Review

Farnesyl Protein Transferase Inhibitors as Potential Cancer Chemopreventives

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Abstract

Among the most important targets for chemopreventive intervention and drug development are deregulated signal transduction pathways. Ras proteins serve as central connectors between signals generated at the plasma membrane and nuclear effectors; thus, disrupting the Ras signaling pathway could have significant potential as a cancer chemopreventive strategy. Target organs for Ras-based chemopreventive strategies include those associated with activating ras mutations (e.g., colorectum, pancreas, and lung) and those carrying aberrations in upstream element(s), such as growth factors and their receptors. Ras proteins require posttranslational modification with a farnesyl moiety for both normal and oncogenic activity. Inhibitors of the enzyme that catalyzes this reaction, farnesyl protein transferase (FPT) should, therefore, inhibit Ras-dependent proliferative activity in cancerous and precancerous lesions (J. B. Gibbs et al., Cell, 77: 175-178, 1994). Because growth factor networks are redundant, selective inhibition of signaling pathways activated in precancerous and cancerous cells should be possible.

Requirements for Ras farnesylation inhibitors include: specificity for FPT compared with other prenyl transferases; specificity for FPT compared with other farnesy1 PP-utilizing enzymes; ability to specifically inhibit processing of mutant K-ras (the most commonly mutated ras gene in human cancers); high potency; selective activity in intact cells; activity in vivo; and lack of toxicity. Numerous FPT inhibitors have been identified through random screening of natural products and by rational design of analogues of the two substrates, farnesyl PP, and the COOH-terminal CAAX motif of Ras tetrapeptides. A possible testing strategy for developing FPT inhibitors as chemopreventive agents includes the following steps: (a) determine FPT inhibitory activity in vitro; (b) evaluate selectivity (relative to other protein prenyl transferases and FPT-utilizing enzymes); (c) determine inhibition of Ras-mediated effects in intact cells; (d) determine inhibition of Ras-mediated effects in vivo (e.g., in nude mouse tumor xenografts); and (e) determine chemopreventive efficacy in vivo (e.g., in carcinogen-induced A/J mouse lung, rat colon, or hamster pancreas).

Mechanism-based Strategies in the Development of Cancer Chemopreventive Agents: Signal Transduction Pathways

This paper is the second in a series on mechanism-based strategies used by the National Cancer Institute's Chemoprevention Branch for development of cancer chemopreventive drugs. The reader is referred to the first paper in this series (1) and our previous reviews (2-4) for a general discussion of chemoprevention and the rationale for this approach. One of the mechanism-based strategies that can be targeted for cancer chemopreventive drug development is interference with deregulated cellular growth controls.

Cells respond to signals from extracellular stimuli via a complicated network of highly regulated events collectively referred to as signal transduction pathways. Stimulation of these pathways results in changes in transcriptional activity (5, 6). Whereas normal cells respond appropriately to extracellular stimuli, many precancerous and cancerous cells have lost this ability and display aberrant signaling (7).

Many sites on the deregulated signaling pathways are potential targets for chemopreventive intervention (8-10). Blocking signal transduction pathways at the cell membrane via inhibition of receptor protein tyrosine kinases, specifically EGFR,2 was the subject of the previous paper in this series (1). Interference with mediators, specifically activated Ras proteins, is the subject of this paper.

Targeting Specific Cytoplasmic Intracellular Mediators: Ras Proteins

ras genes code for M, 21,000 proteins that belong to a large superfamily of GTP-binding proteins that cycle between an active GTP-bound and an inactive GDP-bound state. Four human ras genes have been identified: H-, K-4A, K-4B, and N-ras (11). Normal Ras proteins serve as molecular switches in the mitogenic signal transduction pathway and are crucial regulators of many physiological functions including cell growth and differentiation. Activating mutations in ras genes result in

2 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FPT, farnesyl protein transferase; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; MNU, N-methyl-N'-nitrosourea; AOM, azoxymethane; AML, acute myeloid leukemia; ACF, aberrant crypt foci; FPP, farnesyl PP; GGTase I and II, geranylgeranyl protein transferase types I and II, respectively; HMG-CoA, hydroxymethylglutaryl-CoA.

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proteins that are “stuck” in the active GTP-bound state and constitutively transmit growth signals (11, 12). Oncogenic activity can also result from overexpression of normal Ras proteins (13). As discussed below (see “Specific Human Cancers Associated with Activating ras Lesions”), oncogenic mutations in ras genes have been identified in a variety of human tumors (14).

Because Ras proteins are central connectors between signals generated at the plasma membrane and nuclear effectors (see “Ras and Signal Transduction”), disrupting Ras signaling has significant potential as a cancer chemopreventive strategy. Possible methods include using antisense oligonucleotides that block mutant ras genes or disrupting interactions of Ras with downstream effector molecules (15).

Another strategy is based on the observation that Ras proteins require posttranslational modification with a farnesyl moiety for oncogenic activity (16–18). Inhibitors of the enzyme that catalyzes this reaction, FPT, should therefore inhibit Ras-dependent proliferative activity in cancerous and precancerous lesions (19). The rationale for using Ras antagonists as chemopreventive agents, analysis of potential clinically relevant target organs, and identification of inhibitors with specificity toward FPT are discussed.

Ras and Signal Transduction
An important role for Ras proteins is as cytoplasmic mediators of signaling by extracellular stimuli including growth factors such as EGF, PDGF, and IGF and cytokines such as the interleukins (Fig. 1). Ras proteins can be activated by a variety of signals, including receptor tyrosine kinases (e.g., for EGF, PDGF, and IGF), receptor-associated tyrosine kinases (for cytokines), and G-protein-coupled seven-member serpentine receptors (20–23). The most extensively studied pathways are via activation by growth factors and their receptors, particularly EGF-R. Ligand binding to receptor tyrosine kinases causes receptor dimerization and autophosphorylation. The phosphorylated tyrosine residue binds growth factor receptor binding protein 2, which in turn complexes with the RasGEF FPT. Translocation of FPT to the plasma membrane in the vicinity of Ras results in Ras activation (21).

