain carcinomas of the lung and bronchus, breast, prostate, and colon and rectum remain high, and 5-yr survival rates for many cancer patients are still very low: for cancers of the brain, 32%; esophagus, 14%; liver, 6%; lung and bronchus, 15%; pancreas, 4%; stomach, 22%; and multiple myeloma, 29% *(2)*. Obviously, cancer remains a formidable public health problem.

A "war on cancer" was proclaimed about 30 yr ago. There was great hope and anticipation of reducing mortality rates for common forms of cancer by half by the year 2000 *(3–6)*. Although this stated goal of the US National Cancer Institute (NCI), National Institutes of Health (NIH), has not been attained, much progress has been made, and basic research has further elucidated mechanisms whereby normal cells and tissues become malignant *(5)*.

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Cancer is considered the end stage of a chronic disease process characterized by abnormal cell and tissue differentiation *(6)*. This process of carcinogenesis eventually leads to the final outcome of invasive and metastatic cancer. Recent advances in defining cellular and molecular levels of carcinogenesis, along with a growing body of experimental, epidemiological, and clinical trial data, have led to the development of cancer chemoprevention, a relatively new strategy in preventing cancer *(7–10)*. Cancer chemoprevention is defined as the use of synthetic or natural agents to inhibit, retard, or reverse the process of carcinogenesis *(7–11)*.

Invasive cancer derives from complex interactions of exogenous (environmental) and/or endogenous (e.g., genetic, hormonal, and immunological) factors *(5,12,13)*. Carcinogenesis is progressive, and this progression in precancer is characterized by the appearance of specific molecular and more general genotypic damage associated with increasingly severe dysplastic phenotypes *(11)*. The development of this phenomenon may be represented by three stages that often overlap: initiation, promotion, and progression phases *(10)*. Initiation, an irreversible event, begins when normal cells are exposed to a carcinogen and their genomic DNA undergoes damage that remains unrepaired or misrepaired *(10)*. Promotion, an expansion of the damaged cells, leads to the appearance of benign tumors. Progression, an irreversible process, produces a new clone of tumor cells with increased proliferative capacity, invasiveness, and metastatic potential *(10)*. Transitions between successive stages are believed to be enhanced or suppressed by various factors *(5)*.

Most human cancers seem to be potentially preventable because of controllable or removable causative exogenous factors, such as cigarette smoking, dietary factors, environmental and occupational chemicals, lifestyle and socioeconomic factors, radiation, and specific microorganisms *(5,13)*. These exogenous factors offer the most likely opportunities for interventions targeted to primary prevention—that is, elimination of or avoiding exposure to these environmental factors *(10)*. In addition, however, as a serious and practical approach to the control of cancer, cancer chemoprevention can play an integral role in the overall strategy geared toward reducing the incidence of cancer *(3,6,8,14)*.

Cancer chemopreventive agents, based on their individual underlying mechanisms of action, may be classified into three categories: inhibitors of carcinogen formation, blocking (antiinitiation) agents, and suppressing (antiproliferation/antiprogression) agents *(8,10,15–17)*. Many inhibitors of carcinogen formation, such as ascorbic acid *(18)*, phenols (caffeic acid and ferulic acid) *(19)*, sulfhydryl compounds (*N*-acetyl-L-cysteine) *(20)*, and amino acids (proline and thioproline) *(21)*, act to prevent formation of nitrosamines from secondary amines and nitrite in an acidic environment *(16)*.

Chemoprevention strategies address four goals: inhibition of carcinogens, logical intervention for persons at genetic risk, treatment of precancerous lesions, and translation of leads from dietary epidemiology to intervention strategies *(22)*. Rational and successful implementation of chemopreventive strategies relies intrinsically on tests for efficacy and mechanistic assays, as well as availability of promising chemopreventive agents, reliable intermediate biomarkers, and appropriate clinical cohorts to discover safe and effective drugs for primary and secondary prevention of human cancers *(23)*. Established in the early 1980s, the NCI's Chemoprevention Program has since evaluated more than 1000 potential chemopreventive agents or agent combinations, including more than 40 compounds in about 100 clinical trials *(24)*. Included among a number of plant-derived natural products in this group of potential cancer chemopreventive agents are *S*-allyl-L-cysteine, curcumin, epigallocatechin gallate, genistein, lycopene, perillyl alcohol, and a mixture of soy isoflavones, all of dietary origin *(24)*. This program generally begins by identifying candidate agents through in vitro bioassay screening, epidemiology, and other scientific efforts *(13,15)*. Once potential leads have been identified, mechanistic evaluations through additional in vitro and ex vivo assays are important to assess efficacy, and for planning further tests in animal models to design regimens for clinical testing and use *(17,25,26)*. Agents judged to have potential as human cancer chemopreventive agents are subjected to preclinical toxicology and pharmacokinetic studies *(27)*, followed by Phase I clinical safety and pharmacokinetic trials *(28)*. The most successful agents subsequently progress to clinical chemoprevention trials *(23,26)*.

