Published OnlineFirst June 16, 2009; DOI: 10.1158/0008-5472.CAN-09-0491

Research Article

[6]-Gingerol Suppresses Colon Cancer Growth by Targeting Leukotriene A_4 Hydrolase

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Abstract

[6]-Gingerol, a natural component of ginger, exhibits anti-inflammatory and antitumorigenic activities. Despite its potential efficacy in cancer, the mechanism by which [6]-gingerol exerts its chemopreventive effects remains elusive. The leukotriene A_4 hydrolase (LTA_4H) protein is regarded as a relevant target for cancer therapy. Our *in silico* prediction using a reverse-docking approach revealed that LTA_4H might be a potential target of [6]-gingerol. We supported our prediction by showing that [6]-gingerol suppresses anchorage-independent cancer cell growth by inhibiting LTA_4H activity in HCT116 colorectal cancer cells. We showed that [6]-gingerol effectively suppressed tumor growth *in vivo* in nude mice, an effect that was mediated by inhibition of LTA_4H activity. Collectively, these findings indicate a crucial role of LTA_4H in cancer and also support the anticancer efficacy of [6]-gingerol targeting of LTA_4H for the prevention of colorectal cancer. [Cancer Res 2009;69(13):5584–91]

Introduction

Chemoprevention by plant-derived compounds or dietary phytochemicals has emerged as an accessible and promising approach to cancer control and management (1). Of the many phytochemicals displaying a wide array of biochemical and pharmacologic activities, [6]-gingerol, the major pharmacologically active component of ginger, was reported to exhibit antioxidant and anti-inflammatory properties and exert substantial anticarcinogenic and antimutagenic activities (2). Several lines of evidence suggest that [6]-gingerol is effective in the suppression of the transformation, hyperproliferation, and inflammatory processes that initiate and promote carcinogenesis, as well as the later steps of carcinogenesis, namely, angiogenesis and metastasis (3–7). Despite its anticancer activity against several human cancers, the exact molecular mechanism by which [6]-gingerol exerts its chemopreventive effects is not fully understood. Identification of molecular and cellular targets, which are associated with the suppression of cell malignancy, is important in the prevention of cancer and will provide a better understanding of anticancer mechanisms. Therefore, the delineation of the molecular mechanism of action exerted by [6]-gingerol merits further investigation.

The leukotrienes compose a class of structurally related paracrine hormones derived from the oxidative metabolism of arachidonic acid and are implicated in human cancer and chronic inflammation (8, 9). Leukotrienes are found at high levels in most inflammatory lesions and are involved in the physiologic changes that are characteristic of the inflammatory process (10). Previous studies showed that leukotrienes, such as leukotriene B_4 (LTB_4), a potent chemoattractant that induces a vigorous inflammatory response, are implicated in cancer development (11–14). Because LTB_4 was shown to play a role in carcinogenesis, recent studies focused on leukotriene A_4 hydrolase (LTA_4H) as an attractive target for chemoprevention and cancer therapy (15). LTA_4H is a bifunctional zinc enzyme that catalyzes the final rate-limiting step in the biosynthesis of LTB_4. Besides catalyzing the production of LTB_4, LTA_4H also possesses aminopeptidase activity (16). Although few physiologic substrates have been identified, the suggestion was made that LTA_4H might participate in the processing of peptides related to inflammation and carcinogenesis. LTA_4H was shown to exhibit high levels of protein expression in certain types of cancers, and its inhibition leads to reduced cancer incidence in animal models (17, 18). The analysis of the cocrystal structure of LTA_4H with its inhibitor has provided excellent opportunities for structure-based drug development (19).

Here we found that LTA_4H is overexpressed in several human cancer cell lines, including colorectal cancers. Knockdown of LTA_4H provided new direct evidence showing that LTA_4H is implicated in the anchorage-independent growth of HCT116 colon cancer cells. Moreover, our findings showed that [6]-gingerol suppresses tumor growth of HCT116 cells implanted in nude mice by inhibiting the enzymatic activity of LTA_4H. These data indicate that LTA_4H might be a highly desirable target for the prevention of colorectal cancers.

Materials and Methods

Reagents. [6]-Gingerol (98% purity verified by TLC) was from Dalton Chemical Laboratories. Basal medium Eagle (BME), gentamicin, and L-glutamine were purchased from Life Technologies, Inc. CNBr-Sepharose 4B was purchased from Amersham Pharmacia Biotech. The LTA_4H human recombinant protein and its antibody for Western blot analysis were purchased from Cayman Chemical. The 29-mer small hairpin RNA (shRNA) construct against LTA_4H used in this study was from OriGene Technologies, Inc.

Cell culture and transfection. H520, H1299, HCT15, and LNCaP cells were cultivated in RPMI supplemented with 10% fetal bovine serum (FBS) and antibiotics in a 5% CO_2 incubator. HCT116, HT29, and SKBR3 cells were maintained in McCoy's 5A medium. For transfection experiments, jetPEI (Qbiogen, Inc.) transfection reagent was used following the manufacturer's instructions.

***In silico* target identification.** To find the potential binding proteins of [6]-gingerol, the potential drug target database (PDTD; ref. 20; v. 2007) was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-0491

