

A Useful Approach to Identify Novel Small-Molecule Inhibitors of Wnt-Dependent Transcription

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Abstract

The Wnt signaling pathway is frequently deregulated in cancer due to mutations in genes encoding *APC*, *β-catenin*, and *axin*. To identify small-molecule inhibitors of Wnt signaling as potential therapeutics, a diverse chemical library was screened using a transcription factor reporter cell line in which the activity of the pathway was induced at the level of Disheveled protein. A series of deconvolution studies was used to focus on three compound series that selectively killed cancer cell lines with constitutive Wnt signaling. Activities of the compounds included the ability to induce degradation of β -catenin that had been stabilized by a glycogen synthase kinase-3 (GSK-3) inhibitor. This screen illustrates a practical approach to identify small-molecule inhibitors of Wnt signaling that can seed the development of agents suitable to treat patients with Wnt-dependent tumors. *Cancer Res*; 70(14): 5963–73. ©2010 AACR.

Introduction

The Wnt signaling pathway is activated by Wnt ligands at multiple stages of metazoan development and controls the differentiation and/or proliferation of stem cells in multiple tissues. The “canonical” Wnt/ β -catenin pathway is activated following Wnt ligand binding to a complex comprising Frizzled (Fz) and LRP5/6 receptors and ultimately activates β -catenin/transcription factor–dependent transcription (Fig. 1A). Key steps in the pathway include the formation of a ligand-activated receptor complex, the inhibition of intracellular β -catenin turnover, and the formation of a nuclear β -catenin/transcription factor (TCF) transcription complex. Wnt/ β -catenin signaling activates (and represses) transcription of genes whose promoters contain binding sites for TCF-transcription factors. Target genes include key developmental and oncogenic targets (reviewed in ref. 1).

The Wnt signaling pathway is considered a key therapeutic target for cancer (1). Mutations to “core” Wnt signaling components, including β -catenin, adenomatous polyposis coli

(APC), and Axin, inappropriately activate the β -catenin/TCF branch of the Wnt signaling pathway in cancers arising in a number of tissues, including colon and liver. Many other cancers show evidence of inappropriate Wnt pathway activation, including raised levels of β -catenin (2). Unfortunately, the known intracellular components of the core Wnt pathway are not good targets for small-molecule inhibitors, as many interactions comprise extended protein-protein interfaces. Furthermore, drugs that target core components may induce side effects because the function of key components (e.g., β -catenin) is required for tissue homeostasis in multiple organs, including the immune system and intestine (3).

Small molecules that target “noncore” Wnt signaling components have recently been identified (4). Of particular note, ICG001 blocks the interaction between β -catenin and the noncore CREB binding protein (CBP) leading to a reduction of adenoma formation in mouse models of colon cancer (5). The β -catenin–CBP interaction was shown to promote stem/progenitor marker expression, whereas the related β -catenin–p300 interaction, which was not subject to blockade by ICG001, promoted expression of genes involved in proliferative responses such as *CMYC* (6). The key point from this example is that compounds that target noncore components may be able to regulate distinct subsets of therapeutically relevant transcriptional targets. The recently discovered small molecules XAV939 and IWR2 inhibit Wnt signaling by blocking tankyrase activity, which is required for degradation of the β -catenin scaffold protein Axin (3, 7).

One of the main challenges in targeting noncore Wnt signaling molecules is knowing which protein to target. A large number of potential target genes that regulate Wnt/ β -catenin signaling have been identified in whole-genome RNAi screens, mass spectrometry, and yeast two-hybrid studies (8–10), and it remains to be seen which protein should be the focus of

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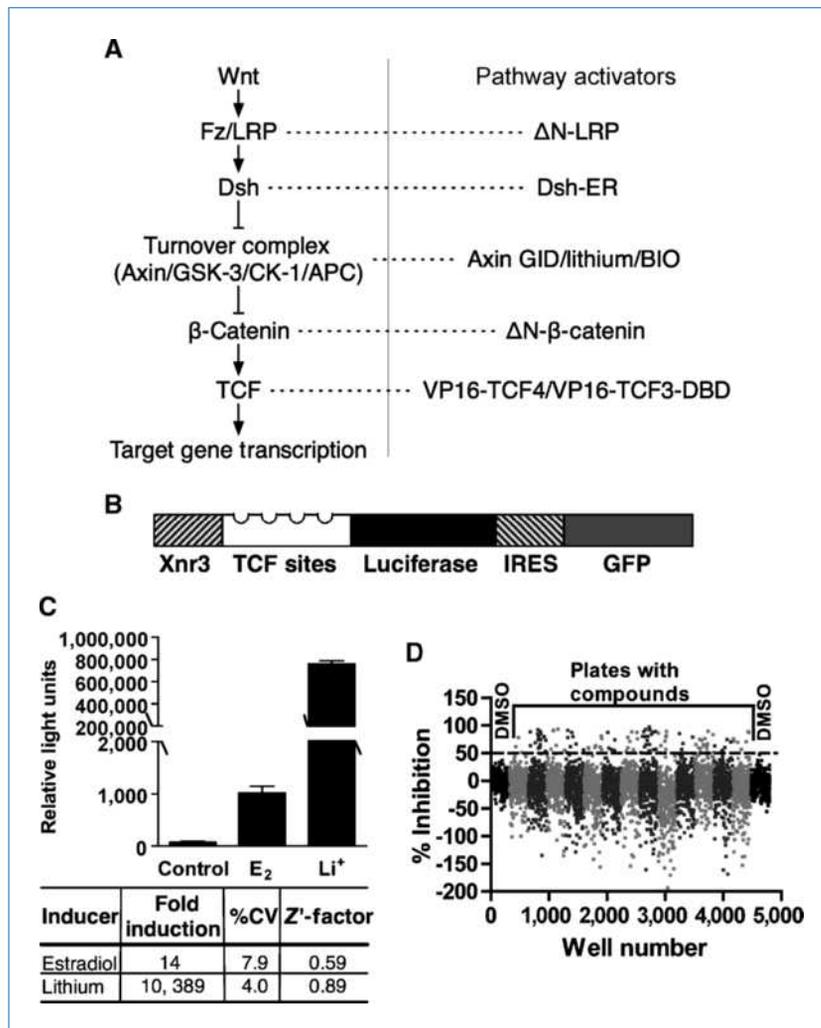