Several potential downstream targets of Ras have been identified, most significantly the protein Raf. Activated Ras recruits Raf to the plasma membrane, resulting in a series of phosphorylation and activation events along the MAPK cascade and leading ultimately to activation of several transcription factors and their target genes. However, Raf-mediated responses cannot account for all of the consequences observed in Ras-activated cells; other possible Ras target proteins include MEKK1 (MAPK/ERK kinase), phosphatidylinositol-3-hydroxy kinase [PI3K], PI200AP, RafGDS, and protein kinase C (PKC) (see text for references). GF, growth factor; IL-1, interleukin 1; RTK, receptor tyrosine kinase; Grb2, growth factor receptor binding protein 2; GEF, growth enhancing factor; TK, tyrosine kinase; SMOR, seven-member serpentine receptor; JNK/SAPKs, Janus-activated kinase/stress-activated protein kinases; NF, neurofibromatosis gene; MAPKK, MAPK kinase; TCF, ternary complex factor.

Fig. 1. Signal transduction pathways: Ras-mediation. Ras proteins can be activated by a variety of signals, including receptor tyrosine kinases. The most extensively studied pathway is activation by EGF-R. Ligand binding to EGF-R causes receptor dimerization and autophosphorylation. The phosphorylated tyrosine residue of EGF-R binds growth factor receptor binding protein 2, which in turn complexes with the RasGEF, FPT. Translocation of FPT to the plasma membrane in the vicinity of Ras results in Ras activation. Several potential downstream targets of Ras have been identified, most significantly the protein Raf. Activated Ras recruits Raf to the plasma membrane, resulting in a series of phosphorylation and activation events along the MAPK cascade and leading ultimately to activation of several transcription factors and their target genes. However, Raf-mediated responses cannot account for all of the consequences observed in Ras-activated cells; other possible Ras target proteins include MEKK1 (MAPK/ERK kinase), phosphatidylinositol-3-hydroxy kinase [PI3K], PI200AP, RafGDS, and protein kinase C (PKC) (see text for references). GF, growth factor; IL-1, interleukin 1; RTK, receptor tyrosine kinase; Grb2, growth factor receptor binding protein 2; GEF, growth enhancing factor; TK, tyrosine kinase; SMOR, seven-member serpentine receptor; JNK/SAPKs, Janus-activated kinase/stress-activated protein kinases; NF, neurofibromatosis gene; MAPKK, MAPK kinase; TCF, ternary complex factor.
Association of Deregulated Ras Activity with Carcinogenesis

General Considerations. The association of activated ras genes with oncogenic transformation in experimental animals (26) and in humans (14, 26, 27) is well established. The activation of ras genes is generally associated with mutations at codons 12, 13, and 61 (27). Although overexpression of normal Ras proteins also leads to transforming activity (27), mutational activation has been observed more consistently in human cancers (28), and interpretation of studies examining overexpression has been complicated by methodological problems, leading to inconclusive results (28).

Patterns of ras mutations may vary across both species and target organs. For example, ras mutations are rare occurrences (<5%) in human breast cancer; however, ~90% of MNU-induced rat mammary cancers carry codon 12 H-ras mutations and ~50% also harbor codon 12 K-ras mutations (29). AOM-induced rat colon tumors (30), N-nitrosobis(2-oxopro- pyl)amine-induced hamster pancreatic carcinomas (31, 32), and human cancers at both of these target sites carry a high percentage of K-ras mutations (14). Similarly, lung adenocarcinomas in humans and similar carcino-gen-induced lesions in mice have high frequencies of K-ras mutations (26, 33, 34).

The specific ras gene altered can also vary across species. In general, H-ras oncogenes are activated in numerous animal cancer models (26) but are infrequently activated in human tumors (14, 26, 27). Furthermore, different target organs within a species show varied patterns of individual ras oncogene activation. In humans, most carcinomas (colon, pancreas, and lung) harbor activated K-ras genes, whereas N-ras mutations have been associated with myeloid leukemia (35).

Another factor implicating ras mutation and overexpression in carcinogenesis is correlation to cancer prognosis. However, the clinical significance of ras mutation and overexpression without consideration of other co-occurring molecular and phenotypic changes is not clear (14). Colon and lung tumors and melanoma do not show a strong correlation of ras mutation or overexpression frequency to disease severity (14), although mutant ras has been associated with a more aggressively transformed phenotype in human fibrosarcoma and colon carcinoma cells (36) and colorectal adenomas (14). The most informative association of ras mutation to cancer prognosis has been observed in leukemia patients. In patients with AML, ras mutations present during active disease usually cannot be detected during remission (14, 37) and are sometimes seen in relapse (14, 38). Also, myelodysplastic syndrome patients with ras mutations appear to have a higher probability of progressing to AML (14, 39).

Target Organs for Chemopreventive Intervention with Ras Processing Inhibitors: Aberrations in Elements Upstream of Ras in the Signal Transduction Pathway. Numerous additional target organs for Ras-based chemopreventive strategies can also be envisioned based on the central role of Ras proteins in the signal transduction pathway. Activation of upstream element(s), such as growth factors and their receptors, could lead to deregulated signaling via Ras proteins (21, 45). Blocking signals at the level of Ras protein may provide a viable means of inhibiting deregulated upstream elements. An in-depth review of all these potential target organs is beyond the scope of this article. However, targets may include, for example, cancers associated with deregulated signaling via EGFR in lung, cervix, and prostate (1, p185ErbB-2 in breast and ovary (23), and PDGF receptor in glioblastomas (23). Importantly, based on this hypothesis, some of the major target organs that do not harbor ras mutations, such as breast cancers, may be amenable to chemopreventive strategies that block Ras activity (45).

Specific Human Cancers Associated with Activating ras Lesions

Colorectum. Since the original reports by Bos et al. (46) and Forrester et al. (47) in 1987, considerable evidence has accumulated for the involvement of ras mutations, particularly K-ras, in colorectal cancer development. McLeod et al. (48) collated results of many studies and found that 40% (377 of 940) of sporadic cancers and 37% (20 of 54) of familial adenomatous polyposis-associated cancers harbor K-ras mutations. In cancers in ulcerative colitis patients, mutations are either common or infrequent, depending on the study. Bhattachar and Saraga (49) combined the results of five studies and reported that 30% (17 of 58) carcinomas examined from ulcerative colitis patients carried K-ras mutations.

