Lipoxygenase Inhibitors as Potential Cancer Chemopreventives

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Abstract
Mounting evidence suggests that lipoxygenase (LO)-catalyzed products have a profound influence on the development and progression of human cancers. Compared with normal tissues, significantly elevated levels of LO metabolites have been found in lung, prostate, breast, colon, and skin cancer cells, as well as in cells from patients with both acute and chronic leukemias. LO-mediated products elicit diverse biological activities needed for neoplastic cell growth, influencing growth factor and transcription factor activation, oncogene induction, stimulation of tumor cell adhesion, and regulation of apoptotic cell death. Agents that block LO-catalyzed activity may be effective in preventing cancer by interfering with signaling events needed for tumor growth. In fact, in a few studies, LO inhibitors have prevented carcinogen-induced lung adenomas and rat mammary gland cancers.

During the past 10 years, pharmacological agents that specifically inhibit the LO-mediated signaling pathways are now commercially available to treat inflammatory diseases such as asthma, arthritis, and psoriasis. These well-characterized agents, representing two general drug effect mechanisms, are considered good candidates for clinical chemoprevention studies. One mechanism is inhibition of LO activity (5-LO and associated enzymes, or 12-LO); the second is leukotriene receptor antagonist. Although the receptor antagonists have high potential in treating asthma and other diseases where drug effects are clearly mediated by the leukotriene receptors, enzyme activity inhibitors may be better candidates for chemopreventive intervention, because inhibition of these enzymes directly reduces fatty acid metabolite production, with concomitant damping of the associated inflammatory, proliferative, and metastatic activities that contribute to carcinogenesis. However, because receptor antagonists have aerosol formulations and possible antiproliferative activity, they may also have potential, particularly in the lung, where topical application of such formulations is feasible.

Introduction
Eicosanoids derived from the arachidonic acid cascade have been implicated in the pathogenesis of a variety of human diseases, including cancer, and are now believed to play important roles in tumor promotion, progression, and metastatic disease. Although most attention has focused on PGs2 and other COX-derived metabolites, mounting evidence suggests that LO-catalyzed products, LTs, and HETEs also exert profound biological effects on the development and progression of human cancers. For example, 12-LO mRNA expression has been well-documented in many types of solid tumor cells, including those of prostate, colon, and epidermoid carcinoma (1, 2). Also, 12(S)-HETE3 production by some tumor cells, including prostate cells, has been positively correlated to their metastatic potential (3, 4). Additionally, studies show that 12(S)-HETE is a critical intracellular signaling molecule, stimulating PKC and eliciting the biological actions of many growth factors and cytokines that regulate transcription factor activation and induction of oncogenes or other gene products needed for neoplastic cell growth (5–7). Some of these include EGF, FGF, PDGF, tumor necrosis factor, granulocyte-macrophage colony-stimulating factor, and both IL-1 and IL-3 (8–12). Additionally, PKC activation by 12(S)-HETE mediates the release and secretion of cathespin B, a cysteine protease involved in tumor metastasis and invasion, particularly in colon cancer cells (13). Furthermore, tumor cell synthesis of 12(S)-HETE stimulates adhesion by increasing the surface expression of integrin receptors (14, 15). Besides 12(S)-
HETE, other LO metabolites, particularly the 5-LO products (5-HETEs), have been implicated in cancer development. For example, data published recently show that 5-HETE directly stimulates prostate cancer cell growth (16). Like 12(S)-HETE, these molecules are capable of exerting pleiotropic effects on normal and malignant cells through autocrine- and paracrine-mediated mechanisms. Taken together, these data imply that LO products, particularly those of 5- and 12-LO, may play important roles in modulating cancer development. On the basis of this information, pharmaceutical agents that directly interfere with the production of LO metabolites or antagonize the signaling functions of LO products may be effective in preventing cancer. Table 1 lists potential chemopreventive mechanisms associated with inhibition of the LO pathway.

Over the past 10 years, pharmacological agents that specifically inhibit the LO metabolic pathway have been developed to treat inflammatory diseases such as asthma, ulcerative colitis, arthritis, and psoriasis. As inflammatory mediators, LTs elicit a number of reactions, including vessel wall adhesion, smooth muscle contraction, granulocyte degranulation, chemotaxis, and increased mucous secretion and vascular permeability (17). These agents include 5-LO inhibitors of the N-hydroxyurea series developed by Abbott [Leutrol, Zyflo (Zileuton), and ABT-761] and, among others, the redox inhibitor AA-861 (Takeda Chemical), the non-redox inhibitor ZD2138 (Zeniaca), agents that interact with the FLAP, including MK-0591 (Merck) and BAY-X1005 (Bayer), and LTβ4 receptor antagonists SC 41930 (Searle), Accolate (zafirlukast; Zeneca), Singulair (montelukast; Merck), and Uftair (pranlukast; Otsuka/Smith-Kline Beecham). Bacillecin and esculetin are among several experimental compounds demonstrating 12-LO inhibitory activity that may show promise as antiproliferative agents. This report discusses the current clinical status of these and other agents and methods to assess their cancer chemopreventive potential and offers an overview of the LO biosynthetic pathway, its metabolic products, and their significance in human disease and the development of cancer.

**LO Biochemistry and Molecular Biology**

The LOs comprise a family of non-heme iron-containing dioxygenases that catalyze the stereospecific oxygenation of the 5-, 12-, or 15-carbon atoms of arachidonic acid (17–19). In cells, arachidonic acid is esterified to membrane phospholipids in the sn2 position. As depicted in Fig. 1, the reaction begins with the intracellular release of arachidonic acid, mediated by either PLA2, or by the combined actions of phospholipase C and diacylglycerol kinase or phospholipase D and PLA2 (18). In leukocytes, cytokines including IL-1 and tumor necrosis factor can activate PLA2 by stimulating a phospholipase-activating protein (20). Once released, arachidonic acid is either converted by catalytic action of 5-, 12-, or 15-LOs into the corresponding HETEs or is metabolized into LTs or lipoxins through additional sequential reactions, which depend on the biosynthetic capacity of each specific cell type (see below).

