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Therapeutics, Targets, and Chemical Biology

Cancer Research

A Novel Tankyrase Small-Molecule Inhibitor Suppresses *APC* Mutation–Driven Colorectal Tumor Growth

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Abstract

Most colorectal cancers (CRC) are initiated by mutations of *APC*, leading to increased β-catenin–mediated signaling. However, continued requirement of Wnt/β-catenin signaling for tumor progression in the context of acquired *KRAS* and other mutations is less well-established. To attenuate Wnt/β-catenin signaling in tumors, we have developed potent and specific small-molecule tankyrase inhibitors, G007-LK and G244-LM, that reduce Wnt/β-catenin signaling by preventing poly(ADP-ribosyl)ation-dependent AXIN degradation, thereby promoting β-catenin destabilization. We show that novel tankyrase inhibitors completely block ligand-driven Wnt/β-catenin signaling in cell culture and display approximately 50% inhibition of *APC* mutation–driven signaling in most CRC cell lines. It was previously unknown whether the level of AXIN protein stabilization by tankyrase inhibition is sufficient to impact tumor growth in the absence of normal *APC* activity. Compound G007-LK displays favorable pharmacokinetic properties and inhibits *in vivo* tumor growth in a subset of *APC*-mutant CRC xenograft models. In the xenograft model most sensitive to tankyrase inhibitor, COLO-320DM, G007-LK inhibits cell-cycle progression, reduces colony formation, and induces differentiation, suggesting that β-catenin–dependent maintenance of an undifferentiated state may be blocked by tankyrase inhibition. The full potential of the antitumor activity of G007-LK may be limited by intestinal toxicity associated with inhibition of Wnt/β-catenin signaling and cell proliferation in intestinal crypts. These results establish proof-of-concept antitumor efficacy for tankyrase inhibitors in *APC*-mutant CRC models and uncover potential diagnostic and safety concerns to be overcome as tankyrase inhibitors are advanced into the clinic. *Cancer Res; 73(10); 3132–44. ©2013 AACR.*

Introduction

Despite detailed understanding of the molecular mechanisms of Wnt/β-catenin signaling and long-standing knowledge of highly prevalent Wnt pathway activation in colorectal cancers (CRC), no targeted therapeutics have advanced to clinical testing for *APC*-mutant CRCs, although Wnt pathway inhibitors have entered clinical trials for other indications (1, 2). The cellular level of transcriptional coactivator β-catenin is regulated by its destruction through β-TrCP–dependent ubiquitylation and proteasomal degradation (3). β-Catenin is stabilized in approximately 75% of colorectal tumors by inactivating mutations in the destruction complex scaffolding protein APC and in an additional 5% by phosphodegron mutations in β-catenin (4, 5). These mutations likely initiate tumor development as they arise at the microadenoma stage, whereas mutations in additional oncogenes and tumor suppressors, including *KRAS*, *p53*, and *SMAD4*, are found in larger adenomas and adenocarcinomas (4, 6). Mouse *Apc* deletion specifically in $Lgr5^+$ intestinal stem cells produces neoplasia (7). In mouse small intestine, ligand-induced Wnt/β-catenin signaling in intestinal stem cells maintains multipotent stem cell self-renewal (8). In an analogous fashion in a mouse inducible *Apc* deletion model of intestinal cancer, $Lgr5^+$ adenoma cells constitute 5% to 10% of tumor cells and give rise to additional stem cell–like cancer cells as well as differentiated cell types (9).

Even with the subsequent accumulation of mutations in other pathways during intestinal tumor progression, at least some CRC models maintain a continued requirement for activated Wnt/β-catenin signaling. β-catenin mediates cell proliferation in established CRC cell lines, such that β-catenin knockdown inhibits colony formation and induce G_1 cell-cycle arrest (10–12). Furthermore, in *APC*- or β-catenin–mutant xenograft tumor models, reduction of β-catenin expression can inhibit tumor growth (12–14). Importantly, the antitumor

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efficacy of inducible β-catenin RNA interference (RNAi) is associated with an induction of biomarkers for intestinal cell–type differentiation and cell-cycle arrest (13). However, β-catenin is not required for cell proliferation and tumor growth in all APC-mutant CRC models, and the determinants of these differential requirements have not been defined.

The scaffolding protein AXIN is the concentration-limiting component of the β-catenin destruction complex (15). AXIN1 and AXIN2 protein levels are constitutively suppressed through poly(ADP-ribosyl)ation (PARsylation) by tankyrases TNKS1 and TNKS2 (16), which directs AXIN ubiquitylation by RNF146 and proteasomal degradation (17, 18). Cell-based screens for Wnt/β-catenin signaling inhibition have identified small-molecule tankyrase inhibitors that block PARsylation activity (16, 19–25). Tankyrase inhibitors stabilize AXIN by preventing its PARsylation and ubiquitylation, thereby enhancing β-catenin degradation (16). Wnt/β-catenin signaling inhibition by tankyrase inhibitors appears to be complete for cell lines that contain wild-type *APC* and are stimulated with exogenous Wnt (16, 19, 21–23). However, the extent of signaling inhibition in *APC*- and β-catenin–mutant cells has not been surveyed in a systematic way for CRC cell lines' and inhibition levels as well as cell survival and proliferation effects appear to differ between tankyrase inhibitors, possibly due to off-target effects.

With the identification of a readily druggable target in the Wnt/β-catenin signaling pathway that acts at the level of β-catenin degradation, it now becomes important to establish whether tankyrase inhibitors exhibit antitumor efficacy in preclinical CRC models *in vivo*. Initial results in genetically engineered mouse *Apc*-mutant models suggest that adenoma formation may be prevented or slowed (21, 22). In addition, the therapeutic window of tankyrase inhibitors will need to be defined as potent inhibition of Wnt/β-catenin signaling in intestine produces significant adverse effects (26, 27). Here, we show that tankyrase inhibitors optimized for potency, selectivity, and *in vivo* stability can sufficiently stabilize AXIN to reduce Wnt/β-catenin signaling and xenograft tumor growth of a subset of *APC*-mutant CRC cell lines. Our studies also reveal intrinsic resistance to inhibition of Wnt/β-catenin signaling and tumor growth by tankyrase inhibitors in some CRC models, as well as intestinal toxicity for doses near the high end of the efficacious concentration range.

Materials and Methods

Cell and biochemical assays

All cell lines were purchased from American Type Culture Collection, cultured according to the supplier's recommendations, and validated for *APC* genotype by sequencing. TOPbrite and SV40 luciferase reporter cell lines and assays for luciferase activity, cell viability, and proliferation were reported previously (17, 28). RNAi and quantitative RT-PCR (qPCR) expression analysis was carried out as described previously (17). Intestinal crypt organoids were isolated and cultured as previously described (29). Tankyrase auto-PARsylation and PARP histone PARsylation biochemical assays were conducted in duplicate by BPS Bioscience following the BPS PARP Assay Kit protocols.

Mouse *in vivo* studies

For pharmacokinetic analysis, three CD-1 mice were each given a single 1 mg/kg i.v. dose [vehicle: 10% dimethyl sulfoxide (DMSO), 60% PEG400, 30% saline] or 5 mg/kg intraperitoneal (i.p.) dose (vehicle: 15% DMSO, 17.5% Cremophor EL, 8.75% ethanol, 8.75% Miglyol 810N, 50% PBS) of G007-LK or G244-LM. In the toxicology study, G007-LK was administered at doses of 0, 60, and 100 mg/kg to female CD-1 mice (6 per group) i.p. once daily for 14 days.

