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Mechanistic Considerations in Chemopreventive Drug Development

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Abstract This overview of the potential mechanisms of chemopreventive activity will provide the conceptual groundwork for chemopreventive drug discovery, leading to structure-activity and mechanistic studies that identify and evaluate new agents. Possible mechanisms of chemopreventive activity with examples of promising agents include carcinogen blocking activities such as inhibition of carcinogen uptake (calcium), inhibition of formation or activation of carcinogen (arylalkyl isothiocyanates, DHEA, NSAIDs, polyphenols), deactivation or detoxification of carcinogen (oltipraz, other GSH-enhancing agents), preventing carcinogen binding to DNA (oltipraz, polyphenols), and enhancing the level or fidelity of DNA repair (NAC, protease inhibitors). Chemopreventive antioxidant activities include scavenging reactive electrophiles (GSH-enhancing agents), scavenging oxygen radicals (polyphenols, vitamin E), and inhibiting arachidonic acid metabolism (glycyrrhetinic acid, NAC, NSAIDs, polyphenols, tamoxifen). Antiproliferation/antiprogression activities include modulation of signal transduction (glycyrrhetinic acid, NSAIDs, polyphenols, retinoids, tamoxifen), modulation of hormonal and growth factor activity (NSAIDs, retinoids, tamoxifen), inhibition of aberrant oncogene activity (genistein, NSAIDs, monoterpenes), inhibition of polyamine metabolism (DFMO, retinoids, tamoxifen), induction of terminal differentiation (calcium, retinoids, vitamin D_3), restoration of immune response (NSAIDs, selenium, vitamin E), enhancing intercellular communication (carotenoids, retinoids), restoration of tumor suppressor function, induction of programmed cell death (apoptosis) (butyric acid, genistein, retinoids, tamoxifen), correction of DNA methylation imbalances (folic acid), inhibition of angiogenesis (genistein, retinoids, tamoxifen), inhibition of basement membrane degradation (protease inhibitors), and activation of antimetastasis genes.

A systematic drug development program for chemopreventive agents is only possible with continuing research into mechanisms of action and thoughtful application of the mechanisms to new drug design and discovery. One approach is to construct pharmacological activity profiles for promising agents. These profiles are compared among the promising agents and with untested compounds to identify similarities. Classical structure-activity studies are used to find optimal agents (high efficacy with low toxicity) based on good lead agents. Studies evaluating tissue-specific and pharmacokinetic parameters are very important. A final approach is design of mechanism-based assays and identification of mechanism-based intermediate biomarkers for evaluation of chemopreventive efficacy. 1994 Wiley-Liss, Inc. •

Key words: Chemoprevention, drug development, mechanism of action, cancer

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Chemoprevention is the administration of agents to prevent induction, inhibit, or delay progression of cancers [1]. The basic cancer-related chemical and biological sciences, pathology, and epidemiology have contributed to the understanding that mutagenesis and uncontrolled proliferation are the important general mechanisms of carcinogenesis. This understanding suggests that cancer chemoprevention may be accomplished by interfering with these mechanisms. Elucidation of specific pathways involved in mutagenesis and proliferation provides fruitful leads for the development of chemopreventive agents. Since Wattenberg's pioneering studies in the early 1970's, a great deal of progress has been made in developing chemopreventive agents for clinical use [2–8]. More than 1,500 naturally occurring and synthetic chemicals have been tested for chemopreventive activity in experimental models [9,10]. These models, including animal cancers, animal and human cells subject to transformation, precancerous lesions, mutagenicity, DNA binding, and enzyme activities, are described elsewhere [10–20]. Numerous tested chemical structural and biological activity classes have shown activity. Examples of structural classes are arylalkyl isothiocyanates, cinnamyl compounds, flavonoids, glucarates, indoles, polyphenols, retinoids and carotenoids, and thiols and sulfides. Biological activity classes include antihormones, antiinflammatory agents, antioxidants, arachidonic acid (AA)[1] metabolism inhibitors, glutathione (GSH) inducers, ornithine decarboxylase (ODC) inhibitors, and protein kinase C (PKC) inhibitors. This paper examines the potential mechanisms of chemoprevention vested in these structural and pharmacological classes providing the conceptual groundwork for chemopreventive drug discovery that leads to structure-activity and mechanistic studies to identify and evaluate new agents.

MECHANISMS OF INHIBITION

Table I lists potential mechanisms of chemoprevention and promising chemopreventive agents associated with these mechanisms. These mechanisms are not yet fully understood; the evidence for their involvement in carcinogenesis inhibition is presented briefly below and has been described in several comprehensive reviews [particularly 4–6,10,21–28]. It should be noted throughout the following discussion that correlation to carcinogenesis inhibition does not necessarily confirm that the mechanism being reviewed is the most important or the only mechanism by which that carcinogenic activity is being inhibited. As will be evident in the descriptions below, many of the chemopreventive agents have potential for acting by multiple mechanisms. Agents used as examples are usually those with considerable promise as chemopreventives. Most are being or will soon be evaluated in clinical chemoprevention trials [7,8]. The experimental and epidemiological data demonstrating the chemopreventive efficacy of these agents has been reviewed extensively [*e.g.*, 9,10]; therefore, unless otherwise stated, the data cited below are from one of the reviews [10] instead of the publications containing the primary data. It should be noted that the review does contain comprehensive citations to original experimental data; new primary data publications not covered are cited in this paper.

CARCINOGEN BLOCKING ACTIVITIES

Inhibit Carcinogen Uptake

Agents with this activity appear to react directly with putative carcinogens, both initiators and promoters. For example, calcium inhibits the promotion of colon tumors induced in rats by azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH) and high dietary fat. It also inhibits colonic hyperproliferation induced in rats by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), in mice by deoxycholic acid, fatty acids, or cholic acid, and in rats and mice by a Western "stress" diet—*i.e.*, a diet low in calcium and vitamin D and high in fat and phosphate. A partial explanation of these effects is that calcium binds to excess bile and free fatty acids that irritate the colon lumen and promote the formation of tumors [29–32]. Such data suggest a potential for other sequestering and chelating agents as chemopreventives, particularly in the colon.

Inhibit Formation or Activation of Carcinogen

Vitamin C inhibition of the formation of carcinogenic *N*-nitroso compounds is probably the best known representative of this mechanism [24]. For example, in mice, vitamin C inhibited the induction of lung tumors by the combination of methylurea

[1]Please see Appendix A: Abbreviations at the end of this Supplement.

