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Selective targeting of mutant adenomatous polyposis coli (*APC*) in colorectal cancer

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Abstract

Mutations in the adenomatous polyposis coli (*APC*) gene are common in colorectal cancer (CRC), and more than 90% of those mutations generate stable truncated gene products. We describe a chemical screen using normal human colonic epithelial cells (HCECs) and a series of oncogenically progressed HCECs containing a truncated APC protein. With this screen, we identified a small molecule, TASIN-1 (truncated APC selective inhibitor-1), that specifically kills cells with APC truncations but spares normal and cancer cells with wild-type APC. TASIN-1 exerts its cytotoxic effects through inhibition of cholesterol biosynthesis. In vivo administration of

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Author contributions: L.Z. designed and performed experiments, analyzed data, and wrote the manuscript; P.C.T., D.N., J.K.D.B., and W.W. demonstrated that cholesterol could rescue TASIN-1 toxicity and implicated EBP as the direct target; U.E. generated the isogenic HCEC lines, initiated the screen, and edited the manuscript; W.W. synthesized TASIN-1; Y.-A.M. performed cholesterol biosynthesis assay; B.P. provided guidance, data analysis and cheminformatics support, and experimental resources for the compound screen; N.S.W. performed the pharmacokinetic analysis; W.E.W. edited the manuscript; S.B.K. designed and supervised biological experiments and edited the manuscript; D.N. supervised the target identification experiments; J.K.D.B. designed and supervised chemistry experiments and edited the manuscript; J.W.S. supervised this project and edited the manuscript.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Competing interests: J.W.S., D.N., and J.K.D.B. are founding members of Elizabeth Therapeutics that is expanding TASIN analogs for clinical development. The other authors declare that they have no competing interests.

TASIN-1 inhibits tumor growth of CRC cells with truncated APC but not APC wild-type CRC cells in xenograft models and in a genetically engineered CRC mouse model with minimal toxicity. TASIN-1 represents a potential therapeutic strategy for prevention and intervention in CRC with mutant APC.

INTRODUCTION

Adenomatous polyposis coli (*APC*) is a multifunctional tumor suppressor gene that is mutated in more than 80% of colon tumors (1). *APC* mutation is believed to be one of the earliest events that contribute to colon cancer initiation (2). The primary function of APC has been attributed to the negative regulation of canonical WNT signaling pathway through proteasomal degradation of β -catenin (3). Additionally, it has been reported that APC functions beyond WNT pathway regulation and is involved in cellular processes related to cell cycle control, migration, differentiation, and apoptosis, all of which might contribute to colon cancer (4-12). Although both alleles are altered in APC-defective colorectal tumors, homozygous deletions of *APC* seem to be very rare. Instead, more than 90% of *APC* mutations generate premature stop codons, resulting in stable truncated gene products, among which mutations at codons 1309 and 1450 are the most highly represented (13, 14). Although the loss of APC tumor-suppressing functions resulting from the mutational loss of the APC C-terminal sequence has been regarded as a critical event in the initiation of colon cancer, there is increasing evidence that APC truncations may exert dominant functions that contribute to colon tumorigenesis. These include enhancement of cell migration, interference with spindle formation, and induction of chromosome instability (15-18). Although this is a highly frequent mutational event in colorectal cancer (CRC), there are currently no therapeutics directly targeting APC truncations. Here, we report the identification of a selective compound, TASIN-1 (truncated APC selective inhibitor-1), which can induce apoptotic cell death in human CRC cells harboring APC truncations in the low nanomolar median inhibitory concentration (IC_{50}) range without affecting normal and cancer cells with wild-type (WT) APC in the high micromolar range. In addition, TASIN-1 inhibits cancer cell growth in human tumor xenografts and in a genetically engineered mouse model of CRC. This compound serves as a platform for further translational development as a putative targeted therapy for colon cancer.

RESULTS

Generation and characterization of isogenic HCEC cell lines

To investigate the functions of truncated APC protein in CRC tumorigenesis, we developed a series of isogenic immortalized human colonic epithelial cell (HCEC) lines (Fig. 1A). 1CT is a line of normal HCECs immortalized with telomerase and cyclin-dependent kinase 4 (CDK4), and we previously showed that these cells are nontransformed and karyotypically diploid, have multipotent stem-like characteristics, and can differentiate in three-dimensional culture conditions (19). 1CTRPA A1309 harbors *TP53* and *APC* knockdowns (>90%), as well as ectopic expression of oncogenic *KRAS*^{V12} and truncated APC1309, whereas 1CTRPA has the same genetic alterations except for the ectopic expression of truncated APC (Fig. 1B). Mutations in genes *TP53*, *KRAS*, and *APC* are key molecular events that

contribute to the initiation and progression of CRC (20). Specifically, this APC truncation (A1309) is strongly selected for in colon cancers (14). We found that ectopic expression of APC truncation promoted a moderate increase in proliferation, enhanced soft agar growth, and increased migration or invasion through Matrigel (fig. S1, A to C). In contrast, ~90% stable knockdown of WT APC does not have any of these effects (fig. S1, A to C), demonstrating that the loss of APC function by itself does not drive colon cancer progression in this experimental cell culture model. These observations support the notion that APC truncations can promote tumorigenic properties, at least in the presence of other genetic alterations. We also observed that transient knockdown of truncated APC in DLD1 cells slowed down cell proliferation and induced caspase activation when cultured in low serum medium (fig. S2).