For xenograft tumor studies, mice were implanted subcutaneously with cells and groups of 10 animals were administered G007-LK i.p. as described in Supplementary Materials and Methods. Male $Apc^{CKO/CKO}$/*Lgr5-creERT2* mice at 10 weeks of age were injected i.p. with 1.5 mg/kg of tamoxifen (Sigma) diluted in ethanol and corn oil (ratio 1:4) and randomized into 2 groups of 5 animals for G007-LK treatment.

Tumor RNA and protein expression analyses

Tumor RNA and protein lysates were prepared as described in Supplementary Materials and Methods. All genes used to evaluate Wnt/β-catenin signaling in tumors and cell lines were validated by qPCR using β-catenin RNAi in the respective cell line in culture.

Results

Tankyrase inhibitors reduce Wnt/β-catenin signaling in a subset of *APC*-mutant CRC cell lines

Through structure–activity relationship studies to be published elsewhere, we developed analogues of TNKS1/2 small-molecule inhibitors in 2 distinct chemical series possessing significantly enhanced potency for Wnt/β-catenin signaling inhibition in cell-based assays, relative to previously published inhibitors (Fig. 1A and 1B; Supplementary Table S1). G007-LK is an analogue of JW74 (21), which binds the adenosine site of the tankyrase NAD^+-binding pocket (25), and G244-LM is an analogue of XAV939 (16), which binds the nicotinamide site of the pocket (20). Tankyrases are members of the PARP superfamily that encompasses 15 other enzymes with a conserved catalytic domain (30). In biochemical assays of auto-PARslyation activity, G007-LK is more selective than a G244-LM analogue or XAV939 in inhibiting TNKS1/2 relative to the 7 PARP enzymes tested (Supplementary Table S1). G007-LK and G244-LM completely inhibit Wnt3a-induced signaling in human HEK293 (Fig. 1B) and mouse 10T1/2 (Supplementary Fig. S1A) cells as assayed by a T-cell factor (TCF)-driven luciferase reporter (TOPbrite) and also by endogenous expression of Wnt/β-catenin target genes in 10T1/2 cells. Both inhibitors only partially reduce (by ∼50%) β-catenin signaling driven by APC mutation in the CRC cell line HCT-15, as measured by either the Wnt/β-catenin signaling reporter or endogenous target gene expression (Fig. 1B), whereas β-catenin RNAi nearly completely inhibits signaling (Fig. 1C). When activity levels of β-catenin in HCT-15 cells are lowered by β-catenin siRNA at limiting concentrations, G244-LM treatment additively blocks Wnt/β-catenin signaling (Fig. 1C). At all levels of partial β-catenin knockdown, tankyrase inhibition produced 30% to 56% inhibition of the remaining Wnt/β-catenin signaling reporter or *AXIN2* mRNA expression.

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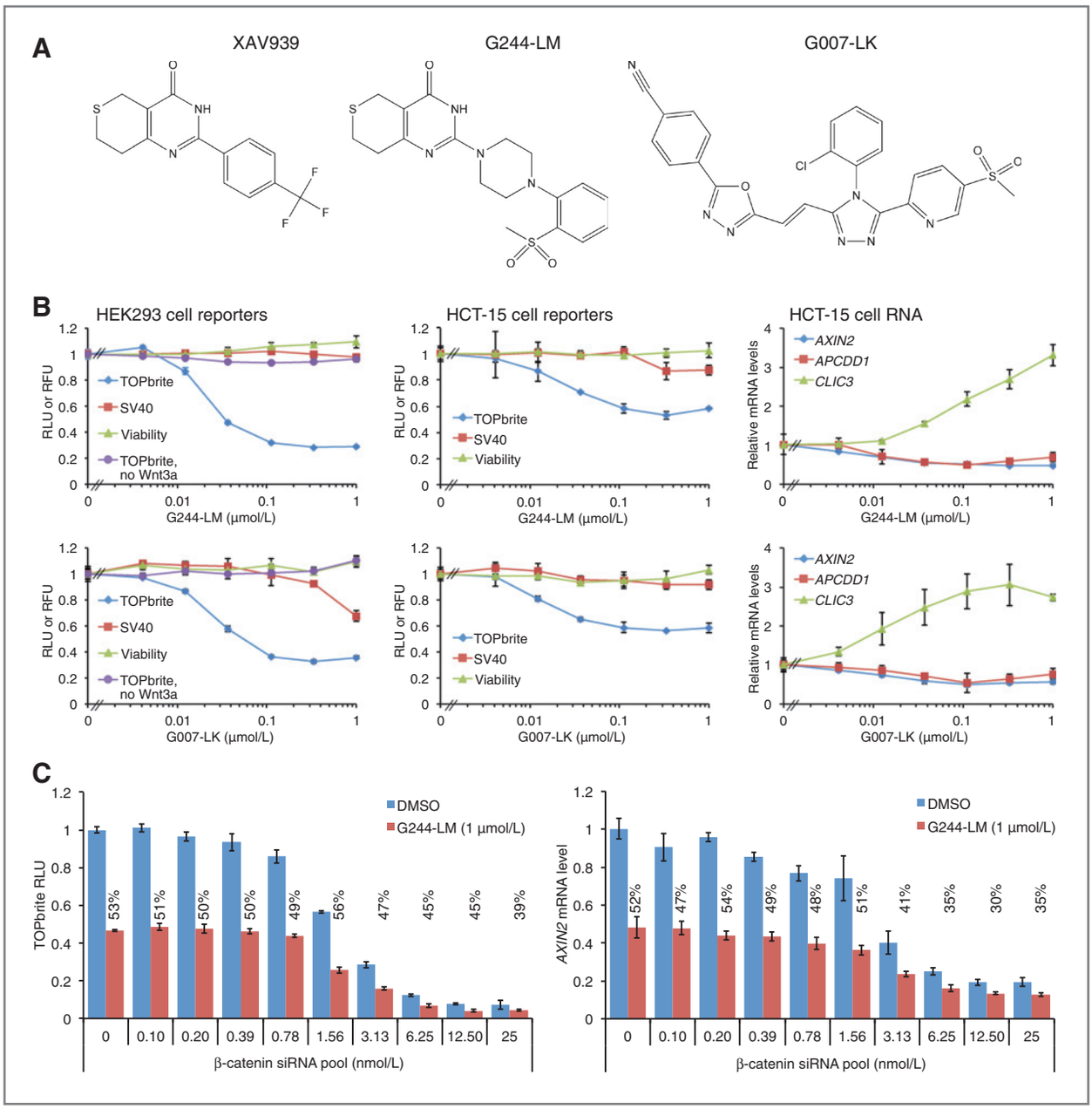

Figure 1. Tankyrase inhibitors partially block mutation-driven Wnt/β-catenin signaling in CRC cell lines. A, chemical structures of 3 tankyrase inhibitors. B, dose–response curves for TOPbrite Wnt signaling and control SV40 luciferase reporters, and for Wnt target gene mRNA expression in HEK293 and HCT-15 cells treated with G244-LM (top graphs) or G007-LK (bottom graphs; RLU, relative luminescence units; RFU, relative fluorescence units). HEK293 cells were stimulated with Wnt3a except where indicated. C, HCT-15 cell TOPbrite Wnt reporter (left graph) and AXIN2 mRNA expression (right graph) after β-catenin RNAi at the indicated siRNA concentrations and treatment with either 1 μmol/L G244-LM or control DMSO. The percentage values above the G244-LM bars show the level of inhibition mediated by G244-LM normalized to the DMSO treatment value at the same β-catenin siRNA concentration.