Published OnlineFirst June 16, 2009; DOI: 10.1158/0008-5472.CAN-09-0491

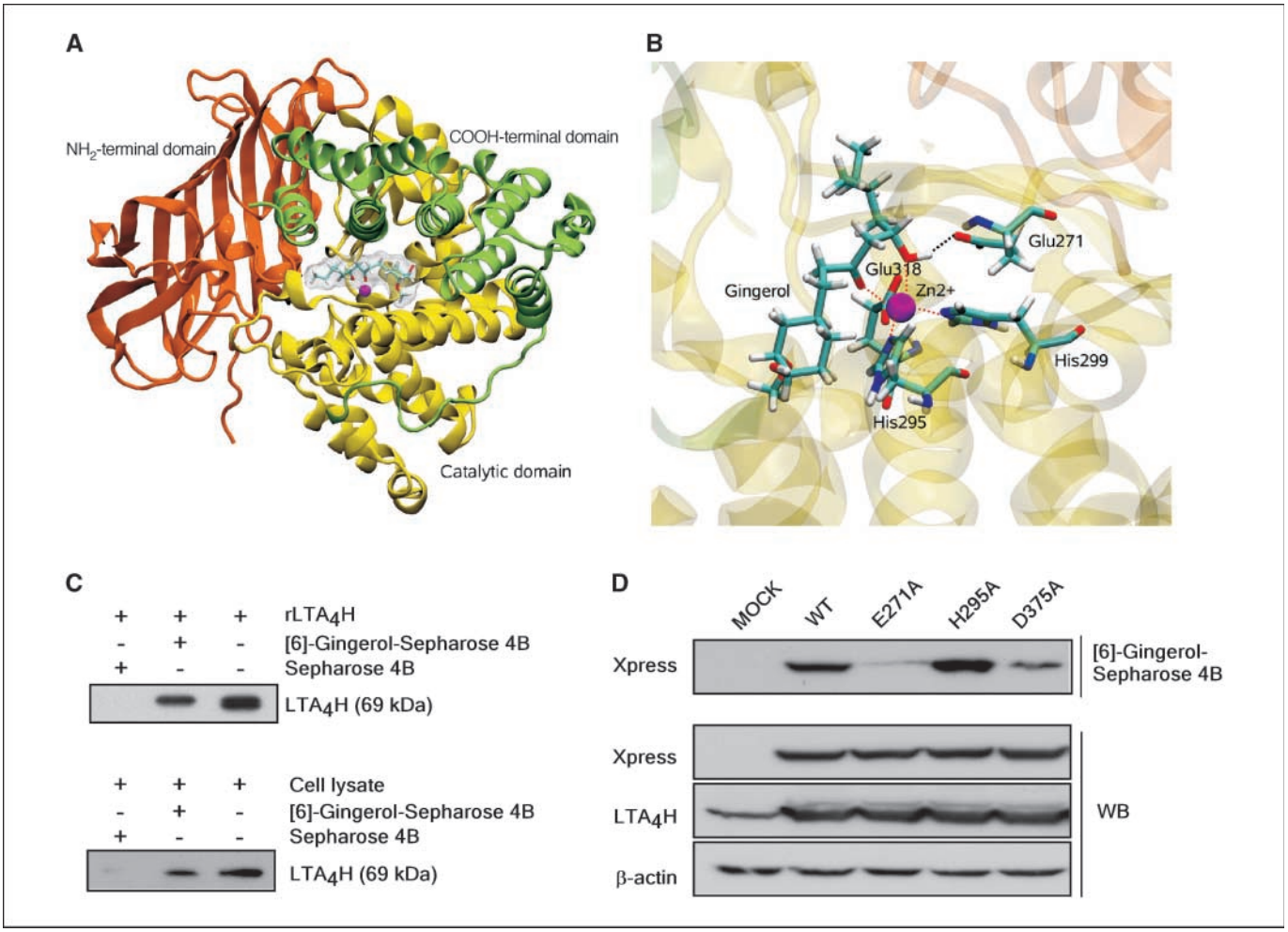

Figure 1. [6]-Gingerol specifically binds with Glu271 of LTA_4H. *A,* proposed molecular model of [6]-gingerol binding with LTA_4H. The catalytic, NH_2-terminal, and COOH-terminal LTA_4H domains are in cartoon representation in yellow, orange, and green colors, respectively. [6]-Gingerol is depicted in stick and transparent surface area, and the zinc ion is represented as a purple sphere. *B,* close-up view of the interactions of [6]-gingerol within the LTA_4H catalytic site. The hydrogen bond between the ligand and Glu271 (both in stick representation) is shown as a dotted black line. The amino acids coordinating the zinc ion are in stick representation and the rest of the catalytic domain is shown as a transparent cartoon. *C,* [6]-gingerol specifically binds with LTA_4H *in vitro* and *ex vivo*. The *in vitro* (*top*) and *ex vivo* (*bottom*) binding of [6]-gingerol with LTA_4H was confirmed by pull-down assay using [6]-gingerol-Sepharose 4B beads and subsequent Western blot analysis. *D,* LTA_4H from cell lysates was pulled down using [6]-gingerol-Sepharose 4B beads, and the binding affinity with [6]-gingerol was determined by Western blot analysis.

used. The PDTD contains structural information (e.g., active site) of more than 830 known or potential protein drug targets. [6]-Gingerol was docked to each target in PDTD with the reverse docking tool TarFisDock (21). More details on the reverse docking procedure are given elsewhere (21–25). The protein "hits" identified through the reverse docking method (i.e., the top 2% of ranked list) are considered as potential target candidates for further validation studies.

Molecular modeling. Considering the structural similarity between [6]-gingerol and bestatin, the LTA_4H crystal structure (PDB code 1HS6) was chosen for further docking studies, which were carried out using the Maestro suite of software (Maestro, version 7.5, Schrödinger). [6]-Gingerol was docked within the LTA_4H binding site using the QM-Polarized ligand docking (26).

Soft agar formation assay. Cells (8×10^3 per well) were suspended in BME (1 mL with 10% FBS and 0.33% agar) and plated over a layer of solidified BME/10% FBS/0.5% agar (3.5 mL) with various concentrations of [6]-gingerol. The cultures were maintained at 37°C in a 5% CO_2 incubator for 6 to 7 d, and the colonies were counted under a microscope using the Image-Pro Plus software (v. 4) program (Media Cybernetics).

Western blot analysis. Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech), which were blocked and hybridized with specific primary antibodies. The protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Biosciences Corp.) after hybridization with a horseradish peroxidase–conjugated secondary antibody.

***In vitro* pull-down assay.** Recombinant human LTA_4H (0.5 μg) or endogenous cell lysates (500 μg) were incubated with [6]-gingerol-Sepharose 4B (or Sepharose 4B only as a control) beads (50 μL, 50% slurry) in reaction buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 2 μg/mL bovine serum albumin, 0.02 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 0.02 mmol/L PMSF], and proteins bound to the beads were analyzed by Western blotting.

Cell proliferation assay. Cells were seeded (2×10^3 per well) in 96-well plates. After incubating for various periods of time, 20 μL of CellTiter96 Aqueous One Solution (Promega) were added and then cells were further incubated for 1 h at 37°C in a 5% CO_2 incubator. Absorbance was measured at 492 nm.

LTA_4H enzymatic assay. Aminopeptidase activity was determined by a modification of a published procedure (27). Recombinant human LTA_4H (0.5 μg) was incubated for 15 min at room temperature in assay buffer

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[50 mmol/L Tris-Cl (pH 8.0), 100 mmol/L KCl] in the presence of various concentrations of [6]-gingerol. Then the substrate (L-alanine-4-nitro-anilide hydrochloride, Sigma Chemical Co.) was added to a final concentration of 5 mmol/L. To measure the LTB_4 levels, HCT116 or HT29 colon cancer cells were preincubated with [6]-gingerol for 24 h and then incubated with serum-free medium containing 5 μmol/L calcium ionophore A23187, 1.6 mmol/L $CaCl_2$, and 10 μmol/L arachidonic acid at 37°C for 30 min. Immunoreactive LTB_4 was quantified by ELISA (Cayman Chemical) following the supplier's instructions.