Mutant K-ras oncogenes are clearly associated with the precancerous events in the colorectum. This genetic lesion has been identified in 10–75% of adenomatous polyps, which are well-established precursors of colorectal cancer (41, 46, 50–54), and in dysplastic colorectal tissues from ulcerative colitis patients (49). The large variations reported in overall ras mutation frequency in adenomatous polyps might result from a
number of factors, including differences in patient populations reflecting disparate etiological factors, assay sensitivity, or specific ras mutations analyzed.

An important factor contributing to variations in mutation frequency is the characteristics of the adenomas themselves. Increased prevalence of ras mutations in adenomas has been associated with parameters that correlate to increased malignant potential including severity of dysplasia (51, 52, 55, 56), increased size (41, 51, 55–57), and increased degree of villous architecture (55, 57, 58). These observations and the usual early occurrence of the K-ras mutations noted above suggest that the presence of K-ras mutations in precancerous colorectal adenomas “marks” these lesions with increased risk for malignant progression. Additionally, K-ras mutations have been detected in the colonic effluent of patients at high risk for developing colorectal cancer; in one patient, the mutation was detected 4 years prior to cancer diagnosis (59, 60).

However, as suggested above, the role of K-ras mutations in colorectal cancer development appears complex. For example, these mutations have been detected in up to 85% (61–64) of ACF (the earliest identified putative precursors of colorectal cancer) in the grossly normal-appearing mucosa of colorectal cancer patients. This incidence is much higher than that in small adenomas (~10%). Additionally, the incidence of K-ras mutations decreased with increasing size in a large number of ACF, although increasing size correlated to increasing adenomatous character of the lesion (61).

Pancreas. The incidence of K-ras mutations in pancreatic cancers far exceeds that in other human cancers. Mutations, usually in codon 12 of the K-ras gene, have been detected in 75–95% of ductal pancreatic adenocarcinomas (65–68). The very high frequency of these genetic lesions in pancreatic cancers, their appearance in a large percentage of small pancreatic adenocarcinomas (69), and the lack of correlation to other tumor stage or grade parameters (66, 70) suggest that they occur during the early stages of carcinogenesis.

Exploring the contribution of K-ras mutations to the natural history of pancreatic cancer has been hampered by the lack of clearly defined precancerous lesions. For example, K-ras mutations have been detected in histological lesions with questionable malignant potential, such as papillary mucinous duct hyperplasia without atypia (71), and in ducts from chronic pancreatitis patients showing papillary hyperplasia in the absence of carcinoma elsewhere within the pancreas (72). Indeed, it has been proposed that K-ras mutations be used to assess the precancerous nature of such lesions (73).

It was reported recently that the specific K-ras codon 12 mutation identified in pancreatic ductal hyperplasia without atypia was not detected in any of the pancreatic carcinomas, despite detection of other codon 12 mutations in 30 of 30 carcinomas. The authors suggested that hyperplasia bearing these genetic lesions might have low malignant potential (74). This observation is very interesting in light of the recent findings in the colorectum cited above, which suggest that ACF carrying K-ras mutations have little potential to progress toward malignancy.

Because pancreatic tissue is not easily accessible, alternative means of detecting K-ras mutations in pancreatic cells is important to potential chemopreventive strategies. K-ras mutations have been detected in pancreatic cancer patients by examining pancreatic (75) and duodenal juice (76) and stool specimens (77). The feasibility of using these techniques to identify lesions that may be amenable to chemopreventive intervention is bolstered by the detection of K-ras mutations in the pancreatic juice of patients who later developed cancer (78). However, K-ras mutations have also been detected in the stool (77) and in pancreatic juice (79) of patients with chronic pancreatitis. As noted above, the significance of these observations is unclear.

Lung. Reynolds et al. (80) collated results of several studies and reported that 32% (76 of 237) lung adenocarcinomas contain ras gene mutations; 67 of 76 were in K-ras. Mutations are most frequently detected at codon 12 (81). Using more sensitive techniques, an increased prevalence (46%) of K-ras mutations has been reported recently in adenocarcinomas (82). ras mutations have also been detected in other non-small cell lung cancers at lower frequencies but are rarely observed in other forms of lung cancer (33).

In various animal tissues, chemical carcinogens including asbestos, 7,12-dimethylbenz(a)anthracene, MNU, vinyl chloride, and benzo(a)pyrene have induced ras mutations (34). The importance of carcinogen-induced ras mutations is emphasized by observations in lung (33). K-ras mutations are much more prevalent in adenocarcinomas from smokers (30%, 41 of 141) than from nonsmokers (5%, 2 of 40; Ref. 81). Furthermore, mutations are as common in former as current smokers, even those who had not smoked for ≥15 years (83). The most frequent type of mutation found in tumors from former smokers is a G→T transversion, which is the same type of mutation induced by the cigarette smoke carcinogen benzo(a)pyrene (83). K-ras mutations have been detected in the normal-appearing bronchial tissue of smokers with non-small cell lung cancers that harbored K-ras mutations (84) and in cytologically negative stored sputum samples from 7 of 15 patients who later developed resectable adenocarcinoma (85). These findings implicate tobacco smoke as a causative agent and suggest that ras mutations may be early genetic lesions during lung adenocarcinoma development.

However, as in other tissues, it appears that K-ras mutations may also occur later but still prior to invasion. Sugio et al. (86) observed K-ras mutations in 4 of 5 areas of carcinoma in situ associated with adenocarcinomas bearing K-ras mutations but not in earlier stages of premalignancy. Li et al. (87) also failed to detect ras mutations in preinvasive bronchial epithelium but found a homogeneous distribution of mutant K-ras in malignant cells of adenocarcinomas, suggesting that the mutations occurred prior to invasion. Larger studies are needed to clarify these findings.

Leukemias. Leukemias are one of few human cancers associated with mutations in ras genes other than K-ras. Mutations, usually in N-ras, have been observed in 25–60% of chronic myelomonocytic leukemia and 30% of adult AML. N-ras mutations have also been observed in 9–40% of myelodysplastic syndromes, stem cells disorders which may progress to AML (35).

Endometrium. Mutations, usually in codon 12 of the K-ras gene, have been reported in 10–30% of endometrial cancers (88–90). Mutation frequency has consistently been shown to be about 10–15% in U.S. women compared with about 20–30% in Japanese women (88, 89, 91), reflecting possible etiological differences.