Of the 11 APC-mutant CRC cell lines tested, 6 showed a reduction in Wnt/β-catenin signaling (29%–76% for expression of β-catenin–activated genes) in response to TNKS1/2 inhibitors (Table 1). Five *APC*-mutant CRC cell lines, 2 of which are derived from the same patient (SW480 and SW620), did not display reduced Wnt/β-catenin signaling in response to tankyrase inhibitor treatment, based on expression of the Wnt/β-catenin signaling reporter and multiple Wnt target genes validated in these cells by β-catenin RNAi (Table 1). Neither the position of the *APC* truncating mutations nor the sensitivity to Wnt/β-catenin signaling activation by *APC* RNAi correlates with sensitivity to TNKS1/2 inhibition (Fig. 2A and Table 1).

In all CRC cell lines tested, G007-LK treatment altered tankyrase protein levels and stabilized AXIN1/2 and RNF146

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Table 1. Differential sensitivity of CRC cells lines to Wnt/β-catenin signaling inhibition by tankyrase inhibitors

	Mutations[a]				
Cell line	*APC* truncation[b]	RAS/RAF	Others	TNKSi transcriptional response[c]	*APC* RNAi Wnt activation[d]
COLO-320DM	811	(wild-type)	p53, SMO	50 (AXIN2), 76 (NKD1)	No
SW403	1197, 1278	KRAS	p53, SMAD4	49 (AXIN2), 53 (NKD1)	Yes
HCT-15	1417	KRAS	FAM123B, PIK3CA, p53, BRCA2, MSH6	58 (AXIN2), 45 (ASCL2)	Yes
DLD-1	1417	KRAS	PIK3CA, SMO	55 (AXIN2)	(not tested)
LS-1034	1309	KRAS	p53, ROR2, TNIK, GRK6, MARK4, others	32 (AXIN2), 49 (APCDD1)	Yes
SW1417	1450	BRAF	PIK3R1, p53, MAP2K4	18 (AXIN2), 29 (SP5)	No
SW480	1338	KRAS	SMAD4	None	No
SW620	1338	KRAS	SMAD4, p53, MAP2K4	None	No
COLO-205	1556	BRAF	SMAD4, p53	None	Yes
LS-411N	789, 1556	BRAF	FBXW7, p53	None	Yes
HT-29	853, 1556	BRAF	PIK3CA, SMAD4, p53	Activation	Yes

NOTE: Cell lines in green or red are sensitive or resistant, respectively, to Wnt/β-catenin signaling inhibition by tankyrase inhibitors.
Abbreviation: TNKSi, tankyrase inhibitor.
[a]Reported by the Wellcome Trust Sanger Institute's Catalog of Somatic Mutations in Cancer (COSMIC).
[b]Codon position of truncation.
[c]Maximal percent inhibition of mRNA level by up to 1 μmol/L G007-LK or G244-LM for AXIN2 and the most sensitive additional gene ("none" indicates that expression of all tested β-catenin-regulated genes was not modulated by tankyrase inhibitors).
[d]Indication whether Wnt target gene expression upon APC RNAi was significantly altered in the direction opposite to that of β-catenin RNAi.

proteins (Fig. 2B; Supplementary Fig. S1B). In contrast to the other cell lines, 2 lines that are resistant to Wnt/β-catenin signaling inhibition by tankyrase inhibitors exhibited stabilization of only one AXIN isoform: AXIN2 but not AXIN1 for COLO-205 cells and AXIN1 but not AXIN2 in LS-411N cells (Supplementary Fig. S1B). G007-LK reduces cytosolic and nuclear β-catenin protein levels in all the *APC*-mutant cell lines in which it inhibits Wnt/β-catenin signaling and also in SW480 cells, but not in the resistant cell lines COLO-205, HT-29, or LS-411N (Fig. 2B; Supplementary Fig. S1B). Quantitating nuclear nonphospho-β-catenin by immunofluorescence reveals that tankyrase inhibitors reduce levels in SW480 cells by up to 48%, similar to the maximal 42% inhibition observed in COLO-320DM cells that are sensitive to tankyrase inhibitors for Wnt/β-catenin signaling inhibition (Fig. 2C). In CRC cells lines LS174T and HCT116 with phosphodegron mutations in β-catenin, G007-LK does not detectably destabilize cytoplasmic or nuclear β-catenin protein (Fig. 2B).

Tankyrase inhibitors can suppress colony formation in CRC lines and growth of *Apc*^{Min} intestinal adenoma spheroids

In cell-based assays, we observed no significant inhibition of cell proliferation or viability with tankyrase inhibitor treatment of CRC lines up to 24 hours (Fig. 1B), as well as no induction of the apoptosis markers *BIM* gene expression (Supplementary Fig. S2C) and caspase-3/7 activities (data not shown). However, cell-cycle analysis revealed that G007-LK and G244-LM reduced the number of COLO-320DM cells in mitosis from 24% to 12% and decreased HCT-15 cells in S-phase from 28% to 18% (Supplementary Fig. S2A). We selected 4 *APC*-mutant CRC cells lines that were sensitive to inhibition of Wnt/β-catenin signaling by tankyrase inhibitors for further characterization of effects on growth using colony formation assays and prolonged compound treatment. COLO-320DM and SW403 cells, but not HCT-15 or DLD-1 cells, showed inhibition of colony formation with either G007-LK or G244-LM (Fig. 3A). Wnt target gene expression analysis indicated that Wnt/β-catenin signaling inhibition was maintained through the end of the treatment in COLO-320DM and HCT-15 cells (Fig. 3B). Intestinal differentiation marker cytokeratin 20 (*KRT20*) expression was induced in both COLO-320DM and HCT-15 cells; however, specific markers for enterocyte (*CA2*) or enterocyte and goblet cell (*TM4SF4*) differentiation (13) display increased expression in COLO-320DM but not HCT-15 cells (Fig. 3B).

As many CRC cell lines exhibit activated receptor tyrosine kinase (RTK) signaling, we tested whether tankyrase and RTK signaling inhibitors could act additively or synergistically in combination. In 4-day treatment assays, we observed very weak enhancement of the effects of MEK1/2 inhibitor GDC-0973 (31) by tankyrase inhibitors on DLD-1 (Supplementary Fig. S2B) and HCT-15 (data not shown) cell proliferation. These interactions were more pronounced in colony formation assays with long-term compound treatment (Fig. 3C). In *KRAS*-mutant DLD-1 and HCT-15 cells, tankyrase inhibitors further reduced the number of colonies formed in the presence