Table I. Possible Mechanisms of Chemoprevention

Carcinogen Blocking Activities

Inhibit Carcinogen Uptake: Calcium
Inhibit Formation or Activation of Carcinogen: Arylalkyl Isothiocyanates, DHEA, NSAIDs, Polyphenols
Deactivate/Detoxify Carcinogen: Oltipraz, Other GSH-Enhancing Agents
Prevent Carcinogen Binding to DNA: Oltipraz, Polyphenols
Increase Level or Fidelity of DNA Repair: NAC, Protease Inhibitors

Antioxidant Activities

Scavenge Reactive Electrophiles: GSH-Enhancing Agents
Scavenge Oxygen Radicals: Polyphenols, Vitamin E
Inhibit Arachidonic Acid Metabolism: Glycyrrhetinic Acid, NAC, NSAIDs, Polyphenols, Tamoxifen

Antiproliferation/Antiprogression

Modulate Signal Transduction: Glycyrrhetinic Acid, NSAIDs, Polyphenols, Retinoids, Tamoxifen
Modulate Hormonal/Growth Factor Activity: NSAIDs, Retinoids, Tamoxifen
Inhibit Oncogene Activity: Genistein, NSAIDs, Monoterpenes
Inhibit Polyamine Metabolism: DFMO, Retinoids,Tamoxifen
Induce Terminal Differentiation: Calcium, Retinoids, Vitamin D_3
Restore Immune Response: NSAIDs, Selenium, Vitamin E
Increase Intercellular Communication: Carotenoids, Retinoids
Restore Tumor Suppressor Function
Induce Programmed Cell Death (Apoptosis): Butyric Acid, Genistein, Retinoids, Tamoxifen
Correct DNA Methylation Imbalances: Folic Acid
Inhibit Angiogenesis: Genistein, Retinoids, Tamoxifen
Inhibit Basement Membrane Degradation: Protease Inhibitors
Activate Antimetastasis Genes

and nitrite; all the compounds were administered orally. Other chemopreventive antioxidants such as vitamin E prevent the formation of nitrosamines from their precursors by scavenging nitrite.

Another method of inhibiting formation of a carcinogen is by preventing metabolic activation of a procarcinogen. Many chemopreventives have this type of activity, including allylic sulfides, arylalkyl isothiocyanates, carbamates, and flavonoids and other polyphenols [reviewed in 10]. For example, the polyphenol ellagic acid is a well-known inhibitor of the cytochrome P-450 enzymes responsible for activating polycyclic aromatic hydrocarbon (PAH) carcinogens such as benzo(*a*)pyrene (B(*a*)P) and 3-methylcholanthrene (MCA). This inhibition is observed in the lungs of strain A mice and in cultured mouse keratinocytes [10]. In accord with this potential mechanism, ellagic acid inhibits PAH-induced skin tumors in mice on both topical and oral administration.

Recent work with arylalkyl and alkyl isothiocyanates by Chung and his colleagues [33] provides a useful model for the application of structure-activity studies to identify or design more potent analogs of good lead agents. The isothiocyanates are known to affect a number of activities associated with nitrosamine metabolism, including the crucial step of α-C-hydroxylation [34–36]. These

compounds are potent inhibitors of nitrosamine-induced tumors. Naturally occurring phenethyl isothiocyanate (PEITC) inhibits the induction of lung tumors in mice and rats by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [36–38]. In the structure-activity study, Chung *et al.* found that synthetic PEITC analogs with longer alkyl chains, such as phenbutyl and, particularly, phenhexyl isothiocyanate (and at high doses the phenoctyl and phendecyl derivatives), are even more effective than their parent in inhibiting NNK-induced lung tumors in mice [33,38–40]. Further, the phenyl moiety is not required for activity. Alkyl isothiocyanates such as hexyl isothiocyanate are also active in the mouse lung model. As for the arylalkyl compounds, activity increases with the length of the alkyl chain, the dodecyl isothiocyanate being more potent than the hexyl analog [33]. Correlations of tumor inhibitory activity with chemical properties associated with longer alkyl chain length indicated that lipophilicity was directly related to potency, while reactivity with GSH was inversely correlated to potency. The investigators interpreted these results as suggesting that potency was determined by ability to bind to hydrophobic cytochrome P-450s (*i.e.*, lipophilicity) and slower metabolism (decreased reactivity with GSH).

Aromatase, a cytochrome P-450-dependent enzyme, catalyzes the first step in estrogen biosynthesis in humans: C-19 hydroxylation and subsequent oxidative cleavage of the androgens androstenedione and testosterone to estrone and estradiol, respectively [41,42]. Several compounds that inhibit aromatase also inhibit chemical carcinogenesis in estrogen-sensitive tissues. For example, 4-hydroxyandrost-4-ene-3,17-dione, a suicide inhibitor of aromatase, inhibits 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced tumors in rat mammary glands [42]. When administered after DMBA, it reduces tumor multiplicity and size and causes regression of existing tumors [43]. CGS 18320b [butanedioic acid, complexed with 4,4'-(1*H*-imidazolylmethylene)bis(benzonitrile) (1:2)] is a competitive inhibitor of aromatase; it reduces tumor incidence in rat mammary glands when administered at the same time as and after the carcinogen, DMBA [44].

Deactivate/Detoxify Carcinogens

Carcinogen deactivation and detoxification is generally regarded as a very important mechanism of carcinogenesis inhibition [4,6]; enhancement of this detoxification may prove to be an important strategy for chemoprevention. Two metabolic pathways are critical. The first is the introduction or exposure of polar groups (*e.g.*, hydroxyl groups) on xenobiotic compounds via the phase I metabolic enzymes, which are primarily the microsomal mixed-function oxidases. In many cases, the polar groups become substrates for conjugation. The second pathway is via the phase II metabolic enzymes responsible for conjugation and the formation of glucuronides, GSH conjugates, and sulfates. Chemopreventive agents that can act via this mechanism have been divided into two groups. Type A compounds are those that primarily enhance the activity of phase II enzymes, particularly GSH-*S*-transferases and also UDP-glucuronyltransferase [4,6,45,46]. Type B inhibitors induce increases in microsomal mixed-function oxidase activities, while also increasing the activity of the major phase II enzymes. Type A compounds are now considered more promising chemopreventive agents [4,6,46], since the enzymes in the microsomal mixed-function oxidase system induced by Type B agents are more likely to increase carcinogen activation.

GSH is a prototype carcinogen scavenger (see also under the more general mechanism of electrophile scavenging below). It reacts spontaneously or via catalysis by GSH-*S*-transferases with numerous activated carcinogens including MNNG, AFB_1 and B(*a*)P-diolepoxide and other activated PAHs [21-,47]. Likewise, GSH protects against mouse skin tumors induced by DMBA/12-*O*-tetradecanoylphorbol-13-acetate (TPA), rat forestomach tumors induced by MNNG, and rat liver tumors induced by aflatoxin B_1 (AFB_1).

A number of promising chemopreventive agents are potent inducers of GSH and GSH-*S*-transferases. Prominent among these compounds are the allylic sulfides, natural products found in onion, garlic, and other members of the *Allium* genus. Oltipraz[5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] is a potent GSH-*S*-transferase inducer with a wide spectrum of chemopreventive activity. *N*-Acetyl-*l*-cysteine (NAC) is essentially a precursor of GSH. NAC shows inhibitory activity in rat colon against DMH- and AOM [48]-induced tumors, in mouse lung against urethane-induced tumors, in rat mammary glands against MNU-induced tumors, and in mouse bladder against *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN)-induced tumors [48].