Mice. Athymic mice [Cr:NIH(S), NIH Swiss nude, 6–9 wk old] were purchased from the National Cancer Institute (NIH) and were maintained under "specific pathogen-free" conditions according to guidelines established by Research Animal Resources, University of Minnesota.

***In vivo* tumor growth.** Mice were divided into three groups: untreated control group (n = 5; 3 males, 2 females), [6]-gingerol group (n = 21; 10 males, 11 females), and vehicle group (n = 20; 10 males, 10 females). A separate group of 5 untreated control mice was maintained as a negative control for comparison of body weights and spontaneous tumor development. For the [6]-gingerol group, 500 μg of [6]-gingerol in ethanol (0.001 μL) suspended in 50 μL autoclaved water were fed to each mouse by gavage in this group three times a week. The dose of [6]-gingerol was based on preliminary pilot studies and also extrapolated from cell culture experiments. For the vehicle-treated group, 0.001 μL of 100% ethanol suspended in 50 μL autoclaved water was fed to each mouse by gavage in this group three times a week. Before tumor cell injection, mice were fed either 500 μg of [6]-gingerol or vehicle (ethanol) three times a week for 2 wk. At the beginning of the 3rd week, HCT116 colon cells (3×10^6) were injected into the right flank of each mouse. Following injection, mice continued to be fed 500 μg [6]-gingerol or vehicle three times a week. Mice were weighed and tumors measured by caliper twice a week. Tumor volume was calculated from measurements of two diameters of the individual tumor according to the formula: tumor volume (mm^3) = [longer diameter × shorter diameter^2]/2. Mice were monitored until tumors reached 1 cm^3 total volume at which time mice were euthanized and tumors extracted. All studies were done according to guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee.

Statistical analysis. All quantitative data are presented as mean value ± SD unless indicated otherwise. The statistical significance of compared measurements was measured using the Student's t test or one-way ANOVA, and $P < 0.05$ was considered significant.

Results

[6]-Gingerol specifically binds with LTA_4H *in vitro* and *ex vivo*. We conducted *in silico* screening using a reverse-docking approach to elucidate potential targets of [6]-gingerol. [6]-Gingerol was reversely screened against the Potential Drug Target Database (PDTD; ref. 20) of ~1,200 protein entries. The top 2% of the ranked list of molecules identified by reverse docking for [6]-gingerol with all potential targets is shown in Supplementary Table S1. Among others, LTA_4H was identified as a possible molecular target for [6]-gingerol (Supplementary Table S1 and Supplementary Discussion). Accumulating evidence supports a functional role for LTA_4H in cancer development, and therefore targeting LTA_4H is regarded as a useful strategy in chemoprevention and cancer therapy (15). Interestingly, our docking model (Fig. 1*A*) showed that [6]-gingerol might bind to LTA_4H in a manner similar to bestatin (19), which is a well-known inhibitor of LTA_4H and other aminopeptidases. In fact, bestatin and [6]-gingerol may share the same localization within the LTA_4H catalytic pocket (Supplementary Fig. S1). Similar to bestatin, [6]-gingerol, with its carbonyl and hydroxyl oxygens, seems to be able to participate in the coordination of the zinc ion with its hydroxyl group to form a hydrogen bond with Glu271 (Fig. 1*B*).

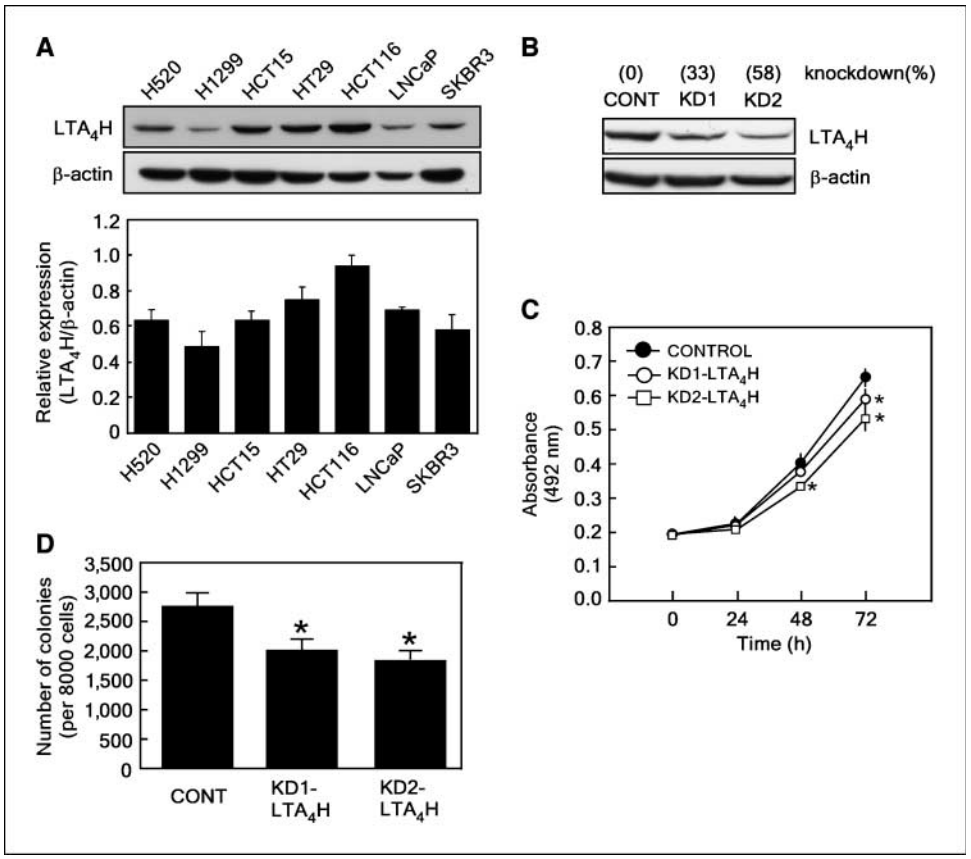

Figure 2. LTA_4H is highly expressed and required for the growth of HCT116 cells. *A,* Western blot analysis of LTA_4H expression in several cancer cell lines. *Top,* H520 and H1299: lung adenocarcinoma; HCT15, HT29, and HCT116: colorectal carcinoma; LNCaP: prostate carcinoma; SKBR3: breast carcinoma. *Bottom,* densitometric analysis of expression level of LTA_4H normalized against β-actin. *B,* HCT116 cells were transfected with shRNA pRS noneffective GFP plasmid (*CONT*) or shRNA pRS LTA_4H plasmid (*KD1, KD2*), and stable colonies were selected by puromycin. Knockdown of LTA_4H was analyzed by Western blot. The percent knockdown was assessed by densitometry (*top*). *C,* using control (*CONT*), KD1-LTA_4H, and KD2-LTA_4H stable cells, cell proliferation was determined at 24-h intervals up to 72 h. *Points,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in proliferation rate compared with control group. *D,* colony formation in soft agar using control, KD1-LTA_4H, or KD2-LTA_4H cells. Cells were grown in soft agar for 6 d and then colonies were counted using a microscope and the Image-Pro PLUS software program (v.4). *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in colony formation versus control cells.

