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Priority Report

Cancer Research

Pharmacological Inhibition of the Wnt Acyltransferase PORCN Prevents Growth of WNT-Driven Mammary Cancer

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Abstract

Porcupine (PORCN) is a membrane bound O-acyltransferase that is required for Wnt palmitoylation, secretion, and biologic activity. All evaluable human Wnts require PORCN for their activity, suggesting that inhibition of PORCN could be an effective treatment for cancers dependent on excess Wnt activity. In this study, we evaluated the PORCN inhibitor Wnt-C59 (C59), to determine its activity and toxicity in cultured cells and mice. C59 inhibits PORCN activity *in vitro* at nanomolar concentrations, as assessed by inhibition of Wnt palmitoylation, Wnt interaction with the carrier protein Wntless/WLS, Wnt secretion, and Wnt activation of β-catenin reporter activity. In mice, C59 displayed good bioavailability, as once daily oral administration was sufficient to maintain blood concentrations well above the IC_{50}. C59 blocked progression of mammary tumors in MMTV-WNT1 transgenic mice while downregulating Wnt/β-catenin target genes. Surprisingly, mice exhibit no apparent toxicity, such that at a therapeutically effective dose there were no pathologic changes in the gut or other tissues. These results offer preclinical proof-of-concept that inhibiting mammalian Wnts can be achieved by targeting PORCN with small-molecule inhibitors such as C59, and that this is a safe and feasible strategy *in vivo*. *Cancer Res; 73(2); 502–7. ©2012 AACR.*

Introduction

Dysregulation of the Wnt signaling cascade has been implicated in multiple disorders including cancer, vascular proliferation, and tissue fibrosis. Wnt autocrine loops and paracrine Wnt secretion from stroma have been shown in multiple settings, even in diseases such as colon cancer that have mutations in downstream components of the Wnt/β-catenin pathway (1, 2). Wnts are upregulated in colorectal cancer cells with mutant APC, in breast cancer cell lines, and in multiple sarcomas (2, 3). The Wnt pathway is activated in several cancers by inactivating mutations in the ubiquitin ligases RNF43 and ZNRF3 that normally downregulate the Wnt receptor Frizzled, and in colorectal cancers by R-spondin gene fusions (4–7). In addition, negative regulators of the Wnt pathway, such as sFRP1 and Dkk1, are epigenetically silenced in multiple cancers. Wnts secreted from cancer cells (8), stromal myofibroblasts (9), and immune cells (10) have been implicated in the process of tumorigenesis and metastasis. If these pathways are important in cancer proliferation and spread, then inhibitors of Wnts may have value as anticancer agents. Specific targeted therapies against Wnts and their receptors, including recombinant Wnt antagonists such as decoy receptors and monoclonal antibodies against individual Wnts, have shown activity in selected settings (8, 11, 12). However, such approaches presuppose a knowledge of which Wnts are important in any given tumor.

An alternative approach to inhibit Wnt autocrine and paracrine signaling is to block the production of all active Wnts. This can be achieved by targeting a key enzyme in Wnt biosynthesis, the membrane bound O-acyltransferase PORCN. PORCN makes a good target because it is essential for the O-palmitoylation of all human Wnts (13–16). PORCN resides in the endoplasmic reticulum, where it adds palmitate to a serine (S209 in human WNT3A) that is completely conserved in all vertebrate Wnts (16). Acylation of S209 is required for the next step in Wnt secretion, binding to the carrier protein WLS (17). Palmitoylation is also essential for WNT to interact with Frizzled receptors outside the cell (13, 18). While genetic ablation of PORCN slows the growth of some tumor lines *in vitro*, PORCN has additional nonenzymatic functions that complicate tests of its role in cancer via a knockout or RNA interference approach (19). As an enzyme, PORCN is an attractive target for small-molecule inhibitors (20–22). Supporting this, we have recently reported that all mammalian Wnt signaling is sensitive to PORCN expression levels, and that small changes in PORCN activity can have significant effects on

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

K.D. Proffitt and B. Madan contributed equally to this work.