GSH-peroxidases (GSH-Px) catalyze the reduction of H_2O_2 and organic hydroperoxides [49–51]; the antioxidant effects of selenium may be related to its function in the enzyme's active site. Although

several studies show that the anticarcinogenic activity of selenium in mouse and rat mammary glands is not mediated by GSH-Px [52–54], in tissues such as colon [55], glandular stomach [56], and skin [50], GSH-Px are thought to play a role.

Another type of inhibition is modulation of the mixed-function oxidases involved in the metabolism of estrogens. Indole-3-carbinol, a compound which occurs naturally in cruciferous vegetables, inhibits the induction of mammary tumors in rats, and induces mixed-function oxidases [57]. Particularly, it induces the activity of the enzymes responsible for 2-hydroxylation of estradiol, leading to increased excretion of estradiol metabolites [58].

Prevent Carcinogen Binding to DNA

DNA-carcinogen adduct formation can be considered a measure of carcinogen exposure [59]. In most cases, it is probably secondary to other mechanisms of carcinogenesis, such as carcinogen activation and formation. Likewise, inhibition of DNA adduct formation is typically an indirect measure of other mechanisms of chemoprevention, particularly inhibition of carcinogen formation and activation and enhancement of carcinogen detoxification. For example, oltipraz prevents the formation of AFB_1-DNA adducts, an effect which has been attributed to increased rates of aflatoxin detoxification by GST [59,60]. Nonetheless, inhibition of DNA adduct formation is a convenient assay for screening potential chemopreventive agents which are expected to modulate carcinogen metabolism.

There is also limited evidence of chemopreventive agents directly obstructing adduct formation. For example, the inhibition of nitroso compound carcinogenesis by ellagic acid has been attributed to blocking the methylation of guanine at the O^6 position [24,61,62]. Presumably, this effect is due to ellagic acid binding to the duplex form of DNA [21,61].

Increase Level or Fidelity of DNA Repair

There are three possible chemopreventive mechanisms that involve DNA repair. First is an increase in the overall level of DNA repair. Second, the enzyme poly(ADP-ribosyl)transferase (ADPRT) is known to be involved in modulation of DNA damage [63,64], and the level of this enzyme is decreased in the presence of carcinogens [65]. For example, NAC prevents the decrease in ADPRT caused by the carcinogen 2-acetylaminofluorene (AAF) [65]. The third mechanism is suppression of error-prone DNA repair. It is known that protease inhibitors depress error-prone repair in bacteria [66], and it has been suggested that they might prevent carcinogenesis by inhibiting an error-prone repair system activated by proteases that, in turn, are induced by tumor promoters [67]. The protease inhibitor best studied as a chemopreventive is Bowman-Birk Soybean Trypsin Inhibitor (BBI), which inhibits DMH-induced tumors in mouse colon and liver, and *N*-methylbenzylnitrosamine (MBN)-induced tumors in rat esophagus.

ANTIOXIDANT ACTIVITIES

Scavenge Reactive Electrophiles

Potent nucleophiles that react directly with carcinogens and other electrophiles may be chemopreventives by this mechanism. The specific reaction of ellagic acid with B(*a*)P-diolepoxide and reactions of GSH with activated carcinogens are described above. NAC is another example of an electrophile scavenger.

Scavenge Oxygen Radicals

There is abundant evidence that activated oxygen species (*i.e.*, singlet oxygen, peroxy radicals, superoxide anion, and hydroxyl radical) are involved in carcinogenesis [*e.g.*, 26,68,69]. Potentially, they act both in initiation and in promotion and progression. For example, oxygen radicals can oxidize DNA bases [70], producing mutagenic lesions [*e.g.*, 71]. Radicals also cause DNA strand breaks and chromosome deletions and rearrangements [72]. Further, they participate in the activation of certain carcinogens, as evidenced by the involvement of peroxidation in activating B(*a*)P-7,8-diol to an intermediate that binds DNA and is mutagenic in bacteria and mammalian cells [26,73]. Kensler [26] has listed carcinogens that form free radicals. Besides PAHs such as B(*a*)P, the list includes aromatic amines (*e.g.*, AAF, azo dyes), hydrazines (*e.g.*, DMH), nitrofurans (*e.g.*, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide) and quinones.

Activated oxygen species most likely play a more important role in tumor promotion and progression. Kensler [26] has summarized the experimental evidence for this involvement. (1) Oxygen radical generating systems (*e.g.*, superoxide anion generation via xanthine oxidase) show activities *in*

vitro similar to those of known tumor promoters. These activities include increasing transformation frequencies of fibroblasts and keratinocytes and increasing transcription of genes associated with early steps in cell proliferation including c-*fos*, c-*myc*, c-*jun*, and ODC [74–76]. (2) Inflammatory cells produce a wide range of reactive oxygen species; there is evidence associating inflammation with cancers in various tissues including stomach, esophagus, colon/rectum, liver, pancreas, mouth, lung, skin, and bladder [77,78, see also the discussion below on AA metabolism]. (3) Tumor promoters stimulate the endogenous production of oxygen radicals in inflammatory cells and keratinocytes. (4) Tumor promoters inhibit endogenous activities that protect against oxidative damage (*e.g.*, GSH-Px, catalase, and superoxide dismutase). (5) Free radical-generating agents such as benzoyl peroxide and butylated hydroxytoluene hydroperoxide are tumor promoters in mouse skin.

There is direct evidence that scavenging activated oxygen species is a chemopreventive mechanism. For example, NAC and other chemopreventive thiols are known to react with hydroxyl radicals [24,79,80]. The reaction of β-carotene with singlet oxygen [*e.g.*, 81,82] and its participation in other free radical-trapping reactions [*e.g.*, 83–88] is well-documented. β-Carotene inhibits DMH-induced colon tumorigenesis in mice and DMBA-induced squamous cell carcinoma in rat salivary glands and in hamster buccal pouch and forestomach. In mouse skin, it also inhibits DMBA-induced tumors and DMBA-initiated tumors promoted by croton oil or TPA. The rationale for the development of β-carotene as a chemopreventive agent was based on case-control epidemiology data from lung cancer patients, a chemical structure indicating ability to scavenge free radicals, and bioconversion to vitamin A.

Phenolic antioxidants are known to scavenge peroxy radicals [24,26,89]; particularly, vitamin E (α-tocopherol) is known to scavenge peroxy radicals [*e.g.*, 24,69,90–93], singlet oxygen [*e.g.*, 51,94], and superoxide radicals [*e.g.*, 24]. Vitamin E inhibits the induction of buccal pouch cancer in hamsters by DMBA and the induction by DMH of colon tumors in mice and of colon and small intestine tumors in rats. Like β-carotene, vitamin E inhibits tumors induced by DMBA/TPA or DMBA/croton oil in mouse skin.