Identification of genotype-selective compounds targeting truncated APC

We used this series of isogenic HCEC lines to carry out a toxicity screen and to identify small molecules that selectively inhibit cell growth of APC-truncated HCECs (1CTRPA A1309) within a 200,000-compound library. The primary positive hits were counter-screened against 1CTRPA A1309, its isogenic precursor 1CTRPA, and normal HCECs (1CT), yielding 14 candidate compounds that selectively killed 1CTRPA A1309 cells (Fig. 1C). Dose-response analysis in two authentic human CRC cell lines, HCT116 (WT APC) and DLD1 (truncated APC1417), resulted in the identification of the lead compound TASIN-1 (Fig. 1D). This compound exhibited potent and selective toxicity toward DLD1 cells ($IC_{50} = 70$ nM) but not toward HCT116 cells ($IC_{50} > 50$ μ M) (Fig. 1E). Sustained treatment with TASIN-1 inhibited soft agar growth only in DLD1 cells (Fig. 1F). However, HCT116 cells showed similar sensitivity to TASIN-1 as DLD1 cells when cultured in medium with 0.1% serum for 7 days (fig. S3).

APC truncation dependency for TASIN-1's cytotoxic effects

To validate APC truncation dependency, we generated two independent stable knockdown DLD1 cell lines expressing shRNAs against truncated APC. Knockdown of truncated APC (>90%) expression desensitized DLD1 cells to TASIN-1 (Fig. 2A), whereas ectopic expression of APC truncation restored the sensitivity of APC knockdown DLD1 cells to TASIN-1 (Fig. 2B), supporting the idea that APC is required for TASIN-1's cytotoxicity. Similar effects were observed in HT29 cells depleted of truncated APC protein (fig. S4A) with or without restoration of APC truncation expression (fig. S4B). In addition, ectopic expression of truncated APC partially sensitized HCT116 (also containing WT APC) to TASIN-1 (fig. S4C). Despite the highly heterogeneous genetic backgrounds, we observed a consistent correlation between TASIN-1 sensitivity and APC status in a panel of CRC cell lines (Fig. 2C and fig. S4D). The stable expression of truncated APC proteins in the CRC cell lines harboring APC mutations was confirmed by Western blot (fig. S4E). TASIN-1 did not affect the viability of HCECs, human bronchial epithelial cells, and BJ fibroblast cells, which are derived from normal tissues, as well as other cancer cell types with WT APC (fig. S4, D and F, and table S1). These results directly implicate APC truncation in the mechanism of action of TASIN-1. We also discovered that TASIN-1 caused poly(adenosine diphosphate-ribose) polymerase 1 (PARP1) cleavage and cytochrome c release from mitochondria and induced caspase 3/7 activity in DLD1 cells but not in HCT116 cells (fig.

S5, A to C), indicative of induction of apoptosis. However, TASIN-1 did not affect WNT pathway activity, unlike IWR-1, which inhibits WNT response via tankyrase interaction and was identified from a compound screen using the same chemical library (fig. S6) (21), demonstrating that TASIN-1 is not directly inhibiting canonical WNT signaling.

Inhibitory effects of TASIN-1 on tumor growth in xenograft mouse models

We next examined the in vivo antitumor activity of TASIN-1 in a xenograft mouse model. Nude mice with established DLD1 and HT29 tumors (~50 to 75 mm³) were treated with intraperitoneal injection of either solvent (control) or TASIN-1 twice daily for 18 days. We observed that TASIN-1 treatment reduced the size of tumor xenografts (Fig. 3A) and reduced tumor growth rates (Fig. 3, B and C) compared with control mice. TASIN-1 did not inhibit tumor growth in HCT116 (WT APC) xenografts (Fig. 3D), demonstrating that TASIN-1 maintains selectivity in vivo. Hematoxylin and eosin and immunohistochemistry (IHC) analysis of residual excised tumors showed that TASIN-1 treatment resulted in the appearance of apoptotic cells with fragmented nuclei and induced an increase in the apoptotic marker cleaved caspase 3 (fig. S7A). Additionally, cleaved PARP1 was detected in tumor lysates from TASIN-1–treated DLD1 xenografts (fig. S7B).