K-ras mutations have also been identified in precancerous endometrial hyperplasia with frequencies correlated to severity of the lesions (88, 89). In one study in which mutations were present in 18% of cancers, the total percentage of K-ras mutations in endometrial hyperplasia was 16% with mutations present in 10% of simple hyperplasia, 14% of complex lesions, and 22% of atypical hyperplastic lesions (88). These results
suggest that although K-ras mutations do not occur in a high percentage of endometrial cancers, when present, they often represent early genetic events during malignant transformation. Cervix. ras mutations appear to be rare in squamous cervical cancers (27, 92, 93). However, expression of unspecified Ras proteins has been reported to increase in high grade and invasive lesions compared with low grade or normal cervical epithelium (94, 95). Based on preliminary results showing progressive increases in Ras expression during lesion progression, Mitchell et al. (96) are investigating Ras expression as a surrogate endpoint for chemoprevention clinical trials in the cervix.

Breast. ras mutations are also rare in human breast cancer (<5%), but overexpression of H-ras proto-oncogene has been reported in up to 70% of breast cancers (97, 98). Furthermore, in one study, expression increased during histological stages of malignant progression and was also significantly higher in patients (n = 18) with hyperplastic changes, who subsequently developed breast cancer (99). Recent evidence suggests that aberrant function of the Ras-related protein TC21R-Ras2 (55% homologous to Ras) may contribute to breast cancer. TC21R-Ras2 activates the MAPK cascade and other downstream elements of the Ras signaling pathway (100). TC21R-Ras2 overexpression or mutation induces transformation in breast epithelial cell lines, and overexpression is seen in the majority of human breast tumor cell lines (101). The significance of these findings awaits confirmation that TC21R-Ras2 is aberrantly regulated in primary breast lesions.

Ras Protein Processing

Because of its central role in signal transduction, Ras protein processing has been studied intensely and reviewed frequently (16–19). Ras protein function depends on association with the inner surface of the plasma membrane. Ras proteins are initially synthesized as cytoplasmic, soluble proteins lacking the conventional transmembrane or hydrophobic domains typical of other membrane-associated proteins. To overcome this, they are posttranslationally modified with a lipophilic C-15 farnesyl moiety in a reaction catalyzed by FPT. Following farnesylation, the Ras COOH-terminal sequence undergoes proteolytic cleavage and carboxymethylation.

Experiments using mutant proteins that cannot undergo these various posttranslational processing events demonstrate that membrane association and farnesylation are critical for Ras transforming activity. Nonfarnesylated mutants of oncogenic Ras are cytosolic and devoid of transforming activity. Further support for the importance of Ras farnesylation comes from studies limiting the availability of FPP, the isoprenoid moiety used in this reaction (17). FPP is a metabolic product of mevalonate, formed via a series of steps from HMG-CoA. The first and rate-limiting step in this pathway is catalyzed by HMG-CoA reductase. Inhibition of HMG-CoA reductase with compactin or lovastatin decreases the availability of FPP, prevents Ras farnesylation, membrane association, cell transformation, and tumorigenesis (102, 103). However, the utility of HMG-CoA reductase inhibitors as Ras farnesylation suppressors is limited because the formation of numerous other isoprenoids in addition to FPP would be affected; additionally, Ras proteins are only a subset of >40 posttranslationally isoprenylated proteins. The majority of these proteins are modified by geranylgeranyl groups rather than by farnesylation; however, inhibition at the level of HMG-CoA reductase would influence all of these isoprenylation reactions (17).

FPT and Related Prenyl Transferases

Two types of cellular prenyl group transfers are the most common and involve transfer of a C-15 farnesyl or a C-20 geranylgeranyl moiety to a cysteine residue via a thioether linkage. Prenylated proteins share characteristic COOH-terminal sequences, which include the CAAX, XXCC, and XCXC motifs (where C is cysteine, A is usually an aliphatic amino acid; X is another amino acid). Three enzymes that catalyze protein prenylation have been identified: FPT, GGTase I, and GGTase II (also called Rap GGTase). GGTase II modifies proteins ending in XXCC and XCXC (19). Until recently, the CAAX tetrapeptide was believed to be the minimum region required for interaction of protein substrates with FPT or GGTase I, with the last residue of the CAAX motif directing enzyme specificity. However, newer studies suggest that enzyme specificity is more complex. Both enzymes form stable non-covalent complexes with FPP and geranylgeranyl PP, when a protein acceptor is not present. FPT only transfers the farnesyl moiety, whereas GGTase will transfer either the farnesyl or geranylgeranyl moiety, depending on which protein acceptor is present (104).

The four cellular Ras proteins are substrates for FPT. In addition, at least eight other cellular proteins undergo farnesylation: nuclear lamins a and b, the Ras-related proteins Rap2 and RhoB, phosphorylase kinase, rhodopsin kinase, cyclic GMP phosphodiesterase α, and the γ subunit of transducin (19). The latter three proteins are involved in vision (105). GGTase I substrates include the γ subunit of mammalian G proteins, Rap1, and CDC42 (106). GGTase II prenylates many Rab proteins, which are involved in protein secretion and endocytosis (106).

Requirements for Specific Ras Farnesylation Inhibitors

Requirements for Ras farnesylation inhibitors include: specificity for FPT compared with GGTases, particularly, GGTase I; specificity for FPT compared with other FPP-utilizing enzymes; ability to specifically inhibit processing of mutant K-ras (the most commonly mutated ras gene in human cancers); high potency; selective activity in intact cells; activity in vitro; and lack of toxicity.

Ras Farnesylation Inhibitors

Two general approaches for identifying FPT inhibitors have been used. The first is random screening of microbial or other products for inhibitory activity. The second is based on rational design of analogues of the two substrates, FPP and the COOH-terminal CAAX motif of Ras tetrapeptides. The discussion below is intended to provide a general overview of the types of structures that have demonstrated inhibitory activity toward FPT. The inhibitors are divided into four categories according to their proposed mechanism of action: (a) FPP competitive inhibitors; (b) CAAX competitive inhibitors; (c) bisubstrate inhibitors; and (d) inhibitors with unknown mechanism(s) of action.