LT production proceeds via 5-LO, which catalyzes the first two steps in the metabolic cascade. 5-LO in the presence of FLAP catalyzes the oxygenation of arachidonic acid into 5-hydroperoxycicosatetraenoic acid, followed by a second reaction in which 5-hydroperoxycicosatetraenoic acid is dehydrated to form the epoxide LTA4. Once formed, LTA4 is further metabolized to either LTB4 via stereoselective hydration by LTA4 hydrolase or to LTC4 through glutathione conjugation catalyzed by LTC4 synthase. Sequential metabolic reactions, catalyzed by γ-glutamyl transferase and a specific membrane-bound dipeptidase, convert LTC4 into LTD4 and LTE4, respectively. These three sulfidopeptide LTs are commonly referred to as the slow-reacting substances of anaphylaxis (21). In the lung, sulfidoepetide LTs are known to act on a single high-affinity, smooth muscle receptor, the cys-LT1 receptor (22), resulting in bronchoconstriction and alterations in vascular permeability and mucous secretion in this tissue (23). Important cellular sources of these LTs include eosinophils, mast cells, and basophils (24).

The intracellular localization of 5-, 12-, and 15-LO proteins varies depending on the enzyme and specific cell type (25). In human PMNs, 5-LO was initially reported to be a soluble, cytosolic protein (26, 27). However, later studies showed that, upon activation with Ca2+, 5-LO translocates from the cytosol to the nuclear membrane (28), where it interacts with FLAP, an Mr 18,000 protein that resides in the nuclear envelope (29, 30). Indeed, reversible shifting of 5-LO protein between soluble and membrane-bound forms has been confirmed by other investigators (28, 31), although with differences in distribution patterns and trafficking in various cell types (32–35). Subcellular localization studies suggest that translocation of 12-LO protein from the cytosol to membrane also occurs upon activation (36, 37), whereas 15-LO appears to exist only in cytosol (38, 39).

Metabolic products of the 5-, 12-, or 15-LO biosynthetic pathways modulate the growth of several normal human cells...
types, including T lymphocytes (40), skin fibroblasts (41), epidermal keratinocytes (42), and glomerular epithelial cells (43). Both LTB₄ and LTC₄ increase in vitro growth of arterial smooth muscle cells (44), airway epithelial cells (45), and mitogen-stimulated lymphocytes (46). Besides these cells, LTs play important roles in regulating both human and murine hematopoiesis (47–50).

The 5-, 12-, and 15-LO genes have been cloned and sequenced in several mammalian species (25, 51). Several isoforms of 12-LO have been characterized, each with distinct genomic sequences, gene structure, immunogenicity, and subtype specificity (51–53). These include human platelet-type 12-LO, referred to as P-12-LO, which metabolizes arachidonic acid into 12(S)-HETE (54); the leukocyte type, or L-12-LO, which converts arachidonic acid or linoleic acid into 12(S)-HETE and also produces small quantities of 15(S)-HETE (55); and 12-LO, a unique epithelial type first isolated from bovine tracheal epithelial cells, which catalyzes the synthesis of both 12(S)- and 15(S)-HETE from arachidonic acid (56, 57). However, these classifications are not precise. P-12-LO has also been identified in human keratinocytes and epidermal carcinoma cells lines, and L-12-LO has been found in peritoneal macrophages, tracheal epithelium, whole brain, and other sources, although it is not expressed in mouse leukocytes (25). Yamamoto et al. (58) recently reviewed the molecular structure, catalytic properties, and biological activities of the 12-LO isoenzymes.

LO Metabolites in Carcinogenesis

Evidence from studies in human cancer cells shows that the LO pathways are involved in carcinogenesis in several major tissues. More importantly, a few studies in animal carcinogenesis models show that inhibition of the LO pathways also may inhibit carcinogenesis.

Lung. Two recent studies found that LO inhibitors (specifically of the 5-LO pathway) have chemopreventive activity in animal lung carcinogenesis. Moody et al. (69) and Rioux and Castonguay (70) showed that the FLAP inhibitor MK 886 (25 mg/kg diet) and 5-LO inhibitor A 79175 (75 mg/kg diet) significantly reduced the multiplicity of NNK-induced tumors in strain A/J mice; A 79175 also reduced tumor incidence. In the same study, aspirin (294 mg/kg diet) reduced tumor multiplicity; the combination of aspirin and A 79175 (i.e., inhibiting both the COX and LO pathways) synergistically lowered tumor incidence and multiplicity. These results strongly suggest that 5-LO pathway inhibitors may have chemopreventive activity in lung.

Supporting evidence links LO metabolites with lung cancer cell growth. Studies conducted by Avis et al. (71) in several human lung cancer cell lines (small cell and non-small cell) found that 5-LO is stimulated by two autocrine growth factors, GRP and IGF, both of which stimulate production of 5-HETE. 5-HETE stimulated the growth of lung cancer cells, whereas cells treated with 5-LO inhibitors NDGA, AA-861, and MK-886 showed decreased proliferation; the COX inhibitor aspirin had little effect. Expression of 5-LO and FLAP mRNA by lung cancer cell lines was confirmed using reverse transcription-PCR, and the presence of 5-LO mRNA was identified in samples of primary lung cancer tissue, including both small cell and non-small cell lung carcinomas.

Also relevant to lung cancer development are studies demonstrating that LOs mediate oxidation of potent carcinogens such as benzidine, o-aminodiphenylamine, and others; this activation can be blocked by adding the LO inhibitors NDGA and esculetin (72). Rat lung LO also oxidizes benzo(a)pyrene (73); LOs have been found in human lung tissue by other investigators (74, 75).

Prostate Cancer. Altered eicosanoid biosynthesis in relation to prostate cancer development has been documented. Initial studies found dramatically reduced levels of arachidonic acid; 10-fold greater turnover in malignant versus benign prostate tissue suggested a possible increase in metabolism via the LO and COX pathways in this tissue (76, 77). Linoleic acid stimulated cell growth in experiments conducted in human prostate cancer cells, whereas indomethacin, esculetin, and piroxicam inhibited it, substantiating the involvement of eicosanoids in prostate cancer cell proliferation (78). Although attention has focused on COX-derived products (79–81), particularly PGE₂ (77), COX inhibitors indomethacin and aspirin failed to reduce human prostate PC3 cell DNA synthesis, although arachidonic acid antagonist eicosatetraynoic acid did reduce synthesis (82, 83), suggesting that LO products are essential in modulating prostate DNA synthesis. However, until 5-LO products are recovered from prostate tissue and their synthesis directly shown to be inhibited by 5-LO inhibitory agents, other possible mechanisms cannot be ruled out.