Non-phenolic antioxidants also scavenge oxygen free radicals. For example, GSH reacts with alkylperoxy radicals [26]. The inhibitory activity of GSH against the promotion of DMBA-induced skin tumors by TPA is cited above. A very interesting group of compounds recently described appear to work by inducing oxygen radical scavenging proteins such as Mn-superoxide dismutase, catalase, and GSH-Px [95]. Examples include the protein-bound polysaccharide PSK-1 and bismuth nitrate.

Antioxidants which are potent inhibitors of AA metabolism perform perhaps the most prominent oxygen free radical scavenging activity associated with inhibition of carcinogenesis. Numerous chemicals with this activity, described below, are proving to be good chemopreventive agents.

Inhibit AA Metabolism

Marnett [96] and Zenser and Davis [28] have reviewed the role of AA metabolism in carcinogenesis. AA is metabolized to prostaglandins (PGs), thromboxanes, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) via oxidative enzymes. Activated oxygen species and alkylperoxy species are formed throughout this process; AA metabolism is increased during inflammation. Two aspects of AA metabolism are associated strongly with carcinogenicity; both are inhibited by antioxidants and antiinflammatory agents.

The first is the PG synthetic pathway, involving the enzyme prostaglandin H synthase (PHS). This enzyme has two activities—cyclooxygenase, which catalyzes the formation of prostaglandin G_2 (PGG_2) from arachidonic acid, and hydroperoxidase, which catalyzes the reduction of PGG_2 to PGH_2. To return to its native state, the hydroperoxidase requires a reducing cosubstrate; procarcinogens, for example arylamino and arylnitro compounds, are such substrates. According to the model proposed, the procarcinogens are activated (oxidized) during catalysis to free radicals and electrophiles that can form adducts with DNA and initiate carcinogenesis. This process can be stopped four ways: (1) at formation of PGG_2 via inhibition of cyclooxygenase, (2) by inhibition of peroxidase activity, (3) by prevention of formation of reactive intermediates, and (4) by scavenging reactive intermediates (*e.g.*, by GSH conjugation). Relevant to these potential mechanisms, cyclooxygenase inhibitors such as nonsteroidal antiinflammatory drugs (NSAIDs—*e.g.*, aspirin, ibuprofen, indomethacin, piroxicam) and certain antioxidants (*e.g.*, flavonoids) are effective inhibitors of carcinogenesis. Additionally, PGH_2 itself breaks down to form a known direct-acting mutagen, malondialdehyde [96]. Thus, inhibition of cyclooxygenase may directly prevent the formation of a potential carcinogen.

The activity demonstrated against colon and bladder cancer in animal models by NSAIDs [48,96–105], which are potent cyclooxygenase inhibitors, suggests the potential for developing chemopreventive drugs based on blocking this enzyme. However, PGs and other PHS products such as thromboxanes have multiple activities, both beneficial and deleterious, some of which are tissue-specific. For example, PGE_2 in the gut promotes protective mucosal secretions; lowered gut PG levels resulting from NSAID administration are associated with one of the major side effects of long-term NSAID treatment, gastrointestinal ulceration and bleeding [*e.g.*, 106]. Likewise, PGs in the kidney and thromboxanes in platelets are important to normal physiological function. Their inhibition is associated with renal tubule toxicity and excessive bleeding, respectively [107]. The development of chemopreventive agents which retain the ability to inhibit the carcinogenesis-associated activities of PGs without depressing protective effects is an attractive strategy. The discovery of an inducible form of cyclooxygenase (COX-2), which is predominant at inflammation sites, in macrophages and in synoviocytes, suggests that such an approach is feasible. In contrast to COX-2, constitutive cyclooxygenase (COX-1) predominates in the stomach, gastrointestinal tract, platelets, and kidney. NSAIDs inhibit both forms of the enzymes, but other compounds inhibit COX-2 selectively—glucocorticoids such as dexamethasone (which, for example, has chemopreventive activity on topical application to mouse skin) and a newly synthesized NSAID, NS-398 [108,109].

The second aspect of AA metabolism associated with carcinogenesis is the burst of PHS and lipoxygenase activity that is seen during inflammation and is stimulated by the tumor promoter TPA. The available evidence suggests that the immediate products of lipoxygenase activity, the HETEs and their hydroperoxy precursors (HPETEs), are more important to tumor promotion than are PGs. Compounds that inhibit lipoxygenase, such as vitamin E, inhibit tumor promotion in mouse skin. Likewise, lipoxygenase inhibitors that are stable one-electron donors which competitively inhibit the production of unstable free radicals and electrophiles by PHS (*e.g.*, curcumin, the flavonoids, and tea polyphenols) also inhibit tumor promotion in mouse skin. Most antiinflammatories that predominantly inhibit cyclooxygenase (*e.g.*, aspirin) are not effective inhibitors of tumor promotion in mouse skin [28].

The release of AA from membrane phospholipids is another control point in the AA metabolic pathway. This release is catalyzed by phospholipases. Position *sn*-2 is the predominant location of AA on phospholipids; phospholipase A_2 catalyzes release of AA from this position. No specific phospholipase inhibitors have been developed as chemopreventive agents. Phospholipase A_2 inhibitors such as glucocorticoids and 4-bromophenacyl bromide do inhibit carcinogenesis. However, these compounds also inhibit other enzymes in the AA pathway, so their inhibition of carcinogenesis cannot be attributed directly to inhibition of phospholipase A_2. Likewise, AA can be released from phospholipids by other mechanisms—*e.g.*, via phospholipase C (PLC) and diacylglycerol (DAG) lipase and increases in intracellular calcium resulting in higher levels of phospholipase activity [28]. Interestingly, the NSAID aspirin may be an inhibitor of PLC [110].

The control of AA release by DAG lipase and phospholipases may be mediated via signal transduction pathways. Compounds that block signal transduction at the membrane level, including tamoxifen, flavonoids, and glycyrrhetinic acid, may inhibit AA metabolism and be chemopreventive by this mechanism. AA metabolites, specifically PGs, also are believed to be involved in signal transduction pathways [28,111,112]. As discussed below, changes in activities in these pathways appear to be integrally involved in cancer promotion and progression. Thus, inhibition of AA metabolism may play a role in controlling these aspects of carcinogenesis, as well as directly blocking carcinogens and tumor promoters. PGE_2 is known to suppress the immune response in certain tumor cells [96,113,114]. Inhibitors of PHS may relieve this suppression.