Inhibitory effects of TASIN-1 on tumor growth in a genetically engineered CRC mouse model

Given the success of TASIN-1 in treating xenografted human tumors, we further tested its antitumor effects in *CPC;Apc* mice, a genetically engineered mouse model that mainly develops colorectal tumors. These mice carry a *CDX2P-NLS* Cre recombinase transgene and a *loxP*-targeted *Apc* allele that deletes exon 14, producing a frameshift at codon 580 and a truncated APC protein (22). Pharmacokinetic analysis revealed that TASIN-1 has a long retention time in mouse large intestinal tissue (Fig. 3E) despite being fairly rapidly cleared from plasma, suggesting that it would be possible to modify the dosing schedule relative to the xenograft experiment. *CPC;Apc* mice that were ~110 days old and had some small polyps (benign tumors) were injected intraperitoneally with either solvent or TASIN-1 (20 mg/kg) twice a week for 90 days. TASIN-1 treatment significantly reduced tumor formation in the colons of *CPC;Apc* mice (Fig. 3, F and G; **P* = 0.001, ***P* = 0.0035, and ****P* = 0.0003). Benign tumors (polyps) that were observed in TASIN-1–treated *CPC;Apc* mice were much smaller than those in the control group (Fig. 3G) and probably pre-existed at the start of the TASIN-1 treatment. Unlike control mice, which did not gain weight over the 90-day treatment, TASIN-1–treated mice gained weight, likely because of the reduced tumor burden (Fig. 3H). Toxicity studies showed that TASIN-1 did not induce obvious histological changes in livers, kidneys, or spleens of treated animals (fig. S8A). In addition, TASIN-1 did not affect serum concentrations of alanine aminotransferase or aspartate aminotransferase (fig. S8B). Moreover, TASIN-1 did not alter blood urea nitrogen, creatinine, or NaCl concentrations and did not have any obvious effects on blood cell counts (fig. S8, B and C). Finally, tumors isolated from TASIN-1–treated mice showed reduced expression of a panel of inflammatory response genes (fig. S9). We have also evaluated TASIN-1's in vivo antitumor activity in older *CPC;Apc* mice (~150 days old) and observed that TASIN-1–treated mice had reduced numbers of polyps of intermediate size and gained weight over 100 days of treatment (Fig. 3, I and J). Together, these in vivo experiments show that TASIN-1

efficiently attenuates tumorigenesis in human xenografts and genetically engineered CRC mouse models without noticeable toxicity.

Inhibition of cholesterol biosynthesis by TASIN-1

We observed that TASIN-1 was only effective in medium with 0.2% fetal bovine serum or fetal calf serum (FCS), which is the culture condition used in our original screen, but not effective in medium with higher (10%) amounts of FCS in HT29 cells (Fig. 4A). TASIN-1 was also ineffective in 2% FCS, but retained its cytotoxicity in the presence of 2% lipoprotein-deficient serum (LPDS) (Fig. 4B). In addition, exogenous addition of low-density lipoprotein (LDL) or cholesterol blocked TASIN-1's cytotoxicity (Fig. 4, C and D). To test whether TASIN-1 exerts its cytotoxicity through inhibition of cholesterol biosynthesis, we examined TASIN-1's effects on the cholesterol biosynthesis rate. TASIN-1 decreased the endogenous cholesterol biosynthesis rate in APC-truncated HT29 and DLD1 cells but not in APC WT HCT116 and RKO cells (Fig. 4, E and F). Similarly, the endogenous cholesterol concentration was visibly decreased in TASIN-1-treated DLD1 but not in HCT116 cells (fig. S10). In addition, we observed that TASIN-1 treatment decreased the cholesterol synthesis rate in cells with truncated APC in our panel (fig. S11).

Identification of EBP as the potential target of TASIN-1

To determine the step at which TASIN-1 inhibited cholesterol biosynthesis, we cotreated HT29 cells with TASIN-1 and various cholesterol biosynthesis pathway intermediates (Fig. 5A). Coadministration of squalene or zymosterol did not fully reverse TASIN-1's effects (Fig. 5B), whereas supplementation of lathosterol reversed TASIN-1's cytotoxicity (Fig. 5B), indicating that emopamil-binding protein (EBP) or 24-dehydrocholesterol reductase (DHCR24) may be the enzymes that TASIN-1 inhibits, because those two enzymes convert zymosterol to lathosterol. Furthermore, cotreatment with desmosterol, which is the substrate for DHCR24, rescued TASIN-1-induced death (Fig. 5B), pinpointing EBP as a potential target of TASIN-1. To confirm that TASIN-1 acts through EBP, we stably knocked down EBP in DLD1 and HCT116 cells and tested their viability in 0.2% serum medium. Although EBP knockdown cells grew fine in 2% serum medium, knockdown of EBP inhibited the growth of DLD1 cells but not HCT116 cells under the 0.2% serum condition, recapitulating TASIN-1's effects (Fig. 5, C to F). Conversely, overexpression of EBP conferred resistance to TASIN-1 in DLD1 cells (Fig. 5, G and H). Together, these results demonstrate that TASIN-1 exerts its killing effects primarily by depleting cholesterol through inhibition of EBP activity. TASIN-1 attenuated tumor burden in *CPC;Apc* mice fed a high-fat diet, and the mice treated with TASIN-1 gained weight, whereas control mice lacked weight gain (fig. S12), suggesting that the *in vivo* antitumor activity of TASIN-1 was not affected by dietary cholesterol content.