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developmental phenotypes (14, 15). A Novartis PORCN inhibitor, LGK974, is in early-phase clinical trials (NCT01351103) although no peer-reviewed published information is available regarding its activity or efficacy. The development of PORCN inhibitors offers the opportunity to directly test if PORCN is a useful target in Wnt-dependent cancers *in vivo*.

Materials and Methods

Extensive additional experimental details are in Supplemental Material.

Reagents

HT1080 and HeLa cells were acquired from the American Type Culture Collection. Cell lines were not tested for authenticity. STF3a cells were previously reported (17). Wnt-C59 was purchased from Cellagen Technology, and is reported in U.S. patent WO/2010/101849. ω-alkynyl palmitic acid (Alk-C16) was synthesized as previously reported (23, 24).

Administration of C59 to mice

C59 was resuspended by sonication for 20 minutes in a mixture of 0.5% methylcellulose and 0.1% Tween-80 for oral administration. MMTV-WNT1 mice were obtained from Jackson Laboratories and backcrossed at least 6 generations to C57/BL6 mice.

Results

C59 is a potent inhibitor of PORCN enzymatic activity

The small-molecule 2-(4-(2-methylpyridin-4-yl)phenyl)-*N*-(4-(pyridin-3-yl)phenyl) acetamide was recently developed and patented by Novartis as a Wnt signaling modulator (25). It is commercially available under the name C59 from at least 2 sources (Cellagen Technology and Biovision), and is claimed to inhibit PORCN enzyme activity at nanomolar concentrations. However, there is no peer-reviewed published information on its efficacy and molecular target. Because a potent, bioavailable, and stable PORCN inhibitor is not yet available we evaluated C59. We find that C59 indeed functions as a bona fide PORCN inhibitor using a number of cell-based assays. C59 inhibits WNT3A-mediated activation of a multimerized TCF-binding site driving luciferase (Super8xTopFlash; STF) with an IC_{50} of 74 pmol/L (Fig. 1A). As expected for a PORCN inhibitor, Wnt secretion into culture medium is completely abrogated by C59 treatment (Fig. 1A, inset). Consistent with C59 targeting PORCN, overexpression of PORCN rescues the inhibition of WNT3A-mediated STF activity, similar to that of an unrelated PORCN inhibitor IWP-1 (refs. 21, 22; Fig. 1B). Wnt acylation is required for binding to the carrier protein WLS (15, 17). WNT3A and WNT8A coimmunoprecipitate with WLS, but this interaction is blocked when cells have been pretreated with C59 (Fig. 1C). Using alkyne palmitic acid and click chemistry