ANTIPROLIFERATIVE ACTIVITIES

Modulate Signal Transduction

Weinstein [22] described carcinogenesis as a progressive series of disorders in the function of signal transduction pathways—signal transduction being the means by which the hormones and growth factors that regulate cell growth, proliferation, and differentiation communicate across cell membranes via receptors and receptor-associated enzymes, then through the cytoplasm and into the nucleus via a network of intermediary molecules known as second messengers [28]. Second messengers include cyclic AMP, inositol 1,4,5-triphosphate, DAG, PGs, and various regulatory proteins

such as the mitogen-activated protein (MAP) kinases. Whitfield [115], Brunton and Workman [116], and Powis [110] have provided recent comprehensive reviews of signal transduction pathways and their potential as targets of chemotherapeutic and chemopreventive drugs. The components of the signal transduction pathways provide many possible sites for chemopreventive activity by restoring normal cellular controls or inhibiting activities that are out of control. In fact, most of the antiproliferation/antiprogression activities important to chemoprevention impact some part of the signal transduction pathways. For example, one of the steps in signal transduction involves activation of PKC by DAG. There is evidence that carcinogenesis may be suppressed by inhibiting this enzyme. The phorbol tumor promoters such as TPA can replace DAG in activating PKC [117]. Chemicals that inhibit PKC, such as the flavonoids and glycyrrhetinic acid, also inhibit TPA-induced tumor promotion in mouse skin.

Further, invocation of the signal transduction pathways also provides a mechanistic rationale for the multiple chemopreventive effects of some classes of agents. For example, agents such as the retinoids, antihormones, and PKC inhibitors which affect activities at the cell membrane level and cytoplasmic and nuclear receptor levels, can also affect other connected events (see also the discussions of AA metabolism above and modulation of hormonal and growth factors and of polyamine metabolism below). Zenser and Davis [28] summarized the evidence that PGs are signal transduction intermediaries. This evidence includes increased PG synthesis observed during human keratinocyte proliferation [118], in MDCK cells treated with carcinogens and tumor promoters [119], in BALB/c 3T3 fibroblasts transformed by MCA [120], during tumor promotion in mouse skin [121], and in malignant tumors [28]. Although this observation is speculative, it is possible that cyclooxygenase inhibitors such as the NSAIDs inhibit tumor promotion by inhibiting these proliferative activities associated with PGs.

Modulate Hormonal/Growth Factor Activity

Chemicals may inhibit the cell growth and proliferation associated with carcinogenesis by directly regulating the induction and activity of specific hormones and growth factors that initiate steps in signal transduction. This regulation may occur at membrane level receptors (for growth factors, peptide hormones, and neurotransmitters) or via cytoplasmic and nuclear receptors (for the steroid superfamily consisting of estrogen, progesterone, retinoid, glucocorticoid, vitamin D, and thyroid receptors) [122]. For example, antiestrogens such as tamoxifen and toremifene bind to nuclear estrogen receptors, preventing the binding and activity of estrogens [123,124]. Both compounds inhibit carcinogen-induced, estrogen-sensitive tumors in rat mammary glands. Tamoxifen also inhibits the induction of kidney tumors in hamsters by estrogen (17β-estradiol).

Phytoestrogens, such as the isoflavone genistein, have antiestrogenic activity. Studies in human breast cancer MCF-7 cells indicate that the antiestrogenic effect may result from slowed translocation of genistein-bound receptor from the cytoplasm to the nucleus compared with that of estradiol-bound receptor [125].

Transforming growth factor β (TGF-β) has antiproliferative activity in both normal and neoplastic cells *in vitro* [126] and in mammary glands [127, 128] and liver [128,129] *in vivo*. Neoplastic cells such as A549 human lung carcinoma cells produce TGF-β, but usually in a latent form that cannot bind to its receptor; these cells are responsive to antiproliferative effects of activated TGF-β [126]. There is evidence from studies in rat intestinal crypt epithelial cells that TGF-β may also promote differentiation [130]. These observations suggest that chemicals that activate TGF-β could also control proliferation in carcinogenesis. Studies with MCF-7 cells and tamoxifen are interesting in this regard. These cells normally produce only small amounts of activated TGF-β, but treatment with tamoxifen or its metabolite, 4-hydroxytamoxifen, increases production up to 20-fold [126,131]. Retinoic acid, which inhibits chemical carcinogenesis, particularly tumor promotion in mouse skin, induces TGF-β_2 in mouse keratinocytes and in mouse skin *in vivo* after topical application. The increase in TGF-β_2 occurs post-transcriptionally. In vitamin A-deficient rats treated with retinoic acid, the level of expression of TGF-β correlates with levels of retinoids in skin, intestine, and respiratory epithelia [128,132].

There is also evidence of cross-regulation among membrane and nuclear receptors. For example, insulin-like growth factor I (IGF-I) stimulates cell replication in various tumors [133,134]. Particularly, human breast cancer cells have membrane receptors for and excrete IGF-I [133,135]. Tamoxifen lowers blood concentrations of IGF-I in breast cancer patients, suggesting that part of its antitumor activity is inhibition of IGF-I [133,135].

Other aspects of receptor activity may be modulated and are possible mechanisms for chemopreventive activity. Generally, receptors are phosphoproteins, and phosphorylation appears to play a role in receptor activation [122]. Thus, chemopreventive agents which inhibit phosphorylation, *i.e.*, inhibit protein kinases, may influence cell proliferation by effects on receptors. Examples are the isoflavone genistein, which is a specific inhibitor of tyrosine kinase [125,136], flavonoids, and glycyrrhetinic acid.

Another mechanism for prevention of hormone-stimulated growth is by deactivation of steroids. In this regard, aromatase inhibitors and modifiers of steroid hydroxylation have been described above under inhibition of carcinogen formation/activation and carcinogen deactivation/detoxification, respectively.

Inhibit Oncogene Activity

During the course of cell proliferation in carcinogenesis, numerous oncogenes are expressed abnormally—possibly as intermediates in the signal transduction pathways. The evidence for oncogene activity in signal transduction in carcinogenesis is based on the similarity of some of their products (protein kinases) to other intermediates [22]. There are several points during activation at which *ras* can be inhibited, and there are data relating this inhibition to chemopreventive activity. First, the membrane receptor-linked enzyme tyrosine kinase is involved in *ras* activation, and kinase inhibitors would be expected to prevent *ras* activation. Particularly interesting are compounds such as genistein [125] which specifically inhibit tyrosine kinase, and thus do not interfere with normal cellular processes mediated by other kinases.

To be activated, RAS must first be farnesylated. *ras* oncogenes are involved in mammary gland carcinogenesis induced by MNU [137] and, to a lesser extent, by DMBA [138]. Gould and his colleagues [139] showed that D-limonene inhibits the progression of mammary tumors induced in rats by MNU or DMBA. They also showed that D-limonene inhibits the farnesylation of small G proteins (21–26 kDa); these experimental data suggest that D-limonene could be preventing oncogene activation by inhibiting post-translational farnesylation of the p21 RAS protein [140]. Perillyl alcohol is an even more potent inhibitor of farnesyl-protein transferase [141]. Recently, several specific farnesylation inhibitors have been described which are structural analogs of the C-terminal tetrapeptide of farnesyl-protein transferase and inhibit the growth of *ras*-dependent tumors [*e.g.*, 142].

