A Novel Tankyrase Inhibitor Decreases Canonical Wnt Signaling in Colon Carcinoma Cells and Reduces Tumor Growth in Conditional APC Mutant Mice

Jo Waaler, Ondrej Machon, Lucie Turnova, Huyen Dinh, Vladimir Korinek, Steven Ray Wilson, Jan Erik Paulsen, Nina Marie Pedersen, Tor J. Eide, Olga Machonova, Dietmar Gradl, Andrey Voronkov, with a multitude of proteins, including the 

junctions (3). In the cytoplasm, cellular functions. At the cell membrane, it is associated with 

b-catenin fate decision, and cell-cycle control (1, 2). The main denom-

nology, Karlsruhe; and 8 Leibniz-Institut F

erations: Oslo University Hospital, SFI-CAST Biomedical Innovation Center, Unit for Cell Signaling, Forskningsparken, Gaustadalleen and Center for Molecular Biology and Neuroscience; Institute for Cancer Research, Department of Biochemistry, The Norwegian Radium Hospital, Stem Cell Innovation Centre, Department of Pathology, Rikshospitalet, Oslo University Hospital, Oslo, Norway; Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic; KIT (Karlsruhe Institute of Technology) Zoological Institute, Cell and Developmental Biology, Karlsruhe; and Leibniz-Institut Fur Molekulare Pharmakologie, FMP, Berlin, Germany

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

Corresponding Authors: Jo Waaler, Oslo University Hospital, SFI-CAST Biomedical Innovation Center, Unit for Cell Signaling, Forskningsparken, Gaustadalleen, 21, 0349 Oslo, Norway and Center for Molecular Biology and Neuroscience, P.O. Box 1105, Oslo-N-0317, Norway. Phone: 47-9514-8669; Fax: 47-2295-8150; E-mail: jo.waaler@rr-research.no; and Stefan Krauss, E-mail: stefan.krauss@rr-research.no

doi: 10.1158/0008-5472.CAN-11-3336

©2012 American Association for Cancer Research.
In the nucleus, β-catenin exerts a number of functions, such as binding to the transcription factor TCF/LEF by replacing Groucho and activation of downstream transcription of genes, including c-Myc, Cyclin D1, and AXIN2 (2). Nuclear β-catenin levels are not only determined by the rate of β-catenin import to the nucleus but also by "nuclear trapping" of β-catenin through factors such as TCF/LEF (17).

In general, mutations in genes encoding central components of the Wnt/β-catenin pathway and the destruction complex lead to the accumulation of nuclear β-catenin and contribute to tumor initiation and progression (18, 19). Wnt-activating mutations are found in a broad range of solid tumors including colon cancer, gastric cancer, hepatocellular carcinoma, breast cancer, medulloblastoma, melanoma, non-small cell lung cancer, pancreas adenocarcinoma, and prostate cancer (20). It has been well established that the majority of intestinal neoplasia with deregulated Wnt signaling harbor truncating mutations in both alleles of the APC tumor suppressor gene (21).

Considerable efforts have been made to identify drugs that inhibit Wnt/β-catenin signaling (18, 22–25). Published inhibitory drugs that interact with biotargets directly associated with canonical Wnt signaling include WAY-316606 for SFRP (26), niclosamide for frizzled (27), NCS668036 for Dsh (28), pyrvinium for AXIN2 (24), XAV939 and IWR-1 for TNKS1/2 (4, 22); 2,4-diaminoquinazoline, quercetin, PKF115-584 and ICG-001 for β-catenin and its binding to TCF or CREB (29). TNKS1/2, in particular, is a promising biotarget for pharmaceutical reagents (4, 6). TNKS1/2 is not only involved in controlling canonical Wnt signaling but has been associated with further cellular processes through either protein poly(ADP-ribosyl)ation (PARsylation) or by protein complex formation. These are (i) telomere maintenance by interacting with TRF1 (33), (ii) spindle formation and stabilization by binding to NuMA (33), and (iii) glucose metabolism by regulating GLUT4 transport through IRAP (33).

In this work, we have identified a novel TNKS inhibitor, JW55, which inhibits the PARP domain of TNKS1/2, leading to the stabilization of AXIN2 followed by increased degradation of β-catenin. JW55 efficiently decreases canonical Wnt signaling in colon carcinoma cell lines, in a Xenopus axis duplication assay, and in tamoxifen-induced tumors in ApcCKO/CKOLgr5-CreERT2+ mice. The identified chemotype JW55 may serve as an attractive start point for the development of novel cancer therapeutics.

