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Resveratrol, a Red Wine Polyphenol, Suppresses Pancreatic Cancer by Inhibiting Leukotriene A_4 Hydrolase

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Abstract

The anticancer effects of red wine have attracted considerable attention. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a well-known polyphenolic compound of red wine with cancer chemopreventive activity. However, the basis for this activity is unclear. We studied leukotriene A_4 hydrolase (LTA_4H) as a relevant target in pancreatic cancer. LTA_4H knockdown limited the formation of leukotriene B_4 (LTB_4), the enzymatic product of LTA_4H, and suppressed anchorage-independent growth of pancreatic cancer cells. An *in silico* shape similarity algorithm predicted that LTA_4H might be a potential target of resveratrol. In support of this idea, we found that resveratrol directly bound to LTA_4H *in vitro* and in cells and suppressed proliferation and anchorage-independent growth of pancreatic cancer by inhibiting LTB_4 production and expression of the LTB_4 receptor 1 (BLT_1). Notably, resveratrol exerted relatively stronger inhibitory effects than bestatin, an established inhibitor of LTA_4H activity, and the inhibitory effects of resveratrol were reduced in cells where LTA_4H was suppressed by shRNA-mediated knockdown. Importantly, resveratrol inhibited tumor formation in a xenograft mouse model of human pancreatic cancer by inhibiting LTA_4H activity. Our findings identify LTA_4H as a functionally important target for mediating the anticancer properties of resveratrol.

Introduction

Considerable attention has been focused on the anticancer effect of red wine (1–3), which contains several types of polyphenolic compounds such as resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) and quercetin (3,3′,4′,5,7-pentahydroxy-flavone; Fig. 1A). Previous reports indicate that resveratrol exerts antitumor effects in the 7,12-dimethylbenz(*a*)anthracene–initiated/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted 2-stage skin cancer model (4). The chemopreventive effects of resveratrol seem to be associated with tumor initiation, promotion, and progression (5). Resveratrol was reported to suppress cell transformation and induce apoptosis through a p53-dependent pathway (6) and ERKs and p38-mediated resveratrol-induced apoptosis (7, 8). Moreover, resveratrol suppresses colon cancer cell

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growth by inhibiting COX-2 activity (9). Resveratrol is a key substance in the cancer preventive activity of red wine (10, 11). However, quercetin also was suggested as an anticarcinogenic polyphenol (12, 13). Previous reports indicated that quercetin suppressed TPA-induced transformation of JB6 P^+ cells (14) and induced apoptosis (15).

Leukotriene A_4 hydrolase (LTA_4H) is a bifunctional zinc metalloenzyme with anion-dependent aminopeptidase and epoxide hydrolase activities (16). LTA_4H is overexpressed in certain human cancers (17). It catalyzes the hydrolysis of the epoxide leukotriene A_4 (LTA_4) to leukotriene B_4 (LTB_4), which stimulates the production of proinflammatory cytokines and mediators (18, 19) and also stimulates cancer cell proliferation (20–22). Moreover, previous studies indicate that inhibiting LTA_4H activity reduces the incidence of cancer and these effects are associated with the inhibition of LTB_4 biosynthesis (23). We previously reported that [6]-gingerol suppresses colon cancer growth by attenuating LTA_4H activity (24).

In the present study, we found that resveratrol directly bound to LTA_4H and suppressed proliferation and anchorage-independent growth by inhibiting LTA_4H activity in pancreatic cancer cells. Moreover, our findings showed that by inhibiting LTA_4H activity, resveratrol suppressed tumor growth of MIA PaCa-2 cells implanted in nude mice. These data suggest that inhibition of LTA_4H activity by resveratrol might be a novel target for the prevention of pancreatic cancer.

Materials and Methods

Chemicals

Resveratrol (99%), quercetin (98%), bestatin (98%), and basal medium Eagle (BME) were from Sigma-Aldrich. Fetal bovine serum (FBS) was from Atlanta Biologicals. CNBr-Sepharose 4B beads were purchased from GE Healthcare UK Ltd. and the CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit was from Promega. The LTA_4H human recombinant protein ($rLTA_4H$), leukotriene B_4 (LTB_4), anti-bodies against LTA_4H and the LTB_4 receptor 1 (BLT_1) for Western blot, and the LTB_4 EIA kit were purchased from Cayman Chemical. The 29mer sh-RNA constructs against LTA_4H were from OriGene Technologies Inc. A methylated derivative of resveratrol, 3,4′,5-triemethoxy-*trans*-stilbene (RSVL3; Fig. 1A), was a gift from Dr. Chi-Tang Ho, Department of Food Science, Rutgers University.

Cell culture and transfection

All cell lines were purchased from American Type Culture Collection and were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. The HCT15, H1299, and LNCaP cell lines were cultured in RPMI-1640 medium/10% FBS. The SK-Br-3 cell line was cultured in McCoy’s 5A medium/10% FBS. The MIA PaCa-2 and PANC-1 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/10% FBS. The HepG2 cell line was maintained in Eagle’s Minimum Essential Medium (MEM)/10% FBS. Human

pancreatic duct–derived (hTERT-HPNE) cells were cultured in M3:5 growth medium [4 parts DMEM to 1 part M3F (INCELL) and 5% FCS; ref. 25]. All cell lines were cultured with antibiotics at 37°C in a CO_2 incubator. For transfection, MIA PaCa-2 cells were seeded (1×10^5) in 6-well plates a day before transfection and expression vectors were transfected transiently using jetPEI (Obiogen Inc.) for 48 hours following the supplier's instructions.