A competitive cancer chemoprevention program titled "Natural Inhibitors of Carcinogenesis" (P01 CA48112) has been supported since 1991 by NCI. The overall theme, and botanical, biological, chemical, biostatistical, and administrative aspects of this program have been summarized previously *(29–33)*. Currently, we provide an update and additional representatives of diverse classes of plant secondary metabolites associated with biological activity in preliminary in vitro assays, with some of these having

been subjected to evaluation in a mouse mammary organ culture model *(34,35)* used as a secondary discriminator to prioritize our leads. Several of these compounds have either significant structural interest or exhibit considerable promise for further development as cancer chemopreventive agents through evaluation in full-term inhibition studies with laboratory animals. Below is a brief outline of our current *modus operandi* in this research program.

Using the resources of the University of Illinois Field Station, the herbarium of the Field Museum of Natural History, Chicago, as well as field collection and commercial sources, sufficient quantities and numbers of plant materials have been procured for investigation in our research program on natural inhibitors of carcinogenesis. Priority in plant material selection, based in part on information contained in the NAPRALERT computer database *(36)*, has been accorded to edible plants, as well as species with reported biological activity relating to cancer chemoprevention, plants with no history of toxicity, and finally plants that had previously been poorly investigated from a phytochemical perspective. A small amount (300 g–1 kg) of each dried plant sample is collected for preliminary investigation.

Crude nonpolar and polar extracts, prepared from each plant obtained, are evaluated for their potential chemopreventive activity using a battery of about 10 short-term in vitro bioassays *(30,32,33)*. Based on the results of these bioassays, selected extracts are further evaluated in an ex vivo mouse mammary organ culture model, as mentioned above. In this assay, test materials are evaluated for their ability to inhibit 7,12-dimethylbenz*(a)*anthracene (DMBA)-induced preneoplastic lesions *(34,35)*. A battery of in vitro bioassays has been developed to monitor inhibition of tumorigenesis at the various stages. For initiation, antimutagenic activity *(37,38)*, antioxidant activity *(38,39)*, and induction of NAD(P)H:quinone reductase (QR) activity *(40,41)* are assayed. For promotion, inhibition of activities such as phorbol ester-induced ornithine decarboxylase (ODC) activity *(42,43)*, cyclooxygenase activity *(44–46)*, phorbol dibutyrate receptor binding *(47)*, and transformation of JB6 mouse epidermal cells *(48)* are assayed. For progression, induction of human leukemia cell differentiation *(49,50)*, inhibition of aromatase activity *(51)*, antiestrogenic and estrogenic effects *(52,53)*, and inhibition of estrone sulfatase activity *(54)* are assayed. Additional examples of leads with significant activities in several of these initial in vitro test systems will be provided later in this review.

In the next stage, plant extracts showing potency and/or selectivity in the in vitro bioassay models are selected for bioassay-guided fractionation to uncover their active principles. Active initial extracts are subjected to solvent partitioning, and chromatography (gravity-, flash-) over standard chromatographic materials, with final purification typically effected by semi-preparative high-performance liquid chromatography (HPLC). Active isolates in the aforementioned bioassays are characterized by the usual physical, spectral, and chromatographic measurements, and every effort is made to obtain unambiguous nuclear magnetic resonance (NMR) assignments for each compound of interest through the use of combinations of conventional one- and two-dimensional NMR methods. We have increasingly used Mosher ester methodology to determine absolute stereochemistry of secondary alcohols (e.g., *41,55*), and recently a simplified method has been developed in our laboratory to prepare Mosher esters directly in NMR tubes, thus obviating the need to purify these derivatives chromatographically *(56)*. In addition, we have capability to perform small-molecule X-ray crystallography (e.g., *56,57*), and some success has been achieved with the use of a liquid chromatography-mass spectroscopy (LC-MS) dereplication method, which has, for example, been applied to the analysis of various antioxidants *(39)* and flavonoids *(58)*.

Pure active compounds are then evaluated in all the in vitro bioassays, and selected compounds are further processed for evaluation in the mouse mammary organ culture (MMOC) model *(34,35)*. On rare occasions, a compound may be rated as active in this ex vivo system even though no significant preliminary in vitro activity is observed *(59)*. Finally, the in vivo cancer chemopreventive activity of highly promising pure plant constituents is evaluated in animal full-term tumorigenesis models, including the two-stage mouse skin model using DMBA as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter, and the rat mammary carcinogenesis model with *N*-methyl-*N*-nitrosourea (MNU) or DMBA as a carcinogen *(60,61)*. Additional in vivo models are used as required.