Materials and Methods

Cell lines and luciferase assays

All cell lines were purchased from American Type Culture Collection and maintained and treated according to the supplier’s recommendations. Reporter assays were carried out as previously described (34).

IncuCyte cell growth measurement assay

A total of 1,000 SW480 or RKO cells were seeded in 96-well plates. The day after, the cell culture medium was exchanged to solutions that contained 0.1% dimethyl sulfoxide (DMSO) or 10 μmol/L JW55 for RKO cells and 0.1% DMSO or 10, 5, or 1 μmol/L JW55 for SW480 cells. All samples consisted of a minimum of 6 replicates. The plate was incubated in an IncuCyte (Essen BioScience) inside a cell culture incubator. Images were captured every second hour to monitor proliferation.

Colony assay

A total of 400 SW480, RKO, or HeLa cells were seeded in 6-cm plates and 1% FBS. The day after, the medium was exchanged

Figure 1. JW55 specifically reduces canonical Wnt signaling in reporter cells and in Xenopus embryos. Reporter controls in 0.1% DMSO. A, left, luciferase reporter activity in HEK293 cells, transiently transfected with NF-κB-Luc and Renilla plasmids and treated with JW55 at 0.1 to 10 μmol/L, +, 30% Wnt3a-CM; –, without Wnt3a-CM. The spotted line shows the IC50 value level. Right, chemical structure of JW55. B, effect of 1 and 10 μmol/L JW55 in NIH/3T3 Shh Light II cells.+; 10 pg XWnt8 and 2 pmol JW55 (black). The axis duplication rate was significantly (*P < 0.001). Collected data from 3 independent assays are shown. Right, representative images of embryos obtained 36 hours after the injection.

Published OnlineFirst March 22, 2012; DOI: 10.1158/0008-5472.CAN-11-3336
Cancer Res; 72(11) June 1, 2012

Cancer Research

embedded in paraffin stained with hematoxylin and eosin (H&E). Fixed colons were
pared in 10% paraformaldehyde (PFA)/PBS solution. Small
small intestines were stained using 1% methylene blue pre-
fixed and the intestines were dissected, washed in PBS,
fixed with 0.2% methylene blue prepared in 10% paraformaldehyde (PFA)/PBS solution. Small
ileum Swiss-rolls were embedded in paraffin and stained with hematoxylin and eosin (H&E). Fixed colons
were randomized into 2 groups and treated with either JW55 (100 mg/kg) or vehicle (DMSO). Daily per oral applications
started the day after and continued for 3 weeks. The mouse
(100 mg/kg) or vehicle (DMSO). Daily per oral applications
were randomized into 2 groups and treated with either JW55
were counted manually with a colony counter (Scienceware). Representa-
tive pictures of colonies were captured with a Leica MZ
Apo Stereomicroscope connected to a Leica DC200 camera
and Adobe Photoshop.

Tumor initiation and treatment in Apc<sup>Cko/Cko</sup>/Lgr5-CreERT2 mice

Seven 12-week old female Apc<sup>Cko/Cko</sup>/Lgr5-CreERT2 mice were injected intraperitoneally with 25 mg/kg of tamoxifen
(Sigma) diluted in an ethanol and corn oil (ratio 1:1). The mice
were randomized into 2 groups and treated with either JW55
(100 mg/kg) or vehicle (DMSO). Daily per oral applications
started the day after and continued for 3 weeks. The mouse
body weight was measured twice a week. The mice were sacrificed and the intestines were dissected, washed in PBS,
and fixed in formaldehyde [10% solution (v/v) in PBS]. The
small intestines were stained using 1% methylene blue pre-
pared in 10% paraformaldehyde (PFA)/PBS solution. Small
ileum Swiss-rolls were embedded in paraffin sectioned and stained with hematoxylin and eosin (H&E). Fixed colons
were embedded in paraffin, sectioned and stained with an anti–
ß-catenin antibody (610153; BD Transduction Laboratories;
see Supplementary Materials and Methods for further details).
The number and size of the intestinal lesions were quantified
by the Ellipse program (ViDiTo).

For other materials and methods, please see previous
descriptions (34) and Supplementary Materials and Methods.