Similarly, it has long been known that nerolidol inhibits conversion of farnesyl pyrophosphate to squalene [143], suggesting that this terpene also might be a competitive inhibitor of other farnesylation reactions such as that of *ras* products. Wattenberg found that nerolidol effectively inhibits the proliferation and progression of colon carcinoma induced in rats by AOM [6,144].

Further, farnesyl pyrophosphate, the substrate for farnesyl-protein transferase, is an intermediate in the synthetic pathway from HMG CoA reductase to cholesterol. Inhibitors of HMG CoA reductase, particularly lovastatin [145], and probably inhibitors of other enzymes along the synthetic route to cholesterol, have been shown to inhibit RAS farnesylation.

Cyclooxygenase inhibitors also might inhibit proliferation in carcinogenesis by inhibition of oncogene expression [28], although the evidence is less direct than for other effects of AA metabolism inhibitors. Expression of the oncogene c-*myc* occurs early in epidermal growth factor (EGF)-induced cell proliferation. PGs are required but not sufficient for c-*myc* expression and DNA synthesis stimulated by EGF in BALB/c 3T3 cells [145]. Indomethacin inhibits both DNA synthesis and oncogene expression in this system; this inhibition is reversed by addition of PGG_2 [28,146,147].

Studies *in vitro* indicate inhibition of oncogene expression as a mechanism for inhibitory activity of protease inhibitors and retinoids. For example, the protease inhibitors 6-aminocaproic acid, antipain, and leupeptin inhibit transformation of NIH-3T3 cells transfected with activated H-*ras* oncogene [148]. 6-Aminocaproic acid inhibits DMH-induced colon tumors in rats; antipain inhibits urethane-induced tumors in mouse lung; and leupeptin inhibits DMBA/croton oil-induced carcinogenesis in mouse skin. Antipain, leupeptin, and BBI suppress c-*myc* expression in normal proliferating mouse fibroblasts [148]. Retinoic acid also inhibits H-*ras*-induced transformation in NIH-3T3 cells [148], and inhibits tumorigenicity in mouse skin.

Inhibit Polyamine Metabolism

Pegg [149] and Verma [27] have reviewed the evidence that polyamines play a significant role in cell proliferation, differentiation, and malignant transformation. The mode of action is not yet known, but it has been suggested that as polycations, the polyamines can stabilize DNA structures;

they have been shown to affect DNA and protein synthesis [27,150]. A critical step in polyamine biosynthesis is the synthesis of putrescine from ornithine that is catalyzed by ODC. There is ample evidence that ODC participates in carcinogenesis. For example, the enzyme is induced during cell transformation by chemical carcinogens [151,152], viruses [153–155] and oncogenes [156–159]. Transfection with vectors for human ODC-induced cell transformation, anchorage-independent growth, and also increased tyrosine phosphorylation in NIH-3T3 cells [160]. Blocking ODC with DFMO or antisense RNA inhibited v-*src* transformation (via inhibition of tyrosine phosphorylation) of rat 2R fibroblasts [160]. This observation led the investigators to suggest that ODC is an oncogene critical to regulation of cell growth and transformation.

Association with cell proliferation during carcinogenesis is also well established. TPA and other tumor promoters increase ODC activity in skin, colon, bladder, and liver [27]. In mouse skin, topically applied TPA causes an approximately 200-fold increase in ODC activity within 4.5 hours after treatment [27]. The increase is dose-dependent and correlates with the ability of the TPA dose to promote skin tumors. Also, the increased ODC activity has been proposed to be specific to tumor promotion, since most carcinogens that are not tumor promoters do not induce ODC [161].

Likewise, chemicals that inhibit induction of or deactivate ODC also inhibit carcinogenesis. Some of the most convincing results demonstrating that inhibition of ODC prevents cancers come from studies with DFMO. DFMO is a specific, mechanism-based irreversible inhibitor of ODC—that is, DFMO is activated by ODC into a form that reacts with the enzyme to inactivate it [149]. DFMO inhibits tumors induced by DMH in mouse colon, AOM in rat colon, DMBA and MNU in rat mammary glands, DMBA/TPA in mouse skin, OH-BBN and MNU in mouse urinary bladder, and OH-BBN in rat urinary bladder.

Verma and his colleagues found that ODC induction by TPA is regulated at the transcription level [162–165]. Regulation occurs in part via signal transduction events at the membrane level. For example, PKC appears to be involved [163,164], as are diverse signal transduction intermediates induced by TPA, including PGs [166], other products of AA metabolism [167], and free radicals [168]. Chemicals that inhibit PKC and AA metabolism and those that scavenge free radicals also may inhibit the induction of ODC; hence, they may be chemopreventives by this mechanism. In this regard, several of the PKC inhibitors, including glycyrrhetinic acid, inhibit ODC induction and tumor promotion in mouse skin. AA metabolism inhibitors also inhibit both ODC induction and TPA-promoted mouse skin tumorigenesis, as do free radical scavengers such as GSH and flavonoids and green tea polyphenols [169].

Vitamin A (retinol) and certain of its derivatives (*i.e.*, retinoids) inhibit carcinogenesis specifically during promotion. There is evidence that the cancer inhibitory activity of these compounds may be mediated partially by regulation of ODC induction. In mouse skin, Verma and Boutwell [170,171] found that inhibition of ODC induction by retinoic acid and analogs such as 13-*cis*-retinoic acid correlates to inhibition of TPA-induced tumor promotion. Analogs which do not inhibit ODC induction also do not inhibit tumor promotion. One of the most active retinoids is 4-HPR. This compound is a potent inhibitor of ODC induction [172] as well as of TPA promotion in mouse skin. It also inhibits mammary tumors in rats induced by DMBA or MNU and urinary bladder tumors in mice induced by OH-BBN when administered after carcinogen treatment is completed.

Inhibition of *S*-adenosyl-*l*-methionine (SAM) decarboxylase is another potential mechanism for inhibiting polyamine biosynthesis that may prove useful in chemoprevention [149,173]. This enzyme, like ODC, is highly regulated in mammalian cells and catalyzes the formation of the polyamines spermidine and spermine from putrescine. Another approach that has been suggested is blocking with non-functional polyamine analogs [174].

Induce Terminal Differentiation

Terminal differentiation is one of the steps in normal, regulated cell proliferation in epithelial tissues. Proliferating cancer cells often have lost the ability to differentiate [115]. These cancer cells are either deficient in or incapable of responding to differentiation signals [115]. Abundant evidence demonstrates that restoring the ability of abnormally proliferating cells to differentiate suppresses carcinogenesis. Several classes of chemopreventives also induce differentiation. Retinoids are the best-studied example. For many years it has been known that vitamin A deficiency causes squamous metaplasia and keratinization—both are signs of uncontrolled proliferation [175]. Studies in hamster trachea [176–178] and various cancer cells [179–187] show that the differentiated phenotype can be restored by treatment with retinoids. Evidence indi-

cates that retinoids control differentiation via intracellular binding proteins (cellular retinol-binding protein and cellular retinoic acid-binding protein) and nuclear receptors [188–192].