2. PHASE 2 ENZYME INDUCTION

Carcinogenesis is a complex and protracted multistage process, yet the entire course can be initiated by a single event wherein a cellular macromolecule is damaged by an endogenous or exogenous agent. Strategies for protecting cells from these initiating events include decreasing metabolic enzymes responsible for generating

reactive species (phase 1 enzymes) while increasing phase 2 enzymes that can deactivate radicals and electrophiles known to intercede in normal cellular processes. Important defenses against electrophile toxicity are provided in the family of phase 2 enzymes, such as glutathione (GSH) *S*-transferases (GSTs) and UDP-glucuronosyltransferases, which catalyze GSH-electrophile or glucuronyl-electrophile conjugates, respectively. Reduction of electrophilic quinones by NAD(P)H:quinone reductase (QR) is another important detoxification pathway, which converts quinones to hydroquinones and reduces oxidative cycling *(62)*. Although phase 1 induction and functionalization of xenobiotics may be required for complete detoxification by the action of phase 2 enzymes, phase 1 enzyme elevation may also be considered a potential cancer risk factor for activation of procarcinogens to reactive species *(63)*. Therefore, an agent that induces phase 2 enzymes selectively would theoretically appear to be a better protector than selective induction of both phase 1 and 2 enzymes. Monofunctional induction of phase 2 enzymes appears to be caused by disrupting the cytoplasmic complex between the actin-bound protein Keap1 and the transcription factor Nrf2, thereby releasing Nrf2 to migrate to the nucleus where it activates the antioxidant response element (ARE) of phase 2 genes and accelerates transcription *(64)*. Alternately, a compound may induce phase 2 enzymes bifunctionally through activation of the aryl hydrocarbon (Ah) receptor-xenobiotic response element (XRE) pathway *(65)*.

QR elevation with in vitro and in vivo systems has been shown to correlate with induction of other protective phase 2 enzymes and provides a reasonable biomarker for the potential chemoprotective effect of test agents against cancer initiation *(66)*. The murine hepatoma cell line Hepa 1c1c7 contains easily measurable inducible QR that provides a reliable, high-throughput system for detecting inducers of phase 2 enzymes *(67)*. This assay can also be used to determine if an agent induces phase 2 enzymes only (monofunctional) or both phase 1 and 2 enzymes (bifunctional). This is accomplished by comparing the induction capability of a compound in wild-type Hepa 1c1c7 cells with that observed in two mutant cell lines designated TAOc1 and BP^rc1, which are defective in a functional Ah receptor or unable to translocate the receptor-ligand complex to the nucleus, respectively *(68)*. Compounds that have similar inducing ability in the wild-type and mutant Hepa lines are considered monofunctional inducers. Activity is expressed by the concentration to double (CD) or quadruple (CQ) QR activity over basal levels, and toxicity is expressed as the concentration to kill 50% (IC_{50}) of the cells. A chemoprotective index ($CI = IC_{50}/CD$) can be expressed to provide a measure of the in vitro therapeutic index of a particular drug candidate.

A variety of natural inducers of phase 2 enzymes have been described, including a number of flavonoids, indoles, isothiocyanates, and dithiolthiones *(69,70)*. Our program has identified a number of natural and synthetic monofunctional and bifunctional inducers of phase 2 enzymes (Table 1, Fig. 1). We have employed a series of methods to further investigate induction patterns and protein and RNA expression, using Western and Northern blotting techniques, RT-PCR, and transient and stable transfection of cells with ARE and XRE reporter genes.

Extracts of an edible fruit from *Physalis philadelphica* Lam. (Solanaceae), commonly called the tomatillo, induced QR equally well in the wild-type Hepa 1c1c7 and mutant cell lines. Tomatillo, used in a variety of Latin American foods, such as enchiladas and salsa verde, are also included in North American sauces and relishes. The active principles were determined to be from a steroidal class of compounds designated withanolides *(71)*. Three withanolides were shown to have potent activity in QR induction, including two known compounds, withaphysacarpin (**1** in Fig. 1) and 24,25-dihydrowithanolide D (**2**, CD = 0.70 μ*M*), as well as a novel substance, 2,3-dihydro-3-methoxywithaphysacarpin (**3**, CD = 7.8 μ*M*) *(72)*. Subsequent analysis of an additional 37 withanolides isolated from a variety of species from Solanaceae revealed 16 withanolides with CD values below 1 μ*M* *(73)*. Of those 16 withanolides, only six exhibited a CI above 10: withaphysalin G (**4**, CD = 0.51 μ*M*), withaphysalin H (**5**, CD = 0.52 μ*M*), withaphysalin J (**6**, CD = 0.39 μ*M*), jaborosalactone P (**7**, CD = 0.75 μ*M*), jaborosalactone 1 (**8**, CD = 0.28 μ*M*), and trechonolide A (**9**, CD = 0.27 μ*M*). Large-scale isolation of selected withanolides from *P. philadelphica* has provided sufficient amounts to begin preliminary animal testing.