Based on their activity in in vitro and in vivo screening assays, some of these drugs may have chemopreventive potential. It should be noted that FPT inhibitors that do not meet all of the requirements set forth above may still be viable chemopreventive drugs under appropriate circumstances.

The most current available animal data have been obtained in models relevant for establishing chemotherapeutic effectiveness, i.e., tumor cell growth inhibition in vivo. Because changes in Ras-mediated signaling occur during the process of carcino-
genetic, (in)effectiveness toward established tumors may not accurately predict (in)activity toward precancerous lesion growth and development. This differential effectiveness has been demonstrated, for example, with retinoids, which are well known chemopreventive agents but have generally been inefficient against established tumors in animal models (107).

**FPP Competitive Inhibitors.** FPP competitive inhibitors (see Table 1) bind to FPT at the FPP binding site and have been identified through both rational drug design (Fig. 2) and random screening (Fig. 3). The most promising inhibitors in this class are expected to be those specific for FPT compared with other FPP-utilizing enzymes, most notably squalene synthase.

Although they are not highly specific for FPT, perillyl alcohol, d-limonene and related metabolites are noteworthy because of the chemopreventive and tumor-shrinking potential they have demonstrated. Perillyl alcohol is a hydroxylated derivative of d-limonene. Both monoterpenes have demonstrated preclinical chemopreventive and chemotherapeutic activity possibly through metabolism to perillic and dihydroperillic acids (108, 109). Both metabolites inhibit FPT and GGTTases directly (110–113) or by selectively decreasing ras levels (114). Perillyl alcohol significantly inhibited AOM-induced colon and small intestine tumor development. In published chemoprevention studies, perillyl alcohol inhibited rat liver (115) and hamster pancreatic (116) tumor development; in chemotherapeutic studies (110, 117, 118), it significantly reduced the growth of established hamster pancreatic tumors, caused regression of established rat mammary gland tumors, and retarded growth of a prostate tumor cell xenograft in athymic nude mice. d-Limonene inhibited growth of mouse lung and skin tumors, rat mammary gland tumors induced by MNU, DMBA, and direct in situ transfer of v-Ha-ras, and rat liver tumorogenesis, as well as regressed established mammary tumors.

### Table 1  FPT inhibitors competitive with FPP*

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<th>Characteristics</th>
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<td>Synthetic compounds</td>
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<td>(α-Hydroxyfarnesyl)phosphonic acid</td>
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<tr>
<td>Cell-free enzyme: Selective cf. GGTTases I, II Noncompetitive with Ras tetrapeptide</td>
<td>156</td>
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<tr>
<td>Compound I (amide analogue of FPP)</td>
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<tr>
<td>Cell-free enzyme: More potent than (α-hydroxyfarnesyl)phosphonic acid. Highly selective cf GGTTases I, II Whole cells: Inactive in H-ras-transformed cells</td>
<td>132, 157</td>
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<tr>
<td>Compound II (hydroxamate analogue of FPP)</td>
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<tr>
<td>Cell-free enzyme: More potent than (α-hydroxyfarnesyl)phosphonic acid. Highly selective cf GGTTases I, II Whole cells: Inactive in H-ras-transformed cells</td>
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<tr>
<td>Compound III (pivolyxymethyl ester analogue of FPP, prodrug of compound II)</td>
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<td>Cell-free enzyme: Inhibit both FPT and GGTTase (more potent toward GGTTase)</td>
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<tr>
<td>Chemopreventive activity: Perillyl alcohol inhibited induction of rat colon and liver, and hamster pancreas tumors</td>
<td>110, 115, 117–128</td>
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<td>Tumor growth inhibition and regression: Perillyl alcohol reduced growth of established hamster pancreatic tumors, regression of established rat mammary gland tumors, and retarded growth of a prostate tumor cell xenograft in athymic nude mice. d-Limonene inhibited growth of mouse lung and skin tumors, rat mammary gland tumors induced by MNU, DMBA, and direct in situ transfer of v-Ha-ras, and rat liver tumorogenesis, as well as regressed established mammary tumors</td>
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<tr>
<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
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<td>Perillyl alcohol, d-limonene and metabolites</td>
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<tr>
<td>Cell-free enzyme: Selective cf. GGTTase and SS. Acyclic more potent than cyclic form</td>
<td>160, 161</td>
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<tr>
<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
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<tr>
<td>Cell-free enzyme: Selective cf. GGTTase I and SS</td>
<td>132, 162</td>
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<td>Whole cells: A isomer inactive in H-ras transformed NIH-3T3 cells</td>
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<tr>
<td>Whole cells: Inhibited growth of K-ras-transformed fibrosarcoma cells (not clear that effect was due to inhibition of Ras processing)</td>
<td>1, 163</td>
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<tr>
<td>Cell-free enzyme: Also inhibits SS (more selective for SS)</td>
<td>132, 165</td>
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<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
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<td><strong>Actinoplamic acids</strong></td>
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<td>Cell-free enzyme: Selective cf. GGTTase and SS. Acyclic more potent than cyclic form</td>
<td>166, 167</td>
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<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
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<td>FPPA1</td>
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<td>Cell-free enzyme: Selective cf. SS</td>
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<td>Cell-free enzyme: α-Fluroration increased inhibitory potency</td>
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<tr>
<td>Cell-free enzyme: Selective cf. GGTTase and SS. Acyclic more potent than cyclic form</td>
<td>160, 161</td>
</tr>
<tr>
<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
<td></td>
</tr>
<tr>
<td>Cell-free enzyme: Selective cf. GGTTase I and SS</td>
<td>132, 162</td>
</tr>
<tr>
<td>Whole cells: A isomer inactive in H-ras transformed NIH-3T3 cells</td>
<td></td>
</tr>
<tr>
<td><strong>Manumycin</strong></td>
<td></td>
</tr>
<tr>
<td>Cell-free enzyme: Selective cf. GGTTase</td>
<td>1, 163</td>
</tr>
<tr>
<td>Whole cells: Inhibited growth of K-ras-transformed fibrosarcoma cells (not clear that effect was due to inhibition of Ras processing)</td>
<td></td>
</tr>
<tr>
<td>Perillyl alcohol, d-limonene and metabolites</td>
<td></td>
</tr>
<tr>
<td><strong>Compound III (pivolyxymethyl ester analogue of FPP, prodrug of compound II)</strong></td>
<td></td>
</tr>
<tr>
<td>Cell-free enzyme: Inhibit both FPT and GGTTase (more potent toward GGTTase)</td>
<td></td>
</tr>
<tr>
<td>Chemopreventive activity: Perillyl alcohol inhibited induction of rat colon and liver, and hamster pancreas tumors</td>
<td>110, 115, 117–128</td>
</tr>
<tr>
<td>Tumor growth inhibition and regression: Perillyl alcohol reduced growth of established hamster pancreatic tumors, regression of established rat mammary gland tumors, and retarded growth of a prostate tumor cell xenograft in athymic nude mice. d-Limonene inhibited growth of mouse lung and skin tumors, rat mammary gland tumors induced by MNU, DMBA, and direct in situ transfer of v-Ha-ras, and rat liver tumorogenesis, as well as regressed established mammary tumors</td>
<td></td>
</tr>
<tr>
<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
<td></td>
</tr>
<tr>
<td><strong>Perillyl alcohol, d-limonene and metabolites</strong></td>
<td></td>
</tr>
<tr>
<td>Cell-free enzyme: Selective cf. GGTTase and SS. Acyclic more potent than cyclic form</td>
<td>160, 161</td>
</tr>
<tr>
<td>Whole cells: Inactive in H-ras-transformed NIH-3T3 cells</td>
<td></td>
</tr>
</tbody>
</table>