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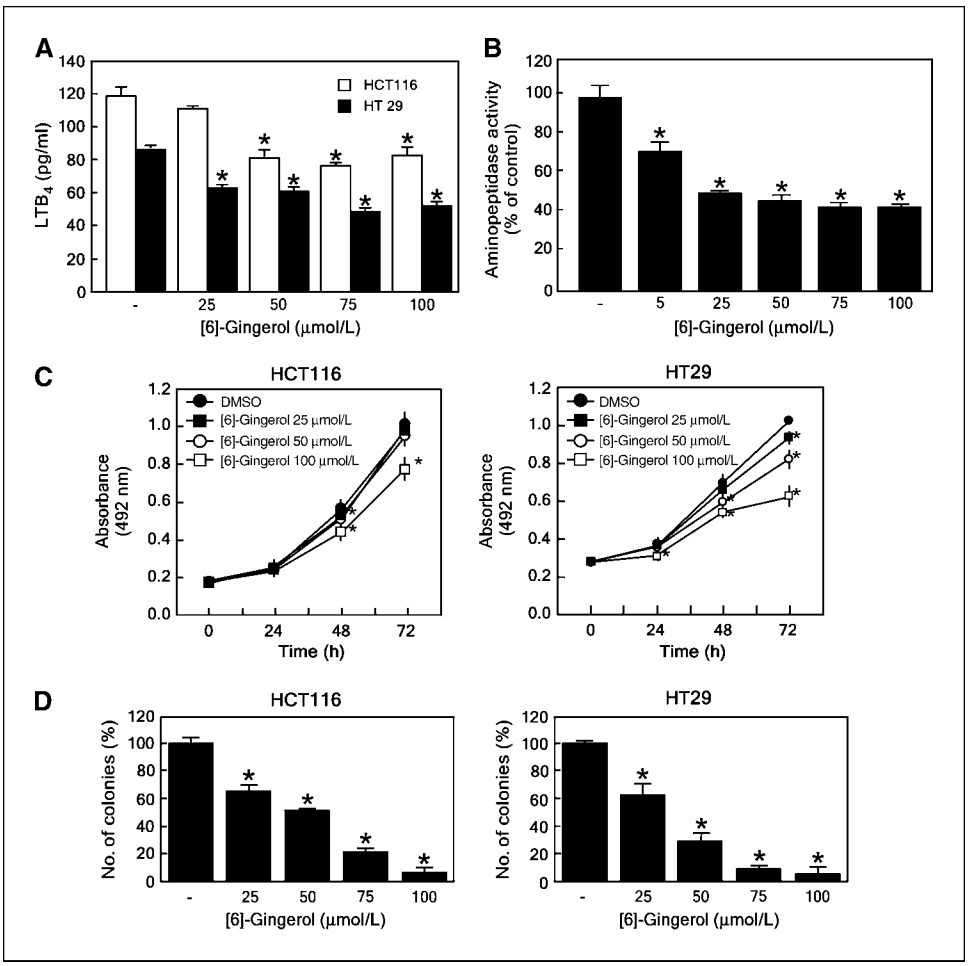

Figure 3. [6]-Gingerol inhibits LTA_4H activity and suppresses the growth of colon cancer cells. *A,* secretion of LTB_4 in HT29 or HCT116 cells was quantified by ELISA. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in LTB_4 secretion in [6]-gingerol–treated cells compared with the DMSO-treated group. *B,* aminopeptidase activity was determined in a spectrophotometric assay at 405 nm. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in activity in cells treated with [6]-gingerol compared with the DMSO-treated group. *C,* [6]-Gingerol suppresses anchorage-dependent growth of HCT116 or HT29 cells. Cell proliferation was estimated by MTS assay. Absorbance (A_{492}) was read at 24-h intervals up to 72 h. *Points,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in proliferative rate compared with control group. *D,* [6]-gingerol inhibits anchorage-independent growth of HCT116 or HT29 cells. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, significant decrease in colony formation in cells treated with [6]-gingerol compared with the DMSO-treated group.

To confirm this prediction, we performed an *in vitro* pull-down assay using [6]-gingerol–conjugated to Sepharose 4B beads. Results revealed that recombinant LTA_4H binds with [6]-gingerol-Sepharose 4B beads, but not with Sepharose 4B beads alone (Fig. 1*C, top*) *in vitro*. We also confirmed the *ex vivo* binding of [6]-gingerol with endogenous LTA_4H in HCT116 cells (Fig. 1*C, bottom*). These results clearly support our hypothesis that LTA_4H is a target for [6]-gingerol *in vitro* and *ex vivo*.

To further identify the amino acid residues of LTA_4H that are required for its binding with [6]-gingerol, we constructed full-length wild-type (WT) LTA_4H, mock, and three LTA_4H mutants, including [E271A]LTA_4H, [H295A]LTA_4H, and [D375A]LTA_4H. The mutants were based on the molecular modeling results, which suggested that Glu271 of LTA_4H might be involved in the binding of [6]-gingerol. The WT, mock, and mutant plasmids of LTA_4H were transfected into HEK293 cells to determine whether the substitution of alanine (Ala) for Glu271, His295, or Asp375 would affect the binding affinity of LTA_4H with [6]-gingerol. The ectopically expressed WT LTA_4H interacted strongly with [6]-gingerol (Fig. 1*D*). In contrast, the [E271A]LTA_4H mutant displayed a markedly reduced binding affinity with [6]-gingerol compared with the WT, H295A, or D375A mutant (Fig. 1*D*). This result indicated that the Glu271 residue is required for LTA_4H binding with [6]-gingerol confirming the docking results (Fig. 1*A* and *B*). The docking model showed no direct interaction between [6]-gingerol and His295, and indeed, as expected, [H295A]LTA_4H did not disrupt the protein-ligand binding (Fig. 1*B* and *D*). The [D375A]LTA_4H mutant showed a small reduction in binding affinity with [6]-gingerol, which might be due to a structural change in the binding pocket that could only partially affect the binding.