The whole flowering and fruiting parts of the medicinal plant *Tephrosia purpurea* were shown to have several moderate to strongly QR active compounds *(41,74)*. Isolation of 7,4′-dihydroxy-3′,5′-dimethoxyisoflavone (**10**), (+)-tephropurpurin (**11**), (+)-purpurin (**12**), pongamol (**13**), lanceolatin B (**14**), (–)-maackiain (**15**), (–)-3-hydroxy-4-methoxy-8,9-methylenedioxypterocarpan (**16**), and (–)-medicarpin (**17**) were the result of activity-guided fractionation with QR. The chalcone (+)-tephropurpurin (CD = 0.15 μ*M*, IC_{50} = 13.4 μ*M*, CI = 89) exhibited a QR value nearly three times higher

Table 1
Quinone Reductase Inducing Ability of Natural and Synthetic Compounds

	Compound	*CD*[a] *(μM)*	IC_{50}[b] *(μM)*	*CI*[c]
1	withaphysacarpin	0.43	4.8	11
2	24,25-dihydrowithanolide D	0.70	5.5	8
3	2,3-dihydro-3-methoxywithaphysacarpin	7.8	46.9	6
4	withaphysalin G	0.51	9.8	19
5	withaphysalin H	0.52	5.2	10
6	withaphysalin J	0.39	11	28
7	jaborosalactone P	0.75	42.7	57
8	jaborosalactone 1	0.28	8.1	29
9	trechonolide A	0.27	7.7	29
10	7,4′-dihydroxy-3′,5′-dimethoxyisoflavone	17.2	63.7	4
11	(+)-tephropurpurin	0.15	13.4	89
12	(+)-purpurin	5.6	50.7	9
13	pongamol	6.1	18.7	3
14	lanceolatin B	22.9	76.3	3
15	(−)-maackiain	8.8	70.4	8
16	(−)-3-hydroxy-4-methoxy-8,9-methylenedioxypterocarpan	14.7	63.7	4
17	(−)-medicarpin	13.7	74	5
18	brassinin	4.0	ND	ND
19	cyclobrassinin	1.2	ND	ND
20	spirobrassinin	7.9	ND	ND
21	*N*-ethyl-2,3-dihydrobrassinin	0.13	ND	ND
22	*S*-selenomethylbrassinin	0.5	ND	ND
23	sulforamate	0.26	34.9	134
24	oxomate	0.96	67	70
25	4′-iodoflavone	0.01	>165	>17,000
26	4′-bromoflavone	0.01	>165	>17,000
27	4′-chloroflavone	0.02	>78	>5000
28	4′-trifluoromethylflavone	0.03	90	3000
29	2′-hydroxy-2-methoxychalcone	0.31	17	55
30	2′-hydroxy-2,6-dimethoxychalcone	0.53	14	26
31	2′-hydroxy-2-methylchalcone	0.67	36	54
32	2′-hydroxy-2-nitrochalcone	0.30	11	37
	sulforaphane[d]	0.23	9.9	42

[a] Concentration to double QR activity in Hepa 1c1c7 cells.

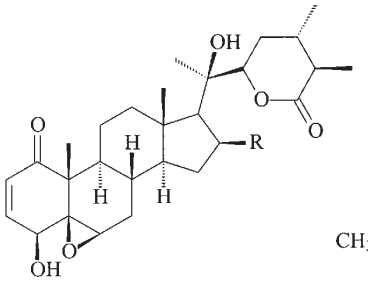
[b] Concentration to inhibit Hepa 1c1c7 cell growth by 50%.

[c] Chemoprevention Index (IC_{50}/CD).

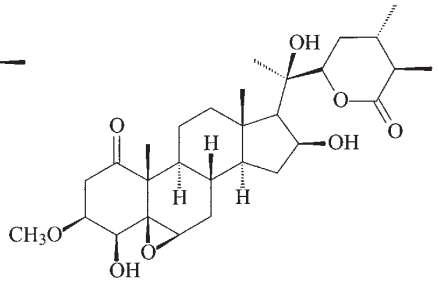
[d] QR assay positive control.

than sulforaphane (CD = 0.23 μ*M*, IC_{50} = 9.9 μ*M*, CI = 42), but with similar toxicity.