* See Figs. 2 and 3 for representative structures. SS, squalene synthase; DMBA, 7,12-dimethyl benz(a)anthracene.
CAAX Competitive Inhibitors. Numerous tetrapeptides that conform to the CAAX consensus sequence act as alternative substrates for FPT and result in competitive inhibition of Ras farnesylation in vitro. Some tetrapeptides inhibit FPT without serving as alternative substrates and are thus true inhibitors of the enzyme. Structure activity analyses showed that the aromatic residue in the A2 position of the CAAX sequence (129) and a positive charge on the cysteine amino group (130) are the most potent inhibitors. However, these tetrapeptides are inactive in whole cells, probably because they are unable to enter cells efficiently or are easily degraded, or both. These tetrapeptide inhibitors are not discussed further here. Several research groups have developed promising CAAX mimetics, which are listed in Table 2 (Fig. 4). Bisubstrate inhibitors are also being developed (Table 3 and Fig. 5).

Inhibitors with Unknown Mechanism(s) of Action. A number of natural product FPT inhibitors have been identified, the mechanism(s) of action of which have not been determined. These are described in Table 4; their structures appear in Fig. 6.

Approach to Developing Ras Farnesylation Inhibitors as Chemopreventive Agents

A number of in vitro and in vivo tests may be used in the development of Ras farnesylation inhibitors as potential chemopreventive agents. Based on differences in the affinities of the Ras proteins for FPT, it is particularly important to establish inhibitory activity toward K-Ras, the form of Ras most often mutated in human cancers. A possible testing strategy is outlined below (Table 5). Priorities for further development would be based on results at each step in the order that follows.

Determine Inhibition of FPT Activity. Inhibitory activity can be determined by measuring incorporation of [3H]FPP into Ras proteins or Ras-related peptides in a reaction catalyzed by isolated or recombinant FPT (131).

Evaluate Selectivity for FPT. The selectivity of the inhibitor toward FPT relative to GGTases can be measured in vitro using GGTases I and II isolated from several sources, such as bovine brain (132); recombinant human GGTase I (133) can also be used. Inhibition of GGTase I and II activity can be measured via incorporation of [3H]GGT into Ras-CAI (Cys-Ala-Ile-Leu) and Ypt-GGCC, respectively (132). If the drug is competitive with respect to FPT, selectivity toward FPT relative to squalene synthase should also be determined (134).

Determine Inhibition of Ras-mediated Effects in Intact Cells. Selective inhibition of Ras processing and effects on Ras-mediated proliferation and transformation should be examined in whole cells. Specificity for FPT in intact cells involves establishing inhibition of prenylation of farnesylated as compared with geranylgeranylated proteins upon incubation with [3H]mevalonate. Often these experiments are performed in the presence of an HMG-CoA reductase inhibitor to prevent isotopic dilution of [3H]mevalonate. Because cells are relatively impermeable to mevalonate, Met-18b-2 cells (CHO cells with efficient mevalonate uptake) or cells transfected with cloned mutant Mev cDNA, which facilitates cellular uptake, can be used. Inhibition of ras-mediated cellular effects, such as inhibition of anchorage-independent and -dependent growth, and reversal of morphological transformation should be established (135).

Determine Inhibition of Ras-mediated Effects in Vivo. Activity of FPT inhibitors on the growth of Ras-dependent tumors
can be evaluated in nude mice injected with transformed rodent or human cells carrying mutant ras genes (133).

**Determine Chemopreventive Efficacy in Vivo.** Potentially, mouse lung is the most efficient model for evaluating FPT inhibitors. In A/J (or A/J F1) mice, virtually all chemically induced tumors have mutated K-ras (136). Using 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone as the carcinogen, >90% of lung tumors have K-ras, and approximately ~50% of liver tumors have Ha-ras mutations. The relatively small size of the mice and high tumor multiplicity allow testing with small amounts of drug. Alternative models are AOM-induced rat colon tumors (30) and N-nitrosobis(2-oxopropyl)amine-induced hamster pancreatic carcinomas (31, 32). Both are considered good models for human cancers at these target sites and carry high percentages of K-ras mutations. Further, K-ras mutations have clearly been associated with the development of human cancers at these target sites (41, 44, 50–60, 65–70). A caveat is that K-ras is preferentially activated by geranylgeranylation, not farnesylation. Tests for chemopreventive efficacy in other established animal tumor models where high levels of proliferation are particularly important to carcinogenesis, e.g., rat and mouse bladder, may logically follow. See Steele et al. (137) for a description of the animal models currently used in the National Cancer Institute Chemoprevention Branch drug development program.