Knockdown of LTA_4H inhibits anchorage-independent growth of HCT116 colon cancer cells. Previous immunohistochemical analysis suggested that LTA_4H is highly expressed in several human cancers including esophageal adenocarcinomas (15). To determine whether LTA_4H activity is directly associated with the tumorigenic properties of cancer cells, we first evaluated the expression of LTA_4H in several human cancer cell lines. Compared with other cancer cell lines, LTA_4H expression was relatively higher in colorectal cancer cell lines, especially in HCT116 cells (Fig. 2*A*). These data suggested that LTA_4H might be associated with the tumorigenic potential of colorectal cancer cells. Based on the finding that LTA_4H is highly expressed in HCT116 cells, we investigated the function of LTA_4H in the growth of this cell line. To assess the effects of LTA_4H inhibition on HCT116 colorectal cancer cell growth, we established two stable HCT116 clones (KD1-LTA_4H and KD2-LTA_4H) that express shRNAs targeting different sequences of LTA_4H. The specificity of shRNA targeting of LTA_4H was confirmed by Western blot analysis. Notably, a substantially reduced expression level of LTA_4H was observed in clone KD2-LTA_4H (KD2) compared with the control cells that express GFP-shRNA (Fig. 2*B*). Additional results indicated that the rate of proliferation of KD2-LTA_4H cells was delayed compared with control cells (Fig. 2*C*).

Based on the finding that knockdown of LTA_4H is associated with a reduced proliferation rate, we examined whether knockdown of

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LTA_4H would affect cell growth under anchorage-independent conditions. Anchorage-independent growth ability is an *in vitro* indicator and a key characteristic of the transformed cell phenotype (28). Our results revealed that the knockdown of LTA_4H in HCT116 cells by shRNA (KD1 or KD2) resulted in fewer colonies being formed in soft agar compared with control cells (Fig. 2*D*). These results suggest that blocking LTA_4H in HCT116 colon cancer cells reduces the malignant potential of these cells.

[6]-Gingerol inhibits LTA_4H activity and suppresses colon cancer cell growth. Based on our results showing that [6]-gingerol directly binds with LTA_4H, we then investigated whether [6]-gingerol inhibits LTA_4H enzyme activity. We first measured the secreted LTB_4 levels in HCT116 and HT29 cells. Results showed that [6]-gingerol suppresses LTB_4 production in both cell lines (Fig. 3*A*). Moreover, the inhibitory effect of [6]-gingerol against aminopeptidase activity was further evaluated *in vitro* by using a *p*-nitroanilide derivative of alanine (Ala-p-NA) as substrate. The aminopeptidase activity of LTA_4H was also potently suppressed by [6]-gingerol (Fig. 3*B*).

Next, we evaluated the effect of [6]-gingerol treatment on proliferation of the colorectal cancer cell lines HCT116 and HT29. Data indicate that [6]-gingerol treatment significantly inhibits HCT116 cell growth at 100 μmol/L (Fig. 3*C, left*) or HT29 growth in a dose-dependent manner (Fig. 3*C, right*). In addition, we examined the effect of [6]-gingerol on anchorage-independent growth of HCT116 or HT29 cells, which highly express LTA_4H. Cells were cultured for 6 days in medium containing various concentrations (0–100 μmol/L) of [6]-gingerol. Control (DMSO-treated) cells grew readily and formed many colonies in soft agar (Fig. 3*D*) in both HCT116 (Fig. 3*D, left*) and HT29 cells (Fig. 3*D, right*). On the other hand, [6]-gingerol–treated cells showed an impaired anchorage-independent growth capability, leading to a significant dose-dependent reduction in colony formation (Fig. 3*D*).

LTA_4H activity enhances anchorage-independent growth of HCT116 cells. Our data indicated that the Glu271 residue of LTA_4H was required for binding with [6]-gingerol. Previous data presented by others (29) suggested that Glu271 is the recognition site for the NH_2-terminal amino group of the peptidase substrate. We therefore determined whether blocking the aminopeptidase activity of LTA_4H would have an effect on its ability to induce anchorage-independent cell growth. WT or [E271A]LTA_4H was transiently transfected into HCT116 cells and aminopeptidase activity was measured. As expected, the expression of WT-LTA_4H, but not of [E271A]LTA_4H, strongly increased the aminopeptidase activity, indicating the importance of the Glu271 residue of LTA_4H in its aminopeptidase activity (Fig. 4*A*). To determine whether the aminopeptidase activity of LTA_4H is involved in anchorage-independent cell growth, we transfected WT or [E271A]LTA_4H