Additional studies by Anderson et al. (84) show reduced DNA synthesis and growth inhibition of prostate cancer cells with specific 5-LO inhibitors (A63162; Abbott), further supporting involvement of the LO metabolic pathway in prostate cancer growth. Likewise, recent work by Ghosh and Myers (16) using PC-3 cells provides convincing evidence that the 5-LO metabolic pathway stimulates prostate cancer cell growth.
specifically, 5-HETE, particularly the 5-oxo-eicosatetraenoic form, stimulates PC-3 cell growth similarly to arachidonic acid; LTs had no effect. 5-HETEs also effectively reversed growth inhibition produced by MK-886 (Merck), a FLAP inhibitor. Both MK-886 and AA-861 effectively blocked prostate tumor proliferation induced by arachidonic acid, whereas the COX inhibitor ibuprofen and 12-LO inhibitors baicalin and BHPF were ineffective.

Besides 5-LO products, other LO metabolites have been implicated in prostate tumor growth. The 12-LO metabolite 12(S)-HETE plays a critical role in prostate tumor metastasis and invasion (1). In 122 matched normal and cancerous prostate tissues, 12-LO mRNA expression was confined to prostate epithelial cells and elevated in malignant cells (3). Also, elevated levels of 12(S)-HETE mRNA significantly correlated with advanced stage, poor differentiation, and invasive potential of prostate cancer cells. Indeed, addition of 12(S)-HETE to rat Dunning R3327 and human prostate adenocarcinoma cells significantly increased their cellular motility and ability to invade basement membrane and correlated with their metastatic potential (85–87). Additional biochemical evidence demonstrated that 12(S)-HETE stimulates secretion of cathepsin B, which is involved in tumor metastases and integrin expression in other tumor cells (13), providing support for the importance of LO products in the development and spread of prostate and other human cancers. More recently, Liu et al. (6) have shown that enhancement of prostate tumor cell invasion by 12(S)-HETE involves selective activation of membrane-associated PKCα. Furthermore, the ability of PKC inhibitor calphostin C to block the 12-LO-stimulated release of cathepsin B lends credence to the observation that 12(S)-HETE acts via activation of PKC (7, 88).

Among several possible mechanisms by which LO inhibition may reduce prostate PC-3 cell proliferation is by altering the concentration of second messengers needed for continued cell growth by shunting other arachidonic acid metabolites into alternate pathways linked to signal transduction mechanisms (89, 90). For example, agents that inhibit 12-LO and block 12-HETE production may result in widespread interference in signal transduction by preventing PKC activation (91, 92). LO inhibition may further affect the expression of proto-oncogenes or their products, including EGF, FGF, TGF-β, N- and K-ras, c-myc, int-2, IGF, and the retinoblastoma gene, also directly or indirectly associated with normal or transformed prostate epithelial cell growth (93, 94). Whether inhibitors of LO biosynthesis can influence expression of these factors, however, has not yet been determined.

Breast. Reports published over the past two decades support a growth-regulatory role for fatty acid metabolites and particularly for the arachidonic acid-derived eicosanoids in the etiology of mammary carcinogenesis (95). Feeding studies conducted in rodent models of mammary tumorigenesis showed that diets rich in linoleic acid, n-6 polyunsaturated fatty acid, and arachidonic acid precursor, stimulate mammary tumor growth induced by either MNU or DMBA (96–99). Conversely, dietary supplementation with n-3 polyunsaturated fatty acid, particularly fish oils, retarded mammary tumor growth and metastasis in a variety of animal tumor models (100). Studies by Abou-El-Ela et al. (101) using the DMBA mammary gland tumor model found that mammary tissue of rats fed 20% menhaden oil produced lower levels of LTβ and PGE₂ compared with groups receiving 20% corn oil or primrose oil, supporting the theory that PGs and LTs may be involved in mammary carcinogenesis.

Additional evidence implicating eicosanoids in human breast carcinogenesis stems from analysis of benign and malignant breast tissue. Markedly elevated levels of both COX and LO metabolites have been documented in human breast cancer tissue compared with tissue from patients with benign disease (102). Recently, Natajaran et al. (103) studied 12-LO mRNA expression in matched, normal, nontumorous and cancerous breast tissue from a small group of patients. In cancerous tissue, 12-LO mRNA expression increased 3–30-fold compared with normal tissue, which contained barely detectable levels. Similarly, greater 12-LO mRNA amounts were documented in two breast cancer cell lines (MCF-7 and COH-BR1) compared with a noncancerous breast epithelial cell line (MCF-10).

Stimulation of breast cancer cell growth by linoleic acid has further been substantiated in vitro in several human breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF-7; Refs. 104–106); the inhibitory action of other n-3 fatty-acids including docosahexaenoic acid (22:6) and eicosapentaenoic acid (20:5), have also been reported in these cells (104–106). Likewise, increased secretion of both LO and COX products, including 12- and 15-HETE and PGE₂, has been documented in human breast cancer cells (MDA-MB-435) treated with 2.7 µM linoleic acid (104). In the same study, linoleic acid enhanced the invasive capacity of breast cancer cells; this effect could be completely blocked by adding esculetin (20 µM), an inhibitor of 5- and 12-LO, but not by adding the COX-specific inhibitor piroxicam. Interestingly, adding 0.1 µM 12-HETE mimicked the stimulatory effect of linoleic acid on cell invasion, whereas PGE₂ and 5-HETE had no effect. Collectively, these data support the concept that enhanced 12-HETE, not PG, is involved in the etiology of breast cancer cell metastasis.

Conflicting reports on the relative importance of LO and COX in breast cancer have been published. For example, some investigators found that COX inhibitors such as indomethacin and flurbiprofen suppress mammary tumor development in rodent models of mammary carcinogenesis (107–109); other studies conducted with these agents, particularly indomethacin, yielded inconsistent results (110) or reported no correlatable effect of COX inhibition to tumor growth (111). Consistent with these findings, in vitro studies have not conclusively associated COX inhibition with reduced mammary tumor cell growth (106, 112, 113). The LO inhibitors such as NDGA (0.1% diet; Ref. 114) and esculetin (0.03% diet; Ref. 115–117) inhibit rat mammary tumor development induced in vivo by MNU or DMBA, lending credence to the hypothesis that LO products are important in mammary tumor development. COX-specific inhibitors produced mixed results in these studies. For example, the relatively selective COX-2 inhibitor nabumetone (0.03% diet) inhibited MNU-induced mammary tumor incidence and multiplicity (105, 117), whereas the COX-1-specific inhibitor piroxicam (0.03% diet) did not inhibit DMBA-induced tumors (116).