Shape similarity methodology

A shape similarity method was used to search for biological targets of resveratrol and quercetin on the basis of their respective structures. The PHASE module of Schrödinger's molecular modeling software package was used to perform the shape similarity search (26). The atom-type information from the queries was also used not only to consider shape similarity but also to align potential pharmacophore points between the queries and the targets. The target library comprised crystallized ligands taken from the Protein Data Bank (PDB; ref. 27). To provide more structure orientations for possible alignment, the maximum number of conformers per molecule in the library to be generated was set to 100 while retaining at most 10 conformers per rotatable bond. The top aligned structure for each molecule searched was returned, when conformers with a Tanimoto similarity coefficient below 0.7 were filtered out (28). A PDB ID was associated with each aligned target molecule to make searching the online PDB archive for the experimental determined structure containing the "hit" ligand possible. In this way, LTA_4H (PDB 3cho) was identified as a potential protein target because of its shape and pharmacophore similarity with 2-amino-*N*-[4-(phenyl methoxy)phenyl]-acetamide, a known LTA_4H potent inhibitor, and both the polyphenols, resveratrol and quercetin.

Computer modeling

During the course of this project, the crystal structure of LTA_4H bound to resveratrol (PDB 3fts) was solved along with 20 other structures bound with small molecular fragments. An alignment of all available 39 LTA_4H crystal structures showed that an overall less than 1 Å root mean square deviation (RMSD) existed among them. The structural similarity search permitted us to choose only the crystal structure of resveratrol bound to LTA_4H for docking studies. The protein was prepared for docking following the standard procedure outlined in Schrödinger's GLIDE docking package. A total of 8 different docking protocols were tested to determine which could best reproduce the crystal structure orientations of all the crystallized LTA_4H ligands extracted from 36 of the crystal structures. This method of validation was used to ensure a higher degree of accuracy in the orientation of the docked quercetin molecule.

The crystal structure orientations of all ligands, with the exception of resveratrol and quercetin, were used as their starting orientation for docking. Resveratrol and quercetin were both built from the beginning and energetically minimized followed by 10,000 step conformational searches and then reminimized creating the most energetically favorable conformation needed for docking studies. For the protein, all crystallographic waters were deleted and a 30-$Å^3$ grid was generated on the binding site of resveratrol to define the protein receptor.

Four GLIDE docking protocols, including docking with standard precision (SP) or extra precision (XP) and with or without the catalytic Zn metal, were employed. These preliminary docking runs were followed by the more CPU-intensive induced-fit docking methods, which were conducted with SP docking, a Zn metal, and either with or without waters. The final 2 docking protocols tested used the induced-fit XP docking with no waters but with or without the Zn metal. A visual comparison between each returned docking pose to its starting crystal structure orientation was done and used as the measure for correct docking.

In vitro and *ex vivo* pull-down assays

Recombinant LTA_4H (0.5 μg) or lysates from MIA PaCa-2 cells (500 μg) were mixed with resveratrol-, RSVL3-, or quercetin-conjugated Sepharose 4B beads or with Sepharose 4B beads alone as a control (50 μL, 50% suspension) and binding was determined by Western blot (24).

LTB_4 production assay

Cells were seeded (1×10^5) into 6-well plates and cultured for 24 hours. The cells were treated with culture medium containing different concentrations of resveratrol, RSVL3, quercetin, or bestatin for 48 hours at 37°C in a CO_2 incubator. LTB_4 production in the medium was quantified using the LTB_4 EIA kit following the supplier's instructions.

Proliferation and anchorage-independent growth assay

For proliferation, cells were seeded (2×10^3) into 96-well plates and treated with different concentrations of resveratrol, RSVL3, quercetin, or LTB_4. After incubation for various (24, 48, or 72 hours) amounts of time in a 5% CO_2 incubator, proliferation was determined as described (24). For anchorage-independent growth, cells (8×10^3/well) were suspended in 1 mL of BME supplemented with 10% FBS and 0.33% agar and plated with various concentrations of resveratrol, RSVL3, quercetin, bestatin, or LTB_4 on 3 mL of solidified BME supplemented with 10% FBS and 0.5% agar with different concentrations of resveratrol, RSVL3, quercetin, bestatin, or LTB_4. Colonies were determined as described (24).

In vivo xenograft mouse model

Athymic nude mice [Cr:NIH(S), NIH Swiss nude, 6–8 weeks old] were purchased from Charles River. Animals were maintained under "specific pathogen free" conditions and all animal studies were conducted according to guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee. Animals were acclimated for 2 weeks before the study and had free access to food and water. The animals were housed in climate-controlled quarters with a 12-hour light/12-hour dark cycle.

Animals were randomly assigned to the following groups: vehicle group ($n = 15$), 10 mg/kg resveratrol group ($n = 15$), 50 mg/kg resveratrol group ($n = 15$), and 50 mg/kg resveratrol control group ($n = 15$). Each mouse was administrated resveratrol (10 or 50 mg/kg body weight in 100 μL of 70% ethanol in autoclaved water as vehicle) or only vehicle 5× per week by oral gavage. After 2 weeks of treatment, MIA PaCa-2 cells (1×10^6 cells) were

injected subcutaneously into the right flank of mice in the respective groups. Following injection, mice continued to be administered resveratrol or vehicle. Mice in the 50 mg/kg resveratrol control group were not injected with cells but maintained for comparison of body weight and tumor development. Mice were weighed and tumors measured by caliper 3× per week. Tumor volume was calculated from measurements of 2 diameters of the individual tumor according to the following formula: tumor volume (mm^3) = (length × width × height × 0.52). Mice were monitored until tumors reached 1-cm^3 total volume, at which time mice were euthanized and tumors were extracted.

Statistical analysis

All quantitative data are expressed as means ± SE or SD as indicated. The Student's *t* test or a 1-way ANOVA was used for statistical analysis. A probability of $P < 0.05$ was used as the criterion for statistical significance.