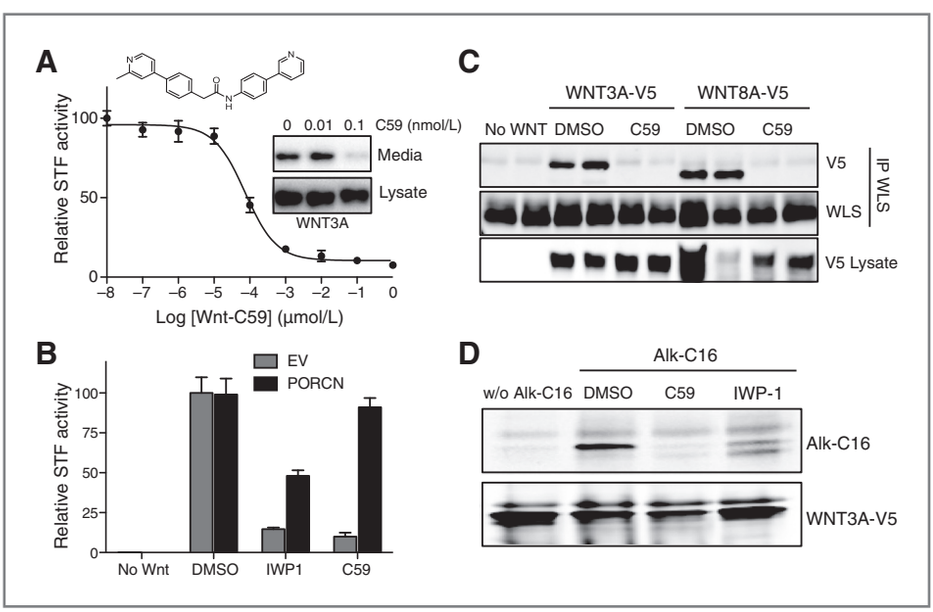

Figure 1. C59 is a bona fide inhibitor of PORCN activity. A, C59 is a potent inhibitor of Wnt/β-catenin signaling. HEK293 cells constitutively expressing WNT3A and the β-catenin reporter STF were treated with C59 or dimethyl sulfoxide (DMSO). After 48 hours, luciferase activity was measured. Error bars represent SD. Structure of C59 is shown above. Inset, WNT3A secretion into culture medium was blocked by 0.1 nmol/L C59. Uncut immunoblots are shown in Supplementary Fig. S1A. B, PORCN overexpression reverses the effects of C59. HT1080 cells were transfected with empty vector (EV) or mPORCN-D expression plasmids followed by treatment with C59 (1 nmol/L) or IWP1 (1 μmol/L). Luciferase activity was measured after 24 hours. Error bars represent SD. C, C59 blocks the palmitoylation-dependent Wnt–WLS interaction. HeLa cells were transfected with either WNT3A-V5 or WNT8A-V5 plasmids, then treated with DMSO or C59 (10 nmol/L). WLS was immunoprecipitated and precipitates were probed for WLS and V5. Uncut immunoblots are shown in Supplementary Fig. S1B. D, C59 blocks palmitoylation of Wnts. Alk-C16 was added to HeLa cells transfected with WNT3A-V5 and cotreated with either DMSO, C59 (100 nmol/L), or IWP1 (1 μmol/L). Lysates were prepared and Wnt was immunoprecipitated with antibody to V5. Click chemistry was conducted to attach azido-biotin to alkyne-palmitate groups. Finally, samples were separated by SDS-PAGE and probed for biotin and WNT3A-V5. This result was reproduced in HT1080 cells (Supplementary Fig. S1C).

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(23, 24), we find that C59 prevents incorporation of palmitate into WNT3A, consistent with inhibition of PORCN activity (Fig. 1D). C59 inhibits the activity of all splice variants of murine PORCN (Fig. 2A). In preliminary studies, we found that very high concentrations of C59 were required to produce developmental phenotypes in *Xenopus* embryogenesis. Consistent with this, while *Xenopus laevis* PORCN was active when expressed in PORCN-null human cells, its activity was resistant to inhibition by C59 (Fig. 2A). Because the *Xenopus* protein is 77% identical to human PORCN, this provides genetic evidence that PORCN is the molecular target of C59, suggests a mechanism for C59 drug resistance to emerge, and indicates that less related MBOAT proteins would also be unaffected by C59. Showing that inhibition of PORCN is likely to prevent all Wnt-mediated signaling, we found that 9 of 9 β-catenin activating Wnts and 4 of 4 additional noncanonical Wnts lost activity when cells were treated with C59 (Fig. 2B and C). In summary, C59 is a nanomolar inhibitor of mammalian PORCN acyltransferase activity and blocks activation of all evaluated human Wnts. Thus, we anticipate that C59 administration will prevent all human and murine Wnt-dependent signaling.