Additional support for the role of LO products in breast cancer development includes recently published data on the ability of 12-HETE to mediate the proliferative effects of estrogen and linoleic acid in a human breast cancer cell line (118). In this study, MCF-7 breast cancer cells, estrogen dependent and unresponsive to growth stimulation with linoleic acid, were transfected with 12-LO cDNA and grew in the absence of estrogen, showing reduced expression of estrogen receptor mRNA and protein compared with the parental cell line. Moreover, 12-LO-transfected cells grew more rapidly than the parental cells (36.2 ± 3.0 x 10⁴ versus 12.4 ± 1.1 x 10⁴) when cultured in media containing linoleic acid. Injection of 12-LO-transfected MCF-7 cells into mammary fat pads of ovariecto-
mized mice produced small solid tumors in 100% of estrogen-supplemented mice and 43% of unsupplemented mice. However, additional studies will be needed to explain the amplified response to estrogen in cells overexpressing 12-LO and to further determine whether 12-LO transfection increases the metastatic potential of these cells.

More recently, using reverse transcription-PCR and Northern blot analysis, identification and characterization of a 15-LO identical to the 15-LO gene product present in human pulmonary tissue has been reported in BT-20 human breast carcinoma cells (119). BT-20 cells overexpress both the EGF receptor and the homologous erbB-2 (neu) oncogene product, two factors identified as possible adverse prognostic indicators in human breast cancer (120, 121). Adding indomethacin to these cells had no effect on EGF and TGF-α, but significantly decreasing both growth rate and tumor volume after body weight, BWA4C was the most effective inhibitor, significantly reducing cell numbers within 72 h (140, 143). Likewise, A63162 (Abbott) to these cells reduced DNA labeling and enhanced cell growth in cancerous breast tissue. On the other hand, reducing 13-HODE via LO inhibition may potentially inhibit the mitogenic response and block further proliferation of breast cancer tissue.

Other in vitro studies postulated that LO metabolism may be involved in modulating tumor cell adhesion. In one study conducted in the human breast carcinoma cell line MDA-MB-435, exogenous NDGA dramatically inhibited adhesion to collagen IV induced by either A23187 or arachidonic acid, whereas indomethacin had no effect, implying that COX is not involved in this process (122). Consistent with these findings, previous reports showed that linoleic acid potentiates the metastatic potential of this cell line both in vitro and in vivo (123, 124) and also stimulates cell adhesion to fibronectin in another human breast cancer cell line (125).

Colon. Besides overwhelming data linking PGs to the etiology of colon carcinogenesis (126), several recent lines of evidence suggest that LO products may also be involved in this process. Specifically, in vitro studies conducted in two colon cancer cell lines (HT-29 and HCT-15) reported time- and dose-dependent stimulation of cell proliferation by LTB4 and 12(R)-HETE, a P450-derived product (127). In the same study, SC41930, a competitive LTB4 antagonist, inhibited LTB4-induced growth stimulation in HT-29 cells. Similar inhibition occurred in murine colon adenocarcinoma cell lines MAC16, MAC13, and MAC26 treated with other 5-LO inhibitors, including BWA4C and BWB70C at micromolar concentrations (IC50 <10 μM), whereas Zileuton (IC50, 40 μM) was less effective (128). The same study further analyzed in vivo activities of these agents in male NMRI mice transplanted with fragments of MAC26 or MAC16 colon tumors. At 25 mg/kg body weight, BWA4C was the most effective inhibitor, significantly decreasing both growth rate and tumor volume after 8–13 days of treatment.

Additional evidence supporting LT biosynthesis in colon cancer development stems from biochemical and genetic studies. The ability of colonic epithelial cell lines to synthesize some LTs, including LTB4 and LTA4, has been confirmed in human HT-29 (129, 130) and CaCo-2 cells (131) and in rat colonic crypt epithelial cells (132). Northern blot analysis of total RNA revealed low levels of mRNA transcripts for both 5-LO and FLAP (129), suggesting that intestinal cells have a limited capacity to synthesize LTs. The low recovery of LO enzyme transcripts is consistent with the theory that LTs serve as intracellular messengers rather than inflammatory mediators in this tissue. More recently, platelet-type 12-LO mRNA has been recovered from a human colon carcinoma cell line (2); treatment of these cells with 12(S)-HETE resulted in up-regulation of 12-LO mRNA and protein (133). Collectively, these data strongly suggest that LO metabolism may play a role in colon tumor proliferation.

Skin. Considerable evidence suggests that LOs are involved in epidermal tumor development. Compared with normal epidermis, large quantities of 12(S)-HETE (50–60-fold greater) were found in papillomas and carcinomas induced by DMBA and TPA in a mouse skin tumor model (134). In the same study, 12-LO enzyme activity was elevated 6-fold in papillomas and 3-fold in carcinomas compared with normal tissue. Moreover, expression of platelet-type 12-LO has been confirmed in both normal human epidermis (135) and human epidermoid A431 carcinoma cells (66). 12(S)-HETE overproduction in papillomas may be a mechanism for progression to malignant carcinoma. Recent studies found that EGF and TPA up-regulate expression of 12-LO mRNA in the human A431 cell line (35, 66, 136). Overexpression of Ha-ras in these cells increased the transcription of 12-LO in a dose- and time-dependent manner that correlated to the cellular expression of ras protein (137). Although this study provides evidence that ras can activate 12-LO gene transcription directly, it did not determine whether agents that inhibit 12-LO would be effective in preventing ras-mediated stimulation of intracellular signaling pathways.