**Discussion and Conclusions**

The use of FPT inhibitors represents a rational, targeted approach for the development of mechanism-based chemopreventive drugs. Activated ras genes have been associated with the early stages of carcinogenesis in several organs of high interest to the Chemoprevention Branch, including colon, pancreas, and lung. Advances in analytical techniques are allowing detection of these molecular alterations in increasingly smaller numbers of cells, thus facilitating detection at earlier stages of carcinogenesis (138). Of practical importance, mutant K-ras genes have been detected in stool and sputum samples of patients whose cancerous and precancerous lesions harbor ras mutations. This suggests that such mutations would be detectable by noninvasive techniques at an early stage when the probability for successful chemopreventive intervention is high.

In light of the role of Ras proteins as central connectors between signals generated at the plasma membrane and nuclear effectors, Ras-based prevention strategies could have broad-based clinical potential. Thus, besides targeting activated Ras proteins, target organs may include those in which Ras signaling pathways are constitutively activated but which do not bear activating ras lesions per se. This could result from aberrations in elements upstream of Ras in the signal transduction pathway, such as receptor tyrosine kinases. One of the most important examples for chemopreventive purposes is breast cancer.
<table>
<thead>
<tr>
<th>Table 2  CAAX competitive inhibitorsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>BZA-2B</strong></td>
</tr>
<tr>
<td><em>BZA-5B</em> (carboxymethylated prodrug for BZA-2B)</td>
</tr>
<tr>
<td><strong>B581</strong></td>
</tr>
<tr>
<td><strong>B956</strong></td>
</tr>
<tr>
<td><strong>B1086</strong> (methyl ester of B956)</td>
</tr>
<tr>
<td><strong>FTI-276</strong></td>
</tr>
<tr>
<td><strong>FTI-277</strong> (methyl ester of FTI-276)</td>
</tr>
<tr>
<td><strong>L-739,749</strong> (methyl ester of L-739,750)</td>
</tr>
<tr>
<td><strong>L-739,750</strong></td>
</tr>
<tr>
<td><strong>L-744,832</strong> (isopropyl ester of L-739,750)</td>
</tr>
<tr>
<td><strong>PD 083716</strong> (pentapeptide) and related dipeptides</td>
</tr>
<tr>
<td><strong>Pseudopeptide amides (no carboxyl moiety)</strong></td>
</tr>
<tr>
<td><strong>SCH 44342</strong> (not peptide)</td>
</tr>
</tbody>
</table>

*a* See Fig. 4 for representative structures.
though only ~5% of human breast cancers harbor mutant ras
genes, deregulated signaling mediated by the upstream ele-
ments EGFR (139, 140) and p185erbB-2 receptor tyrosine
kinases have been associated with breast cancer development
(141). The observation that many tumor cell lines harboring
activated tyrosine kinases and wild-type ras are very sensitive
to growth inhibition with FPT inhibitors (142) lends credence
to the feasibility of this approach. Demonstrating activity of these
inhibitors in experimental animals with tumors carrying wild-
type ras, but with aberrations in upstream elements, will further
establish the utility of this approach.

A possible pitfall to the use of FPT inhibitors is toxicity
associated with inhibition of constitutive, wild-type Ras (143).
However, chemoprevention may not require total Ras blockade,
and lower doses permitting activity sufficient for normal cel-
lar processes while damping carcinogenesis-associated hyper-
activity may be possible. Moreover, the potential utility of
FPT inhibitors as chemopreventive agents is supported by their
apparent lack of toxicity. They have shown remarkable selec-
tivity toward inhibiting the growth of transformed cells in
culture as well as in animal studies. Explanations for these
effects have been suggested: (a) nonfarnesylated oncogenic Ras
proteins exhibit a dominant negative phenotype that could
contribute to specificity. When activated Ras proteins are cyto-
solic they can act as inhibitors of membrane-bound Ras (19,
144). This could explain the observation that incomplete inhi-
bition of Ras farnesylation can result in complete inhibition of

---

**Table 3** BisObject inhibitor

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMS-186511</strong></td>
<td>16, 175, 176</td>
</tr>
<tr>
<td>Cell-free enzyme: Selective cf. GGTase 1</td>
<td></td>
</tr>
<tr>
<td>Whole cells: Selective inhibition of Ras processing cf. geranylgeranylation and myristoylation; selective growth inhibition and morphological expression inhibition in ras-transformed cf. normal cells and cells transformed with geranylgeraniylation- or myristoylation-dependent ras mutants; blocks neurofibromatosis type I malignant phenotype (associated with up-regulation of wild-type Ras)</td>
<td></td>
</tr>
</tbody>
</table>

* See Fig. 5 for representative structures.
Table 4  Inhibitors with unknown mechanisms of action

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Characteristics</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barceloneic acid (from Phoma)</td>
<td>Cell-free enzyme: Selectivity unknown</td>
<td>177</td>
</tr>
<tr>
<td>Cylindrol A (from Cylindrocarpon lucidum)</td>
<td>Cell-free enzyme: Selective cf. GGTages and SS; noncompetitive with both FPP and Ras-CVL peptide</td>
<td>178</td>
</tr>
<tr>
<td>Fusidienol</td>
<td>Cell-free enzyme: Selective cf. GGTages and SS; noncompetitive with both FPP and Ras-CVL peptide</td>
<td>179</td>
</tr>
<tr>
<td>Gliotoxins (gliotoxin and acetylgliotoxin)</td>
<td>Cell-free enzyme: Inhibited partially purified FPT, not selective for FPT</td>
<td>106, 180</td>
</tr>
<tr>
<td>Nonadrides (CP 225,917 and CP 263,114 from unidentified fungus in juniper)</td>
<td>Cell-free enzyme: Also inhibits SS</td>
<td>181</td>
</tr>
<tr>
<td>Patulin</td>
<td>Cell-free enzyme: Inhibits partially purified FPT</td>
<td>182, 183</td>
</tr>
<tr>
<td>Pepticinnamins (from Streptomyces OH-4652)</td>
<td>Cell-free enzyme: Inhibits partially purified FPT</td>
<td>184</td>
</tr>
<tr>
<td>Preussomerins</td>
<td>Cell-free enzyme: Inhibits partially purified FPT</td>
<td>185</td>
</tr>
<tr>
<td>SCH 58450 (from Streptomyces)</td>
<td>Cell-free enzyme: Selective cf. GGTagase I</td>
<td>186</td>
</tr>
<tr>
<td>Streptonigrins (10'-desmethoxy-streptonigrin, streptonigrin)</td>
<td>Cell-free enzyme: Inhibited partially purified FPT (10'-desmethoxy 3× &gt; streptonigrin and 5× &lt; acutely toxic)</td>
<td>187</td>
</tr>
</tbody>
</table>

* See Fig. 6 for representative structures.