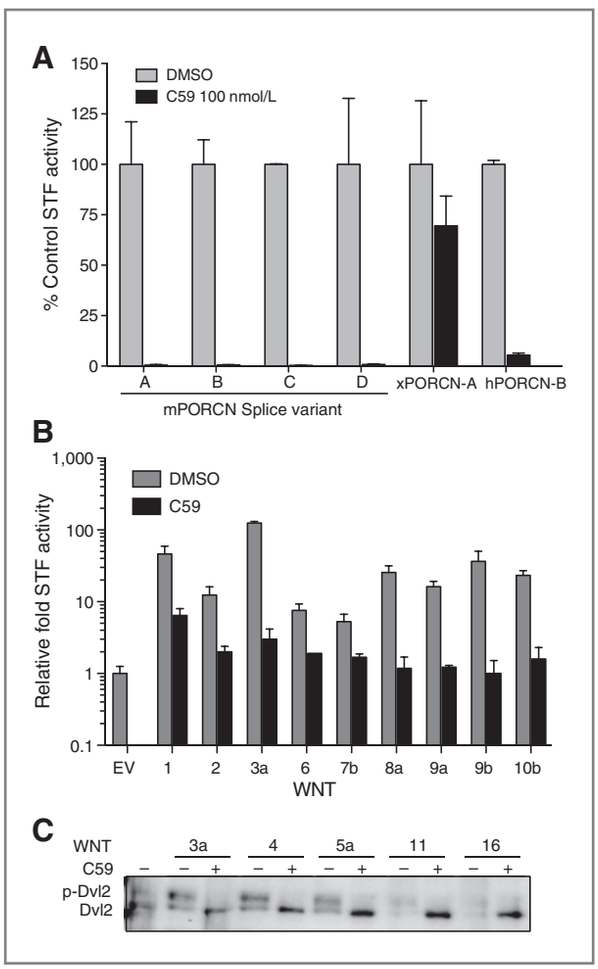

Figure 2. C59 is a general mammalian PORCN/WNT inhibitor. A, all PORCN isoforms are inhibited by C59. PORCN-null HT1080 cells (14) were transfected with 200 pg of the indicated PORCN expression plasmids, along with WNT3A, STF reporter, and mCherry as transfection control. Six hours after transfection, cells were treated with C59 or DMSO as indicated and the following day assayed for luciferase. *Xenopus* PORCN was resistant to the inhibitory effects of C59. B, all canonical Wnts are inhibited by C59. STF luciferase assay was conducted as in A except with wild-type HT1080 cells. Data is presented as fold activation over transfection with no Wnt. Cells were treated with 10 nmol/L C59 or DMSO. Data is presented as mean ± SD. C, noncanonical Wnts are inhibited by C59. Dvl2 mobility shift was assessed in HT1080 cells transfected with the indicated Wnts in the presence or absence of 10 nmol/L C59.

Wnt autocrine loops have been reported in multiple cancer cell lines, and secreted Wnt inhibitors like sFRPs and Frzb have growth inhibitory effects on cancer cell lines as well (2, 11, 26, 27). We therefore assessed the effects of C59 on cancer cell proliferation *in vitro*. C59 does not significantly inhibit the proliferation of any of 46 tested cancer cell lines *in vitro* at concentrations that completely inhibit PORCN (Supplementary Table S1). Inhibition of proliferation of a few cell lines at more than 1.5 μmol/L (20,000-fold above the IC_{50}) is likely to be a cell-type–specific off-target effect. This overall lack of toxicity indicates that Wnt secretion is not essential for most cells to proliferate in 2-dimensional culture. Our results with C59 differ from studies on the inhibitory effects of secreted Wnt inhibitors on proliferation, which we speculate may be due to the reported additional activities of these inhibitors beyond the Wnt pathway (28).

C59 can be administered to mice and prevents tumor growth

To test the role of Wnt signaling *in vivo*, we assessed the bioavailability and *in vivo* half-life of C59 in mice. After either intravenous (2.5 mg/kg) or oral administration (5 mg/kg), the compound half-life in blood was approximately 1.94 hours. Notably, C59 concentration remained greater than 10-fold above the *in vitro* IC_{50} for at least 16 hours following a single oral dose (Fig. 3A). On the basis of the pharmacokinetic profiling, C59 was administered once daily to test its efficacy in treating established Wnt-driven tumors. In mice carrying a mouse mammary tumor virus (MMTV)-WNT1 transgene, overexpression of murine WNT1 causes a high incidence of mammary adenocarcinomas beginning at 10 weeks of age (29). Notably, tumors arising in these mice remain Wnt dependent but have diverse molecular phenotypes and growth rates consistent with the hypothesis that WNT1 expands a vulnerable population that then undergoes second hits (30, 31).