Induction of cholesterol depletion in truncated APC cells by defective SREBP2 activation in response to TASIN-1

To explore potential mechanisms of TASIN-1's selective toxicity to APC-truncated CRC cells, we evaluated the cellular response to TASIN-1-induced cholesterol depletion in HT29 (truncated APC) and HCT116 (WT APC) cells. A dual-luciferase assay was performed after cotransfecting HT29 and HCT116 cells with a plasmid encoding firefly luciferase under the

control of the sterol response element (SRE)–dependent promoter and a plasmid encoding Renilla luciferase driven by a constitutive thymidine kinase promoter. As shown in Fig. 6A, TASIN-1 treatment induced sterol-regulated luciferase activity in HCT116 cells but not in HT29 cells, similar to triparanol, which is an inhibitor of DHCR24. The amount of nuclear sterol regulatory element-binding protein 2 (SREBP2) was increased 8 hours after treatment in HCT116 cells, but not in HT29 cells (Fig. 6B). SREBP2 is a master regulator of cholesterol metabolism and homeostasis (23). When the intracellular cholesterol concentration drops, the SCAP (SREBP cleavage-activating protein)–SREBP complex is transported to the Golgi, where SREBPs are cleaved. The nuclear fragment is then translocated into the nucleus and activates SRE-regulated genes (24). TASIN-1 treatment increased the expression of a panel of SREBP2 target genes in HCT116, but not in HT29 or HCT116 cells with ectopic expression of APC1309 (HCT116 A1309) (Fig. 6, C to E). The SRE luciferase assay also showed that treatment with simvastatin, an inhibitor of HMGCR, induced SRE activity in the two WT APC cells but not in the two mutant APC cell lines or HCT116 A1309 cells (Fig. 6F). These results suggest that DLD1 and HT29 cells, and perhaps most APC-truncated cancer cells, are defective in responding to cholesterol depletion, which APC WT cells (HCT116 and RKO) can overcome by inducing SREBP2 activation and up-regulation of downstream target genes. In agreement, simvastatin also exhibited selective toxicity toward DLD1 and HT29 cells compared to HCT116 cells but was less potent than TASIN-1 (fig. S13).

DISCUSSION

Comprehensive screening of the entire coding region of *APC* in 41 CRC cell lines revealed that there may be interdependence between the “two hits” at *APC* in both sporadic and familial colorectal tumors. Specifically, tumors with an *APC* mutation within the “mutation cluster region (MCR),” especially those close to codon 1300, show allelic loss, whereas tumors with mutations outside this region tend to harbor truncating mutations (25). Mutations within the MCR are associated with the most severe intestinal phenotypes, whereas mutations 5′ or 3′ to the MCR result in reduced polyp multiplicity relative to the severe phenotype (26). It has been proposed that the strong selection for the retention of the truncated APC protein is primarily, if not completely, caused by the requirement for “optimal” β -catenin concentrations for tumor development (27–29). This hypothesis is both supported and contradicted by observations from mouse models expressing shorter or longer truncated APC proteins (30). For example, *Apc*^{1322T/+} and *Apc*^{e1–15/+} mice, which have decreased WNT signaling compared to *Apc*^{Min/+} mice, display increased polyp multiplicity relative to *Apc*^{Min/+} mice (31, 32). In addition, *Apc*^{1572T/+} mice do not develop intestinal polyps, although *Apc*^{1572T/1572T} embryonic stem cells have elevated WNT signaling (33). In contrast, *Apc*^{716/+} mice exhibit higher activation of WNT signaling and harbor more polyps compared to *Apc*^{Min/+} mice (34). Therefore, at the present time, it remains hard to pin down the additional mechanism underlying the selective advantage for the retention of the truncated APC protein.

There have been increasing reports suggesting that APC truncations may exert dominant functions that contribute to colon cancer tumorigenesis (15, 17, 18, 35). Here, we provide robust evidence showing that ectopic expression of APC truncations confers tumorigenic

properties compared to isogenic cell lines with only the depletion of APC. We also observed that transient knockdown of truncated APC in DLD1 cells slowed down proliferation rates and resulted in induction of apoptosis when cultured in low serum medium (more physiological conditions), suggesting that expression of truncated APC protein can promote cell survival pathways to which CRC cells gradually become “addicted” for cell growth, especially in nutrient-starved conditions that may exist in the emerging tumor microenvironment.