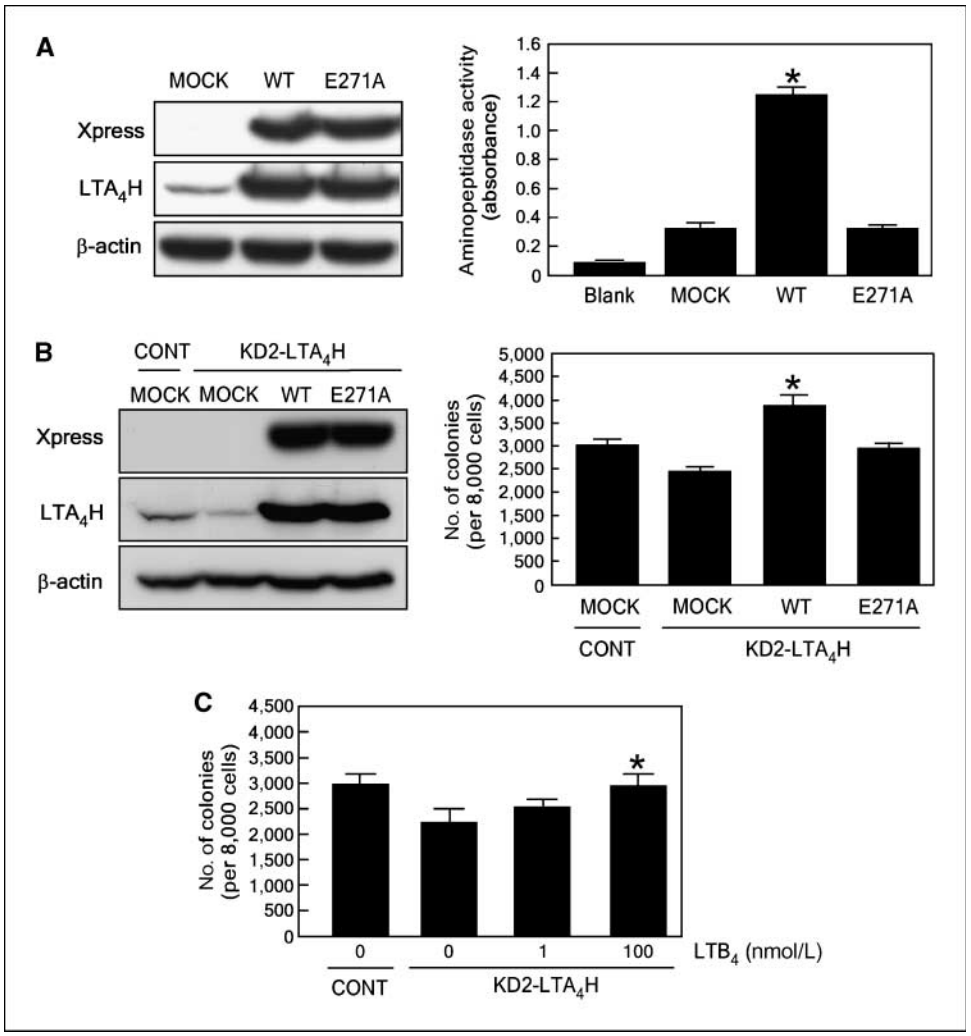

Figure 4. LTA_4H activity is required for HCT116 cell growth in soft agar. *A,* HCT116 cells were transfected with a pcDNA4A-Mock, pcDNA4A-LTA_4H, or mutant [E271A]LTA_4H plasmid and LTA_4H expression level was confirmed by Western blot analysis using an antibody against Xpress or LTA_4H. β-Actin was used as a loading control (*left*). Cell lysates (50 μg) were incubated with 5 mmol/L L-alanine-4-nitro-anilide hydrochloride and aminopeptidase activity was determined with a spectrophotometer at 405 nm (*right*). *, $P < 0.01$, versus MOCK. *B,* stable KD-LTA_4H cells were transfected with a pcDNA4A-LTA_4H or [E271A]LTA_4H plasmid, and expression level was confirmed by Western blot analysis (*left*). *Right,* colony formation of KD2-LTA_4H cells transfected with WT or [E271A]LTA_4H. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.01$, WT-transfected cells compared with MOCK-transfected cells. *C,* soft agar formation assay of KD2-LTA_4H cells treated with the indicated dose of LTB_4. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.05$, versus LTB_4 nontreated control.

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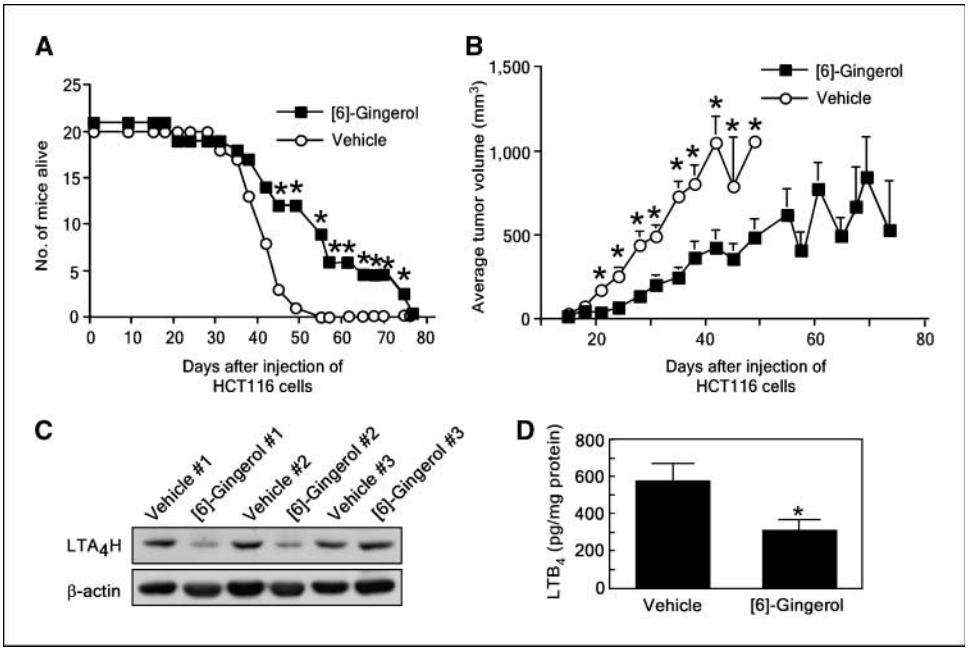

Figure 5. [6]-Gingerol suppresses tumor growth through inhibition of LTB_4 production. *A,* mice fed [6]-gingerol survived significantly longer than did mice fed vehicle. According to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee, mice were to be euthanized when tumor size reached 1 cm^3. Thus, based on this stipulation, mice fed [6]-gingerol survived significantly longer than those fed vehicle (ethanol). All vehicle-treated mice were euthanized by day 50 (after injection) because of attaining maximal allowable tumor size. The study was terminated at day 75 after injection when all but one [6]-gingerol–fed mouse had reached the maximal allowable tumor size of 1 cm^3 (*, $P < 0.05$). *B,* total mean (average) tumor volume in the [6]-gingerol–treated group increased significantly less than that of the vehicle-treated group. Tumor volume was measured and recorded twice a week for the duration of the study (*, $P < 0.001$). *Points,* mean; *bars,* SE (*A* and *B*). Significant differences were determined by one-way ANOVA. *C,* expression of LTA_4H in vehicle- or [6]-gingerol–treated tumor tissues ($n = 3$). *D,* analysis of vehicle- or [6]-gingerol–treated LTB_4 tissue level by ELISA. The amount of LTB_4 is expressed as picograms per milligram of protein ($n = 3$).

into KD2-LTA_4H cells and assessed colony formation in soft agar. Although an enhanced number of colonies was observed in WT and [E271A]LTA_4H–transfected cells compared with mock-transfected cells, a higher level of recovery in colony formation was detected in WT compared with [E271A]LTA_4H–transfected cells, which lack aminopeptidase activity (Fig. 4*B*). These results imply that the aminopeptidase activity of LTA_4H might be necessary for anchorage-independent growth of HCT116 cells. In addition, treatment of KD2-LTA_4H cells with LTB_4 also enhanced soft agar colony formation, indicating that the level of LTB_4 that is produced by the epoxide hydrolase activity of LTA_4H contributes to anchorage-independent growth of HCT116 cells (Fig. 4*C*).