To test the *in vivo* efficacy of C59, we transplanted fragments from 2 independent primary MMTV-WNT1 tumors orthotopically into nude mice. Following development of palpable tumors, mice were treated with either vehicle or C59, 10 mg/kg/d for 17 days. C59 administration arrested or reversed tumor growth in all treated mice ($n = 22$; Fig. 3B). After 17 days of treatment, the tumors were removed and further analyzed. Final tumor weights were significantly different (Fig. 3C). To confirm that C59 was active in immunocompetent mice, we monitored a colony of female nulliparous Bl6 MMTV-WNT1 mice for tumor development. When tumors became palpable, the mice were treated with either vehicle or C59 (5 mg/kg/d). While the number of mice enrolled in this study

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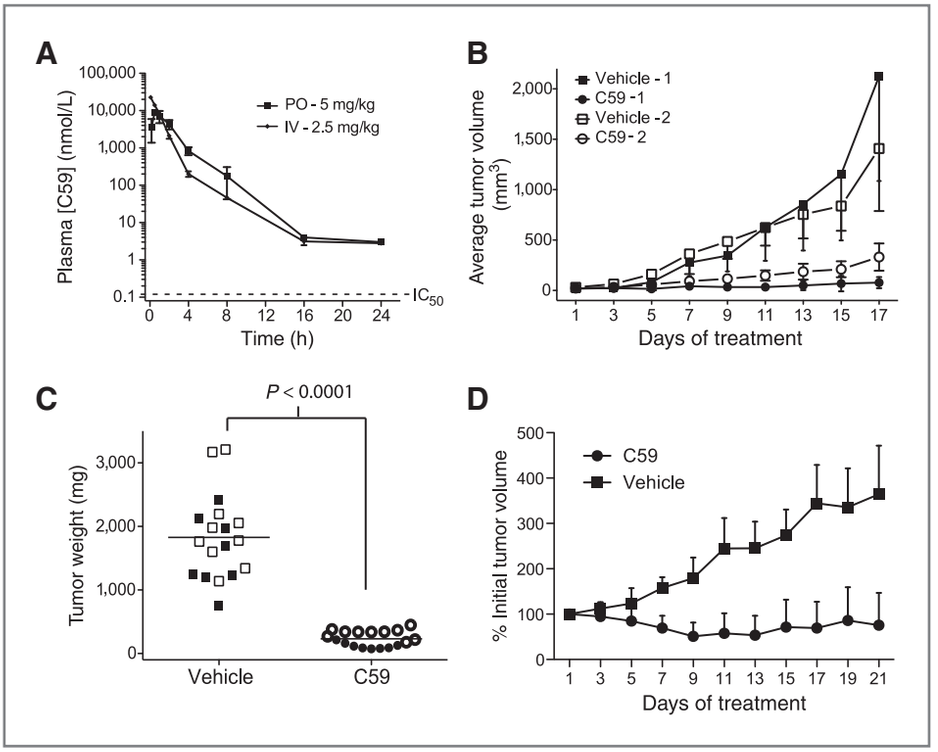

Figure 3. C59 is bioavailable and prevents MMTV-WNT1 tumor growth. A, C59 is bioavailable. Mice were given a single dose of 2.5 mg/kg C59 intravenously or 5 mg/kg orally. At times indicated after treatment, mice were sacrificed and C59 plasma concentration was measured by liquid chromatography/tandem mass spectrometry. Dotted line indicates calculated IC_{50}. Error bars represent SD. B, C59 prevents growth of MMTV-WNT1 tumors. Female nude mice orthotopically transplanted with independent MMTV-WNT1 tumors were treated with vehicle (line 1, $n = 8$; line 2, $n = 10$) or C59 10 mg/kg (line 1, $n = 10$; line 2, $n = 12$) once daily for 17 days. Tumor volumes were measured on alternate days. Data is presented as mean ± SD. $P < 0.001$ (d7-17) using 2-tailed *t* test. C, C59 significantly decreased tumor weight. Tumor weights at sacrifice from the transplanted mice are shown. Data analyzed using 2-tailed *t* test. D, C59 prevents growth of primary MMTV-WNT1 tumors. Female virgin MMTV-WNT1 mice with measurable mammary tumors were treated with vehicle (6 mice) or 5 mg/kg C59 (5 mice) for 21 days. Data represents change in tumor volume. Data is presented as mean ± SEM. $P < 0.05$ from days 7 to 21 using 2-tailed *t* test.