Cruciferous vegetables (such as Brussels sprouts, cauliflower, and Chinese cabbage) provided the indole dithiocarbamate brassinin (**18**) and several natural and synthetic analogs, which were shown to induce QR and GST activities with in vitro and in vivo models *(75,76)*. Of 27 brassinin derivatives tested, cyclobrassinin (**19**,



1: R=OH
2: R=H



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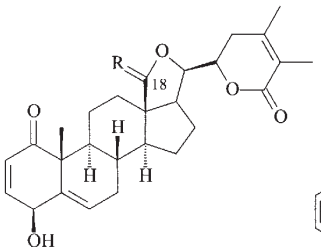
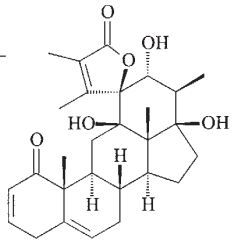


4: R=H, OH (18*R/S*)
5: R=H, OCH_3 (18*R*)
6: R=O



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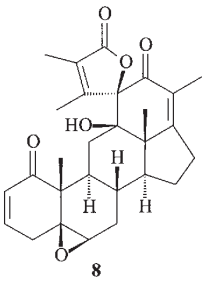

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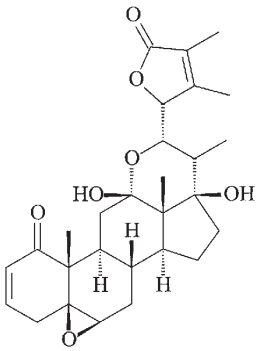

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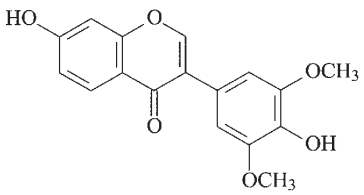

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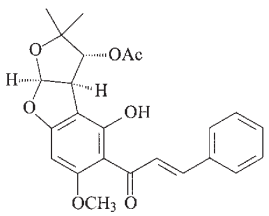

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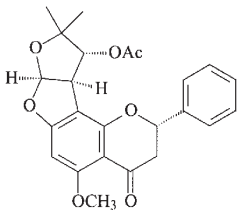

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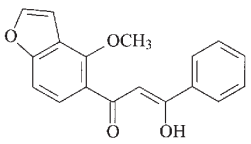

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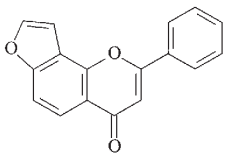

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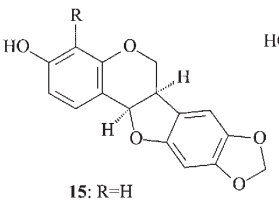
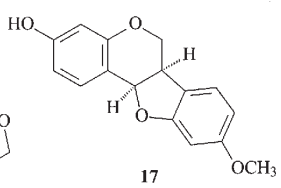


15: R=H
16: R=OCH_3



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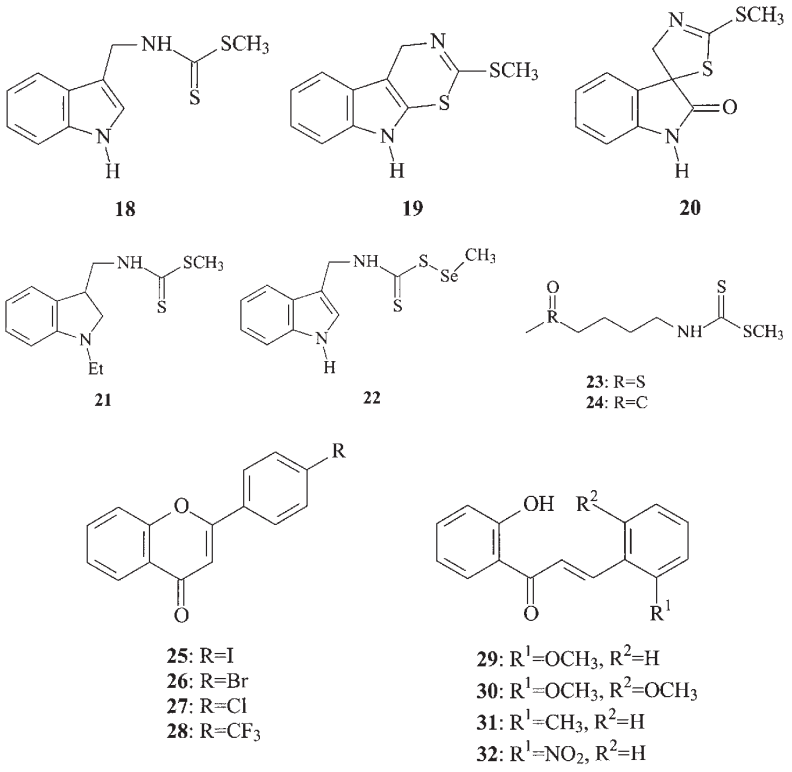

Fig. 1. Active leads identified through utilization of the quinone reductase assay.

CD = 1.2 μ*M*), spirobrassinin (**20**, CD = 7.9 μ*M*), *N*-ethyl-2,3-dihydrobrassinin (**21**, CD = 0.13 μ*M*), and *S*-selenomethylbrassinin (**22**, CD = 0.5 μ*M*), were most active. Brassinin and derivatives were not active QR inducers in the mutant cell lines; therefore, the indole class of compounds are likely to be bifunctional in nature.

