


ORIGINAL ARTICLE

RK-287107, a potent and specific tankyrase inhibitor, blocks colorectal cancer cell growth in a preclinical model

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Aberrant activation of Wnt/ β -catenin signaling causes tumorigenesis and promotes the proliferation of colorectal cancer cells. Porcupine inhibitors, which block secretion of Wnt ligands, may have only limited clinical impact for the treatment of colorectal cancer, because most colorectal cancer is caused by loss-of-function mutations of the tumor suppressor adenomatous polyposis coli (APC) downstream of Wnt ligands. Tankyrase poly(ADP-ribosyl)ates (PARylates) Axin, a negative regulator of β -catenin. This post-translational modification causes ubiquitin-dependent degradation of Axin, resulting in β -catenin accumulation. Tankyrase inhibitors downregulate β -catenin and suppress the growth of APC-mutated colorectal cancer cells. Herein, we report a novel tankyrase-specific inhibitor RK-287107, which inhibits tankyrase-1 and -2 four- and eight-fold more potently, respectively, than G007-LK, a tankyrase inhibitor that has been previously reported as effective in mouse xenograft models. RK-287107 causes Axin2 accumulation and downregulates β -catenin, T-cell factor/lymphoid enhancer factor reporter activity and the target gene expression in colorectal cancer cells harboring the shortly truncated APC mutations. Consistently, RK-287107 inhibits the growth of APC-mutated (β -catenin-dependent) colorectal cancer COLO-320DM and SW403 cells but not the APC-wild (β -catenin-independent) colorectal cancer RKO cells. Intraperitoneal or oral administration of RK-287107 suppresses COLO-320DM tumor growth in NOD-SCID mice. Rates of tumor growth inhibition showed good correlation with the behavior of pharmacodynamic biomarkers, such as Axin2 accumulation and MYC downregulation. These observations indicate that RK-287107 exerts a proof-of-concept antitumor effect, and thus may have potential for tankyrase-directed molecular cancer therapy.

KEYWORDS

colorectal cancer, mouse xenograft model, poly(ADP-ribose) polymerase, tankyrase inhibitor, Wnt/ β -catenin signaling

1 | INTRODUCTION

Wnt/ β -catenin signaling is activated in over 90% of human colorectal cancer. Especially, loss-of-function mutations of the tumor suppressor adenomatous polyposis coli (*APC*), a key negative regulator of Wnt/ β -catenin signaling, are found in 90% of patients.¹ During colorectal carcinogenesis, *APC* mutations are found in precancerous lesions of the intestine and cause initial clonal evolution in the very early stages of the so-called adenoma-carcinoma sequence.² Normally, Wnt/ β -catenin signaling is activated by Wnt ligands. In the absence of Wnt ligands, β -catenin is scaffolded by the destruction complex, which is constituted by Axin, *APC*, casein kinase 1 (CK1) and glycogen synthase kinase 3 β (GSK3 β), and phosphorylated by CK1 and GSK3 β . This phosphorylation within the destruction complex leads to β -transducin repeat-containing protein (β TrCP)-mediated ubiquitination and proteasomal degradation of β -catenin. When Wnt ligands bind to Frizzled, a Wnt receptor, Axin associates with Frizzled and β TrCP dissociates from the destruction complex and β -catenin.³ Then, the accumulated β -catenin binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor in the nucleus, resulting in upregulation of the target genes, such as *MYC*, *AXIN2* and *APCDD1*. In most colorectal cancer, *APC* function is disrupted and Wnt/ β -catenin signaling is dysregulated.

Based on this background, it has been postulated that Wnt/ β -catenin signaling is a promising therapeutic target for colorectal cancer. However, there have been no clinical anticancer drugs that selectively target this pathway.⁴ LGK974,⁵ a porcupine inhibitor, which targets Wnt-activated cancer, has been examined in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT01351103). Because LGK974 inhibits palmitoylation, which is required for activation and secretion of Wnt ligands,⁵ it may be applicable to Wnt-driven cancer without *APC* loss-of-function or *CTNNB1* (gene for β -catenin) gain-of-function mutations. However, 90% of colorectal cancer has *APC* mutations and upregulates Wnt/ β -catenin signaling downstream of porcupine.¹ Therefore, porcupine inhibitors may not be mechanistically suitable as anticancer drugs for most colorectal cancer.

Tankyrases (tankyrase-1/TNKS and tankyrase-2/TNKS2, also known as PARP5a/ARTD5 and PARP5b/ARTD6) are members of the poly(ADP-ribose) polymerase (PARP) family proteins, which have PARP catalytic domains.^{6,7} Although single knockout mice of either TNKS or TNKS2 are viable, their double knockout mice are embryonic lethal, supporting that these two genes are functionally redundant.⁸ Accordingly, the functional differences of TNKS and TNKS2 remain unknown. Tankyrase poly(ADP-ribosyl)ates (PARylates) target proteins, that is, tankyrases add multiple ADP-ribose moieties sequentially to target proteins using NAD⁺ as a substrate. Tankyrase has multiple ANK repeat clusters (ARC), through which it binds various target proteins.⁹⁻¹¹ One of them is Axin, which anchors β -catenin to prevent its translocation into the nucleus.¹² Tankyrase PARylates Axin and the PARylated Axin are recognized by the ubiquitin ligase RNF146, leading to proteasomal degradation of Axin.¹³⁻¹⁶ Consequently, β -catenin is dissociated from the destruction complex and binds to the TCF/LEF

transcription factor, which upregulates expression of the β -catenin target gene. Thus, tankyrases are positive regulators of the canonical Wnt/ β -catenin signaling.

Given that tankyrase has PARP activity, its inhibition by small compounds will provide a therapeutic opportunity for Wnt-driven cancer. XAV939, the first reported tankyrase-selective PARP inhibitor, induces Axin stabilization and suppresses Wnt/ β -catenin signaling in colorectal cancer SW480 cells harboring a truncated *APC* mutation.¹⁵ IWR-1 is another tankyrase inhibitor, initially identified as an Axin stabilizer.¹⁷ Although XAV939 and IWR-1 are not suitable for in vivo experiments owing to their pharmacokinetic problems, several tankyrase-specific inhibitors for in vivo administration have been developed, including G007-LK, NVP-TNKS656, K-756, AZ1366, G-631 and JW55.¹⁸⁻²³ G007-LK inhibits proliferation of the colorectal cancer cells COLO-320DM and SW403, both harboring truncated *APC* mutations, but not HCT-15 and DLD-1 cells, which also have truncated *APC* mutants.¹⁸ G007-LK suppresses tumor growth in mouse xenograft models with two *APC*-mutant colorectal cancer cells.¹⁸ Recently, we have reported that colorectal cancer cells harboring "short" truncated *APC* mutations lacking all seven β -catenin-binding 20-amino acid repeats (20-AAR) (hereinafter, referred to as "short *APC*") show sensitivity to tankyrase inhibitors using 25 colorectal cancer cells (9 established cell lines and 16 patient-derived cancer cell lines), although several patient-derived cancer cell lines also showed sensitivity to tankyrase inhibitors in spite of the partial retention of 20-AAR in the mutant *APC*.²⁴ So far, none of these tankyrase inhibitors have been the subject of clinical trials.

Herein, we have developed a novel, potent and selective tankyrase inhibitor, RK-287107. RK-287107 inhibits proliferation of colorectal cancer cells that harbor short *APC* mutations. Orally given RK-287107 at tolerable doses suppressed tumor growth in a mouse xenograft model, suggesting that RK-287107 is a lead compound for the development of clinical tankyrase inhibitors.