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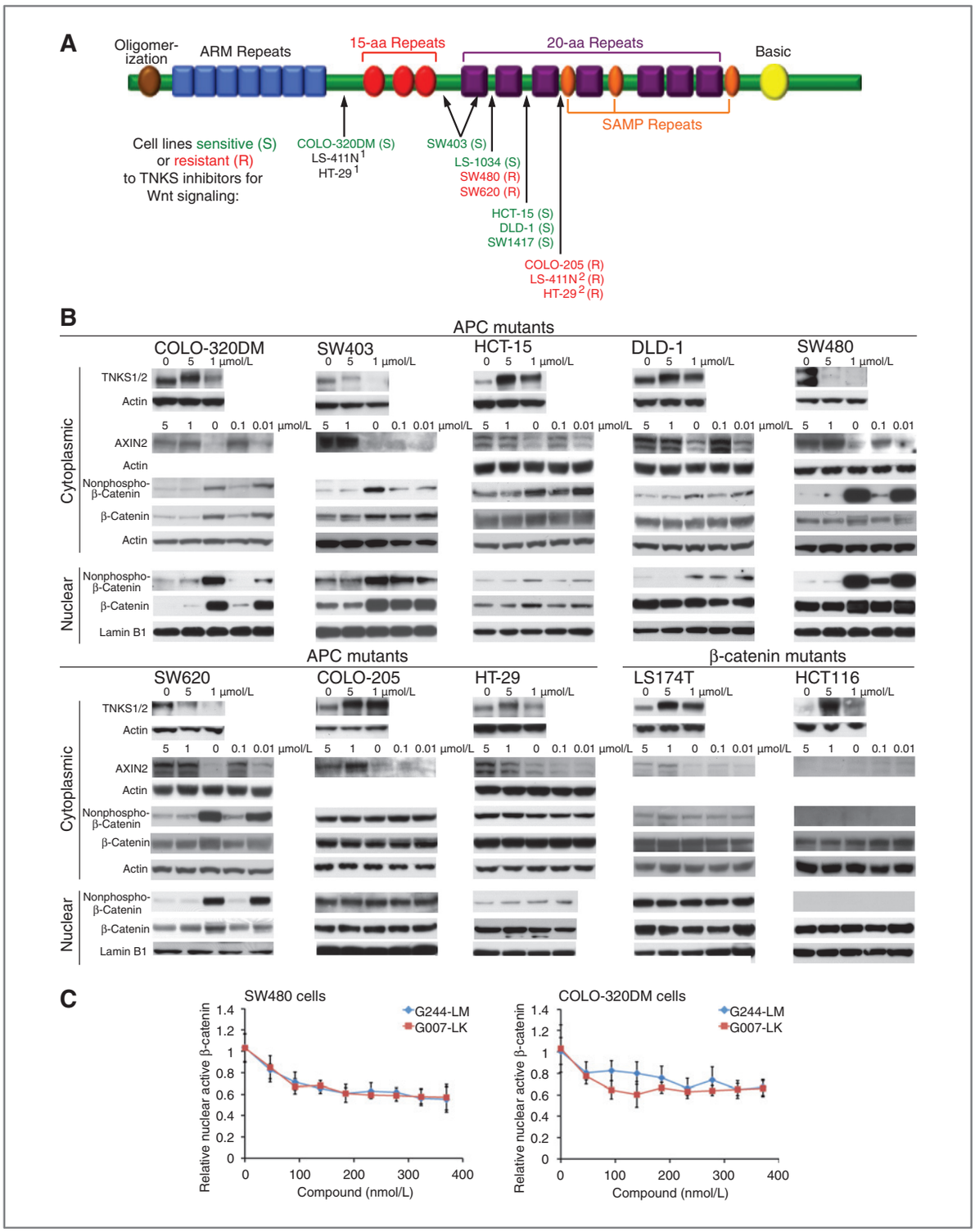

Figure 2. Tankyrase inhibitor effects on Wnt/β-catenin signaling in CRC cell lines. A, schematic representation of truncating mutations in the APC protein for cell lines sensitive (S, green) or resistant (R, red) to Wnt/β-catenin signaling inhibition by tankyrase inhibitors. LS-411N and HT-29 cells contain the 2 mutations shown (1 and 2) on different alleles, and all other cell lines are hemizygous for the single mutation indicated. The positions of domains known to bind β-catenin (15- and 20-amino acid repeats) or AXIN (SAMP repeats) are depicted. B, Western immunoblot analysis of APC- and β-catenin–mutant CRC cell lines treated with G007-LK for 24 hours. Cell lysates were separated into cytoplasmic and nuclear fractions, for which actin and lamin B1 protein levels, respectively, are shown as sample loading controls. C, nuclear nonphospho-β-catenin levels as measured by immunofluorescence after tankyrase inhibitor treatment of SW480 (left graph) or COLO-320DM (right graph) cells for 24 hours.

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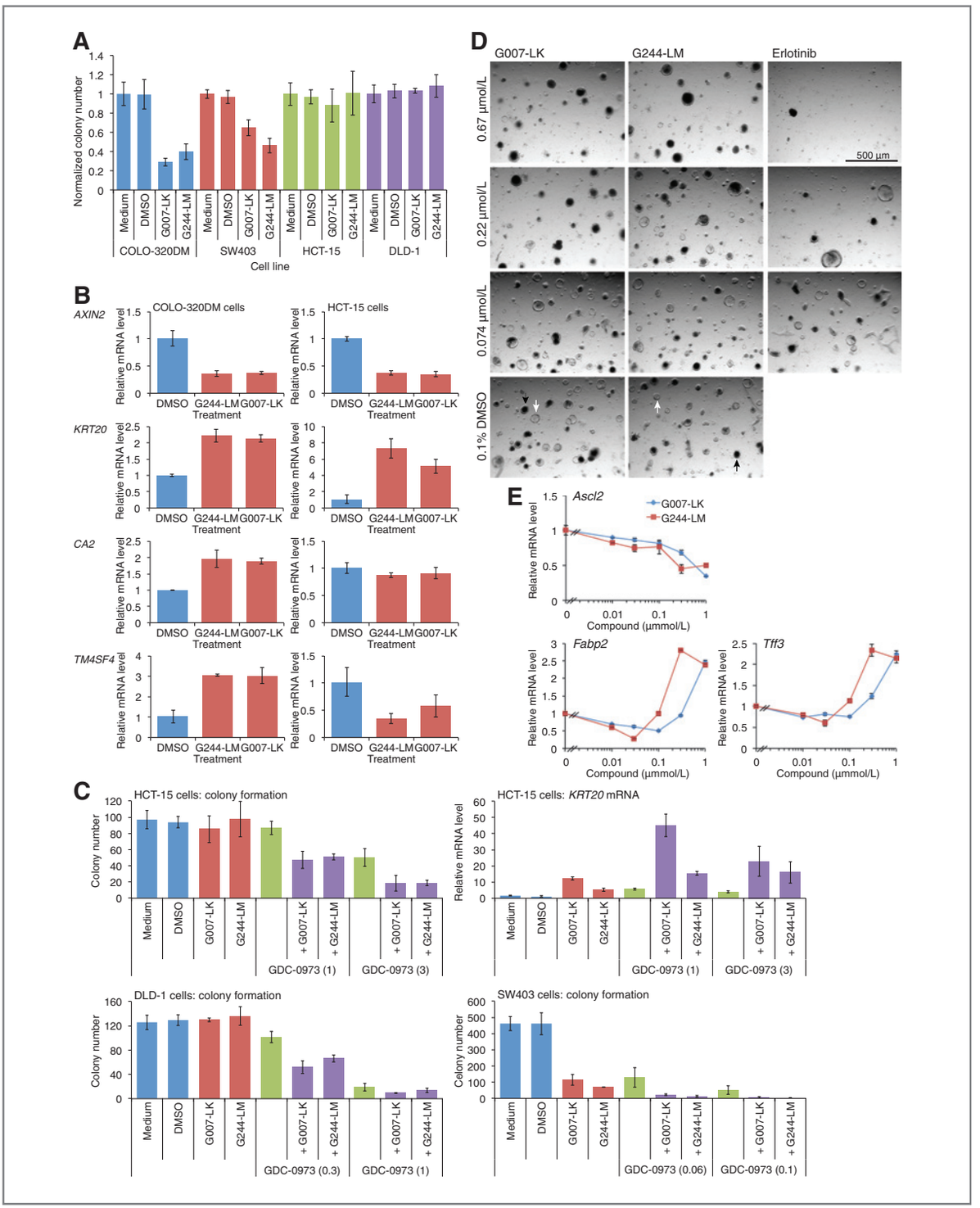

Figure 3. Tankyrase inhibitor effects on APC-mutant cells and adenomas in culture. A, colony formation for cell lines treated for 7 to 13 days with 0.2 µmol/L tankyrase inhibitors. Colony numbers are normalized to the average values of control growth medium wells for each cell line. B, RNA expression levels of *AXIN2* and intestinal differentiation genes for COLO-320DM (left graphs) and HCT-15 (right graphs) cells treated with 1 µmol/L tankyrase inhibitor for 9 days. C, colony formation for cells treated with tankyrase inhibitor (0.2 µmol/L G244-LM or G007-LK) and MEK inhibitor (GDC-0973 at the indicated µmol/L concentrations in parentheses) individually or in combination for 7 to 13 days. *KRT20* RNA expression is also shown for HCT-15 cell treatments for 4 days (top right graph). D, Apc^{Min} intestinal adenoma spheroid images after 5 days of compound treatments. Examples of spheroid cysts with central lumens filled with either clear fluid or presumably apoptotic cells are marked by white or black arrows, respectively, in the control DMSO-treated images. E, Wnt/β-catenin signaling (*Ascl2*) and intestinal cell differentiation (*Fabp2* and *Tff3*) gene expression in Apc^{Min} adenoma spheroids treated with tankyrase inhibitors for 4 days.