Calcium and vitamin D_3 are well-known differentiating agents that also inhibit carcinogenesis [115]. Calcium induces differentiation in epithelial tissues [193] including rat esophagus [194], mouse skin [195], and human mammary gland [196,197] and colon [193]. Vitamin D_3 induces differentiation in human colon [193,198], human and mouse myeloid leukemia cells [193,199–205], mouse skin cells [202], mouse melanoma cells [202], and other cells [202,206,207]. It has been suggested that the effects of the two chemicals on differentiation may be mediated by the same signal transduction pathway, involving the vitamin D_3 nuclear receptor with calcium as the messenger [115,193].

Restore Immune Response

De Flora and Ramel [21] commented on the importance of the immune response in inhibiting carcinogenesis. For example, they described the results of Feramisco *et al.* [208], who found that antibodies to oncogene products or oncoproteins are important in the inhibition of cell transformation and tumor growth.

PGE_2 is known to suppress immune response in certain tumor cells [96,113,114]. Cyclooxygenase inhibitors diminish the immune suppression [209–219]. Marnett [96] suggests that this effect on immune suppression may be part of the mechanism by which cyclooxygenase inhibitors reduce tumor growth, as seen in several animal tumor models including colon and Lewis lung carcinoma [220].

Retinoids also are known to be immunostimulants [191,221]. Retinoic acid increases cell-mediated and natural killer (NK) cell cytotoxicity; retinoids also cause some leukemia cells to differentiate to mature granulocytes comparable to mature neutrophils [180]. Hill and Grubbs [191] suggested that these effects might be partially responsible for the activity of retinoids against established tumors.

Pharmacological doses of vitamin E fed with normal, well-balanced animal diets increase humoral antibody production, especially IgG; this effect has been observed repeatedly in chickens, mice, turkeys, guinea pigs, and rabbits [222]. Vitamin E also stimulates cell-mediated immunity, as evidenced by enhanced mitogenesis and mixed lymphocyte response in spleen cells from mice fed vitamin E [223]. In particular, vitamin E prevents the carcinogen-induced decrease in the density of macrophage-equivalent cells (Langerhans cells) in the buccal pouch of DMBA-treated hamsters [224]. Likewise, vitamin E inhibits the induction of tumors by DMBA in hamster buccal pouch. Locally injected vitamin E also causes the regression of DMBA-induced buccal pouch tumors; the regression is associated with the induction of tumor necrosis factor-α in macrophages [225].

The role of selenium in mediating immune response suggests that the broad spectrum activity of selenium in inhibiting chemical carcinogenesis may be attributed partially to stimulation of the immune system. Kiremidjian-Schumacher and Stozky [226] have reviewed the many effects selenium has on the immune system, including nonspecific, humoral, and cell-mediated immunity. In general, selenium deficiency causes immunosuppression, while supplementation with low doses of selenium restores and increases immune response. Perhaps most important in inhibiting tumorigenesis is the effect of selenium on the cytotoxicity of immune system cells. Compared with normal cells, both T and NK lymphocytes from selenium-deficient mice have decreased ability to destroy tumor cells *in vitro*. Supplementation with 0.5 or 2 ppm selenium enhances the ability of rat NK cells to kill tumor cells. The role of immunostimulation in carcinogenesis inhibition by selenium has been studied only to a limited extent and has not been confirmed. However, the potent inhibitory activity of selenium compounds against DMBA-induced tumors in rat mammary glands is suggestive, since the immunosuppresive effects of DMBA are well-documented.

Increase Intercellular Communication

Gap junctions are the cell components that coordinate intercellular communication. They are composed of pores, or channels, in the cellular membranes that join channels of adjacent cells; these pores are regulated and, when open, allow passage of molecules up to about 1,000 daltons in size [227,228]. Bertram and Lowenstein [228–231] have proposed that gap junctions allow growth regulatory signals to move between cells. There is evidence from studies *in vitro* that inhibition of gap junctional intercellular communication occurs in the proliferative phase of carcinogenesis. Klaunig and Ruch [227] have reviewed this evidence. For example, they cited numerous reports that TPA, other phorbol ester tumor promoters, and mezerein inhibit gap junctional communication in Chinese hamster embryo V79 fibroblasts, mouse HEL-37

epidermal cells, human FL cells and fibroblasts, BALB/c 3T3 mouse fibroblasts, NIH-3T3 mouse fibroblasts, C3H10T1/2 mouse fibroblasts, rat liver epithelial cells, and primary hepatocytes. They noted that in several of the studies, the ability to inhibit intercellular communication correlates to tumor promoting activity *in vivo*. Also, TPA decreases the size and number of gap junctions in cells *in vitro* and in mouse skin. Further, several nonphorbol tumor promoters and nongenotoxic carcinogens are inhibitors of gap junctional communication *in vitro*. The communication inhibitors include such compounds as anthralin, benzoyl peroxide, bile acids, and phenobarbital.

Research carried out by Bertram and his colleagues on C3H10T1/2 cells strengthens the concept that carcinogenesis may be inhibited by enhancing intercellular communication. They found that carotenoids [232] such as β-carotene and canthaxanthine, and retinoids [233] such as [*E*]-4(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)-benzoic acid and vitamin A, enhance communication in C3H10T1/2 cells initiated with MCA. The enhancement of communication correlates to inhibition of transformation in these cells.

To date, only limited data suggest the potential for inhibiting chemical carcinogenesis by the other antiproliferative/antiprogression mechanisms listed in Table I, but the possibilities exist and warrant consideration here.

Restore Tumor Suppressor Function

Over the past few years, several so-called "tumor suppressor" genes have been found. These genes are involved in controlling proliferation and differentiation in cells. Particularly, their function is associated with control of abnormal growth in carcinogenesis. Several of these genes have been identified and implicated in pathogenesis by the presence of mutated or otherwise dysfunctional forms in specific cancers. For example, the tumor suppressor *Rb* is involved in retinoblastoma, osteosarcoma, and tumors in lung, bladder, prostate, and breast [234–238]; p53 in adenocarcinomas in colon and breast, human T-cell leukemias, glioblastomas, sarcomas, and tumors in lung and liver [*e.g.*, 238–247]; WT in Wilm's tumor [248–250]; and DCC in colon tumors [251]. Friedmann [244] has reviewed the research demonstrating the potential for treating cancer patients with exogenous functional tumor suppressor genes to inhibit tumor growth and spread. Possibly, it also will be found that chemicals can modulate the expression and activity of tumor suppressors and inhibit carcinogenesis by this mechanism.