Other Sites. In squamous epithelial carcinomas of the head and neck, 12- and 15-HETE are major arachidonic acid metabolites (138). Also, 12(S)-HETE is the predominant metabolic product of metastatic B16 melanoma cells (139). Additionally, excess LT production, specifically LTC4, has been documented in cells from patients with both acute and chronic leukemias (140–142). Adding 5-LO inhibitors SC 41661A (Searle) and A63162 (Abbott) to these cells reduced DNA labeling and decreased cell numbers within 72 h (140, 143). Likewise, growth inhibition with other LO inhibitors, including piriprost, NDGA, and BW755C, has been demonstrated in several malignant human hematopoietic cell lines; the COX inhibitor indomethacin lacked a suppressive effect (47, 144). These data imply that LO products are essential for the in vitro growth of malignant hematopoietic cells.

Pharmacological Agents

As suggested in the discussions above, two general classes of agents inhibit LO pathways. First are the (usually) specific inhibitors of 5-LO and FLAP, and (usually) nonspecific inhibitors of 12-LO; second are LT receptor antagonists. Although the latter class of agents has high potential to treat asthma and other diseases where drug effects are clearly mediated by LT receptors, inhibitors of specific enzymes in the LO pathway may be better candidates for chemopreventive intervention. Unlike receptor antagonism, inhibition of these enzymes results directly in reducing the production of fatty acid metabolites with concomitant damping of the associated inflammatory, proliferative and metastatic activities that contribute to carcinogenesis. In the following paragraphs, both enzyme and receptor inhibitors are described. However, because they are commercially available and, hence, well characterized, and because they have aerosol formulations and possible antiprofllorative activity, the receptor antagonists may also have che-
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mopreventive potential, particularly in lung, where topical application of such formulations is feasible.

Initial 5-LO inhibitors were nonspecific antioxidants that lacked oral bioavailability, produced adverse physiological effects, or interfered with biological processes. Early 5-LO inhibitory compounds, such as phenidone, and various analogues including BW755C, A53612, and IC1 207968, induced methemoglobinemia, precluding their further clinical development (145). Identifying natural products with LO inhibitory activity, such as caffeic acid, NDGA, and vignafuran, inspired pharmaceutical researchers to develop other compounds and analogues of greater potency and enzyme specificity. These efforts led to development of specific 5-LO inhibitors, FLAP inhibitors, peptide LT receptor antagonists, and LTBA receptor antagonists (Table 2 and Figs. 1-5). Experimental agents demonstrating 12-LO inhibitory activity (Table 3) are shown in Fig. 6. The following discussion addresses each class of compounds and includes some agents that have undergone clinical evaluation.

5-LO Inhibitors

First Generation N-Hydroxyurea Derivatives. Zileuton [Leutrol, N-(1-benzo(b)-thien-2yl) ethyl-N-hydroxyurea], a specific 5-LO inhibitor of the N-hydroxyurea series developed by Abbott, represents the first p.o. active LT inhibitor to show clinical efficacy in humans (145). Zileuton reportedly inhibits 5-LO via iron chelation but is devoid of 12- and 15-LO inhibitory activity (146). After extensive clinical evaluation, Zileuton received FDA approval in December 1996 for treating bronchial asthma in patients 12 years and older and was launched in the United States in January 1997. At oral doses of 600 mg four times daily, Zileuton produces moderate airway function improvement in asthmatics within 30 min. An ex vivo Zileuton in the ionophore-stimulated neutrophil assay. A single 40-mg oral dose in health volunteers produced a 117-fold shift in the LTD4-induced bronchoconstriction response curve (243).

A78773 was ~30-fold more potent than Zileuton in the ionophore-stimulated neutrophil assay. Ex vivo evaluation of A78773 after a single oral dose of 0.5 mg/kg body weight revealed complete inhibition of LTB4 production (95%) for up to 5 h and 70% inhibition for 12 h. At the same dose level, Zileuton showed 70% inhibition for 2 h but steadily declined thereafter (148). The antiproliferative activity of A78773 has not been determined.

A79175, the (R)-enantiomer of A78773, exhibited similar potency to A78773 but showed greater resistance to glucuronidation. A79175 entered Phase I clinical trials but was suspended after the discovery of synthetic intermediates of the (4-fluoro-phenyl)methyl-2-thienyl series represented by A85761 (ABT-761), which showed greater stability. In a Phase I study of ABT-761, a single oral dose of 200 mg produced a plasma half-life of 15 h with plasma levels of 1 μg/ml for up to 72 h after dosing (149). The extended duration of action demonstrated by ABT-761 may be therapeutically beneficial in chemoprevention trials where sustained 5-LO inhibition is desirable.

Redox Inhibitors. One of the most widely studied redox inhibitors is docebenone or 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861), a lipophilic quinone structurally resembling coenzyme Q developed by Takeda Chemical Industries, Ltd. (Osaka, Japan). AA-861 is a potent competitive inhibitor of 5-LO but has no effect on either 12-LO
or COX at concentrations <10 μM. In epidermal homogenates of CD-1 mice, AA-861 is a potent inhibitor of epidermal 12-LO (IC₅₀ 1.9 μM; Refs. 150 and 151).

In addition to its anti-inflammatory properties, AA-861 (15 μM/mouse) strongly suppressed skin tumor formation induced by DMBA and TPA (10 nmol/mouse) and produced dose-dependent inhibition of TPA-stimulated ODC activity in the mouse at concentrations of 1–30 μM (151). Additional in vivo studies further showed that at 30 mg/kg body weight administered i.p., AA-861 prevented TPA-induced increase in ODC activity in liver, spleen, and kidneys of CD-1 mice (152).

Tsukada et al. (153) first reported the antiproliferative activity of AA-861 in a study of three human leukemia cell lines, K562, Molt4B, and HL60, and the human cervical cell line HeLa. In this study, AA-861 (6.7 and 20 μM) markedly reduced the growth of leukemia cells but had no effect on cervical HeLa cells. Similarly, at 5–50 μM, AA-861 produced dose-dependent growth inhibition in murine mastocytoma cells (P815; Ref. 154). More recently, AA-861 at concentrations of 5–10 μM significantly reduced the growth and proliferation of human small cell lung cancer (NCI-H209, H345, H82, and N417) and non-small cell lung cancer (NCI-H1155, H23) cell lines stimulated by either of two autocrine growth factors, IGF and GRP (71). Profiles of arachidonic acid metabolism in AA-861-treated cells showed consistent inhibition of 5-HETE, LTD₄, and arachidonic acid metabolism. Another study induced apoptosis in rat Walker (W256) carcinomasarcoma cells with AA-861 at concentrations of ≥20 μM (155).