Results

Resveratrol specifically binds to LTA_4H

To elucidate potential targets of resveratrol or quercetin, we first conducted *in silico* screening by using a shape similarity approach. Resveratrol and quercetin (Fig. 1A) were screened against all the crystallized ligands available from the PDB. Screening results showed that both polyphenols were very similar to 2-amino-*N*-[4-(phenylmethoxy)phenyl]acetamide, a known LTA_4H inhibitor, which implied that LTA_4H was a possible molecular target for resveratrol or quercetin. The crystal structure of resveratrol bound to LTA_4H (PDB 3fts) was recently solved during a fragment screen crystallography research program that was undertaken to find novel inhibitors of LTA_4H (29). The fragment-based crystallography program yielded 20 LTA_4H structures bound to a variety of molecular fragments increasing the total number of available LTA_4H crystal structures to 39. Our shape similarity search found the ligand crystallized to LTA_4H (PDB 3cho). However, we wanted to know whether the new 3fts structure might be a more appropriate protein for our docking experiments. An alignment of all 39 structures showed that the protein backbone RMSD was less than 1 Å (Supplementary Fig. S1A). This finding provided the necessary validation to choose the resveratrol/LTA_4H complex (PDB 3fts) for docking calculations.

The alignment yielded additional insights into the structure of LTA_4H. The binding groove seems to be very hydrophilic and therefore capable of binding an inordinate number of waters (Supplementary Fig. S1A). The clustering of the water molecules shows that they possess a highly precise location of binding unless specifically displaced by a bound ligand. The size and dimension of the L-shape binding groove is seen when creating surface representations of all the bound ligands (Fig. 1B). The crystallized orientations of resveratrol (PDB 3fts) and bestatin (i.e., the first solved LTA_4H inhibitor crystal complex, PDB 1hs6) show 2 different binding modes observed for all the inhibitors (Supplementary Fig. S1B). Inhibitors of LTA_4H bind by interacting either with the catalytic Zn ion (bestatin) or with the back portion of the binding groove (resveratrol). The binding location of resveratrol is

considered to be the back because the opening into the binding site is near the Zn molecule. The preferred binding of quercetin was the same as that for resveratrol (Fig. 1C).

To validate the results of the shape similarity search, we conducted *in vitro* pull-down assays by using resveratrol- or quercetin-conjugated Sepharose 4B beads. The initial computer docking data using the PDB 3cho crystal structure showed that the hydroxyl groups of resveratrol are important for binding to LTA_4H. We compared the binding of RSVL3, a methylated derivative of resveratrol (Fig. 1A). Our data showed that recombinant LTA_4H ($rLTA_4H$) bound to resveratrol- or quercetin-conjugated Sepharose 4B beads but not to RSVL3-conjugated beads *in vitro* (Fig. 1D, top). We then used MIA PaCa-2 cell lysates to perform an *ex vivo* binding assay. Results indicated that resveratrol-conjugated Sepharose 4B beads bound to endogenous LTA_4H whereas RSVL3- or quercetin-conjugated beads did not (Fig. 1D, bottom). These results suggest that resveratrol might be a more effective agent against LTA_4H than quercetin and its hydroxyl groups are required for the binding.

LTA_4H expression and LTB_4 production are increased in pancreatic cancer cells

We then determined the abundance of LTA_4H in several cancer cell lines and found that LTA_4H expression was relatively higher in pancreatic, breast, and hepatocellular carcinoma cell lines (Fig. 2A). On the basis of findings that LTB_4 stimulates cancer cell proliferation (20–22), we measured LTB_4 production in several cancer cell lines. Our results showed that MIA PaCa-2 pancreatic cancer cells produced relatively large amounts of LTB_4 compared with other cancer cell lines (Fig. 2B). We also determined expression levels of LTA_4H in normal pancreatic cells (hTERT-HPNE cells) and an additional pancreatic cancer cell line PANC-1. LTA_4H expression is higher in PANC-1 and MIA PaCa-2 cells than in hTERT-HPNE cells (Supplementary Fig. S3A), suggesting that LTA_4H might be associated with pancreatic cancer development.

LTA_4H activity is required for growth of MIA PaCa-2 cells

To investigate the role of LTA_4H in pancreatic cancer cells, we created LTA_4H knockdown MIA PaCa-2 cells that express small hairpin RNA (shRNA) targeting LTA_4H. The expression levels of LTA_4H and BLT_1, a specific receptor for LTB_4, were substantially reduced by knockdown of LTA_4H compared with sh-mock cells (Fig. 3A) and LTB_4 production also reduced in sh-LTA_4H cells (Fig. 3B). Previous findings indicate that inhibition of LTB_4 production can suppress pancreatic cancer growth (30). Our data indicated that knockdown of LTA_4H reduced anchorage-independent cell growth (Fig. 3C) and inhibited proliferation (Supplementary Fig. S2A) compared with sh-mock cells. LTB_4 was reported to stimulate pancreatic cancer cell growth mediated by extracellular signal regulated kinases (ERK; ref. 31). Our results also showed that LTB_4 stimulated anchorage-independent growth (Fig. 3D) and proliferation of MIA PaCa-2 cells (Supplementary Fig. 2B). Moreover, phosphorylation of ERKs (Thr202, Tyr204) was increased by LTB_4 treatment (Supplementary Fig. S2C). These results suggest that LTA_4H is associated with pancreatic cancer cell growth mediated through ERKs signaling.