2 | MATERIALS AND METHODS

2.1 | Chemical compounds, screening and in vitro PARP assay

Yeast high-throughput screening for compounds that repress the growth inhibitory effect of tankyrase-1 overexpression on fission yeast was carried out as described.²⁵ Hit compounds were subjected to in vitro PARP assays. Detailed description is given in Doc S1.

2.2 | Cell culture and proliferation assays

Human colorectal cancer cell lines COLO-320DM, HCC2998, HCT-116 and DLD-1 were maintained in RPMI-1640 medium with 10% heat-inactivated FBS as described previously.^{24,26} Human colorectal cancer cell lines SW403 and RKO were maintained in DMEM medium with 10% FBS. Cell proliferation was evaluated by MTT and BrdU assays. Detailed description is given in Doc S1.

2.3 | Luciferase reporter assay

Conditioned media derived from the cultures of L cells and L-Wnt3A cells were obtained according to ATCC protocol. Reporter assay was carried out as described in Doc S1.

2.4 | Western blot analysis

Cell lysates were prepared and western blot analysis was carried out as previously described.⁹ Detailed description is given in Doc S1.

2.5 | Immunofluorescence staining

Immunofluorescence staining was carried out as described previously²⁴ using anti-non-phosphorylated β -catenin antibody (D13A1, 1:500; Cell Signaling Technology, Danvers, MA, USA). Fluorescence images were quantified with ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.6 | Quantitative reverse transcription (qRT)-PCR and transcriptome analyses

Total RNAs were prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany). qRT-PCR and microarray analyses were done as described in Doc S1. The gene expression data have been deposited in the Gene Expression Omnibus (GEO) (GSE113965).

2.7 | In vivo xenograft experiments

All animal procedures were carried out in the animal experiment room at the Japanese Foundation for Cancer Research (JFCR) according to protocols approved by the JFCR Animal Care and Use Committee. COLO-320DM cells were suspended in Matrigel (Corning, Corning, NY, USA) and HBSS in a 1:1 ratio (5.2×10^6 cells/100 μ L/mouse) and were implanted s.c. in the rear right flank of 6-week-old female NOD.CB17-Prkdc^{scid}/J mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan). The length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated using the equation: $TV = (L \times W^2)/2$. When tumor volumes reached approximately 59–120 mm³ (59–104 mm³ and 71–120 mm³ for i.p. and i.p./p.o. experiments, respectively), mice were separated into groups of 10 and six animals, respectively, with similarly sized tumors, and treatment was started the day after grouping. For i.p. administration, dosing solution contained 15% DMSO, 17.5% Cremophor, 8.75% ethanol, 8.75% PEG-40 hydrogenated castor oil and 50% PBS. For oral administration, RK-287107 was milled and suspended in sterilized 0.5% (w/v) methyl cellulose in water. Tumor growth inhibition coefficient (% TGI) was calculated according to the following equation: $\% TGI = [(V_{\text{vehicle}} - V_{\text{experiment}})/V_{\text{vehicle}}] \times 100$, where V is relative tumor volume on the final day. Pharmacodynamic and pharmacokinetic analyses were carried out as described in Doc S1.

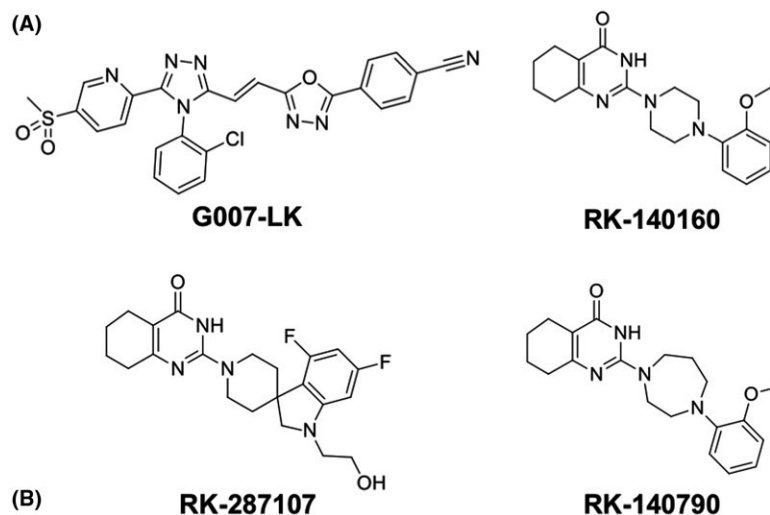
3 | RESULTS

3.1 | RK-287107 inhibits tankyrase-1 and -2 but not PARP1 enzyme activities

Using the high-throughput screening of nearly 140 000 small molecule compounds that repress the growth-suppressing effect of tankyrase-1 overexpression on fission yeast cells²⁵ and subsequent PARP assay, we identified a compound, RK-140160, as a tankyrase inhibitor (Figure 1A). IC₅₀ values of RK-140160 for tankyrase-1 and tankyrase-2 PARP activities in vitro were 42.2 and 42.3 nmol/L, respectively, which were almost equivalent to or lower than those of G007-LK, a previously reported tankyrase-selective inhibitor¹⁸ (Figure 1B). RK-140160 at concentrations of up to 20 μ mol/L did not inhibit PARP1. Therefore, by using RK-140160 as a lead compound, we synthesized a series of structurally related derivatives and developed a novel compound, RK-287107, as a more potent, and selective tankyrase inhibitor (Figure 1B,C). RK-287107 inhibited tankyrase-1 and tankyrase-2 in vitro with IC₅₀ values of 14.3 and 10.6 nmol/L, respectively, whereas it had no effect on PARP1 enzyme activity at up to 20 μ mol/L. As a negative control, RK-140790 is another derivative that had a closely similar structure to RK-140160 but retained much less ability to inhibit tankyrases. The IC₅₀ values of RK-140790 for tankyrase-1 and tankyrase-2 were 428-fold and 414-fold higher than those of RK-287107, respectively. Detailed information on the yeast high-throughput screening, synthetic routes and chemical properties of RK-287107 and its derivatives will be described elsewhere (F. Shirai, T. Tsumura, Y. Yashiroda, H. Yuki, H. Niwa, S. Sato, T. Chikada, Y. Koda, K. Washizuka, N. Yoshimoto, M. Abe, T. Onuki, Y. Mazaki, C. Hirama, T. Fukami, H. Watanabe, T. Honma, T. Umehara, M. Shirouzu, M. Okue, Y. Kano, T. Watanabe, K. Kitamura, E. Shitara, Y. Muramatsu, H. Yoshida, A. Mizutani, H. Seimiya, M. Yoshida, H. Koyama, manuscript in preparation).