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of GDC-0973 by 28% to 64%. These effects were associated with increased expression of intestinal differentiation markers such as *KRT20* (Fig. 3C) but did not correlate with either enhanced expression of apoptosis marker *BIM* or with MEK inhibitor effects on β-catenin–regulated gene expression (Supplementary Fig. S2C). Tankyrase and MEK inhibitors additively enhanced the reduction in colony formation by either treatment alone in *KRAS*-mutant SW403 cells (Fig. 3C). COLO-320DM cells lacking RTK pathway mutations were insensitive to GDC-0973, and MEK inhibition did not further enhance the reduction of colony formation by tankyrase inhibitors (Supplementary Fig. S2C).

Growth of mouse intestinal organoids cultured *ex vivo* requires exogenously supplied R-spondin (for Wnt/β-catenin signaling amplification) and EGF (29). Organoid growth is suppressed by tankyrase inhibitors G007-LK and G244-LM with IC_{50} values of 0.08 and 0.11 μmol/L, respectively (Supplementary Fig. S2D). Organoids cultured from Apc^{Min} mouse small intestine adenomas are constitutively activated for Wnt/β-catenin signaling, thereby bypassing the requirement for R-spondin, and grow mostly as spheroid cysts devoid of differentiated cell types and containing a central fluid-filled lumen (8). Tankyrase inhibitors blocked growth of spheroids with this morphology (Fig. 3D). Inhibition of tankyrase in Apc^{Min} spheroids also reduced expression of Wnt target gene *Ascl2* and induced expression of intestinal cell–type differentiation markers *Fabp2* for enterocytes and *Tff3* for goblet cells (Fig. 3E). Interestingly, spheroids with lumens filled with apparently apoptotic cells (29) seemed to increase in size with tankyrase inhibitor treatment, consistent with the induction of differentiated cells that are eventually shed into the central lumen. In contrast, EGFR inhibitor erlotinib reduced the number of spheroids with both lumen morphologies (Fig. 3D).

G007-LK exhibits antitumor efficacy in xenograft and genetically engineered CRC models

Pharmacokinetic analysis in mice indicates that G007-LK administered i.p. at a dose of 5 mg/kg yields compound exposure in blood above 1 μmol/L for 9 hours whereas G244-LM is much less metabolically stable (Fig. 4A; Supplementary Table S2). A single 50 mg/kg i.p. dose of G007-LK in mice maintained compound exposure in plasma and HCT-15 cell xenograft tumors at greater than 0.5 μmol/L for at least 16 hours (Supplementary Fig. S3A) and also stabilized AXIN1 and AXIN2 proteins in tumors for at least 24 hours (Fig. 4B). Wnt transcriptional target genes were also regulated in tumors by the compound at these time points (Fig. 4C).

We have tested 4 xenograft models of *APC*-mutant CRC cell lines that are sensitive to inhibition of Wnt/β-catenin signaling pharmacodynamic markers by G007-LK in cell culture and find that 2 models show antitumor efficacy. In the COLO-320DM model, xenograft tumors showed G007-LK concentration-dependent growth inhibition with either daily or twice daily dosing (Fig. 4D; Supplementary Table S3). Percent body weight loss for these mice remained above 10% at doses up to 20 mg/kg twice daily or 40 mg/kg daily, at which concentrations we achieved 61% and 48% tumor growth inhibition, respectively (Fig. 4D; Supplementary Table S3). In COLO-320DM tumors at 21 days of treatment, TNKS1 and TNKS2 protein levels were reduced, AXIN1 and AXIN2 were stabilized, and β-catenin level was decreased (Fig. 4E). For 4 tankyrase substrates other than AXIN1/2 (32–34), no consistent change in protein levels was seen in treated COLO-320DM tumors (Fig. 4E). Also, the Axin-βTrCP substrate plakoglobin (35) was not altered by G007-LK treatment in COLO-320DM tumors (Fig. 4E).

Wnt/β-catenin signaling was clearly inhibited in a dose-dependent manner in the efficacy study tumors as indicated by reduced expression of β-catenin–activated genes *NKD1*, *APCDD1*, and *TNFRSF19* (*TROY*), as well as increased expression of β-catenin–repressed gene *CLIC3* (Fig. 4F). Consistent with induction of intestinal cell differentiation mediating tumor growth inhibition, G007-LK treatment increased expression of *KRT20* and *TM4SF4* in COLO-320DM tumors (Fig. 4F). Some markers for enterocyte differentiation (*VIL1*, *CAMP*) were induced, whereas others were repressed (*FABP2*, *ALPI*) or unchanged (*CA2*, *LCT*; Fig. 4F and data not shown). None of the goblet or enteroendocrine cell differentiation markers tested showed a change in expression levels in G007-LK–treated tumors (data not shown).

G007-LK also showed antitumor efficacy for SW403 cell xenografts in mice treated either daily or twice daily, with up to 71% tumor growth inhibition at doses that produce less than 10% change in body weight (Fig. 5A and Supplementary Table S3). In treated SW403 tumors at the end of the study, AXIN1 and AXIN2 proteins were stabilized (Fig. 5B), and Wnt/β-catenin signaling was inhibited on the basis of the regulation of Wnt target genes validated in SW403 cells in culture by β-catenin RNAi (Fig. 5C). In the genetically engineered mouse model $Apc^{CKO/CKO}$ *Lgr5-CreERT2*, excision of a floxed *Apc* allele truncates the protein at codon 580, producing multiple adenomas when Cre recombinase expression in $Lgr5^{+}$ cells is activated by tamoxifen (22). G007-LK dosed at 50 mg/kg daily from the day after tamoxifen treatment reduced the size and multiplicity of adenomas in the small intestine that expressed high levels of β-catenin (Supplementary Fig. S3B and S3C).

In contrast to the antitumor efficacy observed for G007-LK in COLO-320DM and SW403 xenograft models, HCT-15 and DLD-1 tumors were not sensitive to tumor growth inhibition by the tankyrase inhibitor administered daily (Fig. 5D) or twice daily (data not shown), even though compound exposure in plasma and tumors was similar in all of the efficacy studies (Supplementary Fig. S3D). Despite the lack of antitumor activity, inhibition of expression of Wnt target genes *AXIN2* and *APCDD1*, as well as induction of intestinal differentiation markers *KRT20*, *TM4SF4*, and *FABP2* was observed in HCT-15 xenografts (Fig. 5E).