Despite the prevalence of APC truncations in CRC, there are currently no therapeutics directly targeting them. Previous work has shown that combinatorial treatment with all-*trans*-retinyl acetate (RAc) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively kill APC-deficient CRC cells by repressing c-FLIP (cellular Fas-associated death domain protein-like interleukin-1 β -converting enzyme-like inhibitory protein) expression induced by β -catenin activation (36). The investigators observed that this combinatorial treatment exerted synergistic effects with small interfering RNA against APC, namely, the loss of function of WT APC in immortalized HCECs and induced apoptosis specifically in Apc^{Min/+} mice, which mainly develop polyps in the small intestine. In contrast, we used isogenic HCECs with or without ectopic expression of truncated APC to screen for small molecules targeting a dependency generated by a gain of function of the truncated APC protein. We identified a candidate small molecule, TASIN-1, which specifically kills HCECs and CRC cell lines with truncated APC through induction of apoptotic cell death while sparing normal and cancer cells expressing WT APC. Stable knockdown of truncated APC conferred resistance to TASIN-1 in DLD1 and HT29 cells to a lesser extent compared with WT APC cells, whereas ectopic expression of APC truncation partially sensitized HCT116 cells to TASIN-1. These results suggest that the presence of truncated APC is required but not sufficient for TASIN-1’s cytotoxicity. This compound was able to inhibit tumor growth in both a human CRC xenografted mouse model and a genetically engineered CRC mouse model, *CPC;Apc* mice, with no apparent toxicity. The reason for selection of *CPC;Apc* mice is that these mice have conditional inactivation of APC (expression of APC580) in the colon epithelial cells and mainly develop colorectal tumors, which are similar to human CRC, whereas the commonly used Apc^{Min/+} mice mainly develop small intestinal tumors and carcinomas are rare (22). One limitation of the current study is the lack of patient-derived xenograft mouse models carrying WT or truncated APC to evaluate TASIN-1’s cytotoxicity.

Mechanistic investigations of TASIN-1 identified synthetic lethal interactions between the endogenous cholesterol synthesis pathway and APC truncations. Statins, a class of HMGCR inhibitors, induce apoptosis in some human CRC cells and attenuate colitis-associated colon cancer in mice (37-39). However, epidemiological studies have reported conflicting results regarding the association between the use of statins and a reduced incidence of CRC, partially because of the pleiotropic effects of statins (40-45). TASIN-1 shows better potency and selective cell killing of truncated APC cells compared to simvastatin. This difference may partially result from the changes in different sterol intermediates induced by these reagents targeting different steps in the pathway. The dynamic range of sensitivity profiles toward TASIN-1 in a panel of CRC cells suggests that TASIN-1’s effects are highly context-dependent. All CRC lines harbor mutations in other oncogenes or tumor suppressor genes

such as *KRAS*, *PI3K*, *TP53*, etc., which contribute to aberrant lipogenesis and regulation (46). The involvement of these oncogenic events suggests that the underlying mechanisms of lipogenesis regulation and mode of action of cholesterol-lowering drugs in cancer cells are complex, and this partially contributes to the differential sensitivity toward TASIN-1. Here, there was a good correlation between cell sensitivity and the degree of cholesterol synthesis reduction induced by TASIN-1 treatment. Moreover, rescue experiments with various sterol intermediates pinpointed EBP as a potential target of TASIN-1. A proteomics approach using biotinylated analogs and in vitro binding assays will be necessary to assess the direct interaction between TASIN-1 and EBP protein. Additional mechanistic investigations are warranted to delineate the relationship between truncated APC and cholesterol biosynthesis pathway.

Our results can also be interpreted to suggest that selective toxicity toward truncated APC cells may be partially a consequence of their defect in feedback regulation of SREBP2 activation in response to TASIN-1-induced cholesterol depletion. Unlike HCT116 and RKO cells, DLD1 and HT29 cells were defective in activating SRE transcriptional activity when treated with simvastatin. HCT116 A1309 cells were also defective in the activation of SRE activity in response to simvastatin, supporting the idea that APC truncations may be involved in the cholesterol feedback regulatory pathway. Similarly, it has been reported that the loss of classic feedback regulation of the mevalonate pathway may contribute to sensitivity to statins in multiple myeloma cells (47). Aberrations in sterol-mediated feedback have been documented in several tumor types (48-51). Our study shows that CRC cells expressing mutant APC are defective in cholesterol homeostasis regulation in a lipid-deficient environment and thus provides the rationale for using more selective cholesterol-lowering drugs to treat APC-mutant CRC cells. However, the detailed mechanism underlying this SREBP2 feedback defect remains to be determined. It is worth noting that HCT116 cells showed similar sensitivity to TASIN-1 as DLD1 cells when cultured in medium with lower serum concentration for a longer period of time. Therefore, WT APC cells may also respond to TASIN-1 depending on the availability of lipid in the environment and exposure time to TASIN-1. The fact that TASIN-1 did not reduce tumor growth in the HCT116 xenograft mouse model may be partially due to the short half-life of TASIN-1 in plasma, limiting the amount and exposure time of TASIN-1 at the tumor site. In this context, the immediate feedback activation of SREBP2 may compensate for TASIN-1-induced cholesterol depletion in HCT116 xenografts.