Resveratrol suppresses proliferation and anchorage-independent cell growth by inhibiting LTA_4H activity

We then compared the effects of resveratrol, RSVL3, or quercetin on several aspects of MIA PaCa-2 pancreatic cancer cells. Bestatin, a well-known inhibitor of LTA_4H activity (32), was used as a positive control. Our data showed that resveratrol suppressed LTB_4 production in a dose-dependent manner whereas RSVL3 or quercetin had no effect (Fig. 4A). We then determined the effect of resveratrol on proliferation and anchorage-independent growth. Results indicated that resveratrol suppressed proliferation whereas the RSVL3 or quercetin was much less effective (Fig. 4B). Resveratrol strongly suppressed anchorage-independent cell growth, whereas RSVL3 (30 μmol/L) had a slight effect and quercetin (up to 30 μmol/L) exerted no effect (Fig. 4C). The inhibitory effect of resveratrol on anchorage-independent cell growth was stronger than that of bestatin (30 μmol/L). Resveratrol also suppressed proliferation (Supplementary Fig. S3B) and anchorage-independent growth in PANC-1 pancreatic cancer cells (Supplementary Fig. S3C). LTB_4 is known to contribute to BLT_1 expression (33) and therefore resveratrol might suppress BLT_1 expression due to its inhibition of LTB_4 production. Our data showed that resveratrol, indeed, inhibited the expression level of BLT_1 dose-dependently (Fig. 5A, left) and the effect was stronger than that of bestatin (Fig. 5A, right). In contrast, RSVL3 or quercetin exerted no effect (Fig. 5A, right). On the other hand, resveratrol did not affect LTA_4H protein expression (Fig. 5A). These results suggest that resveratrol suppresses pancreatic cancer cell growth by inhibiting LTA_4H activity and is more potent than bestatin.

The effects of resveratrol are reduced by knockdown of LTA_4H

To investigate whether the effects of resveratrol are mediated directly through LTA_4H, we compared the effects of MIA PaCa-2 cells transfected with an sh-mock or sh-LTA_4H plasmid. Resveratrol (30 μmol/L) suppressed proliferation in sh-mock cells but had less effect in sh-LTA_4H cells (Fig. 5B). In anchorage-independent growth, the inhibitory effect of resveratrol was also reduced in sh-LTA_4H cells compared with sh-mock cells (Fig. 5C). These results suggest that LTA_4H is a direct target for resveratrol to suppress growth of pancreatic cancer cells.

Resveratrol suppresses tumor growth by inhibiting LTA_4H activity

We then determined whether resveratrol could suppress tumor development in a xenograft model *in vivo*. Oral administration of resveratrol (10 or 50 mg/kg body weight) resulted in a significant inhibition of tumor growth compared with the vehicle-treated group. In the vehicle group, 10 of 15 (66.7%) mice developed tumors, whereas only 6 of 15 (40%) or 4 or 15 (26.7%) mice developed tumors in the 10 or 50 mg/kg resveratrol group, respectively (Fig. 6A). The average tumor volume per mouse was also decreased from 337 mm^3 in the vehicle group to 223 or 147 mm^3 in the 10 mg/kg or 50 kg/kg resveratrol group, respectively (Fig. 6B). To further determine whether the antitumor effect of resveratrol *in vivo* was associated with LTA_4H, tumor extracts were prepared and analyzed for LTA_4H expression and LTB_4 production. Results indicated that resveratrol suppressed LTB_4 production in tumors (Fig. 6C). On the other hand, resveratrol did not affect LTA_4H protein expression

(Fig. 6D). Overall, these results suggest that resveratrol might serve as an effective agent against pancreatic cancer by targeting LTA_4H.

Discussion

Pancreatic cancer is the fourth leading cause of cancer death in the United States, with a dismal 5-year survival rate of 5% (34). In recent years, many dietary compounds have been recognized as anticancer agents and previous studies indicate that resveratrol suppresses growth of pancreatic cancer cells (35–37). However, the molecular mechanisms underlying the effect of resveratrol are unknown. Our results herein show a role for resveratrol as a chemopreventive and chemotherapeutic agent against pancreatic cancer and strongly suggest that LTA_4H is an important target.

Previous reports indicate that inactivating LTA_4H results in the suppression of inflammatory diseases in animal models (18, 19), making LTA_4H a recognized anti-inflammatory target. Recently, a high expression of LTA_4H (17) and increased cancer cell proliferation induced by LTB_4 (31) were reported. Thus, LTA_4H and LTB_4 are possible targets for suppressing cancer cell progression (17). Our data showed that the LTA_4H protein level and LTB_4 production are higher in MIA PaCa-2 pancreatic cancer cells than other cancer cells (Fig. 2). Moreover, anchorage-independent growth of MIA PaCa-2 cells was decreased by knockdown of LTA_4H (Fig. 3C) and enhanced by treatment with LTB_4 (Fig. 3D). These results suggest that LTA_4H activity might play an important role in pancreatic cancer cell growth.

The anticancer activities of resveratrol were first shown by Jang and colleagues (5) in a 2-stage mouse skin cancer model. Our results herein indicate that resveratrol directly binds to LTA_4H (Fig. 1C) and strongly suppresses anchorage-independent growth of MIA PaCa-2 cells by inhibiting LTA_4H activity (Fig. 4) and BLT_1 expression (Fig. 5A). Recently, Davies and colleagues (29) reported that resveratrol directly bound to LTA_4H as determined by X-ray crystallography. This report strongly supports our findings that LTA_4H is a direct target of resveratrol. Harikumar and colleagues (36) reported that resveratrol suppressed pancreatic cancer cell growth through inhibition of NF-κB activation. LTB_4 is reported to increase NF-κB DNA binding activity (38). These reports suggest that suppression of pancreatic cancer growth by resveratrol is associated with inhibition of LTA_4H activity. On the other hand, resveratrol is reported to have no effect on growth of PANC-1 cells (39). In that report, cell density was 10-fold higher than in our study and might explain why resveratrol could not suppress cell growth. Others (40) reported that resveratrol had no effect on BOP-induced pancreatic carcinogenesis in hamsters. In this case, hamsters were fed resveratrol in their diet whereas we fed resveratrol by gavage, a method that more tightly controls the dose consumed.