Induce Programmed Cell Death (Apoptosis)

Apoptosis is a well-regulated function of the normal cell cycle; it requires gene transcription and translation [252,253]. Tumor suppressors, such as wild-type p53 [254,255] and growth factors, particularly TGF-β_1 [253,256], have been implicated as inducers of apoptosis. Programmed cell death has been described as the complement to mitosis in the maintenance, growth, and involution of tissues; it is the process by which damaged and excessive cells are eliminated [253]. Apoptosis is inhibited by tumor promoters such as TPA [255,257,258] and phenobarbital [255,259] and other chemicals that stimulate cell proliferation such as hormones [253,260–263]. These data suggest that induction of apoptosis may inhibit tumor formation. Further, chemicals that inhibit tumor promotion may act by inducing or preventing inhibition of apoptosis. As yet there is no published direct evidence that this mode of action is operative. However, hamster pancreatic cancers regress when apoptosis is induced [253,264], and the chemopreventive agent tamoxifen also induces programmed cell death in human mammary cancer MCF-7 cells [253].

Correct DNA Methylation Imbalances

Wainfan and Poirier [265] reviewed their work and that of others indicating that changes in DNA methylation patterns are involved in carcinogenesis. Methyl-deficient diets cause fatty livers, increased cell turnover, and promote the development of carcinogen-induced liver tumors in rats and mice [265–269]. Conversely, methyl-rich (fortified with choline and methionine) diets prevent or reduce these effects [265,270–272]. Changes in gene expression—increased expression of protooncogenes and decreased expression of growth factors and growth factor receptors in liver—appear in animals on methyl-deficient diets [265,273–276]. These effects are similar to those seen in rodents given tumor promoting chemicals [265,277–279], and they are reversible on methyl replacement [265,280]. Most importantly, the increased protooncogene expression correlates to hypomethylation of the genes in animals fed methyl-deficient diets for one week or longer [265,274,280–282]. Essentially, these observations support the hypothesis that hypomethylation of DNA results in alterations

in the expression of genes involved in cell growth and regulation [265,269,271]. This observation, in turn, supports the more general concept that changes in DNA methylation, including hypomethylation as well as mutation and steric blocking, can affect carcinogenesis [265,283–286]. There is only very limited evidence associating chemicals that affect DNA methylation with inhibition of carcinogenesis. Methionine, which is involved with choline, folic acid, and vitamin B_{12} in regulating intracellular methyl metabolism [265], inhibits DMBA- and MNU-induced mammary gland cancers in rats. Also, folic acid inhibits isoniazid-induced lung tumors in mice.

Inhibit Angiogenesis

Angiogenesis is the process leading to formation of new blood vessels. In normal tissue, it is a highly regulated process essential to reproduction, development, and wound repair [287]. In carcinogenesis, it is required in tumor growth and involved in metastasis [288]. A study in transgenic mice (strain RIP1-Tag 2) indicates that angiogenesis also may occur before tumors are formed—*i.e.*, in hyperplasia [289]. There is indirect evidence that certain chemicals that inhibit carcinogenesis may inhibit angiogenesis. For example, PGE_1 and PGE_2 are angiogenic [290,291]. Therefore, compounds that inhibit PG synthesis may inhibit carcinogenesis by inhibiting angiogenesis.

The last two activities, inhibition of basement membrane degradation and activation of antimetastasis genes, occur very late in carcinogenesis.

Inhibit Basement Membrane Degradation

Tumor cells produce various enzymes that destroy the basement membrane which acts as a barrier against malignant cancer cells and prevents cancer spread. These enzymes include the proteases collagenase, cathepsin B, and plasminogen activators [148,292]. Protease inhibitors are known to act against thrombin and type IV collagenase, which are among the proteases hypothesized to participate in the destruction of basement membranes during cancer invasion [148]. Thus, protease inhibitors that inhibit carcinogenesis may derive their effects, in part, by inhibiting basement membrane degradation.

Activate Antimetastasis Genes

In the past few years, evidence has accumulated that tumor invasion and metastasis are controlled by effector genes just as are the other phases of tumor cell proliferation [293]. Several genes have been identified which are involved in the suppression of metastasis [293]. One of these is *nm*23, which apparently produces nucleoside diphosphate kinase [294]. Levels of *nm*23 correlate inversely with the prognosis and metastatic state in human breast cancer [295,296]. At this time, there is no substantial evidence that chemicals which inhibit carcinogenesis will induce the antimetastasis genes; however, it is possible that chemicals which increase the expression of the genes or enhance levels of their products (*e.g.*, nucleoside diphosphate kinase) may inhibit the progression of cancers by these mechanisms.

FUTURE DIRECTIONS FOR MECHANISTIC STUDIES IN CHEMOPREVENTIVE DRUG DEVELOPMENT

The systematic drug development program envisioned for chemopreventives is only possible with continuing research into mechanisms of action and thoughtful application of the mechanisms to new drug design and discovery. Described below are several approaches to mechanistic evaluation that may be pursued.

As noted previously, many of the most promising agents demonstrate a wide spectrum of potential chemopreventive and other pharmacological activities. An interesting possibility is to map activity profiles or patterns for these agents. These profiles could be compared among the agents to identify activities that appear to be most important. Profiles of untested agents could also be compared with those of well-established chemopreventives. Grever and colleagues [297] have described a similar approach to potential cancer chemotherapeutics. In fact, mechanistic profiles have been applied to chemopreventive agents in the development of peptide gel maps of several promising agents (Drs. Leigh and Norman Anderson, Large Scale Biology, Inc.). The objective of the mapping is to identify a protein or protein pattern common to active compounds. The pattern may be specific to a pharmacological or structural class or be the same across several classes. For example, oltipraz and several analogs were mapped, and a protein marker was found that correlated to chemopreventive activity. An extension of this methodology is the elucidation of the marker proteins.

Another approach is structure-activity relationship (SAR) analyses such as that of Chung *et al.*

[33] with arylalkyl and alkyl isothiocyanates described above. As in this example, a primary use of SAR would be to optimize the activity of good lead agents, comparing the effects of varying substituents and other chemical properties on chemopreventive activity. Conversely, modifications of agents to lower toxicity might also be evaluated by SAR.

Besides chemical structure and chemoprevention-related pharmacological effects *per se*, mechanistic models of carcinogenesis considering tissue specific effects and pharmacokinetics will be important in developing and improving the clinical efficacy of agents. For example, these parameters may be useful in selecting combinations of agents with complementary chemopreventive effects. Also, such models can lead to identification of target-specific agents with reduced toxicity, as in the case of the COX-2 inhibitors described above, which have high antiiflammatory activity and low gut toxicity compared with COX-1 inhibitors.

A final and important aspect of mechanism-based studies is in defining appropriate biological and pharmacological test systems to evaluate chemopreventive agents. This aspect includes, for example, identifying enzyme assays based on mechanistic considerations (*e.g.*, ODC or tyrosine kinase inhibition) for initial screening of agents, and selecting intermediate biomarkers of cancer (*e.g.*, proliferation and genetic changes) for use in short-term clinical trials.

The large body of information on carcinogenesis and chemopreventive mechanisms that already exists and has been summarized in this paper provides a strong base for future studies. Moreover, the progress that has been made to date in applying mechanistic studies suggests the high promise of such studies for evaluating and developing chemopreventive agents for clinical use.

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