Our study has translational relevance in that we have identified a genotype-selective compound that has synthetic lethality with the cholesterol biosynthesis pathway specifically in truncated APC cells in vitro and in vivo, with no apparent toxicity. Considering the high prevalence of APC mutations in patients with CRC, targeting truncated APC should be an effective therapeutic strategy for prevention and intervention of CRC and a potential marker for stratifying patients in future personalized medicine clinical trials.

MATERIALS AND METHODS

Study design

The aim of this study was to identify small molecules that can selectively kill CRC cells expressing truncated APC. Isogenic immortalized HCECs were used in a 200,000-compound high-throughput screen. In vivo antitumor activity of the lead compound was evaluated using both xenografts and a genetically engineered CRC mouse model. Evaluation of drug toxicity was performed by IHC staining and serum analysis, evaluated in a blinded manner. All the animal studies were performed according to the guidelines of the University of Texas (UT) Southwestern Institutional Animal Care and Use Committee (IACUC). Sample size and replicates are specified in each figure legend.

Compound screen

The UT Southwestern Compound Library is composed of ~230,000 small molecules (ChemBridge Inc.) arrayed in DMSO in 384-well plates at a concentration of 5 mM and stored at -20°C . Cells were seeded at a density of 400 cells per well in 384-well plates in HCEC medium supplemented with 0.2% serum. After 24 hours, compounds were added to a final concentration of $2.5\text{ }\mu\text{M}$ in 0.5% (v/v) DMSO in a one-compound-per-well format. Plates were incubated for 96 hours under physiologic oxygen conditions (2% O_2), and the CellTiter-Glo assay was performed to measure cell viability. Experimental values were normalized to the mean of vehicle control on the same plate. Compounds with z score lower than -3 were defined as primary hits. A detailed description of the screen is provided in the Supplementary Materials.

Cell culture

The culture conditions of HCECs and their isogenic series have been reported elsewhere (19). Human cancer cell lines (DLD1, HCT116, RKO, HT29, SW480, SW620, HT55, T84, etc.) and 293FT cells (American Type Culture Collection) were cultured in basal medium supplemented with 10% serum. NCI-H508, SW403, and SW948 cell lines were provided by J. Pollack (Stanford University, CA).

Plasmids

CDK4, hTERT (human telomerase reverse transcriptase), pSRZ-shTP53, and pBABE-hyg-*KRAS*^{V12} were described previously (52). shRNAs targeting APC (clone ID: TRCN0000010297 and TRCN0000040097, GE Dharmacon) or EBP (clone ID: V2LHS_250591 and V2LHS_197457, Open Biosystems) were in pLKO.1 vector and pGIPZ vector, respectively. The lentiviral APC1309 constructs were cut from pcDNA5 plasmids and cloned into pLenti6 vector. EBP cDNA (clone ID: IOH11243, Life Technologies Inc.) was cloned into pLX303 vector using Gateway cloning system (Thermo Fisher). phRL-TK (encoding the Renilla luciferase gene) and pSRE-Luc plasmids were constructed as previously described (53).

SRE assay

Cells were transfected with 200 ng of pSRE-firefly luciferase and 200 ng of pTK Renilla luciferase plasmids in medium with 10% serum. After 24 hours, cells were replaced with HCEC + 0.2% serum medium with or without 2.5 μ M TASIN-1 and 5 μ M triparanol or simvastatin. Luciferase activity was measured using a Dual-Luciferase Assay kit (Promega) 24 hours later.

Cholesterol synthesis

CRC cells were treated with DMSO or 2.5 μ M TASIN-1 in 0.2% serum medium for different amounts of time, followed by incubation with 14 C-labeled acetate (250 μ Ci) for 6 hours in the presence or absence of TASIN-1. Cholesterol synthesis rate was determined as previously described (54).

Animal experiments

Subcutaneous xenografts were established in 5- to 6-week-old female athymic nude mice [National Cancer Institute (NCI)] by inoculation of 2×10^6 CRC cells into both dorsal flanks of each mouse. When the tumors grew to 2 to 3 mm in diameter, the mice were injected intraperitoneally with TASIN-1 at a dose of 40 mg/kg (dissolved in 0.2 ml of solvent containing 10% DMSO and 10% cremophor) or solvent alone twice daily until the tumors grew to about 15 mm in diameter in the control group. Tumors were measured with calipers, and their volumes were calculated using the formula $l \times w^2 \times 0.5$, where l and w represented the length and width of the tumor, respectively. The tumor volume measurements for each mouse at each time point are provided in table S2. The transgenic CRC model, *CDX2P-NLS Cre;Apc^{+/-loxP} (CPC;Apc)* mouse, was provided by E. Fearon (22) and bred and housed in our facilities. Male *CPC;Apc* mice ~110 days old were injected intraperitoneally with either solvent or TASIN-1 (20 mg/kg per injection) twice a week for 90 days. Weights were measured every 15 days over the treatment period. For the experiment with older mice, male *CPC;Apc* mice ~150 days old were injected intraperitoneally with either solvent or TASIN-1 (40 mg/kg per injection) twice a week for 100 days. For the high-fat-diet experiment, male *CPC;Apc* mice around 100 days old were injected with either vehicle or TASIN-1 (40 mg/kg) twice per week for 75 days and were fed a high-fat diet (TD.120156, Teklad; 1.25% cholesterol) over the treatment period. In all the other in vivo experiments, mice were fed on Teklad Global Diet, which contains no cholesterol. These studies were performed according to the guidelines of the UT Southwestern IACUC.