We (9) previously reported that the anticancer effect of resveratrol is mediated directly by COX-2 and RSVL3 had no effect most likely because of its low binding affinity. Our present results indicated that RSVL3 did not bind to LTA_4H (Fig. 1D) and exerts no effect on pancreatic cancer cell growth (Fig. 4). These findings indicate that the anticancer effects of resveratrol on pancreatic cancer are mediated directly through LTA_4H and the hydroxyl

groups of resveratrol seem to be required for binding with LTA_4H. Our results also showed that quercetin bound to $rLTA_4H$ *in vitro* but did not bind to LTA_4H *ex vivo* (Fig. 1D). MEK (MAP/ERK kinase) and Raf were reported as direct molecular targets of quercetin (14). This suggests that quercetin likely binds to other proteins with much higher affinity than does LTA_4H *ex vivo*, which could result in a competition for binding resulting in much weaker binding in cells.

In conclusion, we provided evidence showing that resveratrol suppresses anchorage-independent growth of MIA PaCa-2 pancreatic cancer cells by inhibiting LTA_4H activity. In a xenograft mouse model, our results indicated that promotion of pancreatic cancer could be delayed or suppressed by resveratrol and the effect is associated with the inhibition of LTA_4H activity. Collectively, these findings support the anticancer effect of resveratrol mediated through LTA_4H for the prevention of pancreatic cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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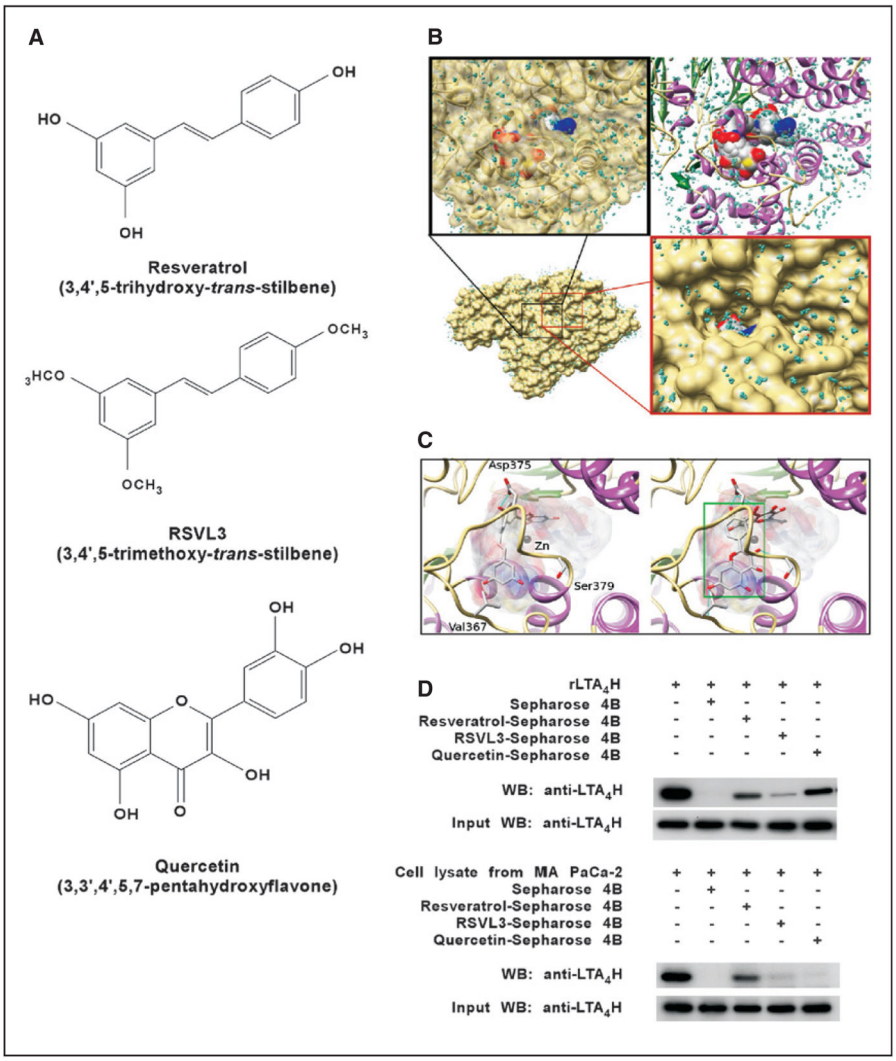

Figure 1.

Resveratrol specifically binds to LTA_4H. A, chemical structures of resveratrol, RSVL3, and quercetin. B, multiple views of the binding pocket of LTA_4H shown through surface representations. The entrance into the binding pocket (red box) is located above the Zn ion. Therefore, inhibitors such as resveratrol, must traverse through the binding pocket, past the Zn ion, and bind in the back-end pocket of the L-shaped cavity, which is nonsurface accessible (black box). C, top-down view of the docked binding orientations of resveratrol and quercetin in LTA_4H. The induced-fit extra-precision (IF-XP) docking protocol returned 2 possible docking modes for both molecules. The resveratrol from the X-ray structure was docked back into the protein structure in the exact same location as was found in the crystal structure. This location was energetically no different than resveratrol binding and interacting with the Zn metal as determined by the IF-XP method. However, the lone XP docking method showed that binding in the back-end pocket (crystal structure binding location) was energetically more favorable (~4 kcal/mol) than the interaction with Zn. Quercetin is similar in shape and size to resveratrol and exhibited different binding modes as well. However, the IF-XP method showed a dramatic difference in binding preference. The