Sulforaphane, an isothiocyanate isolated from broccoli, has potent monofunctional phase 2 enzyme-inducing activity *(77)*. A novel analog, sulforamate (**23**), was designed using the predicted methyl dithiocarbamate product of myrosinase-induced decomposition of the sulforaphane glucosinolate precursor glucoraphanin *(78)*. Sulforamate exhibited similar potency to sulforaphane (CD = 0.26 vs 0.23 μ*M*), but three times less toxicity (IC_{50} = 34.9 vs 9.9 μ*M*). Sulforamate also increased GSH levels twofold in Hepa 1c1c7 and H4IIE rat hepatoma cells and was shown to interact with the ARE of QR and GST Ya without involvement of XRE. However, difficulty in synthesizing either sulforaphane or sulforamate has precluded their development. Therefore, a more synthetically accessible analog was devised. Oxomate (**24**) is a keto-analog to sulforamate and is substantially easier to produce in multikilogram quantities. Oxomate (CD = 0.96 μ*M*) has weaker QR activity compared to sulforaphane and sulforamate in vitro, but toxicity is also substantially reduced (IC_{50} = 67 μ*M*). Oxomate, sulforamate, and sulforaphane have shown similar dose-response patterns for inhibiting DMBA-induced lesions in MMOC, and significant inhibition of DMBA-induced mammary carcinogenesis in Sprague-Dawley rats has been demonstrated *(79)*. Additionally, oxomate significantly reduced tumor multiplicity in DMBA-induced female Sprague-Dawley rats fed at 3% in the diet.

Many flavonoids from a variety of plants have shown moderate QR-inducing capability; however, a systematic analysis of structural characteristics responsible for this activity has not been undertaken. A variety of B-ring substituted 2′-hydroxychalcones and their related flavanones and flavones were examined for QR-inducing activity in cultured Hepa 1c1c7 cells; more than 10 compounds exhibited CD values less than 1 μ*M* with CI values more than 50. The most potent compounds

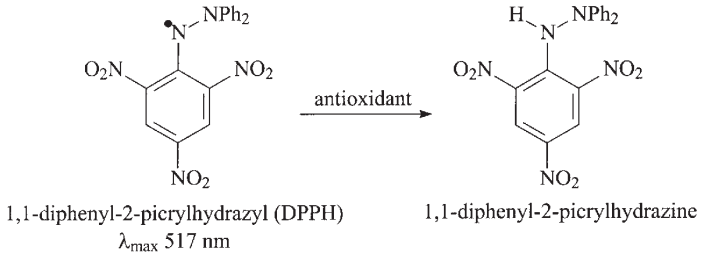

Fig. 2. Antioxidant assay employing DPPH.

were *para*-substituted flavones (**25–28**), followed by several *ortho*-substituted chalcones (**29–32**). 4′-Bromoflavone (**26**, 4′BF) was selected for further development, as it showed potent inducing capability (CD = 10 n*M*) without toxicity in Hepa 1c1c7 or H4IIE cells (IC_{50} >100 μ*M*) to give a CI above 10,000. Additionally, a simple synthesis of 4′BF was devised that allowed cost-effective scale-up of multikilograms of high-purity material. 4′BF was shown to reduce mammary tumor incidence from 89.5 to 30 and 20% in the 2 and 4 g 4′BF/kg diet groups of DMBA-induced female Sprague-Dawley rats, and reduced tumor multiplicity from 2.63 to 0.65 and 0.20, respectively *(40)*.

Use of the QR assay has consistently provided excellent leads, many of which have shown good activity in animal carcinogenesis models and are being considered for preclinical toxicology.

3. ANTIOXIDANTS

Free radicals, species with one or more unpaired electrons, are produced in normal or pathological cell metabolism by ionizing radiation or through transition metal-mediated molecular interactions. In biological systems, reactive oxygen species (ROS) formed by free radical processes are involved in both initiation and promotion of carcinogenesis *(80)*. Hydroxyl radicals, superoxide anions, and hydrogen peroxide are associated with initiation of carcinogenesis by causing heritable DNA damage through mutation of DNA or alteration of DNA repair enzymes *(81)*. Oxygen radicals and related species may also be involved in tumor promotion by signaling expression of protooncogenes and other growth factors *(82)*.

As biological systems have co-evolved with aerobic metabolism to counteract damage from ROS, a strategy of enhancing endogenous antioxidant defense systems with dietary or pharmaceutical agents is a reasonable approach *(83,84)*. Several assay systems have been developed to discover potentially active compounds from synthetic and natural sources *(39)*. It is important to note that a potential antioxidant lead must be evaluated in more than one antioxidant assay due to variability in the different test systems *(85)*.