Tankyrase inhibition reduces Wnt/β-catenin signaling and cell proliferation in normal intestine

As Wnt/β-catenin signaling maintains tissue homeostasis in the intestine, we examined the small intestine of mice treated with tankyrase inhibitor for 21 days in the COLO-320DM xenograft tumor efficacy study. G007-LK inhibited the intestinal Wnt-activated expression of genes *Axin2*, *Lgr5*, and *Olfm4* in a dose-dependent manner (Fig. 6A). The cell proliferation marker *Ki67* mRNA displayed reduced expression only at the

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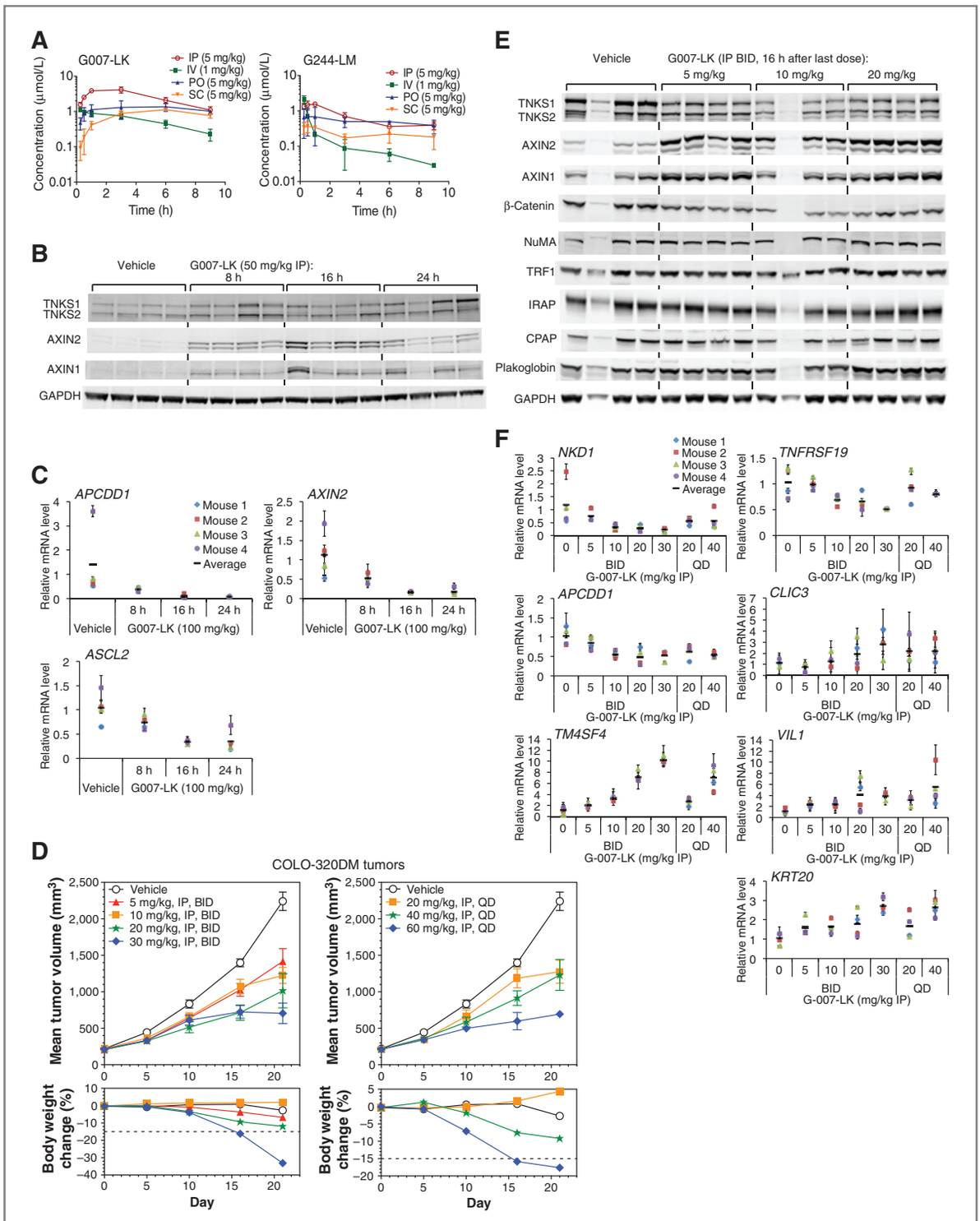

Figure 4. Pharmacokinetic, pharmacodynamic, and antitumor efficacy properties of tankyrase inhibitors. A, compound exposure in plasma of mice administered a single dose of G007-LK (left graph) or G244-LM (right graph) by different routes (PO, peroral). Western immunoblot analysis (B) and Wnt transcriptional target gene expression (C) in HCT-15 cell xenograft tumors at 8, 16, and 24 hours after a single G007-LK i.p. dose. D, COLO-320DM xenograft tumor volume (top graphs) and mouse body weight change (bottom graphs) after G007-LK daily (QD) or twice daily (BID) i.p. dosing for the duration of the study. Group mean ± SEM tumor volumes, as well as linear mixed-effects-fitted curves for percent body weight change, are shown for each group of 10 animals. Western immunoblot (E) and qPCR gene expression (F) analysis of pharmacodynamic markers for the same COLO-320DM tumors as in D collected 16 hours after the last dose. Four mice were randomly selected from the 10 mice in each group for tumor harvesting.

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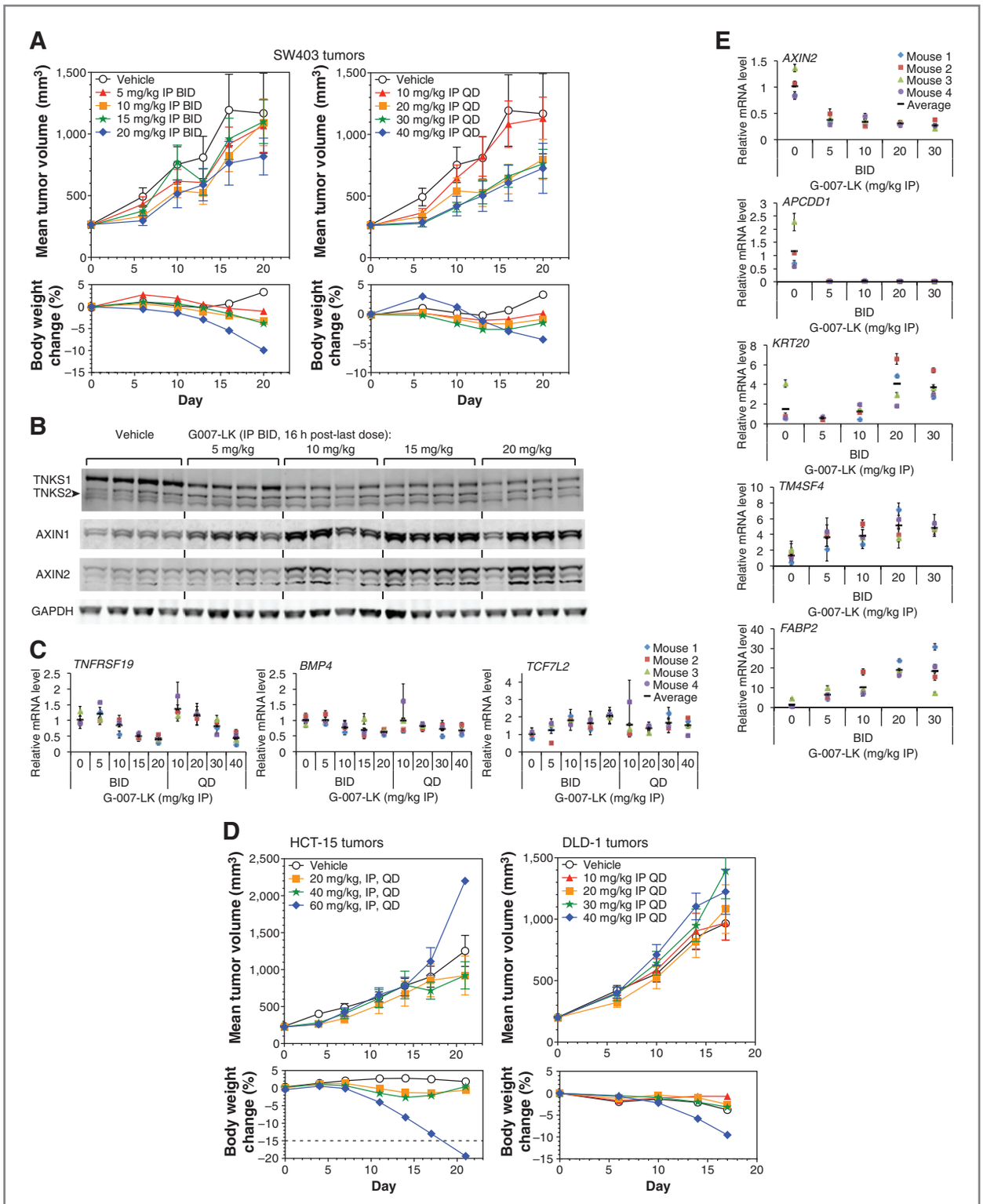