3.2 | RK-287107 shows an antiproliferative effect on colorectal cancer cells harboring short APC mutations

Short APC mutations predict the sensitivity of colorectal cancer cells to tankyrase inhibitors.²⁴ To investigate the effect of RK-287107 on the growth of six human colorectal cancer cell lines with various APC mutations, we carried out MTT assays. COLO-320DM and SW403 cells, both of which had short APC, showed high sensitivity to RK-287107 and G007-LK (Figure 2A). RK-140790, as an inactive negative control, showed only marginal effects, if any, on the growth of these cells. The 50% growth inhibition (GI₅₀) values of RK-287107 and G007-LK on COLO-320DM cells were 0.449 and 0.434 μ mol/L, respectively. Meanwhile, these tankyrase inhibitors did not inhibit the growth of other cell lines, including RKO (APC wild-type, β -catenin-independent), HCT-116 (CTNNB1 gain-of-function mutation), HCC2998 and DLD-1 (APC mutations with partial retention of 20-AARs). To further examine the effect of RK-287107 on cell proliferation, we carried out a BrdU assay in COLO-320DM and



Compound	Enzyme inhibition [IC ₅₀ (nmol/L)]		
	TNKS	TNKS2	PARP1
G007-LK	58.1	85.5	>20 000
RK-140160	42.2	42.3	>20 000
RK-287107	14.3	10.6	>20 000
RK-140790	6117.9	4383.9	>20 000

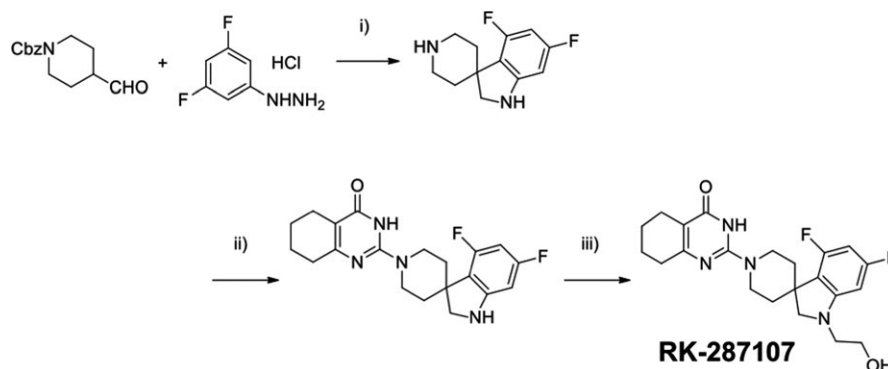


FIGURE 1 In vitro properties of newly identified tankyrase inhibitors. A, Chemical structures of tankyrase inhibitors and a related compound. G007-LK, a previously reported tankyrase inhibitor;¹⁸ RK-140160, tankyrase inhibitor identified by high-throughput screening; RK-287107, highly potent and specific tankyrase inhibitor synthesized by setting RK-140160 as a lead compound; RK-140790, a structurally related compound of RK-140160 without potent activities to inhibit tankyrase. B, poly(ADP-ribose) polymerase (PARP) inhibitory activities of the compounds. Each compound was subjected to PARP assay in vitro. IC₅₀ values are shown. TNKS, tankyrase-1; TNKS2, tankyrase-2. C, Synthetic routes of RK-287107. (i) (1) TFA, CHCl₃, reflux, 3 h; (2) NaBH(OAc)₃, CHCl₃-MeOH, rt, 1 h; (3) TFA, reflux, 3 h; (ii) (1) 1-Amidinopyrazole hydrochloride, Et₃N, CHCl₃, rt, 1 h; (2) Ethyl 2-cyclohexanonecarboxylate, EtONa, EtOH, reflux, 3 h [(i) + (ii), 64% from aldehyde]; (iii) Glycolaldehyde dimer, NaBH(OAc)₃, CHCl₃-AcOH, rt, 14 h (53%)

RKO cells under treatment with RK-287107, G007-LK, RK-140790 and olaparib,^{27,28} a PARP1/2-selective PARP inhibitor. As shown in Figure 2B (left), DNA synthesis in COLO-320DM cells was reduced by RK-287107 and G007-LK in a dose-dependent way. Inhibition of DNA synthesis at higher doses was larger in RK-287107-treated cells than in G007-LK-treated cells. As negative controls, olaparib and RK-140790 showed no effects at concentrations up to 3 μmol/L, and marginally inhibited DNA synthesis at 10 μmol/L (Figure 2B left and right). RKO cells were resistant to these tankyrase inhibitors and olaparib, although 10 μmol/L olaparib inhibited the DNA synthesis

to half that of the basal level (Figure 2B middle). These observations indicate that RK-287107 preferentially inhibits the growth of tankyrase inhibitor-sensitive cells, that is, short APC mutant colorectal cancer cells.

3.3 | RK-287107 stabilizes Axin and downregulates β-catenin signaling

To examine the pharmacodynamic effects of RK-287107 in COLO-320DM cells, we carried out western blot analysis. Tankyrase

PARYlates itself and is consequently ubiquitinated by RNF146, resulting in degradation.^{13,29,30} Therefore, inhibition of tankyrase would block its PARYlation/degradation, leading to protein accumulation. As shown in Figure 3A and Figure S1, RK-287107 and G007-LK caused accumulation of tankyrase and Axin1/2, which are the most direct pharmacodynamic biomarkers observed as a result of the reduced PARYlation and subsequent stabilization. Levels of Axin2 accumulation were more evident than those of Axin1, consistent with a previous report.²⁴ Of note, the extent of Axin2 accumulation was slightly reduced upon higher-dose treatment with tankyrase inhibitors, because *AXIN2* is a transcriptional target of β -catenin (see below). Consequently, downregulation of active (ie, non-phosphorylated) β -catenin was observed both in the western blot analysis and indirect immunofluorescence microscopy (Figure 3B). The immunofluorescence intensities of active β -catenin were 100%, 0.63%, 106%, and 26.4% in DMSO, RK-287107, Olaparib, and G007-LK-treated cells, respectively. Although β -catenin was localized in the nucleus in COLO-320DM cells in a steady state, its fluorescence intensity was reduced by treatment with RK-287107 or G007-LK. Furthermore, these inhibitor-treated cells showed several intracellular foci, which were considered to be degradasomes³¹ (Figure 3B, inset, arrowheads). Olaparib did not affect tankyrase, Axins or β -catenin at all, excluding the possibility that the observed phenomena were caused by PARP1/2 inhibition.

To determine whether RK-287107 suppresses the transcriptional activity of TCF/LEF, a downstream effector of the Wnt/ β -catenin pathway, we carried out TCF/LEF reporter luciferase assays. In COLO-320DM cells, RK-287107 suppressed the reporter activity in a dose-dependent way (Figure 3C, left). Although G007-LK less efficiently suppressed the reporter activity, olaparib had no effect. As HEK293T cells have an intact Wnt/ β -catenin pathway, high TCF/LEF luciferase activity was detected when conditioned medium of L-Wnt3A cells containing Wnt3A ligands was added to HEK293T cells (Figure 3C, right). RK-287107 and G007-LK suppressed TCF/LEF luciferase activity in L-Wnt3A-stimulated HEK293T cells, whereas olaparib only marginally inhibited the activity. Next, we carried out quantitative RT-PCR analyses on mRNA expression of *AXIN2* and *MYC*, transcriptional target genes of Wnt/ β -catenin signaling. We found that RK-287107 and G007-LK, but not olaparib, suppressed the mRNA expression of *AXIN2* and *MYC* in COLO-320DM cells (Figure 3D). These observations indicate that RK-287107 inhibits tankyrase, which leads to suppression of β -catenin signaling.