One assay system is based on the ability of a potential antioxidant to scavenge the stable radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH), producing 1,1-diphenyl-2-picrylhydrazine (Fig. 2). The nitrogen radical of DPPH imparts a deep violet color at 517 nm in solution; this absorbance can be monitored to determine a sample's effectiveness in quenching free radicals. Although this assay has the advantage of high-throughput capability to quickly screen libraries of candidates for potential antioxidant activity, it is based on quenching a nitrogen radical and may yield a high number of false positives. Potential lead compounds from this assay should be confirmed with additional test systems, such as those described below.

A second assay system involves measuring superoxide anion inhibition in TPA-induced cultured HL-60 cells. HL-60 cells can be induced to differentiate with dimethylsulfoxide (DMSO) to give characteristics similar to neutrophils, and stimulation with TPA generates dose-dependent quantities of superoxide anion *(86,87)*. Using cytochrome c as a monitor, the inhibitory effects of test samples can be determined.

A third antioxidant assay uses the xanthine/xanthine oxidase system, where superoxide anion scavenging capacity and inhibition of xanthine oxidase is measured by monitoring formation of uric acid at 295 nm (Fig. 3). Xanthine oxidase (XOD) is an enzyme that catalyzes hypoxanthine to xanthine and uric acid in the presence of molecular oxygen to yield superoxide anion. Inhibition of XOD or quenching of superoxide anion results in dose-dependent reduction of uric acid production.

Using the DPPH assay as a primary screen and validating potential leads with the HL-60/TPA and/or XOD

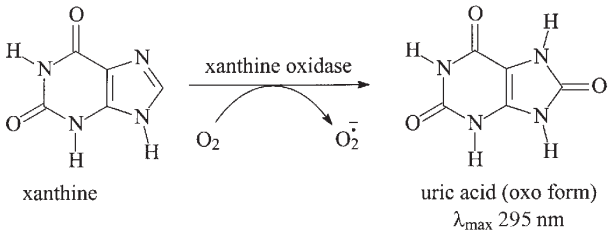

Fig. 3. Antioxidant assay employing xanthine.

systems, a variety of naturally occurring compounds were identified as potential antioxidants (summarized in Table 2). A variety of flavonoids from *Chorizanthe diffusa* Benth. (Polygonaceae) (**33–37**, Fig. 4) were shown to have moderate antioxidant activity in both DPPH and HL-60 antioxidant assays *(38,39)*. An extract of *Mezoneuron cucullatum* Roxb. (Leguminosae) yielded two stilbenes, resveratrol (**38**) and piceatannol (**39**), that were active in the HL-60 antioxidant assay; the flavone apigenin (**40**) and chalcone isoliquiritigenin (**41**) exhibited potent activity in the XOD assay *(39)*. Scirpusin A (**42**), also from *M. cucullatum*, showed only moderate activity in either of the DPPH and XOD assays. *Cerbera manghas* L. (Apocynaceae) produced olivil (**43**), (−)-carinol (**44**), and (+)-cycloolivil (**45**) as moderately active compounds in the DPPH assay *(53)*. Two flavonol glucosides (**46**,**47**) from *Daphniphyllum calycinum* Benth. (Daphniphyllaceae) *(88)*, and an epicatechin (**48**) from *Antirhea acutata* (DC.) Urb. (Rubiaceae) *(89)*, were isolated using the DPPH assay, with **48** showing activity in the cytochrome c reduction assay as well. An ornamental shrub, "smoke tree" or *Continus coggygria* Scop. (Anacardiaceae), yielded gallic acid and gallic acid esters, the aurones sulfuretin (**49**) and sulfurein (**50**), and the biaurone disulfuretin (**51**). The Malaysian island shrub *Gyrinops walla* Gaertn. (Thymelaeaceae) yielded mangiferin (**52**) through bioassay-guided fractionation with the DPPH assay *(90)*.

4. CYCLOOXYGENASE INHIBITION

The involvement of prostaglandins (PGs) and other eicosanoids in the development of human cancer has been known for more than two decades *(91)*. Importantly, an increase in PG synthesis may influence tumor growth in human beings and experimental animals *(92)*; numerous studies have illustrated the effect of PG synthesis on carcinogen metabolism, tumor cell proliferation, and metastatic potential *(93,94)*. As a result, inhibition of PG synthesis has been examined as a means of preventing tumor development *(94,95)*. Two major observations demonstrate the role of PGs in cancer genesis: inhibition of PG synthesis hinders tumor development in animal models and in some human cancers *(93)*, and a direct relationship between the levels of PG synthesized and cancer incidence in both humans and animal models. Moreover, members of the arachidonic acid (AA) metabolizing enzyme family seem to play a significant role in carcinogenesis, since modulation of these pathways results in suppression of tumor growth *(96)*.