Quantitative reverse transcription PCR

Total RNA was isolated from cell lysates or mouse tissue using an RNeasy Plus Universal Mini Kit (Qiagen) according to the manufacturer's protocol. Then, 1 μ g RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Roche). Real-time quantitative PCR reactions were set up in triplicate with Ssofast Master Mix (Bio-rad) and run on a LightCycler 480 (Roche). Restriction fragment length polymorphism analysis for the detection of the mutant *Kras* was performed as described previously (55). All the primers (Sigma) used in this study are listed in tables S3 and S4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Identification of TASIN-1 through a 200,000–small-molecule high-throughput screen. (A) List of isogenic HCECs used in the high-throughput screen. shTP53, p53-specific short hairpin RNA (shRNA); aa, amino acid. (B) Confirmation of ectopic expression of APC truncation, knockdown of WT APC, and expression of p53 and oncogenic *KRAS*^{V12} by Western blot or a restriction digestion assay. The cell line used in our primary screen is highlighted in the red box. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) Flowchart of overall screening strategy. (D) Chemical structure of TASIN-1. (E) Dose-response curves of TASIN-1 in HCT116 and DLD1 cells. The box on the right lists the IC₅₀ value for each cell line. (F) Representative photographs and quantification of HCT116 and DLD1 cells grown in soft agar in the presence or absence of 2.5 μM TASIN-1 for 7 days. Data represent means ± SD, *n* = 6 pooled from two independent experiments. ****P* = 0.0005, Student's *t* test.

Fig. 2. APC truncation dependency for TASIN-1's cytotoxic effects.

(A) Dose-response curves of DLD1 cells expressing nonsilencing (Nsi) shRNA or shRNAs against APC. The figure also shows APC protein expression in cells treated with each shRNA, as well as the IC₅₀ value for each cell line. (B) Dose-response curves of DLD1 APC knockdown cells infected with a lentiviral vector expressing truncated APC A1309. Knockdown and ectopic expression were demonstrated by Western blot. β-Actin was used as the loading control. The IC₅₀ value for each cell line is shown in the lower right. cDNA, complementary DNA. (C) Left: The bar graph shows the surviving fraction for each cell line treated with dimethyl sulfoxide (DMSO) or 2 or 10 μM TASIN-1 for 72 hours. Data represent means ± SD, *n* = 9 pooled from three independent experiments. Right: The chart lists the average IC₅₀ values for each cell line based on two biological replicates. Statistical significance was determined by comparing the average IC₅₀ values of the cell lines with WT APC and cell lines with truncated APC. *****P* = 0.00005, Student's *t* test. The status of APC in each cell line is based on published data (56) and the Cancer Genome Project Database (www.sanger.ac.uk/genetics/CGP).

Fig. 3. Inhibitory effects of TASIN-1 on tumor growth in a xenograft mouse model and a genetically engineered mouse model.

(A) Tumor sizes of TASIN-1–treated DLD1 xenografts (bottom row) are smaller than those of control mice (top row). Scale bar, 10 mm. TASIN-1 reduces the growth rate of DLD1 (B) and HT29 (C) xenografts but not HCT116 xenografts (D). Data in (B) to (D) represent means $\pm$ SD of six to eight tumors. $*P = 0.0052$, $**P = 0.0017$, $***P = 0.0002$, and $****P = 0.0003$ in (B) and $*P = 0.002$, $**P = 0.0002$, $***P = 0.0001$, and $****P = 0.0001$ in (C), Student's *t* test. (E) Pharmacokinetics of TASIN-1. CPC;Apc mice (mixed genders) were injected intraperitoneally with TASIN-1 (20 mg/kg). Plasma and large intestine tissues were collected at different time points ($n = 3$). TASIN-1 concentrations (nanograms per milliliter for plasma and nanograms per gram for tissue) were determined by liquid chromatography–tandem mass spectrometry. (F) Representative photographs of colons from control and TASIN-1–treated CPC;Apc mice. CPC;Apc mice around ~110 days old were injected intraperitoneally with either solvent or TASIN-1 (20 mg/kg) twice per week for 90 days. (G) TASIN-1 treatment reduces the number of benign tumors (polyps) and decreases polyp size in CPC;Apc mice. (H) TASIN-1–treated CPC;Apc mice gain weight, whereas control mice do not. Data in (G) and (H) represent means $\pm$ SD of 8 to 10 mice. $*P = 0.001$, $**P = 0.0035$, and $***P = 0.0003$ in (G) and $*P = 0.0237$, $**P = 0.0001$, and $***P = 0.004$ in (H), Student's *t* test. (I) TASIN-1 treatment reduces the number of polyps of intermediate size in older CPC;Apc mice. CPC;Apc mice were injected with either vehicle or TASIN-1 (40 mg/kg) twice per week for 100 days, starting at age ~150 days. (J) TASIN-1–treated older mice gain weight over the 100-day treatment course, whereas control mice do not. Data in (I) and (J) represent means $\pm$ SD of four mice. $*P = 0.01$ in (I) and $*P = 0.046$ in (J), Student's *t* test.