3.4 | RK-287107 inhibits tumor growth in a mouse xenograft model

We next investigated the therapeutic effect of RK-287107 in vivo by using immunodeficient mouse xenograft models. COLO-320DM cells were s.c. injected into NOD-SCID mice and tumor growth was monitored. As shown in Figure 4A (upper), i.p. administration of RK-287107 at 100 and 300 mg/kg once per day resulted in 32.9% and 44.2% TGI, respectively. Under these treatment conditions, there was no detectable effect on body weight (Figure 4A, lower) or

behavior of the mice. To determine the concentrations of RK-287107 in plasma and tumor tissues 4 hours after drug administration, we carried out liquid chromatography-mass/mass spectrometry (LC-MS/MS) analyses. Plasma concentrations of RK-287107 increased in a dose-dependent way, whereas its concentration in tumor tissues was not significantly changed (Figure 4B).

To evaluate the pharmacodynamic effects of RK-287107 at the protein level, we carried out western blot analysis at 4 hours after the final administration of the compound. As shown in Figure 4C and Figure S2, Axin1 and Axin2 accumulated in RK-287107-treated tumor tissues. In particular, the Axin2 level was increased in a dose-dependent way and inversely correlated with relative tumor size (Pearson's correlation $r = -0.65$) (Figure 4D). We could not detect downregulation of β -catenin presumably because the whole cell extracts, instead of the nuclear extracts, were subjected to the analysis (Figure 4C, see Section 4). RNA expression of *MYC*, one of the β -catenin target genes, in the tumor tissues was downregulated by RK-287107 and showed a moderate correlation with relative tumor size (Pearson's correlation $r = 0.57$) (Figure 4E). Transcriptome analysis with GeneChip microarrays and gene set enrichment analysis (GSEA) showed that β -catenin target gene expression was higher in the vehicle-treated samples than in RK-287107-treated samples, indicating that RK-287107 suppressed the expression of β -catenin target genes (Figure 4F). Together, these observations indicate that RK-287107 suppresses the Wnt/ β -catenin pathway and tumor growth of the xenografted COLO-320DM cells in vivo.

3.5 | RK-287107 is an orally effective tankyrase inhibitor in mice

The above-mentioned antitumor efficacy of RK-287107 was observed under the conditions of repetitive dosage of the compound. This suggests that oral dosage might be the most preferable administration route in a practical therapeutic setting. Therefore, we next carried out a xenograft experiment to determine whether RK-287107 is available for oral dosage. Figure 5A (upper) shows that intraperitoneal (150 mg/kg, twice per day) and oral (300 mg/kg, twice per day) dosage of RK-287107 resulted in 47.2% and 51.9% TGI, respectively. Relative body weights were slightly lower in the oral groups than in the i.p. group, presumably because the former groups were subjected to the mechanical stress of feeding needles (Figure 5A, lower). There was no detectable difference in body weight between the oral, vehicle and RK-287107-treated groups. Pharmacokinetic analysis of plasma and tissue samples indicated that RK-287107 with oral administration showed a higher concentration of the compound than the i.p. administration under these dosage conditions (Figure 5B).

Western blot analysis of the tumor tissues at 4 hours after the final dosage showed that Axin2 was upregulated in the cytoplasmic extracts of the RK-287107-treated groups with i.p. and oral administration (Figure 5C, upper, and Figure S3). Again, the relative tumor volumes showed good inverse correlation with the expression levels of Axin2