Figure 5. G007-LK inhibits growth of SW403, but not HCT-15 or DLD-1, APC-mutant CRC xenograft tumors. A, SW403 cell xenograft tumor volume (top graphs) and mouse body weight change (bottom graphs) for G007-LK administered i.p. twice daily (BID) or daily (QD). Western immunoblot (B) and gene expression (C) analysis of pharmacodynamic markers for SW403 tumors from the study in A collected 16 hours after the final dose from 4 randomly selected mice in each group. D, tumor volumes and body weight changes for HCT-15 (left graphs) and DLD-1 (right graphs) cell xenografts for groups of 10 mice dosed daily with G007-LK. E, Wnt/β-catenin signaling target (*AXIN2* and *APCDD1*) and intestinal differentiation (*KRT20*, *TM4SF4*, and *FABP2*) gene expression in HCT-15 xenograft tumors from the study in D.

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highest dose tested (30 mg/kg twice daily; Fig. 6A). In this dose group, 7 of the 10 animals died or were sacrificed because of excessive weight loss. Tissue sections revealed that the normal Ki67 protein expression at crypt bases was reduced or lost in some regions of the small intestine, even though the tissue architecture was generally preserved, at least in mice that survived to the end of the study (Fig. 6B).

To determine the mechanism of toxicity, we administered high concentrations of G007-LK to non–tumor-bearing mice and carried out histopathology and clinical pathology analyses when mice started to become moribund (10 days at 60 mg/kg i.p. daily or 9 days at 100 mg/kg i.p. daily). Severe necrosis and inflammation of the small intestine was determined to be the cause of moribundity and death. Intestinal epithelial degeneration and sloughing with villus blunting and crypt loss was apparent in the small intestine, especially the ileum (Fig. 6C; Supplementary Fig. S4A). A systemic inflammatory response to epithelial barrier disruption was measured by increased absolute neutrophil count and band neutrophil percentage, as well as decreased blood plasma albumin due to protein-losing enteropathy (Supplementary Fig. S4B).

Discussion

Almost all colorectal tumors carry mutations in APC or β-catenin that lead to activation of Wnt/β-catenin signaling (4, 5), and many CRC cell lines and tumor models maintain a requirement for activated β-catenin signaling for growth (10–14). Tankyrase is currently the most highly validated druggable target in the Wnt/β-catenin signaling pathway that acts at the level of β-catenin stability or function such that inhibitors reduce signaling driven by *APC* mutations. Previously reported tankyrase inhibitors have not been definitively shown to be metabolically stable (16, 19, 21–23), and *in vivo* antitumor efficacy of tankyrase inhibitors in human CRC models has hitherto not been presented. The potent and selective TNKS1/2 inhibitor G007-LK used in the current studies shows greater *in vivo* stability than previously reported inhibitors, and sufficient bioavailability to downregulate Wnt/β-catenin signaling in

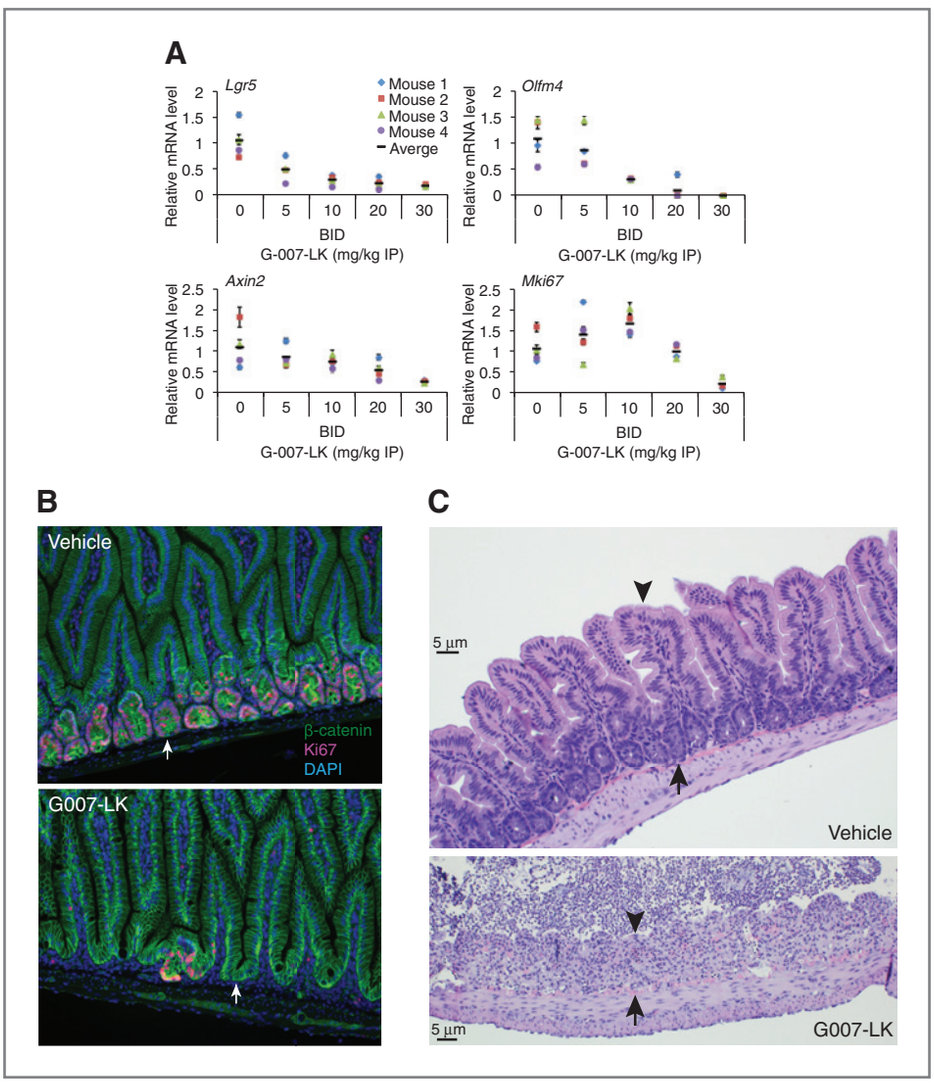

Figure 6. G007-LK inhibits Wnt/β-catenin signaling and disrupts tissue morphology and function in intestine. A, gene expression analysis of small intestine samples of mice from the COLO-320DM xenograft study in Fig. 4D, dosed i.p. twice daily (BID) with G007-LK and harvested 16 hours after the final dose. B, Ki67 (pink) and β-catenin (green) immunofluorescent staining of small intestine sections from mice as in A for vehicle (top) and 30 mg/kg i.p. BID G007-LK (bottom) treatment groups. Intestinal crypt bases (arrows) are oriented toward the bottom of the images. 4′,6-Diamidino-2-phenylindole (DAPI) staining (blue) identifies nuclei, and anti-β-catenin fluorescence is visible at cell junctions but not in crypt base cell nuclei with this staining protocol. C, hematoxylin and eosin staining of small intestine ileum sections from moribund mice after 10 days of treatment with vehicle (top) or G007-LK 60 mg/kg i.p. daily (bottom). Diffuse villous blunting (arrows and arrowheads mark crypt bases and villi tips, respectively) and sloughing of intestinal mucosal epithelia into the lumen (above arrowhead) can be seen in the G007-LK–treated intestine.