was smaller, again even the lower dose of C59 significantly blocked tumor growth (Fig. 3D). Final tumor weights are shown in Supplementary Fig. S2A.

Tumor growth inhibition is associated with decreased Wnt/β-catenin signaling in tumors

To determine whether the inhibition of tumor growth was accompanied by inhibition of Wnt/β-catenin signaling, we examined the expression of selected target genes in the allograft and primary tumors by quantitative reverse-transcription PCR (qRT-PCR). *Axin2*, *Ccnd1*, *c-Myc*, and *Tcf7* transcripts were significantly reduced in tumors from mice treated with C59 (Fig. 4A and Supplementary Fig. S2B). Consistent with a decrease in *c-Myc* and *CyclinD*, treated tumors also had significantly decreased proliferation as indicated by Ki67 staining (Fig. 4B).

A major function of WNT1 is inhibition of the β-catenin destruction complex, and consistent with this, vehicle-treated tumors had abundant β-catenin in cytoplasm and nucleus. In contrast, tumors from C59-treated mice had normal membrane β-catenin staining and markedly decreased cytoplasmic and nuclear β-catenin (Fig. 4C and Supplementary Fig. S3). Suggesting C59 is not toxic to normal tissues at this dose, mice in the treatment group had stable body weight (Supplementary Fig. S2C). Moreover, no signs of toxicity were observed in the multiple tissues histologically examined at the end of the study (Supplementary Fig. S4). Notably, treated mice had normal intestinal morphology and nuclear β-catenin staining was maintained in the crypts (Fig. 4D and Supplementary Fig. S4).

Discussion

In this study, we confirm that the small-molecule C59 is a nanomolar inhibitor of the acyltransferase activity of PORCN, and show that small-molecule–mediated inhibition of PORCN is an effective means for preventing WNT1-driven tumor growth in mice. C59 inhibits palmitoylation of Wnts and is not active against *Xenopus* PORCN. Thus, changes in the primary sequence of PORCN confer resistance to C59, confirming genetically that PORCN is the target of C59. C59 is more than100-fold more potent than the previously reported PORCN inhibitor IWP1. We find no apparent toxicity to cells or mice at a drug concentration that effectively inhibits MMTV-WNT1–driven tumor growth. Intestinal architecture of treated mice appears normal. A similar lack of intestinal toxicity was seen when Wnt signaling was inhibited with Fzd8CRD-Fc (11). We speculate that Wnt-addicted tumors are hypersensitive to