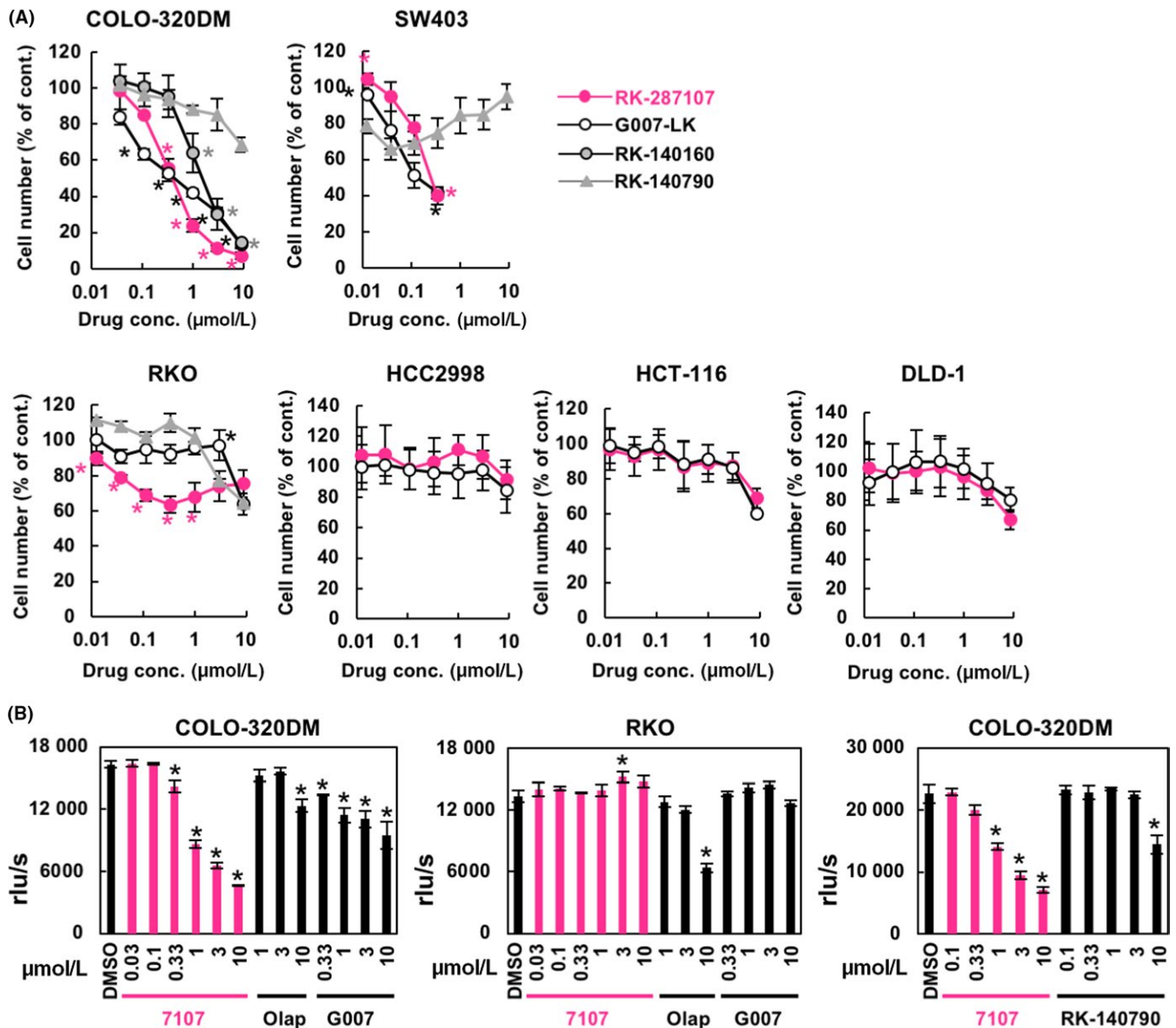
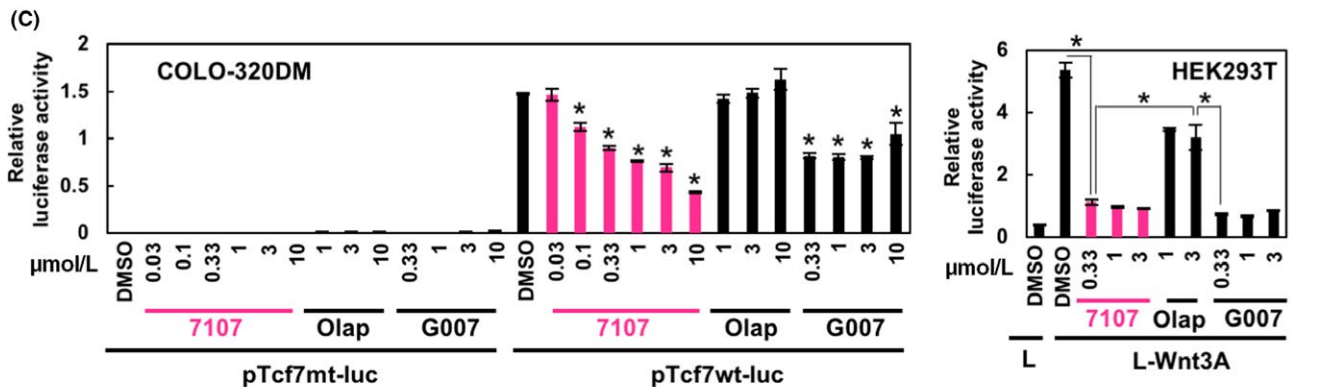
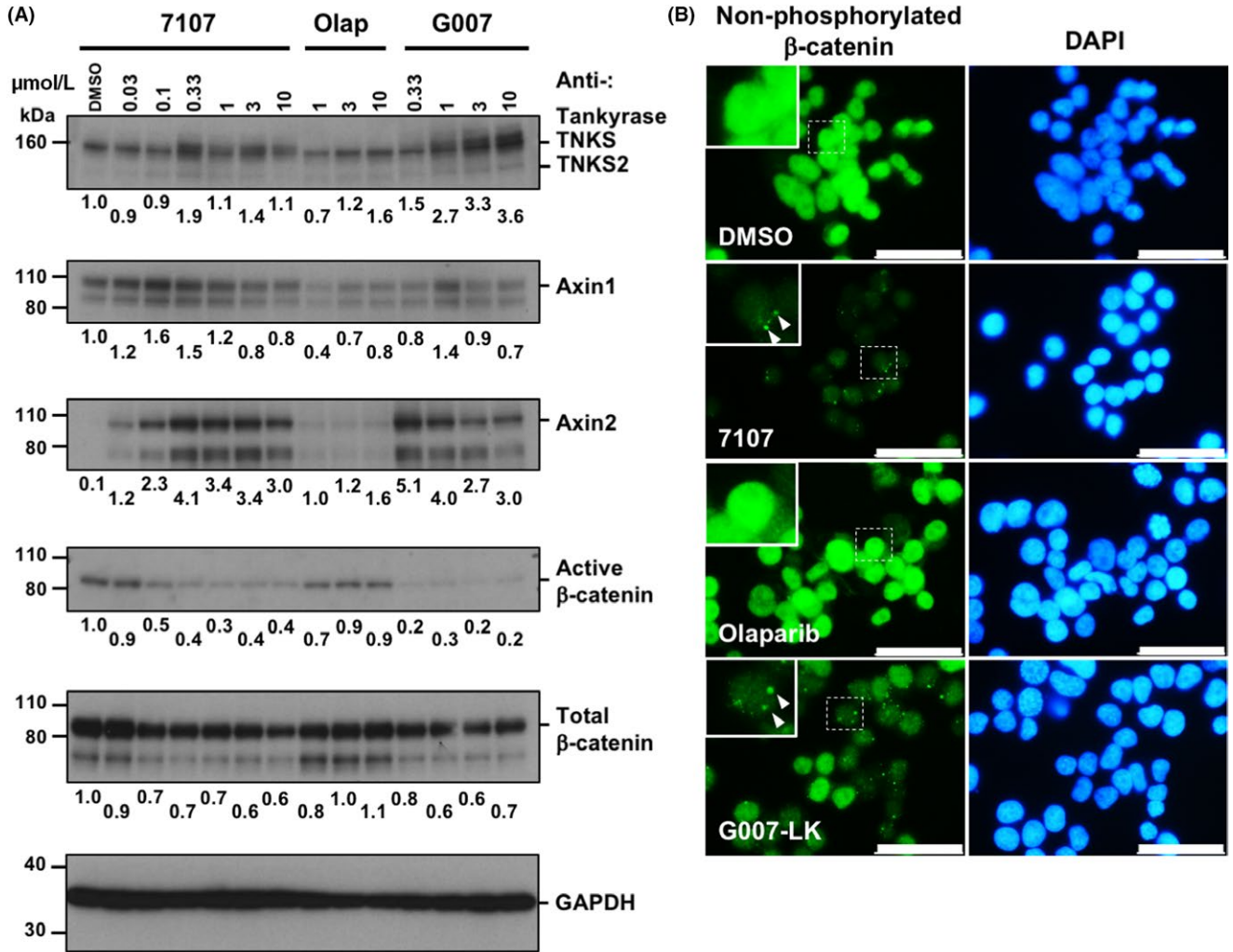


FIGURE 2 Antiproliferative effect of RK-287107 on colorectal cancer cells. A, Effects of RK-287107 and related compounds on cancer cell growth. Cells were treated with the compounds in triplicate for 120 h. Relative cell number was quantitated by MTT assays. Experiments were repeated at least twice and the average values were plotted. * $P < 0.01$ vs RK-140790-treated cells at the same concentration of compounds by Tukey-Kramer test. B, Effects on DNA synthesis. BrdU assays were carried out using COLO-320DM and RKO cells after 48-h treatment with the compounds. 7107, RK-287107; Olap, olaparib; G007, G007-LK. Experiments were repeated at least twice and the representative results are shown. * $P < 0.01$ vs DMSO-treated cells by Tukey-Kramer test. Error bar indicates standard deviation

FIGURE 3 RK-287107 downregulates β -catenin signaling in cultured cells. A, Western blot analysis. COLO-320DM cells were treated with RK-287107, olaparib and G007-LK for 16 h. Whole cell lysates were subjected to western blot analysis. Full-length blots are presented in Figure S1. B, Indirect immunofluorescence staining. COLO-320DM cells were treated with the compounds at 1 μ mol/L for 16 h. Then the cells were subjected to immunofluorescence staining with anti-non-phosphorylated (active) β -catenin antibody. Nuclear DNA was counterstained with DAPI. Scale bar, 20 μ m. Inset, magnified view of the dotted area; arrowhead, degradosome focus. C, T-cell factor (TCF) reporter assay. Cells were treated with the compounds for 30 h after transfection of the reporter vectors. In the case of HEK293T cells, L or L-Wnt3A conditioned media were added to the cells simultaneously with the indicated inhibitors. Luciferase reporter assays were carried out. * $P < .01$ vs pTcf7wt-luc-transfected cells with DMSO or the indicated in the figure by Tukey-Kramer test. D, qRT-PCR assay of β -catenin target genes. COLO-320DM cells were treated with the inhibitors for 48 h. Relative expression levels of *AXIN2* and *MYC* transcripts were determined by normalization with those of *ACTB* expression. Error bar indicates standard deviation. * $P < 0.01$ vs DMSO-treated cells by Tukey-Kramer test