[6]-Gingerol suppresses tumor growth by inhibiting LTA_4H activity *in vivo*. Based on the results of our *ex vivo* and *in vitro* data, we evaluated whether [6]-gingerol could suppress tumor growth *in vivo*. The body weights of [6]-gingerol– or vehicle-treated groups were similar throughout the study (data not shown). The first measurable tumors (minimum of 13.5 mm^3) were observed in both experimental groups on day 15 after injection (data not shown). However, the vehicle-treated group had 13 measurable tumors, whereas only 4 tumors were large enough to be measured in the [6]-gingerol–treated group (data not shown). Furthermore, all mice in the vehicle-treated group had developed measurable tumors by day 28 after injection, whereas all mice (except one) in the [6]-gingerol group did not develop measurable tumors until day 38. Furthermore, results showed that mice fed [6]-gingerol survived significantly longer than those receiving vehicle, implying that tumors grew much slower. Specifically, as of day 49 after injection, all vehicle-treated mice had been euthanized due to tumor size equal to 1 cm^3. On the other hand, at day 49, 11 of the [6]-gingerol–treated mice still had not developed tumors equal to 1 cm^3 (Fig. 5*A*). Collectively, the results presented in Fig. 5*B* show that mean tumor volume in the vehicle-treated group increased significantly faster than that in the [6]-gingerol–treated group ($P < 0.001$). To further determine whether the antitumor effect of [6]-gingerol was associated with inhibition of LTA_4H, tumor extracts from each of the three vehicle-treated and three [6]-gingerol–treated mice (i.e., euthanized on the same day of the experiment) were prepared and analyzed for LTA_4H expression and the production of LTB_4. Western blot analyses revealed that the [6]-gingerol–treated tumor extracts exhibited substantially decreased LTA_4H expression level compared with vehicle-treated tumors (Fig. 5*C*). Consistent with this result, ELISA data showed that [6]-gingerol–treated tumors exhibited a much lower level of LTB_4 production, suggesting that [6]-gingerol inhibits colon tumor formation by suppressing LTA_4H activity *in vivo* (Fig. 5*D*).

Discussion

Colorectal carcinoma, the third leading cause of cancer-related deaths in the United States, is a highly preventable cancer with a transition from precursor to malignant lesion of 10 to 15 years (25). Chemoprevention by consumption of edible phytochemicals has gained considerable attention as a promising strategy for reducing the incidence of colorectal cancer as well as other cancers. Our results herein clearly show a role of [6]-gingerol as a chemopreventive and/or chemotherapeutic agent for colorectal carcinomas and strongly suggest that LTA_4H is a potential therapeutic target of [6]-gingerol. Notably, LTA_4H has long been recognized as an anti-inflammatory target. Its enzymatic product, LTB_4, is widely

Published OnlineFirst June 16, 2009; DOI: 10.1158/0008-5472.CAN-09-0491

implicated in the pathogenesis of several inflammatory diseases, including asthma, psoriasis, rheumatoid arthritis, and bowel disease (30). In addition, previous reports provide evidence supporting a possible role for LTA_4H and LTB_4 in cancer cell progression. Notably, higher expression of LTA_4H (17) and an elevated production level of LTB_4 (31) in colon cancer tissue have been reported. In addition, LTB_4 was reported to stimulate the proliferation of colorectal cancer cells (32). Consistent with these findings, our observations also showed that LTA_4H was highly expressed in most of the human colorectal cancer cell lines tested and knockdown of LTA_4H impaired the growth of HCT116 colon cancer cells, suggesting that LTA_4H might play an important role in the promotion and progression of colorectal carcinomas.

Carcinogenesis is a multistep process accompanying molecular alterations that drive the progressive transformation of normal cells into highly malignant derivatives. One of the noticeable characteristics of malignant cancer cells is the ability to survive and grow in the absence of anchorage to an extracellular matrix (28, 33). Our new evidence showing that HCT116 cells with knockdown of LTA_4H (i.e., KD-LTA_4H cells) were less capable of surviving under anchorage-independent growth conditions suggests a crucial role for LTA_4H in colorectal cancer cell malignancy. We also showed that LTA_4H enhanced HCT116 cell growth in soft agar through its aminopeptidase and epoxide hydrolase activity. Overall, this evidence strongly indicates that inhibition of LTA_4H activity might be a potential target to prevent colorectal carcinoma promotion and progression.

Bestatin, a classic aminopeptidase inhibitor, is known to bind the Glu296 residue of LTA_4H to inhibit both enzyme activities (34). Notably, our results indicate that [6]-gingerol binds to Glu271 and also inhibits both the aminopeptidase and epoxide hydrolase activities of LTA_4H. Because Glu271 is the recognition site for the NH_2-terminal amino group of the peptidase substrate (29), [6]-gingerol could inhibit the binding of known and unknown peptidase substrates to LTA_4H. Although the mechanism is not entirely clear, [6]-gingerol seems to inhibit the epoxide hydrolase activity of LTA_4H in a manner similar to bestatin and results in a reduced anchorage-independent growth of HCT116 cells in soft agar. Recovery experiments using knockdown-LTA_4H cells transfected with wild-type LTA_4H or treated with LTB_4 confirmed that these activities of LTA_4H are required for colony formation in soft agar.

We and others have reported that [6]-gingerol inhibits cell transformation and mouse skin carcinogenesis. Indeed, [6]-gingerol was reported to suppress epidermal growth factor–induced neoplastic transformation in mouse epidermal JB6 cells (3), 7,12-dimethylbenz(*a*)anthracene–induced skin cancer promotion in ICR mice (35), and 12-*O*-tetradecanoylphorbol-13-acetate–induced cyclooxygenase-2 (COX-2) expression in a mouse skin cancer model (4). In addition, [6]-gingerol inhibits angiogenesis and metastasis (5, 6), which suggests a broad anticancer activity of [6]-gingerol mediated by multiple mechanisms in various cancers. Our results herein are noteworthy in that promotion of colorectal carcinoma can be delayed and suppressed by [6]-gingerol *in vivo*. Moreover, the low *in vivo* toxicity and potent tumor inhibitory activity of [6]-gingerol observed in nude mice suggest that [6]-gingerol is an effective chemopreventive agent for colorectal carcinoma. In conclusion, we showed here that LTA_4H is closely associated with colorectal cancer cell growth, promotion, and progression. Moreover, we provided clear evidence showing that [6]-gingerol effectively suppresses anchorage-independent cell growth and *in vivo* tumor growth in HCT116 cancer cell–bearing nude mice by inhibiting LTA_4H activity. Collectively, these findings support the anticancer efficacy of [6]-gingerol through its targeting of LTA_4H for the prevention of colorectal cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/25/09; revised 4/16/09; accepted 4/16/09; published OnlineFirst 6/16/09.