PGs produced by cyclooxygenases (COXs) are represented by a large series of compounds that mainly enhance cancer development and progression, acting as carcinogens or tumor promoters with profound effects on carcinogenesis *(97)*. Most malignant human tumors have a prolonged period of pathological development during which they pass through several preneoplastic and premalignant stages. This situation affords the opportunity of interrupting or reversing tumorigenesis at an early stage *(22,98)*. Thus, the ability to regulate the COX pathway provides a reasonable opportunity for cancer chemoprevention.

Metabolites of AA, e.g., PGs, prostacyclins, and thromboxanes, are produced in many tissues and facilitate a diverse group of physiological and pathophysiological responses. For example, these bioactive lipids are potent mediators of several signal transduction pathways that modulate cellular adhesion, growth, and differentiation *(99)*.

Cleaved from membrane phospholipids by phospholipases, AA can then be metabolized by the COX pathway to produce PGs (Fig. 5). COX, also known as PGH-synthase, is the rate-limiting enzyme in the metabolic conversion of AA to PGs and related eicosanoids. AA is converted to PGH_2 through the action of COX, which exhibits two distinct catalytic activities, cyclooxygenase and peroxidase *(100)*. Subsequently, PGH_2 is converted by cell-specific synthases to products such as PGE_2, $PGF_{2\alpha}$, PGI_2, or thromboxanes *(101)*.

Table 2
Antioxidant Activity of Plant Natural Products

	Compound	*DPPH*[a] *(μg/mL)*	*HL-60*[b] *(μg/mL)*	*XOD*[c] *(μg/mL)*
33	quercetin	15.0	9.3	ND[d]
34	5,8,3′,4′-tetrahydroxy-3,7′-dimethoxyflavone	29.8	>50	ND[d]
35	5,7,3′,4′-tetrahydroxy-3-methoxyflavone	39.2	25.7	ND[d]
36	5,8,3′,4′,5′-pentahydroxy-3,7-dimethoxyflavone	10.4	21.6	ND[d]
37	3′′-*O*-acetylquercitrin	12.0	18.4	ND[d]
38	resveratrol	94.6	6.2	59.1
39	piceatannol	68.4	3.4	10.9
40	apigenin	>200	>20	2.4
41	isoliquiritigenin	>200	>20	8.6
42	scirpusin A	78.0	>20	38.5
43	olivil	37.5	ND[d]	ND[d]
44	(−)-carinol	15.8	ND[d]	ND[d]
45	(+)-cycloolivil	27.5	ND[d]	ND[d]
46	5,6,7,4′-tetrahydroxyflavonol-3-*O*-rutinoside	43.2	ND[d]	ND[d]
47	kaempferol 3-*O*-neohesperidoside	79.6	ND[d]	ND[d]
48	8-[1-(3,4-dihydroxyphenyl)-3-methoxy-3-oxopropyl]epicatechin	29.1[e]	ND[d]	ND[d]
49	sulfuretin	18.7	ND[d]	ND[d]
50	sulfurein	16.1	ND[d]	ND[d]
51	disulfuretin	9.7	ND[d]	ND[d]
52	mangiferin	20.1	ND[d]	ND[d]
	gallic acid[f]	3.6	ND[d]	ND[d]

[a]ED_{50} of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging.

[b]IC_{50} of TPA-induced free radical formation in cultured HL-60 cells.

[c]IC_{50} of XOD (xanthine/xanthine oxidase) activity.

[d]Not determined.

[e]ED_{50} in μ*M*.

[f]Positive control standard.

Two COX isoforms, COX-1 and COX-2, are known. COX-1 is constitutively expressed in many tissues *(101,102)*; PGs produced by COX-1 are thought to mediate housekeeping functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and platelet aggregation *(103–105)*. In contrast to COX-1, the isoform COX-2 is not generally detected in most tissues *(106)*. However, COX-2 is an inducible enzyme expressed in response to pro-inflammatory agents, including cytokines, endotoxins, growth factors, tumor promoters, and mitogens *(107)*. COX-2 is expressed in a few specialized tissues such as brain, testes, and macula densa of the kidney, in the apparent absence of any induction process.

Since COX plays an important role in cancer development, we have tested more than 2000 plant extracts for inhibition of COX-1 and COX-2. The assay is based on measurement of PGE_2 produced in the COX reaction via an enzyme immunoassay (Fig. 6). The effect of test compounds on COX activity is determined by measuring PGE_2 production. Reaction mixtures are prepared in 100 m*M* Tris-HCl buffer, pH 8.0, containing 1 μ*M* heme, 500 μ*M* phenol, 300 μ*M* epinephrine, sufficient amounts of COX-1 or COX-2 to generate 150 ng of PGE_2/mL, and various concentrations of test samples. The reaction is initiated by adding AA (final concentration, 10 μ*M*) and incubating for 10 min at room temperature (final volume, 200 μL). Reactions