Fig. 6. Inhibitors with unknown mechanisms of action.

Ras signaling (145); (b) the redundancy of signaling pathways predicts that inhibition of a specific deregulated pathway in cancerous or precancerous lesions should have minimal effects on normal cell function. Support for this hypothesis comes from studies in which FPT inhibitors diminished growth factor-induced MAPK activation in transformed, but not in normal, cells (146, 147).

Redundancy may be due to continued signaling along
non-Ras-mediated, parallel pathways, such as the Jak/Stat pathway (148). Signaling via Ras-related proteins may also be involved. For example, the Ras-related protein TC21R-Ras1 is able to activate the MAPK cascade and other downstream elements of the Ras signaling pathway (100). Interestingly, these proteins are not sensitive to FPT inhibitors, perhaps because they can be posttranslationally modified by either farnesylation or geranylgeranylation (149). Moreover, recent evidence suggests that FPT inhibitors are capable of differentially inhibiting the four cellular Ras proteins. The K-Ras4B protein has a 50-fold higher affinity for FPT than H-Ras in vitro, and K-Ras4B farnesylation is inhibited by FPT inhibitors only at concentrations 8-fold higher than those active toward H-Ras (44). Thus, selective inhibition of H- versus K-Ras mediated signaling may allow continued growth of normal cells.

However, the latter observations also have significance for chemopreventive strategies, because most human cancers with ras mutations selectively harbor them in the K-ras4B gene (150). The majority of early work with FPT inhibitors used H-ras-transformed cells and xenografts. More recent studies have shown that K-ras-transformed cell lines are generally much less sensitive to growth inhibition by FPT inhibitors than cell lines transformed with other ras genes (15, 151); indeed, in one study, K-ras-transformed cell lines were not more sensitive than those with wild-type ras (151). These observations underscore the necessity of using K-ras4B-transformed cells for testing potential inhibitors both in vitro and in vivo.

Recent studies also suggest that FPT inhibitors are not absolutely selective for Ras but may also affect other transformation-associated proteins. Phenotypic reversions of ras-transformed cells treated with the CAAX mimetic L-734,749 did not correlate to the state of Ras processing but rather to regulation of action stress fiber formation (152). One non-Ras protein that may be affected by FPT inhibitors is RhoB, which is usually geranylgeranylated but may also be farnesylated in vivo (153). Dominant inhibitory mutants of RhoB mimic FPT inhibitor ability to block Ras transformation (154), and the effects of L-734,749 can be suppressed by ectopic expression of farnesylation-independent forms of RhoB (153).

Additionally, data have been presented that K-Ras4B may be resistant to FPT inhibitors because it is posttranslationally modified by geranylgeranylation rather than farnesylation. K-Ras4B, not H-Ras, is a substrate for CAAX GGTagase I in vitro (44); in fact, oncogenic K-Ras4B processing and constitutive activation of MAPK were potently inhibited by a GGTagase 1-selective inhibitor, GGTI-286, but not by the FPT selective inhibitor FTI-277. On the other hand, oncogenic H-Ras was very sensitive to FTI-277 and highly resistant to GGTI-286 (150). These results may be explained by the higher affinity of K-Ras4B compared with H-Ras for FPT. However, the authors suggested that K-Ras4B is geranylgeranylated in cultured cells based on the observation that GGTI-286 inhibited oncogenic K-Ras4B processing and MAPK activation at concentrations that did not affect farnesylation-dependent processing (150). Demonstration that K-Ras4B is geranylgeranylated in vivo, especially under circumstances in which FPT is inhibited, could have a major impact on the design of Ras processing inhibitors.

Increasing knowledge of signaling pathways downstream of Ras suggests other strategies for improving the specificity and selectivity of the Ras processing blockade in preventing carcinogenesis. Particularly, specific inhibitors of downstream carcinogenesis-associated c-Jun or c-Fos activation (see Fig. 1) could be designed to supplement or replace FPT inhibition in certain high-risk tissues. For example, a combination with an inhibitor of downstream pathways may allow lower doses of FPT inhibitor to be used with concomitantly less toxicity to normal cell functions requiring Ras mediation.

Because precancerous and cancerous lesions generally harbor more than one genetic abnormality, drugs showing in vivo efficacy against lesions carrying multiple genetic changes would be especially promising for further clinical development. The FPT inhibitor FTI-276 exhibits antitumor activity against a human xenograft harboring both a K-ras mutation and a p53 deletion (146). This observation, together with their apparent lack of toxicity, suggests that FPT inhibitors indeed have practical chemopreventive potential. Detailed studies to determine the mechanism(s) of action of these drugs is critical for determining further clinical development.

A new picture of Ras processing appears to be emerging that could significantly impact the design of Ras posttranslational processing inhibitors. However, it is clear that tremendous progress has been made in understanding the biochemical processes involved in Ras-mediated signal transduction. The high frequency of ras mutations in selected cancers and precancers, as well as the crucial role of Ras proteins as central components of signal transduction pathways, makes disruption of Ras-mediated signaling a very promising target for chemopreventive drug development.

Weinstein has described carcinogenesis as a progressive disorder in signal transduction (155). In this regard, the significance to chemoprevention of these early and promising studies with FPT inhibitors, as well as those described previously with EGFR inhibitors (1), extends well beyond the individual mechanistic classes. These studies exemplify the drug design strategies that are becoming possible as our understanding of the molecular pathways and elements controlling cell fate increases. Particularly important for chemoprevention are the newly identified opportunities to block early proliferative changes, making use of the signal transduction mechanisms before they are destroyed.

References
Chemical Induction of Cancer: Modulation and Combination Effects, pp. 73-122.


Review: FPT Inhibitors as Cancer Chemopreventives


Farnesyl protein transferase inhibitors as potential cancer chemopreventives.


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