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CRC xenograft tumors. We show G007-LK tumor growth inhibition in 2 *APC*-mutant CRC xenograft models, as well as in a genetically engineered mouse *Apc* mutant model, supporting therapeutic efficacy of tankyrase inhibitors for Wnt pathway mutation–driven cancer.

Whereas tankyrase inhibitors completely blocked Wnt ligand–induced signaling in cells with wild-type *APC* and β-catenin, *APC*-mutant CRC cells lines that were sensitive to tankyrase inhibitors showed only approximately 50% reduction in Wnt signaling relative to β-catenin RNAi. Therefore, wild-type *APC* is likely required for the full β-catenin degrading activity of the AXIN protein that is stabilized by tankyrase inhibition. However, COLO-320DM cells are hemizygous for a truncating *APC* mutation 5′ to all known AXIN or β-catenin–binding sites and lack *APC* activity as measured by *APC* RNAi, yet they are sensitive to β-catenin degradation and inhibition of Wnt/β-catenin signaling by tankyrase inhibitors. This suggests that full-length *APC* function may not be strictly required for stabilized AXIN to degrade or sequester β-catenin. Five of the 11 tested CRC cell lines mutant for *APC* are resistant to Wnt/β-catenin signaling downregulation by tankyrase inhibitors, apparently through at least 2 mechanisms. G007-LK stabilizes AXIN1/2 proteins in all cell lines tested, although possibly to a lesser extent in resistant cell lines COLO-205 and LS411N, but β-catenin protein levels are not reduced by G007-LK in COLO-205, LS-411N, and HT-29 cells. In contrast, tankyrase inhibition reduces cytosolic and nuclear β-catenin levels in SW480 cells. However β-catenin levels in untreated SW480 cells are unusually high, and the remaining levels after G007-LK treatment are still higher than in the other untreated cell lines tested, possibly sufficiently elevated to maintain activated Wnt/β-catenin signaling.

In contrast to the robust and rapid effects on cell proliferation in response to inhibition of RTK signaling, blocking morphogen signaling pathways Wnt, Hedgehog, or Notch generally produces more limited effects (12, 36, 37). However, morphogen pathway inhibitors can still produce potent tumor growth inhibition, in some cases due to effects on tumor cell differentiation, tumor-associated stroma, or angiogenesis (13, 36, 37). Tankyrase inhibitors induce expression of multiple intestinal cell–type differentiation markers in COLO-320DM and HCT-15 cells treated in culture or as xenograft tumors, with some genes induced in only one or the other cell line that might differentially impact growth. G007-LK–treated COLO-320DM tumors are not induced for all markers tested of any specific intestinal cell lineage, therefore specification of a normal differentiated cell type does not seem to account for the inhibition of tumor growth. It will be important to establish in tumors whether effects other than induction of intestinal differentiation contribute to the antitumor efficacy of tankyrase inhibitors.

In *KRAS*-mutant cell lines HCT-15 and DLD-1, tankyrase inhibitors do not show single-agent activity for inhibition of clonogenic cell growth. However, tankyrase inhibitors add to the activity of a MEK inhibitor in reducing colony formation in these cell lines. Interestingly, an activating *KRAS* mutation increases adenoma multiplicity and accelerates tumor progression in *Apc*-mutant mice, possibly through enhancing β-catenin transcriptional target gene regulation (38). Conversely, combined RNAi knockdown of β-catenin and *KRAS* shows greater inhibition of CRC cell proliferation and xenograft tumor growth than RNAi of either single gene, as well as induction of apoptosis (39). However, we observed MEK inhibitor enhancement of intestinal differentiation gene expression, but not Wnt/β-catenin signaling inhibition or apoptosis, by tankyrase inhibitors. Tankyrase inhibition has previously been shown to synergistically enhance EGFR inhibitors for cell proliferation and tumor growth inhibition of non–small cell lung cancer cell lines (40) and to potentiate phophoinositide 3-kinase (PI3K) or AKT inhibitors for apoptosis induction in CRC cell lines (41). Future drug combination tumor studies will address whether this translates *in vivo* to a therapeutic benefit for tankyrase inhibitors in CRC models. In addition, defining determinants of resistance and sensitization to growth inhibition by tankyrase inhibitors will be critical in developing predictive diagnostic biomarkers to realize the full therapeutic potential of tankyrase inhibitors for cancer therapy.

G007-LK appears to exhibit a narrow efficacious concentration range, as we observe 50% to 70% tumor growth inhibition at 20 mg/kg twice daily (or 30–40 mg/kg daily) for COLO-320DM and SW403 xenograft tumors with less than 10% reduction in mouse body weight and significant toxicity at 30 mg/kg twice daily (or 60 mg/kg daily). The dose-limiting toxicity precludes determining whether tankyrase inhibition can achieve tumor stasis or regression. The epithelial degeneration and reduction of crypt proliferation detected in the intestine appears to be the cause of moribundity and is likely an on-target effect of the observed Wnt/β-catenin signaling inhibition in intestine. Ectopic expression of the Wnt/β-catenin signaling antagonist *DKK1* can also attenuate proliferation in intestinal crypts leading to epithelial degeneration (26, 27). Interestingly, this effect is reversible (27) and, in fact, complete ablation of $Lgr5^+$ intestinal stem cells can be tolerated without perturbing the long-term homeostasis of the intestine as new $Lgr5^+$ stem cells appear to be generated from another cell population (42, 43). Future studies will need to address whether the intestinal toxicity of tankyrase inhibitors can be reversible and manageable with intermittent dosing.

The durable effects of G007-LK in COLO-0320DM and SW403 tumors on AXIN stabilization, β-catenin destabilization, and expression of β-catenin–regulated and intestinal differentiation genes is consistent with a mechanism-based tumor growth inhibition. Unexpectedly, we observed a greater reduction in the expression of some β-catenin–activated genes in tumor xenografts compared with cell culture lines treated with G007-LK, possibly due to extended treatment duration. However, intestinal antitumor efficacy can be achieved in at least the Apc^{1638N} mouse model with only a 50% reduction in Wnt/β-catenin signaling through knocking out a single β-catenin allele (44). In addition to AXIN, more than 10 substrates of tankyrase have been identified (32–34). However, the steady-state levels of 4 tankyrase substrates other than AXIN (NuMA, TRF1, IRAP, and CPAP/CENPJ) and 1 AXIN-β-TrCP substrate other than β-catenin (plakoglobin) were not altered in end-of-study COLO-320DM xenograft tumors treated with tankyrase

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inhibitor. The protein stability of TRF1 (32), CPAP (34), and plakoglobin (35) has been shown to be regulated by tankyrase-mediated PARsylation or AXIN-mediated ubiquitylation in some cell lines, suggesting that not all tankyrase functions may be preserved in CRC cells. As we have identified tumor models whose growth is reduced by tankyrase inhibitors, proof-of-mechanism studies can be initiated to show a causal link between AXIN protein stabilization, β-catenin degradation, and growth inhibition.

Note Added in Proof

Since this article was accepted, a structure-activity relationship study for the G007-LK tankyrase inhibitor series has been published (45).

Disclosure of Potential Conflicts of Interest

M. Merchant is employed (other than primary affiliation; e.g., consulting) in Genentech, Inc. as a Scientist and has ownership interest (including patents) in Roche. J. Waaler and S. Krauss have ownership interest (including patents) in patent on compound. No potential conflicts of interest were disclosed by the other authors.

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