Results

JW55 is a potent and selective inhibitor of the canonical
Wnt pathway

To identify chemical compounds that inhibit the canonical
Wnt signaling pathway, a library consisting of 37,000 small
molecules (ChemBioNet) was screened using HEK293 cells
that were stably transfected with a SuperTOP: d1EGFP reporter
(34). The vector contains a synthetic TCF-responsive promoter
(SuperTOP: 7 x TCF binding sites) that initiates the expression
of destabilized d1EGFP upon activation of canonical Wnt
signaling (34). Canonical Wnt signaling was activated using conditioned media (CM) from L Wnt3a expressing cells.
The screen resulted in 77 primary hits including the previously
described compounds JW67 and JW74 (34) and JW55 (Fig. 1A,
right panel). To evaluate the efficacy and specificity of JW55,
the compound was tested in 3 different luciferase reporter
assays. Wnt3a-induced HEK293 cells containing a transiently
transfected ST-Luc (SuperTOP-luciferase) reporter showed
inhibition by JW55 with an IC<sub>50</sub> value of 470 nmol/L (Fig. 1A,
left panel and Supplementary Fig. S1A). In contrast, Shh
Light II cells (Gli1-Luc reporter) that were activated with 50% Shh-CM were not inhibited by 10 or 1 μmol/L JW55. Also,
TNFα–activated HEK293 cells containing a transiently

Figure 2. JW55 inhibits canonical Wnt signaling in CRC cells. A, the
control bars are shown in gray and JW55-treated samples are
shown in black. Left, Inhibition of GSK3ß (25 mmol/L LiCl) in HEK293
cells, transiently transfected with ST-Luc and Renilla, is
counteracted by 0.1 to 10 μmol/L JW55. + , 25 mmol/L LiCl; --, no LiCl.
Controls in 0.1% DMSO. The mean values of 3 independent
assays and SDs are shown. Right, HEK293 cells transiently
transfected with ST-Luc and Renilla plus full-length ß-catenin or da-Cat.
+, with ß-catenin plasmids; --, without ß-catenin. Controls in 0.1%
DMSO. Ten μmol/L JW55 inhibited luciferase activity induced
by ectopic wild-type ß-catenin (*, P < 0.05), whereas da-Cat induction
was unaffected. n = total number of measurements from multiple
independent assays. The SDs are shown as error bars. B, JW55–
mediated reduction of luciferase activity in different CRC cell lines
stably transfected with ST-Luc and Renilla. Left, a dose-dependent
reduction of ST-Luc activation was detected in HCT-15 and SW480
cells harboring mutant APC. Right, JW55-mediated reduction in
HCT116 cells containing a single allele mutation in S45 of ß-catenin.
The mean values and the SE of several independent assays are
shown. C, 25 or 10 μmol/L JW55 reduced the relative expression
of AXIN2, SPF, and NKD1 mRNA in the CRC cells SW480 and DLD-1
as shown by real-time RT-PCR analysis. The means of
3 independent experiments are shown along with error bars
depicting SDs.
transfected NF-κB–Luciferase (NF-κB-Luc) plasmid showed no pathway inhibition in the presence of 10 or 1 μmol/L of JW55 (Fig. 1B and C).

The selectivity and specificity of JW55 was further tested in an in vivo Xenopus axis duplication assay, a highly stringent test for examining inhibitors of canonical Wnt signaling. When injected into the ventral blastomeres of 4-cell stage Xenopus laevis embryos, XWnt8 mRNA induced Wnt signaling resulting in the formation of a second body axis. By using selective Wnt inhibitors, the axis duplication can be inhibited and a normal phenotype restored (34). Coinjection of 10 pg XWnt8 and 2 pmol of JW55 resulted in a significant 51% reduction of the axis duplication incidence in developing frog embryos when compared with the DMSO vehicle control (z-test, \( P = 0.001 \); Fig. 1D, left). This result provided evidence that JW55 acts as a specific and effective inhibitor of canonical Wnt signaling.