Non-Redox Inhibitors. ZD2138 by Zeneca, a selective, p.o.-active 5-LO inhibitor of the methoxytetrahydropyran series, is devoid of redox and iron ligand-binding properties (156). Despite its promising anti-inflammatory profile, Phase II clinical trials carried out in asthmatics had mixed results (145), halting further clinical development of this compound. Whether ZD2138 exerts antiproliferative activity remains to be determined.

FLAP Inhibitors, Indole Series. Extensive screening of indole compounds derived from COX inhibitors indomethacin and sulindac led to development of MK-886 by Merck (157), the first FLAP inhibitor to reach clinical evaluation. MK-886 is believed to work by binding to an arachidonic acid binding site on FLAP, facilitating the transfer of the substrate to 5-LO (158). Asthmatics administered 750 mg of MK-886 p.o. showed only 50% inhibition of LTB₄ in ex vivo-stimulated whole blood and in urinary LTE₄ levels (159). On the basis of these data, further clinical development of MK-886 was discontinued. However, antiproliferative effects of MK-886 were observed recently at micromolar concentrations in malignant cells from patients with chronic myelogenous leukemia (141) and in human lung cancer cells (71). On the basis of these preliminary findings, MK-886 may have some therapeutic potential as a chemopreventive agent.

MK0591 represents a novel 2-indolealkanoic acid derivative and second-generation FLAP inhibitor developed by Merck (160). Like MK-886, MK0591 blocks 5-LO activity by binding to FLAP, thereby preventing 5-LO translocation and activation. Despite its promising biochemical profile, development of MK0591 was discontinued after results from further clinical testing were below anticipated values.

FLAP Inhibitors, Quinoline Series. Optimization of the 2-quinolymethoxyphenyl residue of Revlon’s REV 5901 led to BAY-X1005, a potent, p.o. active inhibitor of 5-LO developed by Bayer AG to treat asthma (161, 162). BAY-X1005 reportedly lacks 12-LO or COX inhibitory activity and is devoid of antioxidant activity (163). The binding of BAY-X1005 to FLAP has been reported to directly correlate with the degree of LTB₄ inhibition (164). Development of BAY-X1005 for...
treating asthma was discontinued after a Phase III clinical evaluation. The antiproliferative capacity of BAY-X1005 has not been reported in the literature.

**LTB₄ Receptor Antagonists.** SC 41930, a potent first generation LTB₄ receptor antagonist developed by Searle (Monsanto; Refs. 165 and 166) has demonstrated potency in a variety of inflammatory models. However, the discovery that SC 41930 inhibits f-MLP-induced superoxide release (167) prompted further research to develop agents with greater potency and selectivity.

Second-generation agents derived from structural analogues of SC 41930 include monomethyl amide SC 53228 (168, 169) and thiazole analogue SC 50605 (7-(3-(2-(cyclopropylmethyl)-3-methoxy-4-(thiazol-4-yl)phenoxy)propoxy)-3,4-dihydro-8-propyl-2H-1-benzopyran-2-carboxylic acid) (167). Potent antagonists of LTB₄ receptors both demonstrate substantially improved pharmacological profiles compared with SC 41930. SC 50605 and SC 53228 have been selected for further clinical evaluation.

**LTD₄ Receptor Antagonists.** Ultair (Pranlukast, ONO-1078, SB205312) or N-(4-oxo-2-(1H-tetrazol-5-yl)-4H-1-benzopyran-8-yl)-4-(4-phenylbutoxy)-benzamide, licensed from Ono Pharmaceuticals by Smithkline Beecham, is the first LTD₄ antagonist to be introduced in the world, having been approved for marketing as an asthma treatment in the United States. Accolate has demonstrated potent activity both in vitro and in vivo (170). Upon receiving FDA approval, the Pulmonary-Allergy Drug Advisory Committee has declared Accolate safe and effective in patients older than 12 years of age with mild to moderate asthma (171). The chemopreventive efficacy of this agent, however, has not been investigated.

**Esculetin and Other Experimental 12-LO Inhibitors (Non-specific).** Esculetin, a coumarin derivative extracted from the bark of Aesculus hippocastanum, is a well-known competitive inhibitor of both the LO and COX metabolic pathways (172-175). At concentrations of 0.1–1 μM, esculetin exerts LO inhibitory activity; levels >10 μM are required for COX inhibition (172, 175). Acute toxicity testing of esculetin in the mouse produced an LD₅₀ of 1450 mg/kg body weight i.p. and 2000 mg/kg when given p.o. (176).

The antiproliferative action of esculetin has been demonstrated in several in vitro models. At concentrations of 3–30 μg/ml, it significantly reduced LTB₄ secretion and produced growth suppression in the MDA-MB-231 human breast cancer cell line (177). Likewise, esculetin at 20 μM completely suppressed production of 12-HETE in estrogen-independent human MDA-MB-435 breast cancer cells stimulated with linoleic acid (104). In the same study, 2 or 20 μM of esculetin completely blocked the invasiveness of cells stimulated with linoleic acid while completely suppressing type IV collagenase (metalloproteinase-9) mRNA expression. Similar growth-suppressive effects of esculetin have been reported in human T-lymphoid leukemia cells (IC₅₀ 3.6 ± 0.3 μM; Ref. 178) and in vascular smooth muscle cells (IC₅₀ 10.9 ± 2.8 μM) stimulated with 5% FCS (179).

In vivo studies have further shown that esculetin significantly inhibits the development of mammary tumors induced by 12-O-tetradecanoyl phorbol acetate (TPA) (177). In a similar study, esculetin at 20 μM completely suppressed the growth of mammary tumors induced by 12-O-tetradecanoyl phorbol acetate (TPA) (177).

**Fig. 4.** LTB₄ receptor antagonists.

**Fig. 5.** Peptidyl LT antagonists.
by DMBA in female Sprague Dawley rats fed either a high-fat (20% soybean oil) or low-fat (0.5% soybean oil) diet (115, 116). Rats administered diets containing 0.03% esculetin showed significant reduction in mammary tumor incidence, tumor growth, and cell kinetics in both the high- and low-fat dietary groups. More recently, esculetin has been effective in reducing the invasive and metastatic activity of malignant tumor cells (180). Murine melanoma cells pretreated with 50 μM esculetin and injected into syngeneic mice produced significantly lower numbers of melanoma colonies on the lung surface and fewer tumor metastases. Thus, esculetin may be an effective chemopreventive agent on the basis of these data; however, additional studies will be needed to assess its efficacy in other tumor models.