docking indicated that the back-end pocket binding (green square) was preferred by 8 kcal/mol over the binding and interaction with the Zn metal. Asp375 played an important role in either anchoring or binding orientation, but hydrogen bonds of quercetin with the protein backbone of Val367 and Ser379 likely caused its better binding energy. Therefore, the preferred binding of quercetin is the same as that for resveratrol, in the back-end binding pocket of LTA_4H. D, binding of resveratrol, RSVL3, and quercetin to LTA_4H. *In vitro* (top) and *ex vivo* (bottom) binding was confirmed by pull-down assay. Recombinant LTA_4H or a lysate prepared from MIA PaCa-2 cells was incubated with resveratrol-, RSVL3-, or quercetin-conjugated Sepharose 4B beads, or with Sepharose 4B beads alone, and the pulled down proteins were analyzed by Western blot (WB).

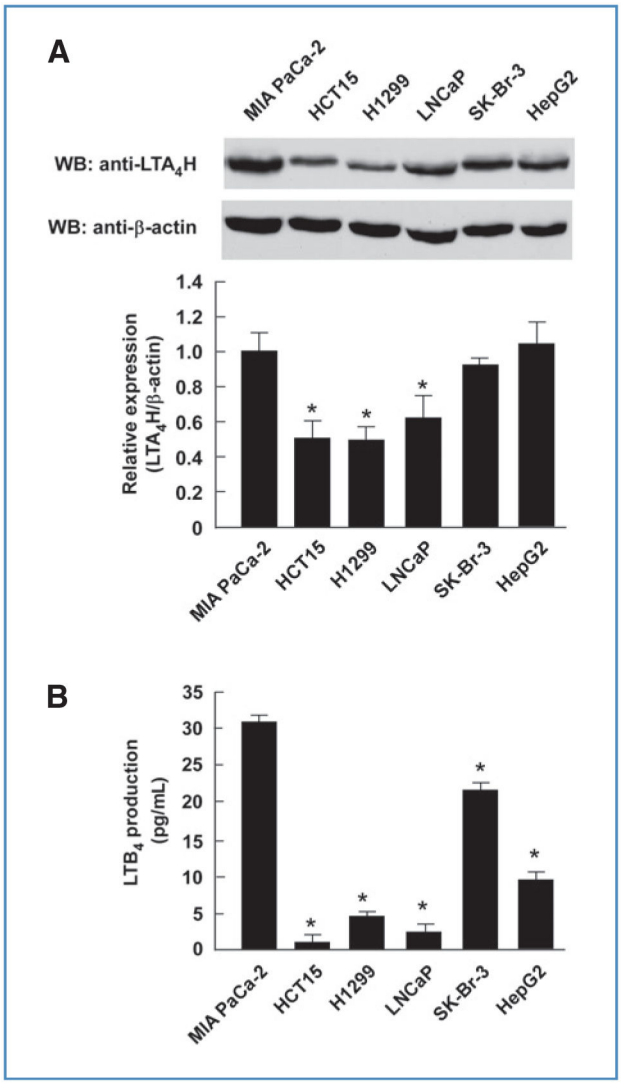

Figure 2.

LTA_4H expression and LTB_4 production are high in MIA PaCa-2 pancreatic cancer cells. A, Western blot (WB) analysis of LTA_4H expression in 6 cancer cell lines: MIA PaCa-2 (pancreatic carcinoma); HCT15 (colorectal carcinoma); H1299 (lung adenocarcinoma); LNCaP (prostate carcinoma); SK-Br-3 (breast carcinoma); and HepG2 (hepatocellular carcinoma). Densitometric analysis of the relative expression level of LTA_4H was normalized against β-actin. B, LTB_4 production is higher in MIA PaCa-2 pancreatic cancer cells than in other cancer cells. Cells were incubated for 48 hours, and LTB_4 production in medium was quantified by ELISA. For A and B, data are represented as means ± SD from 3 different experiments. The asterisk (*) indicates a significantly lower expression or production than the expression levels or activity of MIA PaCa-2 cells ($P < 0.05$).

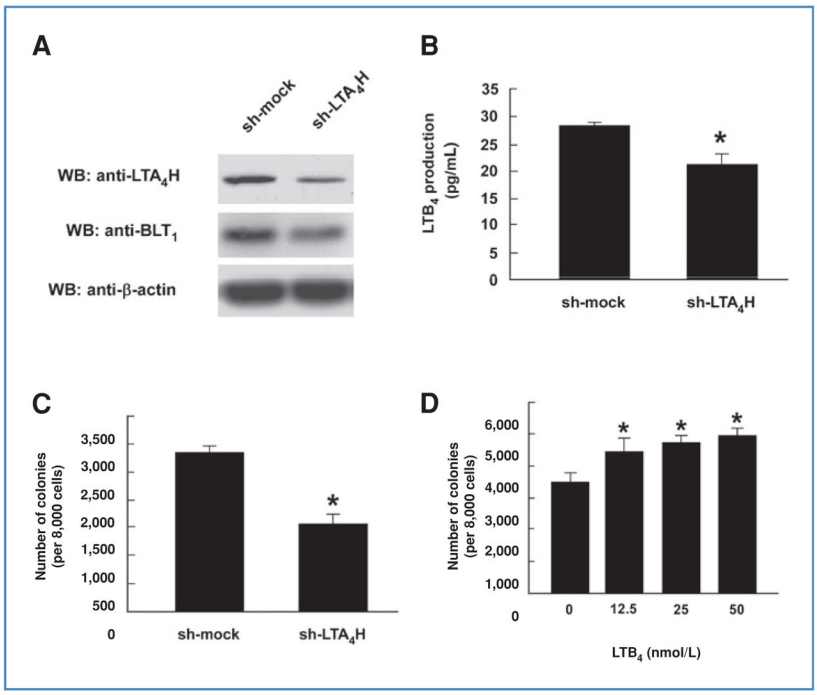

Figure 3.