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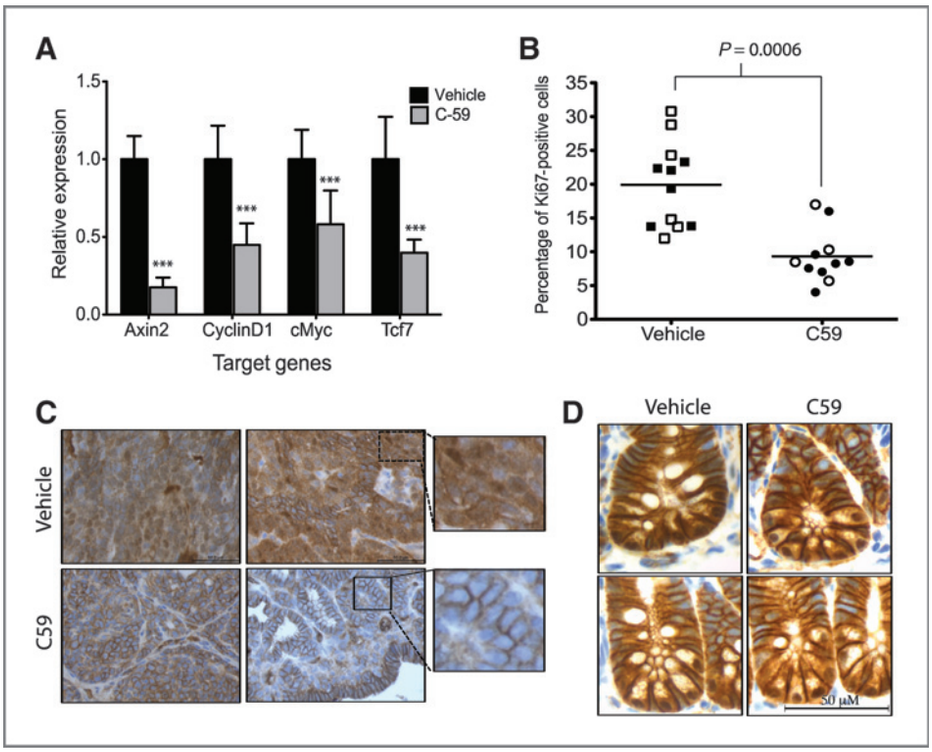

Figure 4. C59 decreases Wnt pathway activity in MMTV-WNT1 tumors. A, C59 inhibits β-catenin target gene expression. Total RNA was isolated from orthotopically transplanted tumors, and transcript levels for *Axin2*, *Ccnd2*, *C-myc*, and *Tcf7* were measured by qRT-PCR. Expression was normalized to *Actb*. ***, $P < 0.001$, 2-tailed *t* test. B, C59 decreases proliferation. Ki67 immunostaining in sections from the primary tumors (open symbols) and orthotopically transplanted tumors (closed symbols) was digitally quantified. Percentages of Ki67-positive nuclei are shown. Data analyzed using 2-tailed *t* test. C, C59 decreases cytoplasmic and nuclear β-catenin in tumors. β-catenin staining in MMTV-WNT1 tumor sections. Two representative samples from each treatment arm are shown. Right, outset, are enlargement of areas indicated in middle. Scale bars, 50 μm. D, C59 at therapeutically effective dose does not affect intestinal nuclear β-catenin. Intestinal sections from mice treated with vehicle or C59 for 21 days were stained for β-catenin.

small reductions in Wnt activity, whereas normal tissues such as intestine are more tolerant of decreases in Wnt signals and/or have alternative pathways for self-renewal.

The Wnt pathways contribute to the progression of various cancers, via both β-catenin–activating mutations and by paracrine and autocrine Wnt signaling. Increased Wnt production has also been identified in diverse nonmalignant diseases. In many cases, the implicated Wnts may be working via non-β-catenin pathways. PORCN inhibitors may therefore have efficacy even in diseases without activated β-catenin. Thus, it is a longstanding goal to identify therapeutics that can effectively target this pathway. Our recent work has confirmed that PORCN is a key node for fine control of total Wnt-dependent cell signaling, further supporting its use as a target (14, 15). As such, specific and bioavailable inhibitors of PORCN represent attractive new molecules that may be of value in the treatment of various cancers, in addition to other Wnt-stimulated diseases.

Disclosure of Potential Conflicts of Interest

D.M. Virshup is a consultant/advisory board member of Experimental Therapeutics Centre, Singapore. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K.D. Proffitt, B. Madan, R.N. Hannoush, D.M. Virshup
Development of methodology: K.D. Proffitt, B. Madan, R.N. Hannoush
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.D. Proffitt, B. Madan, Z. Ke, V. Pendharkar, L. Ding
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.D. Proffitt, B. Madan, Z. Ke, V. Pendharkar, L. Ding, R.N. Hannoush
Writing, review, and/or revision of the manuscript: K.D. Proffitt, B. Madan, M.A. Lee, R.N. Hannoush, D.M. Virshup
Study supervision: M.A. Lee, D.M. Virshup

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