Fig. 4. Inhibition of cholesterol biosynthesis by TASIN-1.

(A) Dose-response curves of HT29 cells treated with TASIN-1 in medium supplemented with 0.2 or 10% FCS. (B) Dose-response curves of HT29 cells treated with TASIN-1 in medium supplemented with 2% LPDS or 2% FCS. (C) HT29 cells were treated with 2.5 μM TASIN-1 in the presence or absence of 2 μg/ml of LDL. Cells were counted after 72 hours of treatment. *** $P = 0.0002$, Student's t test. (D) Dose-response curves of HT29 cells treated with TASIN-1 alone, together with 50 μg/ml of methyl-β-cyclodextrin (MCD) or cholesterol complexed with MCD. Data in (A) to (D) represent means $\pm$ SD, $n = 9$ pooled from three independent experiments. (E) HT29 cells cultured in 0.2% serum medium were treated with or without 2.5 μM TASIN-1 for 2, 18, or 42 hours and labeled with ^{14}C -labeled acetate for 6 hours. Cholesterol synthesis rates were determined after normalization to protein content. (F) Cholesterol synthesis rates were measured in WT APC (HCT116 and RKO) or truncated APC (DLD1 and HT29) CRC cells after 48 hours of exposure to TASIN-1. Data in (E) and (F) represent means $\pm$ SD, $n = 6$ from three independent experiments. * $P = 0.0161$, ** $P = 0.0128$, and *** $P = 0.0108$ in (E) and * $P = 0.0128$, ** $P = 0.037$, *** $P = 0.0109$, and **** $P = 0.0411$ in (F), Student's t test.

Fig. 5. Identification of EBP as the potential target for TASIN-1.

(A) Schematic illustration of cholesterol biosynthesis pathway. Some steps were omitted for simplicity. Modified from (57). HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR, hydroxy-3-methylglutaryl-CoA reductase; SC5D, sterol-C5-desaturase. (B) Dose-response curves of HT29 cells treated with TASIN-1 alone or together with 10 μ g/ml of squalene, zymosterol, lathosterol, or desmosterol. (C and D) Growth curves of DLD1 (C) or HCT116 (D) cells expressing Nsi shRNA or shRNAs against EBP cultured in 0.2% serum medium. (E and F) Knockdown of EBP in DLD1 (E) or HCT116 (F) cells was confirmed by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Data represent means $\pm$ SD, $n = 3$. mRNA, messenger RNA. (G) Dose-response curves of DLD1 cells infected with a lentiviral vector expressing LacZ or EBP. Data in (B) to (D) and (G) represent means $\pm$ SD, $n = 9$ pooled from three independent experiments. (H) Ectopic expression of EBP was demonstrated by Western blot. β -Actin was used as the loading control.

Fig. 6. Defective SREBP2 feedback activation in APC mutant cells in response to TASIN-1-induced cholesterol depletion.

(A) Relative SRE luciferase activity in HCT116 and HT29 cells treated with 2.5 μ M TASIN-1 or 5 μ M triparanol for 24 hours. Data represent means $\pm$ SD, $n = 6$ pooled from three independent experiments. * $P = 0.0029$ and ** $P = 0.003$, Student's t test. (B) HCT116 and HT29 cells were treated with DMSO or with 2.5 μ M TASIN-1 for 8 or 24 hours and subjected to nuclear fractionation and Western blot. Cleaved SREBP2 protein was detected in the nuclear fraction. Lamin A/C was used as the loading control. (C to E) qPCR analysis of the major target genes regulated by SREBP2 in HCT116 (C), HT29 (D), or HCT116 A1309 cells (E) treated with 2.5 μ M TASIN-1 for 24 or 48 hours. Data represent means $\pm$ SD, $n = 9$ pooled from three independent experiments. *LDLR*, LDL receptor; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *HMGCR*, HMG-CoA reductase; *HMGCS1*, HMG-CoA synthase 1; *HMGCS2*, HMG-CoA synthase 2; *FDFT1*, farnesyl-diphosphate farnesyltransferase 1. (F) Relative SRE luciferase activity in WT APC (HCT116 and RKO)

or truncated APC (DLD1 and HT29) as well as HCT116 A1309 cells treated with 5 μ M simvastatin for 24 hours. Data represent means $\pm$ SD, $n = 6$ pooled from three independent experiments. * $P = 0.004$ and ** $P = 0.0026$, Student's t test.

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