JW55 regulates β-catenin stability at the level of the destruction complex

To identify the interference point of JW55 with canonical Wnt signaling, the effect of JW55 was monitored after LiCl-induced activation of the pathway. The activity of GSK3β, which is part of the β-catenin destruction complex, is reduced in the presence of LiCl (35), rendering the N-terminal phosphorylation of β-catenin by GSK3β ineffective. This causes nuclear accumulation of β-catenin and active Wnt signaling. HEK293 cells, transiently transfected with ST-Luc and Renilla, were incubated with 25 mmol/L LiCl and different doses of JW55. The LiCl-activating effect was counteracted by JW55 in a dose-dependent manner with an IC₅₀ value of 360 nmol/L, indicating that JW55 acts at the level or downstream of the destruction complex (Fig. 2A, left and Supplementary Fig. S1B).

Next, the effect of JW55 on regulating the activity of β-catenin was tested. We used (i) wild-type β-catenin and (ii) β-catenin with point mutations in the N-terminal phosphorylation sites (S33, S37, T41, and S45) that resists degradation and functions as dominant active (da-Cat). Plasmids containing full-length β-catenin or da-Cat, along with ST-Luc and Renilla, were transiently transfected in HEK293 cells. The expression of wild-type β-catenin led to an increased ST-Luc reporter activity that could be reduced by 55% when the transfected cells were exposed to 10 μmol/L of JW55 (normality test failed, rank sum test: \( P = 0.001 \); Fig. 2A, right). In contrast, the activation of the pathway by da-Cat could not be inhibited by 10 μmol/L of JW55, further indicating that JW55 acts at the level or downstream of the destruction complex (normality test failed, rank sum test: \( P = 0.457 \); Fig. 2A, right).

JW55 inhibits canonical Wnt signaling in colorectal cancer cell lines in vitro

Mutations in the APC gene, which occur in nearly all colorectal cancers (CRC; ref. 18), lead to ineffective degradation of β-catenin and aberrant upregulation of Wnt signaling. The cell lines SW480 and HCT-15 (mutated in codon 1338 and 1417 of the APC gene, respectively) were stably transfected with ST-Luc and Renilla and incubated at various doses of JW55 for 48 hours. A dose-dependent reduction of luciferase activity was detected in both cell lines. JW55 was effective in the range of 1
to 5 μmol/L in SW480 cells and 0.01 to 5 μmol/L in HCT-15 cells (Fig. 2B, left). Next, HCT116 CRC cells with integrated ST-Luc and Renilla reporters were used to test JW55. HCT116 carries a point mutation in the CK1α-dependent phosphorylation site S45 of one β-catenin allele; however, S45-mutated β-catenin may still be phosphorylated in the remaining GSK3β phosphorylation sites (e.g. S33, S37, and T41), with resulting semi-regulated β-catenin turnover (36). In HCT116 cells, JW55 was effective in the range of 0.01 to 5 μmol/L (Fig. 2B, right). A basal luciferase expression level at approximately 50% was reached after exposure to 5 μmol/L in the CRC cells.

Furthermore, the effect of JW55 on the expression of endogenous Wnt target genes was examined by real-time PCR in the cell lines SW480 and DLD-1. Compared with SW480 cells, the APC mutation in DLD-1 cells results in a more extensive C-terminal deletion (37). In SW480 cells, a dose-dependent decline in the expression of the 3 target genes was observed: AXIN2 (25 μmol/L: 55% and 10 μmol/L: 45%), SP5 (25 μmol/L: 38% and 10 μmol/L: 15%) and NKD1 (25 μmol/L: 31% and 10 μmol/L: 7%; Fig. 2C, left). A similar reduction was observed in DLD-1 cells after exposure to 10 μmol/L of JW55 (AXIN2: 72%, SP5: 38% and NKD1: 66%; Fig. 2C, right). Subsequently, a more extensive Illumina gene expression analysis was carried out in JW55-treated SW480 cells. Several Wnt target genes (Wnt homepage) were differently expressed (Supplementary Table S1). Upregulated genes included (Log2 ≥ 0.5): WISP3, TCF7, PLAUR, EFNB2, and NOTUM. Downregulated genes included (Log2 ≤ −0.5): AXIN2, NKD1, DKK1, MMP7, ID2, GAST, FZD2, EDN1, CYR61, SOX18 and, similar to our previous observations (34), members of the SPANX gene family.

**Figure 4.** JW55 specifically inhibits TNKS1, TNKS2 but not PARP1 in biochemical assays. A, JW55 B, XAV939 (logarithmic scale). C, the calculated IC50 values are displayed in the table. The mean values represent 2 independent experiments and the error bars show SDs. D, immunoblotting of TNKS1/2 after exposures of SW480 cells to JW55, XAV939, or IWR-1.