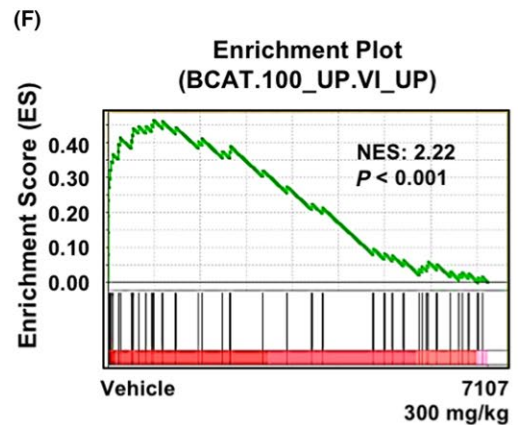
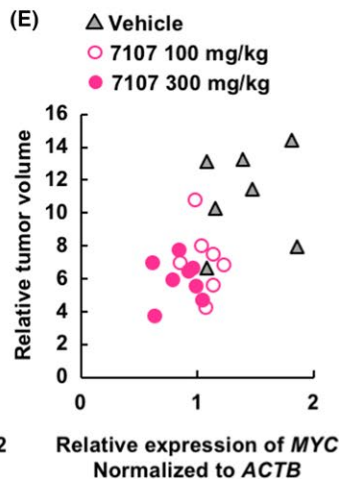
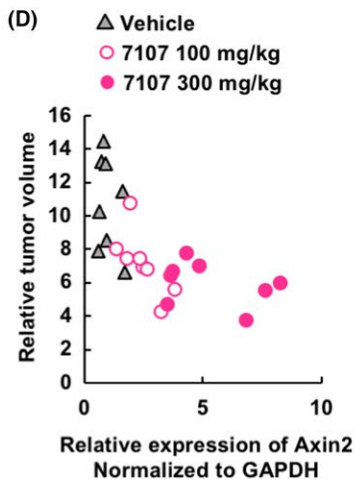
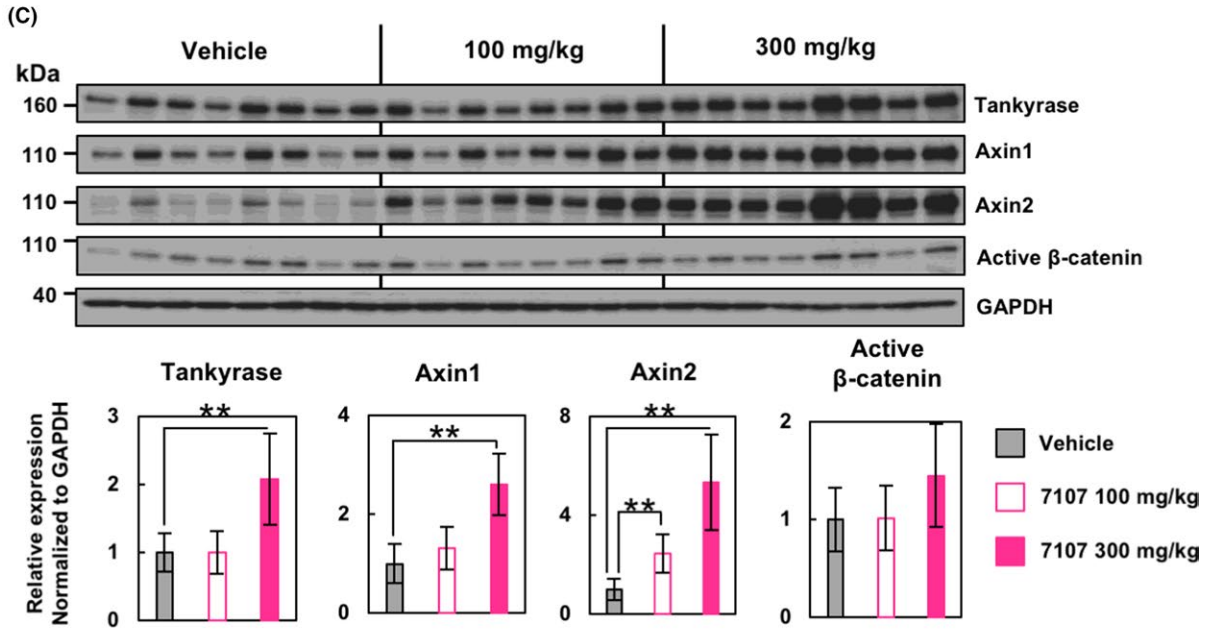
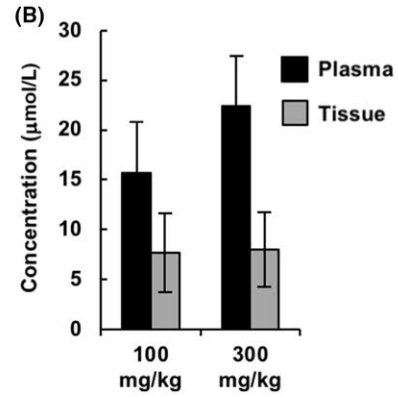
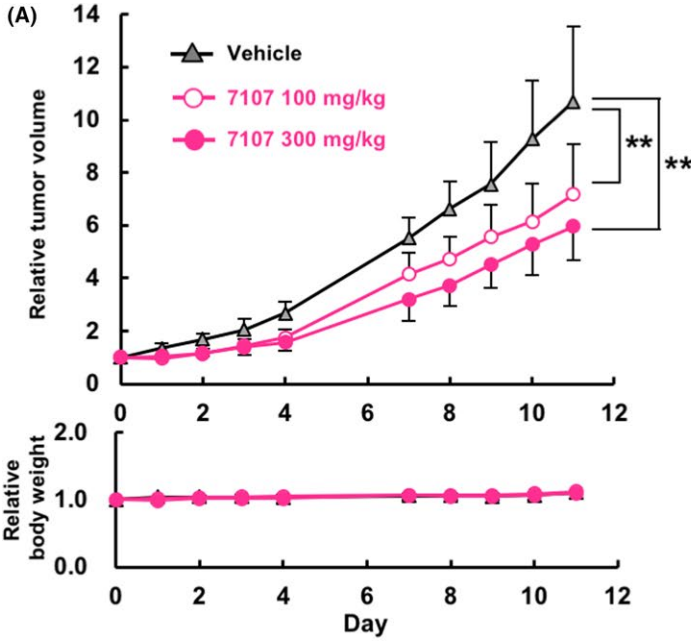


FIGURE 4 In vivo antitumor effect of RK-287107 through repression of β -catenin signaling. A, Tumor growth inhibition by RK-287107 in vivo. NOD-SCID mice were injected s.c. with COLO-320DM cells. RK-287107 was given i.p. under a 5-days on/ 2-days off schedule for 2 weeks at a dose of 100 mg/kg or 300 mg/kg once per day. Error bar indicates standard deviation. $**P < 0.01$ by Tukey-Kramer test. B, Concentration of RK-287107 in plasma and tumor tissue at the sampling point at 4 h from the final dosage of the compound. C, Xenograft tumors in (A) were collected at 4 h from the final administration, and subjected to western blot analysis. Each lane indicates a tumor derived from an independent mouse. Full-length blots are presented in Figure S2. Bottom panels are quantitative representations. $**P < 0.01$ by two-sided Student's paired *t* test. D,E, Scatter plots of Axin2 protein expression normalized to GAPDH (D) or MYC gene expression normalized to ACTB (E) and relative tumor volume of each sample. Samples for qRT-PCR analysis were collected at 4 h from the final dosage. F, Gene Set Enrichment Analysis (GSEA) showing enrichment of the β -catenin pathway-related gene signature in the tumor tissues of the vehicle-treated group vs that of the RK-287107-treated group (300 mg/kg)

protein (Pearson's correlation $r = -0.84$) (Figure 5D). Furthermore, active (ie, non-phosphorylated) β -catenin was downregulated by either oral or i.p. administration of RK-287107 (Figure 5C, lower). These observations indicate that RK-287107 is an orally effective tankyrase inhibitor.