Baicalein, a low molecular weight flavonoid isolated from *Scutellaria baicalensis* Georgy roots, is a key component of Japanese herbal medicine Sho-saiko-to, commonly used to treat chronic liver diseases in Japan. Early studies conducted in rat platelets showed that nanomolar concentrations of baicalein exert potent 12-LO inhibitory activity, whereas much higher levels are required for COX inhibition (181). In rat PMNs, baicalein also reportedly blocks 5-LO and production of LTC4 (182).

The antiproliferative effects of baicalein have been reported in several in vitro models. Mooto *et al.* (183) showed that baicalein (IC$_{50}$ 50 μg/ml) reduced DNA synthesis and inhibited growth in two human hepatoma cell lines (phospholipase C/P/PRF/5 and Hep-G2). Similar growth-suppressive effects of baicalein have been reported by other investigators in various hepatocellular cancer cell lines, particularly HuH-7 cells (184–186). Likewise, treating human breast cancer cells (MCF-7) with baicalein at microgram levels produced potent antiproliferative activity (IC$_{50}$ 5.3 μg/ml; Ref. 187). In human T-lymphoid leukemia cells (CEM) stimulated with 10% FCS, baicalein dose dependently reduced cell proliferation (IC$_{50}$ 4.7 ± 0.5 μM), exhibiting maximal inhibition (91.5 ± 1.4%) at 10$^{-4}$ M (176). In the same study, baicalein reduced the expression of PDGF-A mRNA, and to a lesser degree also affected TGF-β$_1$ mRNA expression, suggesting that baicalein may exert its antiproliferative action via inhibition of growth factor-mediated signaling pathways. Additional studies found that 5–100 μM concentrations of baicalein in rat Walker (W256) carcinoma cells induced apoptosis in a dose-dependent manner (155). Whether baicalein exerts similar antiproliferative activity in vivo, however, remains to be determined. Nevertheless, these initial data suggest that baicalein may be an effective chemopreventive agent.

Another select 12-LO inhibitor is BHPP, one of a series of hydroxamic acids originally developed by Rorer that was found to exert 5-LO inhibitory activity in rat leukocytes (188). Additional studies demonstrated 12-LO inhibitory activity in Lewis lung carcinoma cells and porcine leukocytes (189) and in murine B16a melanoma cells (7).

The antiproliferative action of BHPP has recently been
reported in rat Walker (W256) carcinosarcoma cells. At concentrations of 0.01–100 µM, BHPP demonstrated dose-dependent growth inhibition; at 10 µM, it produced DNA fragmentation and induced apoptosis in treated cells (190). BHPP has been studied in vitro to assess the role of 12-LO and 12(S)-HETE production in tumor cell matrix interactions and analyze integrin αIIbβ3 expression and in vivo in an experimental model of tumor metastasis (2). In these studies, murine B16a HM340 cells of high metastatic potential were exposed to increasing concentrations of BHPP (10⁻⁵–10⁻⁸ M). Cells treated with BHPP showed decreased adhesion to fibronectin and subendothelial matrix and reduced surface expression of αIIbβ3. Oral and i.p. administration of BHPP (50 mg/kg body weight) 30 min before tail vein injection of HM340 cells produced a 40–50% reduction in lung colony formation in syngeneic C57BL/6J mice. Additional testing will be needed to verify the potency of BHPP in models of chemoprevention.

Representative LT inhibitory agents that have been clinically evaluated but not specifically addressed in this report are listed in Table 4. These include WY50295 (Wyeth-Ayerst), Montelukast or MK-476 (Merck Frosst), WY-50295 (Wyeth-Ayerst) 5-LO/LT antagonist Arthritis, asthma, psoriasis, inflammation Discontinued Phase II. In addition to these sources, methods have been developed to purify 5-LO from human leukocytes using ATP-agarose affinity chromatography (191–193). According to this protocol, ~500 µg of protein can be recovered from 8 × 10⁶ cells. Additionally, glycogen-induced rat peritoneal PMNs are an alternate source of 5-LO when cells are harvested and lysed, then centrifuged. The resulting supernatant is used to measure 5-LO activity as above. Using 105,000 × g supernatant fraction, the major arachidonic acid metabolites produced are LTB₄ and 5-HETE.

Once activity profiles are determined in cell-free assays, each agent is further evaluated in whole-cell models, further verifying LO biosynthesis and establishing the inhibitory efficacy of each agent. Examples of intact cell models used in assessing eicosanoid biosynthesis include human whole blood or PMNs, washed platelets (12-LO), and rat peritoneal leukocytes. In these experiments, cells are preincubated in the presence and absence of varying concentrations of inhibitors, then stimulated with ionophore. LO metabolites are extracted and measured using either enzyme immunoassay or RIA or prelabeled with radiolabeled arachidonic acid before separation by TLC or high-performance liquid chromatography.

### Cell Proliferation and DNA Synthesis Assays

Once inhibitory potency has been established in whole-cell models, each agent is further subjected to a series of antiproliferative screens using established cell culture cancer assay systems. These assays should include representative human cell lines for breast, colon, prostate, and lung cancer. Experiments determine LT or HETE-mediated growth stimulation and concentration-dependent inhibition of cell proliferation and DNA synthesis for each agent. Cell proliferation can be measured by the colorimetric method of Mosmann (194) using tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Briefly, cells are plated at a uniform density and allowed to incubate for a given time period (usually several days) in the presence or absence of inhibitors, then processed according to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide protocol. For DNA synthesis analysis, cells are plated at a uniform density, then exposed to [³H]thymidine and allowed to

### Assessing LT Inhibitory Activity

To assess potency and enzyme specificity, pharmacological profiles of each agent are established based on screening data obtained from both cell-free assays and in vitro model systems. For this purpose, each compound is subjected to a series of enzymatic screens to determine 5-, 12-, and 15-LO inhibitory activities. 5-LO measurement is typically conducted in cell lysates from rat (RBL-1) cells. According to this protocol, RBL-1 cells are harvested and lysed by sonication and centrifuged, and the supernatant fraction was used as a source of the enzyme (146). Activity (5-HEc production) is then measured by either RIA or enzyme immunoassay. Alternately, activity can be determined by using [³H]arachidonic acid as substrate, followed by extraction and separation via TLC or reverse-phase high-performance liquid chromatography with on-line radiation detection. In a similar fashion, 12-LO can be assessed in lysates of human platelets or in partially purified preparations available through commercial sources. Likewise, purified forms of rabbit reticulocyte or soybean 15-LO can be obtained through local suppliers.