**Figure 5.** Scheme showing the proposed effect of JW55 on protein stability in the β-catenin destruction complex. The red arrow indicates nuclear translocation of β-catenin and expression Wnt target genes in a Wnt on state. The blue arrows depict the inhibition of the PARP domain of TNKS by JW55, leading to stabilization of the destruction complex, increased N-terminal phosphorylation of β-catenin, and degradation in the proteasome.
JW55 destabilizes β-catenin by increasing cytoplasmic AXIN2 levels

Previous studies have shown that an increase in AXIN2 steady-state levels induced by TNKS1/2 or CK1a inhibitors is accompanied by a decrease in β-catenin concentrations (4, 22, 24, 34). Increased levels of AXIN2 protein promote degradation of β-catenin even in cells with truncated APC (4, 22, 38). Western blot analysis of SW480 cells lysates revealed a dose-dependent increase of cytoplasmic AXIN2 after JW55 treatment (range: 10 μmol/L–100 nmol/L; Fig. 3A and Supplementary Fig. S2). Furthermore, an antibody against the active and nonphosphorylated form of β-catenin (active β-catenin, ABC) identified reduced levels of β-catenin in the cytoplasm of JW55-treated cells (Fig. 3A and Supplementary Fig. S2). In addition, a modest reduction of total β-catenin and a substantial decrease of nuclear ABC were observed, and an increase in phosphorylated β-catenin (pβ-catenin) levels was seen indicating ongoing β-catenin degradation (Fig. 3A and Supplementary Fig. S2).

To gain further insight into the changes in cellular distribution of AXIN2 and β-catenin, JW55-treated SW480 cells were analyzed by immunofluorescence. A general reduction of total β-catenin, both in the cytoplasmic and nuclear compartments, was detected at the doses of 5 and 1 μmol/L (equal shutter speed; Supplementary Fig. S3). Confocal microscopy (equal shutter speeds) revealed, in accordance with the Western blot analysis, that the levels of cytoplasmic AXIN2 were significantly increased (Fig. 3B) and large protein foci, probably representing accumulated destruction complexes, were observed (Fig. 3B, arrows). Clusters of colocalized cytoplasmic β-catenin and AXIN2 have previously been detected in SW480 cells after treatment with the Wnt antagonist JW74 (34). Similar to JW74, enhanced phosphorylation and resulting degradation of β-catenin after JW55 exposure seemed to be orchestrated by stabilization of AXIN2 in the destruction complex (Fig. 3 and 5).

JW55 specifically inhibits the PARylation activity of TNKS1 and TNKS2

By inhibiting the PARP domain of TNKS1/2, XAV939, IWR-1, and JW74 prevent auto-PARylation of TNKS1/2 and PARylation of AXIN2 (4, 22, 39, 40). This leads to a stabilization of
AXIN2, an accumulation of proteins in the destruction complex followed ultimately by an increased degradation of β-catenin (4, 22). To test whether JW55 decreased canonical Wnt signaling by directly inhibiting the PARP domain of TNKS1/2, we carried out biochemical assays for monitoring the activity of TNKS1/2 and PARP. JW55 decreased auto-PARylation of TNKS1/2 in vitro with IC50 values of 1.9 μmol/L and 830 mmol/L, respectively (Fig. 4A and Supplementary Fig. S1C). However, in contrast to XAV939 (ref. 4; Fig. 4A and Supplementary Fig. S1D), but similar to IWR-1 (4), JW55 exhibited no inhibition of PARP1 at doses up to 20 μmol/L (Fig. 4A). Although further details may emerge, present evidence suggests that JW55 decreases canonical Wnt signaling by specifically inhibiting the PARP domain of TNKS1/2 (Fig. 5), although leaving the activity of the PARP domain of at least PARP1 unaffected. Molecular docking of JW55 into the adenosine site of the human PARP domain of TNKS2 using the structure of the PARP domain in complex with IWR-1 (X-ray, PDB code 3UA9) reveals that the binding site and position of JW55 in the model is similar to IWR-1 (40), but different from XAV939 (Supplementary Fig. S4). Binding of JW55 to recombinant TNKS2 protein was also observed by using a fluorescence polarization competition assay, with a structurally similar but inactive analog as a negative control (ref. 41; Supplementary Fig. S5). Interestingly, opposite to the effect of XAV939 (4), a decrease in endogenous TNKS1/2 levels was detected after exposure to JW55 and IWR-1 in SW480 cells.