LTA_4H activity is required for growth of MIA PaCa-2 pancreatic cancer cells. A, expression levels of LTA_4H and BLT_1 are decreased by knockdown of LTA_4H. MIA PaCa-2 cells were transfected transiently with sh-mock or sh-LTA_4H, and cell lysates were analyzed by Western blot. B, LTB_4 production is reduced by knockdown of LTA_4H. Cells were incubated for 48 hours, and LTB_4 production in the medium was quantified by ELISA. C, anchorage-independent growth is decreased in sh-LTA_4H cells. Cells were grown in soft agar for 5 days and then colonies were counted. D, LTB_4 stimulates anchorage-independent growth of MIA PaCa-2 cells. Cells were grown in soft agar with LTB_4 (0, 12.5, 25, or 50 nmol/L) for 5 days and then colonies were counted. For B–D, data are shown as means ± SD from 3 different experiments. For B and C, the asterisk (*) indicates a significant decrease compared with sh-mock cells ($P < 0.05$). For D, the asterisk (*) indicates a significant increase compared with untreated control cells ($P < 0.05$).

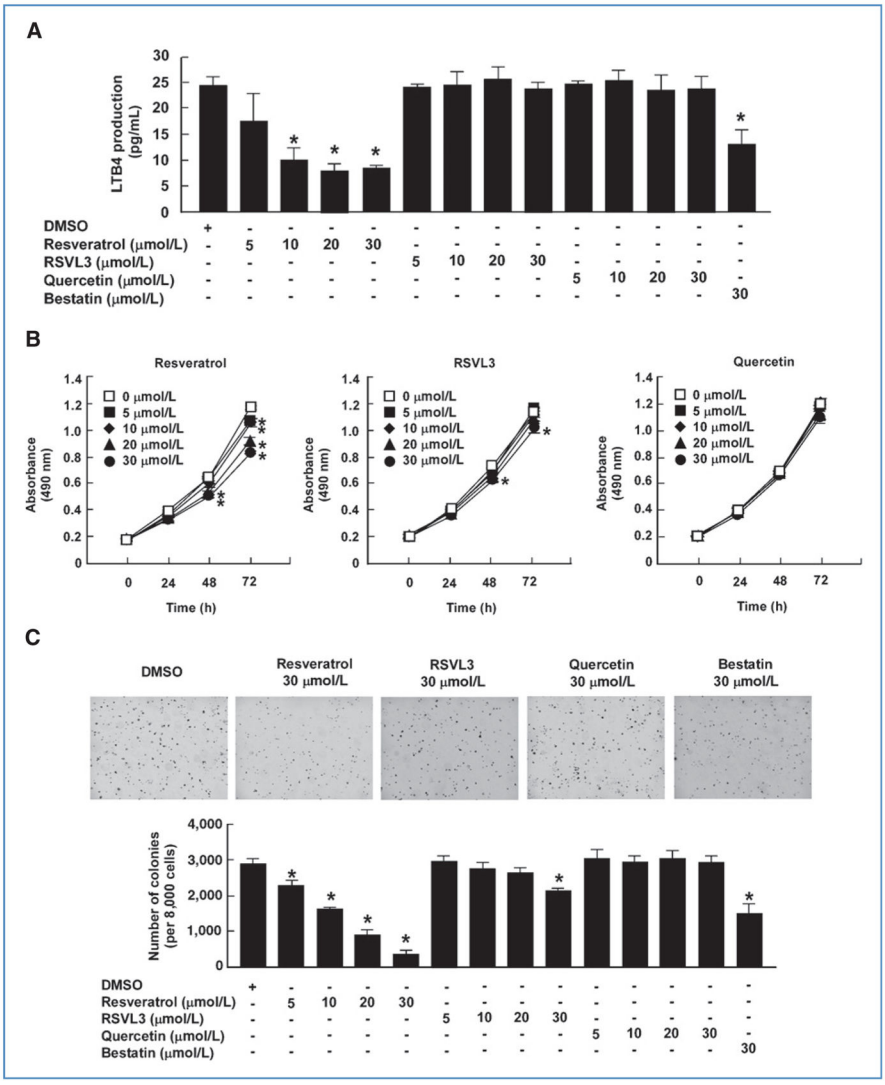

Figure 4.

Resveratrol suppresses proliferation and anchorage-independent growth of pancreatic cancer cells. A, resveratrol inhibits LTB_4 production, whereas RSVL3 and quercetin have no effect. Cells were treated for 48 hours with resveratrol, RSVL3, quercetin (0, 5, 10, 20, or 30 μmol/L), or bestatin (30 μmol/L). LTB_4 production was estimated by ELISA. B, resveratrol, but not RSVL3 or quercetin, inhibits MIA PaCa-2 proliferation. Cells were cultured with resveratrol, RSVL3, or quercetin (0, 5, 10, 20, or 30 μmol/L) and proliferation was estimated at 24-hour intervals up to 72 hours. C, resveratrol, but not RSVL3 or quercetin, suppresses anchorage-independent MIA PaCa-2 cell growth. Cells were grown with resveratrol, RSVL3, quercetin (0, 5, 10, 20, or 30 μmol/L), or bestatin (30 μmol/L) in soft agar for 5 days and then colonies were counted. Data are represented as means ± SD from 3 different experiments. The asterisk (*) indicates a significant decrease compared with untreated control cells ($P < 0.05$).