Grant support: The Hormel Foundation (Austin, MN), Pediatric Pharmaceuticals (Iselin, NJ), Korea Research Foundation Grant (Korean Government; KRF-2007-357-C00084), the 863 Hi-Tech Program of China (grant 2007AA02Z304), and the Shanghai Committee of Science and Technology (Grant 07dz22004).

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References

1. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
2. Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res 1999;428:305–27.
3. Bode AM, Ma WY, Surh YJ, Dong Z. Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. Cancer Res 2001;61:850–3.
4. Kim SO, Kundu JK, Shin YK, et al. [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-κB in phorbol ester-stimulated mouse skin. Oncogene 2005;24:2558–67.
5. Kim EC, Min JK, Kim TY, et al. [6]-Gingerol, a pungent ingredient of ginger, inhibits angiogenesis *in vitro* and *in vivo*. Biochem Biophys Res Commun 2005;335:300–8.
6. Lee HS, Seo EY, Kang NE, Kim WK. [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. J Nutr Biochem 2008;19:313–9.
7. Suzuki F, Kobayashi M, Komatsu Y, Kato A, Pollard RB. Keishi-ka-kei-to, a traditional Chinese herbal medicine, inhibits pulmonary metastasis of B16 melanoma. Anticancer Res 1997;17:873–8.
8. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science 1987;237:1171–6.
9. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 2001;294:1871–5.
10. Fabre JE, Goulet JL, Riche E, et al. Transcellular biosynthesis contributes to the production of leukotrienes during inflammatory responses *in vivo*. J Clin Invest 2002;109:1373–80.
11. Avis I, Hong SH, Martinez A, et al. Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. FASEB J 2001;15:2007–9.
12. Moody TW, Leyton J, Martinez A, Hong S, Malkinson A, Mulshine JL. Lipoxygenase inhibitors prevent lung carcinogenesis and inhibit non-small cell lung cancer growth. Exp Lung Res 1998;24:617–28.
13. Goulet JL, Snouwaert JN, Latour AM, Coffman TM, Koller BH. Altered inflammatory responses in leukotriene-deficient mice. Proc Natl Acad Sci U S A 1994;91:12852–6.
14. Gunning WT, Kramer PM, Steele VE, Pereira MA. Chemoprevention by lipoxygenase and leukotriene pathway inhibitors of vinyl carbamate-induced lung tumors in mice. Cancer Res 2002;62:4199–201.
15. Chen X, Wang S, Wu N, Yang CS. Leukotriene A_4 hydrolase as a target for cancer prevention and therapy. Curr Cancer Drug Targets 2004;4:267–83.
16. Orning L, Krivi G, Fitzpatrick FA. Leukotriene A_4 hydrolase. Inhibition by bestatin and intrinsic aminopeptidase activity establish its functional resemblance to metallohydrolase enzymes. J Biol Chem 1991;266:1375–8.
17. Chen X, Li N, Wang S, et al. Leukotriene A_4 hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. J Natl Cancer Inst 2003;95:1053–61.
18. Arguello M, Paz S, Hernandez E, et al. Leukotriene A_4 hydrolase expression in PEL cells is regulated at the transcriptional level and leads to increased leukotriene B4 production. J Immunol 2006;176:7051–61.
19. Thunnissen MM, Nordlund P, Haeggstrom JZ. Crystal structure of human leukotriene A_4 hydrolase, a bifunctional enzyme in inflammation. Nat Struct Biol 2001;8:131–5.
20. Gao Z, Li H, Zhang H, et al. PDTD: a web-accessible protein database for drug target identification. BMC Bioinformatics 2008;9:104.
21. Aktan F, Henness S, Tran VH, Duke CC, Roufogalis BD, Ammit AJ. Gingerol metabolite and a synthetic analogue Capsarol inhibit macrophage NF-κB-mediated

Published OnlineFirst June 16, 2009; DOI: 10.1158/0008-5472.CAN-09-0491

iNOS gene expression and enzyme activity. Planta Med 2006;72:727–34.
22. Li H, Gao Z, Kang L, et al. TarFisDock: a web server for identifying drug targets with docking approach. Nucleic Acids Res 2006;34:W219–24.
23. Li H, Li C, Gui C, et al. GAsDock: a new approach for rapid flexible docking based on an improved multi-population genetic algorithm. Bioorg Med Chem Lett 2004;14:4671–6.
24. Ewing TJ, Makino S, Skillman AG, Kuntz ID. DOCK 4.0: search strategies for automated molecular docking of flexible molecule databases. J Comput Aided Mol Des 2001;15:411–28.
25. Arber N, Levin B. Chemoprevention of colorectal cancer: ready for routine use? Curr Top Med Chem 2005;5:517–25.
26. Schrödinger Suite 2006 QM-polarized ligand docking protocol. New York: Schrödinger, LLC; p. 2005. Glide version 4.0; Jaguar version 6.5; QSite version 4.0.
27. Rudberg PC, Tholander F, Andberg M, Thunnissen MM, Haeggstrom JZ. Leukotriene A_4 hydrolase: identification of a common carboxylate recognition site for the epoxide hydrolase and aminopeptidase substrates. J Biol Chem 2004;279:27376–82.
28. Freedman VH, Shin SI. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 1974;3:355–9.
29. Rudberg PC, Tholander F, Thunnissen MM, Haeggstrom JZ. Leukotriene A_4 hydrolase/aminopeptidase. Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms. J Biol Chem 2002;277:1398–404.
30. Penning TD. Inhibitors of leukotriene A_4 (LTA_4) hydrolase as potential anti-inflammatory agents. Curr Pharm Des 2001;7:163–79.
31. Dreyling KW, Hoppe U, Peskar BA, Morgenroth K, Kozuschek W, Peskar BM. Leukotriene synthesis by human gastrointestinal tissues. Biochim Biophys Acta 1986;878:184–93.
32. Bortuzzo C, Hanif R, Kashfi K, Staiano-Coico L, Shiff SJ, Rigas B. The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. Biochim Biophys Acta 1996;1300:240–6.
33. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
34. Andberg M, Wetterholm A, Medina JF, Haeggstrom JZ. Leukotriene A_4 hydrolase: a critical role of glutamic acid-296 for the binding of bestatin. Biochem J 2000;345 Pt 3:621–5.
35. Park KK, Chun KS, Lee JM, Lee SS, Surh YJ. Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice. Cancer Lett 1998; 129:139–44.

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Cancer Res 2009;69:5584-5591. Published OnlineFirst June 16, 2009.