4 | DISCUSSION

In the present study, we showed that a novel small-molecule compound, RK-287107, potently inhibits tankyrases but not PARP1 enzyme activity. Through blockade of the β -catenin signaling pathway, RK-287107 suppressed tumor growth of human colorectal cancer cells in mouse xenograft models with either i.p. or oral administration. RK-287107 was optimized from a high-throughput screening hit compound, RK-140160. RK-140160 is structurally similar to XAV939, the first described tankyrase inhibitor, and binds to the nicotinamide subsite of the donor NAD⁺ binding site. Optimization of the spiroindoline substituent, which was predicted to be exposed to the cytosolic space, resulted in improvement of enzymatic and cellular activities, drug metabolism and pharmacokinetics profile of the compounds. Detailed information on the chemical optimization will be described elsewhere (F. Shirai, T. Tsumura, Y. Yashiroda, H. Yuki, H. Niwa, S. Sato, T. Chikada, Y. Koda, K. Washizuka, N. Yoshimoto, M. Abe, T. Onuki, Y. Mazaki, C. Hirama, T. Fukami, H. Watanabe, T. Honma, T. Umehara, M. Shirouzu, M. Okue, Y. Kano, T. Watanabe, K. Kitamura, E. Shitara, Y. Muramatsu, H. Yoshida, A. Mizutani, H. Seimiya, M. Yoshida, H. Koyama, manuscript in preparation).

We previously reported that the status of the APC mutation is a potential biomarker for sensitivity to tankyrase inhibitors in colorectal cancer.²⁴ RK-287107 suppressed proliferation of COLO-320DM and SW403 cells with short APC mutations, which lack 20-AARs, but not that of RKO, HCC2998 and DLD-1 with more than two 20-AARs, indicating that sensitivity to tankyrase inhibitors is predictable by APC mutations (Figure 2A). HCT-116 cells have a constitutively active mutation in the CTNNB1 gene caused by deletion of Ser45, which suggests that HCT-116 has less sensitivity to tankyrase inhibitors. Therefore, it is reasonable that HCT-116 cells did not show sensitivity to tankyrase inhibitors (Figure 2A). This result also indicates that RK-287107 is a tankyrase-specific inhibitor and has fewer off-target effects. RK-287107 reduced the level of active β -catenin in the nuclei of COLO-320DM cells (Figure 3B). We also carried out immunofluorescence staining of

β -catenin using SW403 cells. In these cells, however, β -catenin was detected in the cytoplasm, nuclei, and at the cell-cell junctions. Therefore, it is unclear from our results whether RK-287107 reduces β -catenin in the nuclei in SW403 cells (A. Mizutani, H. Yoshida, H. Seimiya, unpublished data).

In HEK293T cells, we needed to stimulate the cells with external Wnt3A ligand for activation of Wnt signaling (Figure 3C). Because HEK293T cells have WT APC, Axins accumulated upon tankyrase inhibition can cooperate with APC to degrade β -catenin. This observation is consistent with a previous report on another tankyrase inhibitor, XAV939.¹⁵ In colorectal cancer cells, RKO cells retain WT APC and have no abnormalities in the Wnt signaling pathway. In fact, RKO cells do not accumulate β -catenin,²⁴ which explains why these cells are resistant to tankyrase inhibitors, including RK-287107 (Figure 2A). Meanwhile, HCC2998 and DLD-1 cells express the mutant APC with partial 20-AAR deletions. These APC mutants are hypomorphic and exert a dominant-negative effect on Axin-dependent degradation of β -catenin.²⁴ Therefore, in these cells, RK-287107-induced Axin accumulation would not lead to β -catenin degradation, which explains the resistance to tankyrase inhibitors. By contrast, COLO-320DM cells have a "short" APC, which lacks all of the seven 20-AARs. This mutant APC does not contribute to β -catenin degradation, causing hyperactivation of β -catenin signaling.²⁴ Therefore, COLO-320DM cells are highly dependent on β -catenin, and tankyrase inhibitors efficiently downregulate β -catenin and block cell growth.

Over the past decade, various tankyrase inhibitors have been developed,^{15,17-23,32} some of which have been adapted to in vivo administration in preclinical models.¹⁸⁻²³ Among such inhibitors, G007-LK is one of the most studied tankyrase inhibitors in vivo, although it has only been given i.p.¹⁸ It has been reported that G007-LK given at high doses in mice causes body weight loss and lesions in the small intestine, which are considered severe side-effects.¹⁸ It has been reported that another tankyrase inhibitor, G-631, also has severe side-effects on the small intestine.²³ Meanwhile, no body weight alterations or histological changes in the small intestine in mice have been observed on 21-day oral dosage of JW55, a tankyrase-specific inhibitor.²² In our present study, we did not observe any body weight loss in mice with i.p. administration of RK-287107 (Figure 4A). When RK-287107 was given orally, body weight loss was observed; however, the control group, in which the vehicle was given orally, showed equivalent body weight loss to the RK-287107-administered group.