**JW55 reduces growth of SW480 colon cancer cells**

CRC cells can enter cell-cycle arrest as a result of antagonized canonical Wnt signaling (42–44). Various proliferation assays were carried out to see whether JW55-mediated inhibition of canonical Wnt signaling would affect CRC cell growth. First, SW480 cells were incubated with 10 μmol/L JW55 and labeled with bromodeoxyuridine (BrdUrd) and propidium iodide (PI). The subsequent flow cytometry cell-cycle analysis revealed that JW55 treatment lowered the proportion of cells in S phase (from 28.4% in DMSO-treated controls to 22.2%), raised moderately the cell fraction in the G1 phase (from 37.3%–38.8%) and increased the number of cells in G2–M phase (from 34.3%–39%; Fig. 6A).

Next, we monitored the cell proliferation kinetics using IncuCyte. SW480 cells were exposed to 10, 5, or 1 μmol/L of JW55 for 9 days, and the confluency was measured every 2 hours. All doses of JW55 reduced SW480 cell growth and the most robust effect was detected at a dose of 10 μmol/L JW55. The confluency was reduced to 55% relative to the DMSO control at the end of experiment (Fig. 6B). In parallel, the CRC cell line RKO, which contains wild-type APC and β-catenin and exerts Wnt-independent cell growth, was used as a control. RKO cells reached 100% confluency within 7 days and were not affected by treatment with 10 μmol/L JW55 (Fig. 6B).

Furthermore, proliferation of SW480 cells was examined by consecutive passages in the presence of 5, 2.5, or 1 μmol/L JW55. A dose-dependent decrease of cell numbers over 3 passages was noted in SW480 cells in the presence of JW55, whereas HeLa cells (Wnt-independent cervical cancer cells) remained unaffected (Fig. 6C). Finally, SW480 cells were grown under low serum conditions (1% FBS) along with various concentrations of JW55 (5, 1, and 0.1 μmol/L) and the formation of colonies was quantified. We observed a concentration-dependent reduction of colony numbers in SW480 cells and no reduction in colony numbers in the control cell lines HeLa and RKO when cultured in 5 μmol/L JW55 (Fig. 6D, left). All SW480 colonies, which formed in the presence of JW55, were substantially smaller (Fig. 6D, right).

Taken together, these data showed that JW55-mediated inhibition of canonical Wnt signaling resulted in reduced cell-cycle progression, proliferation, and colony formation in the CRC cell line SW480 in vitro.

**JW55 reduces tumor development in conditional Apc knockout mice**

Recently, the leucine-rich-repeat containing G-protein–coupled receptor 5 (Lgr5) was established as a specific marker for intestinal epithelial stem cells (ISC). The knockin mouse Lgr5-Egfp-Ires-CreERT2, further referred to as Lgr5-CreERT2, expresses a tamoxifen-regulated variant of Cre recombinase that is under the control of the Lgr5 locus (45). The Cre-mediated excision of the floxed exon 14 in mice with conditional Apc alleles (CKO) changes the reading frame downstream of the deletion. This results in the production of