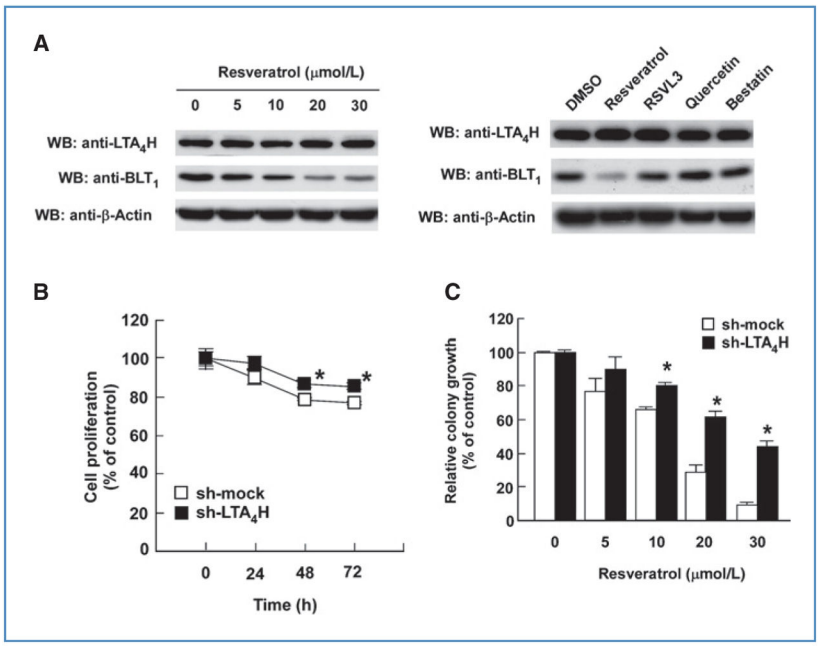

Figure 5.

Resveratrol suppresses BLT_1 expression, and binding to LTA_4H is required for the inhibitory effects of resveratrol. A, resveratrol suppresses the expression levels of BLT_1 but has no effect on LTA_4H expression. Cells were treated with resveratrol, RSVL3, quercetin, or bestatin for 72 hours and cell lysates were analyzed by Western blot (WB). B, resveratrol has less effect on proliferation of sh-LTA_4H cells than that of sh-mock cells. Cells were transfected transiently for 48 hours with sh-mock or sh-LTA_4H. Both cell types were treated with 30 μmol/L of resveratrol and proliferation was measured at 24-hour intervals up to 72 hours. C, resveratrol has less effect on anchorage-independent growth of sh-LTA_4H cells than that of sh-mock cells. Cells were grown in soft agar with resveratrol (0, 5, 10, 20, or 30 μmol/M) for 5 days and colonies were counted. Data are represented as means ± SD from 3 different experiments. The asterisk (*) indicates a significant decrease compared with sh-mock cells ($P < 0.05$).

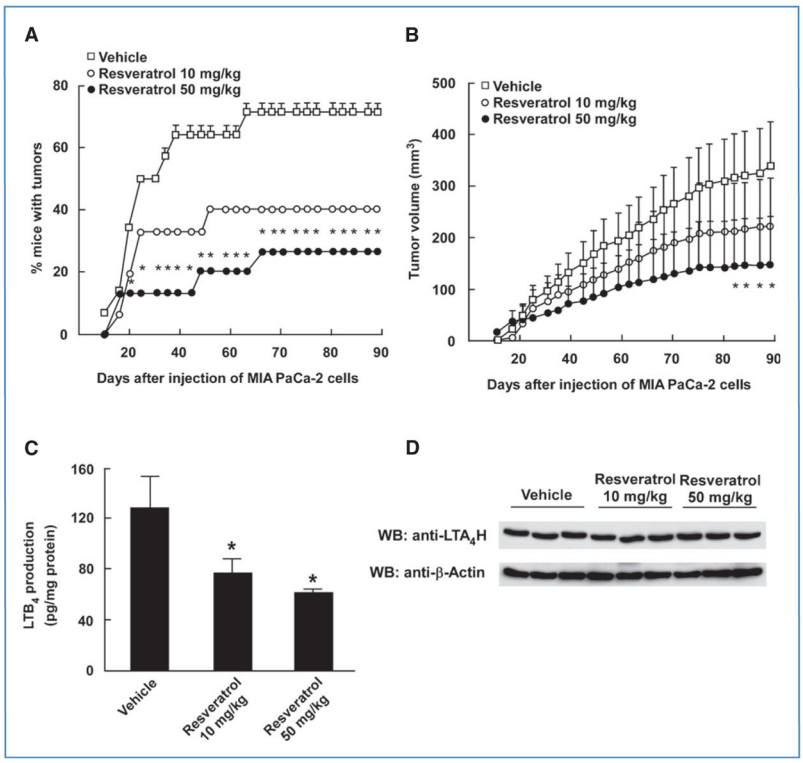

Figure 6.

Resveratrol suppresses tumor growth by inhibiting LTA_4H activity *in vivo*. Athymic nude mice were treated as described in the Materials and Methods section, and tumor volume was measured and recorded 3 times a week. A, resveratrol reduces the number of tumor-bearing mice. B, resveratrol suppresses tumor volume *in vivo*. C, LTB_4 production is reduced in resveratrol-treated tumors. LTB_4 levels were analyzed by ELISA and the amount of LTB_4 is expressed as picograms/milligram of protein. D, expression of LTA_4H in vehicle- or resveratrol-treated tumors ($n = 3$). For A and B, data are represented as means ± SE and differences were determined by 1-way ANOVA. The asterisk (*) indicates a significant decrease versus vehicle-treated groups ($P < 0.05$). For C, data are represented as means ± SD and significance is determined by Student's *t* test. The asterisk (*) indicates a significant decrease versus vehicle group ($P < 0.05$).