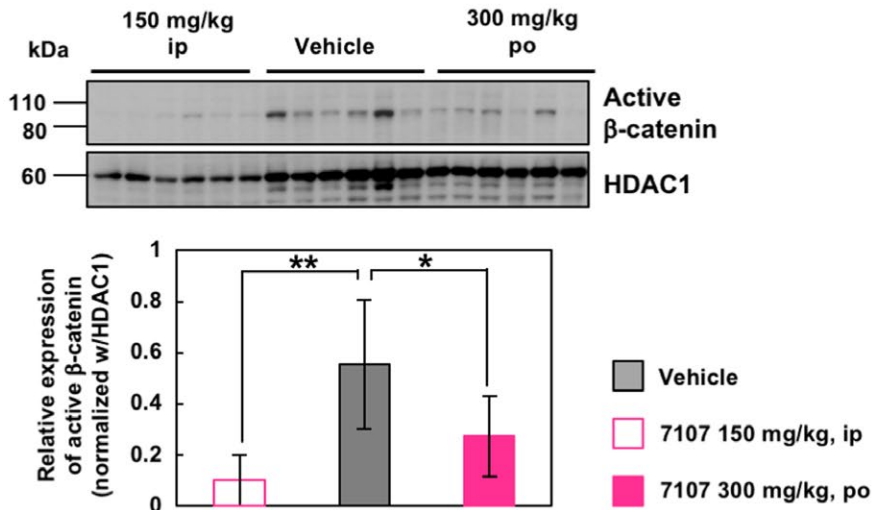
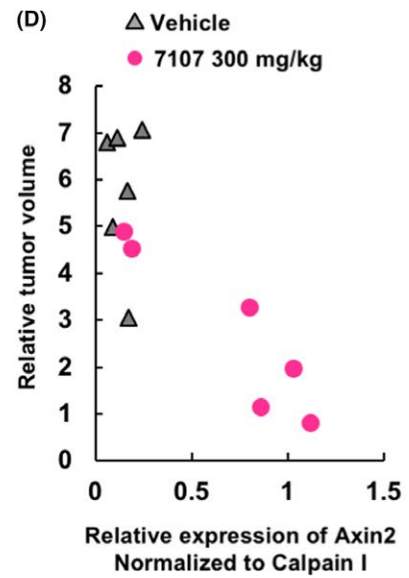
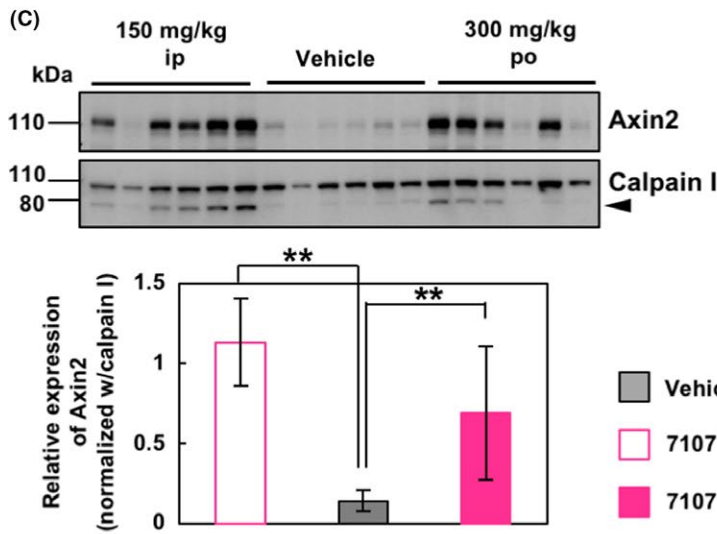
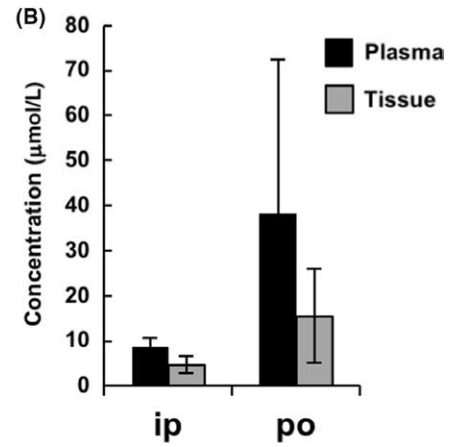
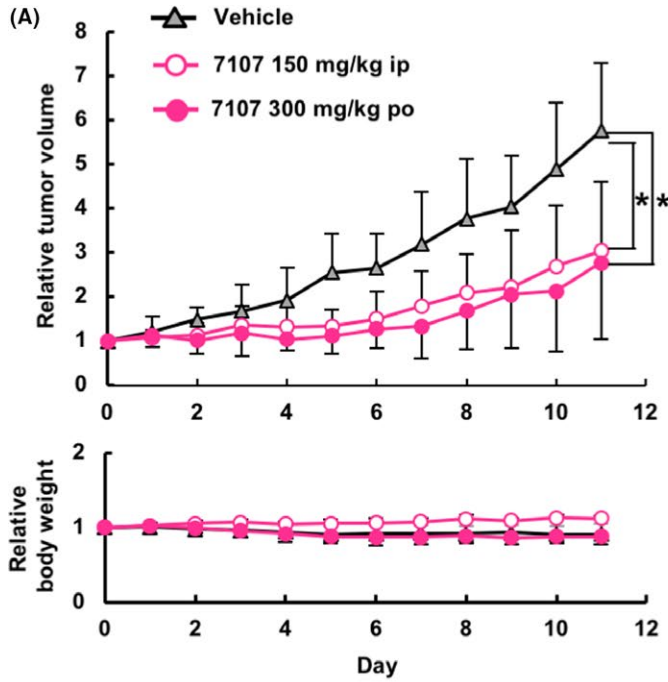


FIGURE 5 Orally given RK-287107 exerts an antitumor effect. A, Tumor growth inhibition by orally given RK-287107. NOD-SCID mice were injected s.c. with COLO-320DM cells before giving RK-287107. RK-287107 was given for 11 days at a dose of 150 mg/kg i.p. or 300 mg/kg p.o. twice a day. Error bar indicates standard deviation. * $P < 0.05$ by Tukey-Kramer test. B, Concentration of RK-287107 in plasma and tumor tissue at the sampling point at 4 h from the final dosage of the compound. Error bar indicates standard deviation. C, Western blot analyses of pharmacodynamic biomarkers in the same xenograft tumors as in (A) collected at 4 h from the final dosage. Cytoplasmic (upper panels) and nuclear (lower panels) extracts from the tumor tissues were subjected to western blot analysis. Full-length blots are presented in Figure S3. Error bar indicates standard deviation. ** $P < 0.01$ and * $P < 0.05$ by two-sided Student's paired t test. D, Scatter plot of Axin2 protein expression normalized to Calpain I and relative tumor volume of each sample

These results indicate that the body weight loss observed in this study was due to the stress of oral dosage and vehicle toxicity. RK-287107 administration did not show obvious body weight loss in mice, whereas G007-LK causes severe body weight loss in mice.¹⁸ One possible reason for this discrepancy would be that the metabolic stability of RK-287107 is much lower than that of G007-LK. After incubation with murine microsomes, percentages of the remaining G007-LK and RK-287107 were 96% and 18%, respectively (our unpublished observations). Because we used rather high doses of RK-287107 (up to 300 mg/kg, Figures 4 and 5), it was mechanistically difficult to further increase the doses. We did not carry out pathological analyses on intestinal toxicity because RK-287107 did not induce body weight loss as compared with the vehicle-treated group.

Knockdown of Axin2, but not Axin1, abolishes tankyrase inhibitor-mediated β -catenin degradation in COLO-320DM cells,²⁴ suggesting that β -catenin downregulation by RK-287107 would mainly be caused by accumulated Axin2. In HCC2998 cells, which have APC mutations that partially retain 20-AARs, tankyrase inhibitor-mediated β -catenin degradation depends on Axin2 but not on Axin1 when mutant APC is depleted by siRNA-mediated knockdown.²⁴ Meanwhile, not only Axin2, but also Axin1, accumulated in RK-287107-treated xenograft tumors (Figure 4C), suggesting that β -catenin degradation caused by tankyrase inhibitors also depends on Axin1 in vivo.

When we used whole cell extracts instead of nuclear extracts, it was technically difficult to clearly detect downregulation of β -catenin in RK-287107-treated xenograft tumors. This might be as a result of the fact that the signal of active β -catenin decreased in a time-dependent way during lysate preparation. Because accumulation of Axin2 was easily detected at 4 hours from the final drug administration in vivo, we routinely evaluated the pharmacodynamic biomarkers at this time point. Meanwhile, degradation of active β -catenin emerged more slowly than Axin2 accumulation (our unpublished observations).

Some melanoma patients respond to immune checkpoint inhibitors (eg, anti-programmed-cell-death 1 antibodies, such as nivolumab and pembrolizumab), whereas others do not respond. In those drug-resistant patients, melanoma-intrinsic β -catenin signaling may often avoid T-cell infiltration in melanoma by means of inhibition of CCL4 secretion.³³ In tumors with activated β -catenin signaling, 14% of cases have an active mutation of CTNNB1, whereas 61% of cases are caused by overexpression of Wnt ligand (WNT7B), receptor (FZD3) or β -catenin itself.³³ These observations suggest that tankyrase inhibitors could provide a novel opportunity for combination therapy with immune checkpoint inhibitors for non-responder patients.

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CONFLICTS OF INTEREST

TC, TT and MO are employees of Meiji Seika Pharma Co., Ltd. Other authors have no competing interests to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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