---

Figure 7. JW55 treatment decreases development of adenomas in conditional Apc knockout mice. A, representative microscopy images showing hematoxylin and eosin (H&E)-stained sections of Swiss-rolls (the distal part of the ileum is centered) showing an extensive decrease in adenoma development in the small intestine of JW55-treated (100 mg/kg) mice when compared with control mice (DMSO). The right panel shows the morphology of a wild-type (wt) ileum. B, tumors in the colon of ApcCKO/CKOLgr5-CreERT2 mice displaying high levels of β-catenin and EphB2, which indicates active Wnt signaling and an ISC-like phenotype. The expression of β-catenin (d, e), EphB2 (f, g), and the proliferation marker Ki67 (h, i) is shown in the colon of vehicle (DMSO) or JW55-treated mice. Microphotographs in d and e are shown in more detail in d’ and e’, respectively. The sections were counterstained with hematoxylin nuclear stain. Epithelial lesions are indicated by black arrows. G, the graphs illustrate quantification of tumor area, count, and size from the ileum (H&E stained) and colon (β-catenin labeled) of JW55-treated or untreated ApcCKO/CKOLgr5-CreERT2 mice. The histograms display the mean values and the error bars show SDs. The top panel shows a statistically significant (*) decrease of average total tumor area in the ileum subsequent to the JW55 treatment (P < 0.003). Bottom panels, left, the colon tumor count was substantially (**) reduced after JW55 exposure (P < 0.057). Middle, the mean total tumor area of the colon was significantly (%) reduced (P < 0.043). Right, the mean area of one tumor was significantly (%) reduced (P < 0.003). D, the phenotype of nonadenomatous colons is not affected by JW55 (right panels) when compared with wild-type tissue (wt, left panels). Representative pictures are shown. The sections were stained with Ki67 (l and m) and EphB2 (j and k) that label proliferating and ISC-like cells localized toward the bottom of the crypts. Cytokeratin 20 (Krt20; n and o) identifies terminally differentiated epithelial cells that are found at the apical surface of the mucosa. The black bars in the bottom right corners of the images represent 1 mm in (a, b, and c), 0.2 mm in (d, e, and f), and 0.1 mm in other images.
a truncated and nonfunctional 580 amino acid (aa) Apc polypeptide (46) that relates to the homozygous mutations seen in the intestinal cancer model mouse ApcMin (multiple intestinal neoplasia, Min; ref. 47). Lgr5-CreERT2 mice were intercrossed with ApcCKO/CKO animals and multiple tumors were observed after tamoxifen injections in the small intestine (ileum) and the colon of ApcCKO/CKO/Lgr5-CreERT2- mice (Fig. 7A and Supplementary Fig S6). In the colon, fewer tumors developed when compared with the general adenoma progress in the small intestine. To evaluate the JW55-mediated decrease of intestinal tumor development in vivo, ApcCKO/CKO/Lgr5-CreERT2+ mice were injected intraperitoneally with a 25 mg/kg single dose of tamoxifen. A day after, daily per oral applications of JW55 (100 mg/kg; 3 females) or vehicle (DMSO; 4 females) were initiated. The dose of 100 mg/kg was chosen to counteract the rapid liver metabolism of JW55 as indicated by the human liver microsome stability analysis (t1/2 = 10.1 minutes; Supplementary Fig S7A). No measurable effects on mouse body weight were noticed throughout the experiment period (Supplementary Fig S7B). After 21 days, the mice were sacrificed and the dissected intestines were embedded in paraffin and sectioned. Immunohistochemical staining revealed that the neoplastic lesions expressed β-catenin and the mouse ISC marker Ephrin type-B receptor 2 (EphB2), indicating aberrant activation of canonical Wnt signaling in the tumor tissue (Fig. 7B; refs. 44, 48–50). The β-catenin–stained colon adenomas contrasted with the surrounding healthy mucosa, and image analysis software (Ellipse) was used to quantify the number and areas of β-catenin–positive lesions in the colon (Supplementary Fig. S7C). As it was impossible to distinguish the borders of individual tumors in the small intestine (ileum), only the total tumor area per mouse was recorded. In the ileum, a significant reduction of the total tumor area was observed after JW55 injections (mean: 2.93 mm2 and median: 2.95 mm2) when compared with the control group (mean: 8.84 mm2 and median: 9.51 mm2; normality test failed, rank sum test: P = 0.003; Fig. 7A and C, top panel). In the colon, the tumor count was substantially reduced in JW55-treated mice (mean: 9.7 and median: 6.0) when evaluated against the control group (mean: 33.8 and median: 26.0; normality test failed, rank sum test: P = 0.057; Fig. 7C, bottom panel). Furthermore, a significant decrease of the total tumor area was noticed after injections with JW55 (mean: 0.022 mm2) when compared with the control group (mean: 0.154 mm2; Students t test: P = 0.009; Fig. 7C, bottom panel). The area of single tumors was significantly reduced after injections with JW55 (mean: 0.0025 mm2) when compared with the untreated group (mean: 0.0049 mm2; Students t test: P = 0.043; Fig. 7C, bottom panel). Interestingly, the proportion of cells that expressed Ki67, a marker of proliferating and ISC-like cells (50), was substantially decreased in adenomas exposed to JW55 when compared with tumors that developed in the control mice (Fig. 7B, panel h and i).