### Table 4 Representative Leukotriene Inhibitors/Antagonists Not Included in Text

<table>
<thead>
<tr>
<th>Agent (developer)</th>
<th>Target</th>
<th>Indication</th>
<th>Clinical status</th>
</tr>
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<tbody>
<tr>
<td>CGS-25019C (Novartis/Ciba-Geigy)</td>
<td>LTB₄ antagonist</td>
<td>Arthritis, asthma, psoriasis, inflammatory bowel disease</td>
<td>Phase II</td>
</tr>
<tr>
<td>SC-52798 (Searle)</td>
<td>LTB₃ antagonist</td>
<td>Asthma, bronchitis, rheumatoid arthritis</td>
<td>Phase II</td>
</tr>
<tr>
<td>WY-50295 (Wyeth-Ayerst)</td>
<td>5-LO/LT antagonist</td>
<td>Arthritis, asthma, psoriasis, inflammation</td>
<td>Discontinued Phase II</td>
</tr>
<tr>
<td>Montelukast/MK-476 (Merck Frosst)</td>
<td>LTA₄ antagonist</td>
<td>Asthma (patients ≥6 years of age)</td>
<td>New Drug Application filed first quarter 1997, approved in Finland September 1997</td>
</tr>
<tr>
<td>WY-50295 (Wyeth-Ayerst)</td>
<td>5-LO/LT antagonist</td>
<td>Arthritis, asthma, psoriasis, inflammatory bowel disease</td>
<td>Discontinued Phase II</td>
</tr>
<tr>
<td>SB-201993 (SmithKline Beecham)</td>
<td>LTA₃ antagonist</td>
<td>Inflammation, psoriasis</td>
<td>Discontinued Phase II</td>
</tr>
<tr>
<td>SC-52798 (Searle)</td>
<td>LTA₃ antagonist</td>
<td>Inflammation, psoriasis, ulcerative colitis</td>
<td>Discontinued Phase II</td>
</tr>
<tr>
<td>VML-2950 (Vanguard/En Lilly)</td>
<td>LTA₄ antagonist</td>
<td>Asthma, inflammation, inflammatory bowel disease, psoriasis, rheumatoid arthritis, ulcerative colitis</td>
<td>Phase II</td>
</tr>
<tr>
<td>SB-201993 (SmithKline Beecham)</td>
<td>LTA₃ antagonist</td>
<td>Inflammation, psoriasis</td>
<td>Phase II</td>
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<tr>
<td>SC-52798 (Searle)</td>
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incubate for 1–4 h in the presence and absence of inhibitors. After incubation, the cells are washed, and residual label is assessed by scintillation counting. Cell viability is determined in the presence of inhibitors to rule out any agent-induced toxic effects by either trypan blue exclusion methodology or by the colorimetric method of Mosmann (194) described above.

**In Vivo Testing.** The chemopreventive efficacy of select inhibitory agents can be further tested in well-established in vivo carcinogen models (195). These include MNU- and DMBA-induced mammary carcinogenesis assays conducted in female Sprague Dawley rats (196–198). Both models require a single administration of the carcinogen; mammary tumors are produced within 65–80 days. The strain A/J mouse lung adenoma model is generally used to evaluate the chemopreventive activity of agents against lung cancer (199). It may be advantageous to administer the LO inhibitors by aerosol inhalation to avert systemic toxicity (200).

Potential inhibitors of colon carcinogenesis have been assessed using rat (F344) and mouse (CF1) species (201, 202). For example, according to established protocols, a single dose of azoxymethane administered s.c. to male F344 rats causes colon adenoma and adenocarcinoma formation in about 70% of treated animals by 40 weeks (202). Short-term colon tumor models include measurement of ACF in portions of rat colon stained with methylene blue (203, 204). ACF, thought to be preneoplastic lesions that eventually give rise to colon tumors, are used frequently as intermediate biomarkers of colon cancer (205). Test agents are generally administered p.o., either before, during, or after a single or multiple dose of azoxymethane. Efficacy is evaluated as a reduction in the number of ACF after histological analysis.

Several models have been described for evaluating effects on prostate carcinogenesis. These include the Noble rat model in which male rats are treated with testosterone and estradiol (206, 207), and a second, more recent model developed by Bosland, whereby Wistar rats are given cyproterone acetate, MNU, and testosterone (208, 209). Evaluation of skin tumor inhibition will be conducted according to a two-stage skin tumorigenesis protocol (210) in which DMBA and TPA induce skin papillomas and squamous cell carcinomas (211, 212). Before initiation with DMBA or after promotion with TPA, test agents are applied topically to the back of SENCAR or CD-1 mice, which are highly susceptible to skin tumor induction.

Once the most appropriate studies are completed, activity profiles of the agents will be compared to identify compounds suitable for chemoprevention trials in human subjects. Note that some test compounds have already demonstrated activity in lung and breast. Agents that can be obtained through commercial or other reliable sources and for which pharmacological and preclinical safety data already exist will be recommended initially for further safety evaluation and clinical testing.

Because LO inhibitors are antiinflammatories, conducting future chemoprevention studies and applications in the lung has a high priority. Although approved pharmaceuticals in this class are bioavailable p.o., as noted above, it could be beneficial to explore the use of inhalant formulations, which would deliver agent locally to the lung, potentially reducing toxicity and allowing higher, more efficacious doses. The prostate is also a cancer target of high interest, because LO inhibitors demonstrate a high rate of arachidonic acid metabolism and anti proliferative activity in prostate cancer cells. If these agents prove to be low-toxicity inhibitors of oxidative fatty acid metabolism, the breast will be a potential target for further study.

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Lipoxygenase Inhibitors as Potential Cancer Chemopreventives

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