Importantly, when examining the healthy mucosa with antibodies against various epithelial cell populations, such as EphB2 for ISCs and Krt20 for terminally differentiated epithelial cells (50), we did not observe changes in its morphology, proliferation, or differentiation after exposure to JW55 (Fig. 7D). In summary, the results obtained from JW55-treated conditional Apc knockout mice indicated that in vivo small-molecular inhibition of TNKS1/2 leads to decreased canonical Wnt signaling followed by reduced adenoma induction and possibly progression of adenoma carcinoma.

Discussion

Altered properties of Wnt/β-catenin signaling are enabling factors for a multitude of diseases, including cancer. Among known interference points in the canonical Wnt signaling pathway, the PARP domains of TNKS1/2 seem to be a particular suitable target for inhibiting the pathway. Blocking the PARP domain of TNKS1/2 with low molecular inhibitors, such as XAV939, IWR-1, and JW74 (4, 34), leads to the stabilization of the destruction complex that triggers increased degradation of β-catenin (19). As predicted for a TNKS inhibitor, we observed a massive cytoplasmic accumulation of AXIN2 after treatment with 500 nmol/L JW55, followed by a significant reduction of β-catenin levels in vitro (Fig. 5). A similar stabilization of AXIN2 was also seen with JW74, XAV939, and IWR-1 (4, 22, 34). However, despite the substantial increased specificity of JW55 to the PARP domain of TNKS1/2 when compared with XAV939, we cannot rule out that JW55 may affect additional mechanisms that contribute to the observed effects in colon cancer cell lines and in ApcCKO/CKO/Lgr5-CreERT2+ mice. For instance pyrvinium decreases Wnt signaling both by stabilizing AXIN2 and through inhibition of CK1ε (24). Furthermore, in complementation to altering canonical Wnt/β-catenin signaling, a JW55-mediated inhibition of TNKS1/2 may induce additional intracellular effects including altered telomere maintenance or spindle formation.

The in vivo treatment of tamoxifen-induced polyposis in ApcCKO/CKO/Lgr5-CreERT2+ mice JW55 showed a profound effect on tumor progression. In comparison with the well-established ApcMin mice (47), this mouse model provides a clearer assay for scoring adenoma formation because the tumor induction and treatment are time controlled. However, as adenoma induction is rather strong, it is difficult to assess later stages of CRC development as mouse mortality does not allow long-term tracking of the tumors. Thus, the presented data further solidify the functional implication of PARylation by TNKS1/2 in a malignant transformation of the colon and the small intestine.

Interestingly, in our setting, the exposure of progenitor cells in a normal crypt to JW55 did not exert visible alterations in the intact mucosa as measured with the markers EphB2, Ki67, and Krt20. However, we cannot exclude that a longer treatment may cause reduction in cell turnover in the intestine epithelium (45). The efficient in vivo reduction of adenomas in mice and CRC growth in vitro by a tankyrase-specific inhibitor further strengthens the importance of TNKS and β-catenin as potential therapeutic biotargets.

TNKS1/2 is a member of a family of 5 PARP proteins in humans, including PARP1, which is an extensively explored target for drug development (33). Particularly in the BRCA1/2 mutant background, PARP1 inhibitors exhibit therapeutic effects in vitro and in vivo. Because PARP proteins are
involved in a range of biologic functions, including genome maintenance, the selectivity of PARP inhibitors may be an issue in the therapeutic setting. Similar to XAV939, JW55 inhibits auto-PARylation of TNKS1 and TNKS2 in a biochemical assay. However, in contrast to XAV939, we see no evidence for the inhibition of PARP1 by JW55, making this chemotype attractive for further development toward a TNKS1/2 selective inhibitor. It remains to be determined in which settings a preferred PARP inhibitor such as for instance olaparib, a broad PARP inhibitor such as XAV939, or a selective TNKS inhibitor such as JW55, JW74, and IWR-1 may be the favorable choice.

Disclosure of Potential Conflicts of Interest
The described chemical compound may have commercial value if further investigated.

References


A Novel Tankyrase Inhibitor Decreases Canonical Wnt Signaling in Colon Carcinoma Cells and Reduces Tumor Growth in Conditional APC Mutant Mice

Jo Waaler, Ondrej Machon, Lucie Tumova, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3336

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/03/22/0008-5472.CAN-11-3336.DC1

Cited articles
This article cites 50 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/11/2822.full#ref-list-1

Citing articles
This article has been cited by 38 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/72/11/2822.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/72/11/2822.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.