Figure 1. Primary screen. A, pathway activators (see text) that were used to induce Wnt/ β -catenin signaling at distinct levels. B, the reporter construct used in the human epithelial HEK293-based cell line. The bicistronic TCF-luciferase-IRES-GFP reporter contained the Wnt response element from the Xnr3 promoter and four TCF binding sites. C, fold induction, coefficients of variation, and Z-factors following 24 h estradiol and lithium induction. D, subset of thirteen 384-well plates (alternate shades of gray) and DMSO control plates (black) from the primary screen data. The dotted line indicates 50% inhibition of estradiol induction.

drug discovery efforts. To overcome this problem, we used a cell-based assay to screen a diverse library of small molecules for regulators of TCF-dependent transcription. We identified a number of chemical regulators that functioned at distinct levels within the Wnt signaling cascade.

Materials and Methods

Materials

The Cancer Research UK Centre for Cancer Therapeutics compound library was used for the primary screen. CCT031374 analogues were either obtained from this library or from commercial sources. Preparation of compounds to follow-up the initial hit matter is described in Supplementary Material. Δ N-LRP, Δ N- β -catenin, VP16-TCF4, Tau-1N4R, c-myc-luciferase, and survivin-luciferase vectors were kind gifts of K. Brennan (University of Manchester, Manchester, United Kingdom), P. Polakis (Genentech, South San Francisco, CA), A. García de Herreros (IMIM-Hospital del Mar, Barcelona, Spain), S. Lovestone (King's College London, London, United Kingdom), B. Vogelstein (Johns Hopkins Kimmel

Cancer Center, Baltimore, MD), and R. Moon (University of Washington School of Medicine, Seattle, WA), respectively. Human tumor cell lines were obtained from American Type Culture Collection, grown for less than 20 passages, and tested regularly for *Mycoplasma* infection (Lonza Mycoalert).

Reporter cell line

A fragment of the Xnr3 enhancer (–180 to –60), four TCF consensus-binding sites, and a c-Fos minimal promoter were inserted into the pUB-bsd blasticidin resistance plasmid (Invitrogen). The *luciferase* gene and the IRES-GFP-SV40 poly(A) sequences from pIRES-hrGFP-2a (Stratagene) were inserted downstream of the promoter (Fig. 1B). The reporter vector was transfected into a stable HA-Dvl2-ER (estrogen receptor)-expressing HEK293 cell line (11). Blasticidin-resistant cells were treated with 9 mmol/L lithium chloride (LiCl) for 16 hours to induce Wnt-dependent expression of green fluorescent protein (GFP). Two rounds of fluorescence-activated cell sorting (FACS) were used to enrich for high-GFP-expressing cell clones. The 7df3 clone was used as the reporter line in the primary screen.

Primary screen

Structurally diverse, low molecular weight compounds (63,040) were added individually to reporter cells at 20 $\mu\text{mol/L}$ 2 hours before addition of 10 $\mu\text{mol/L}$ β -estradiol in 384-well plates. Luciferase assays were carried out 24 hours after β -estradiol addition using SteadyGlo reagent (Promega). Activities of primary hits were reconfirmed in further luciferase assays and counterscreened for inhibition of luciferase enzyme (EasyLite kinase reagent, Perkin-Elmer) and nonspecific cell growth inhibition (Celltiter Blue, Promega).

Secondary assays

Hit compounds (20 $\mu\text{mol/L}$) were assayed in transiently transfected HEK293 cells cotransfected with Topflash (Wnt reporter, Upstate), TK-renilla (control), and cytomegalovirus-driven HA-Dvl2-ER expression plasmids. Twenty-four hours after transfection, cells were stimulated with 3 $\mu\text{mol/L}$ β -estradiol or treated with the glycogen synthase kinase-3 (GSK-3) inhibitor 6-bromoindirubin-3'-oxime (BIO; Calbiochem). Luciferase activity was measured after 24 hours using DualGlo reagent (Promega). For deconvolution assays, HEK293 cells were cotransfected with Topflash and CMV-lacZ reporter (control) plasmids together with constitutively active core components of the pathway: $\Delta\text{N-LRP}$, Axin-GID, $\Delta\text{N-}\beta$ -catenin, and VP16-TCF4. Compounds were added 24 hours after transfection, and reporter activity was analyzed a further 24 hours later using BrightGlo and BetaGlo (Promega). Luciferase activity was normalized to β -galactosidase reporter activity. Experiments with c-myc and survivin-luciferase reporters were carried out in HEK293 cells using Axin-GID as the inducer. SW480 cells were cotransfected with Topflash and CMV-lacZ reporter plasmids 24 hours before compound addition. Reporter assays were conducted after a further 24 hours.

Growth inhibition (GI_{50})

Cells were seeded to give a density of 25% confluence after 24 hours in either 96- or 384-well tissue culture plates. Compounds (0.05–100 $\mu\text{mol/L}$) were added at 24 hours; Cell Titre Blue or WST-1 (Roche) reagent was added after 72 hours (HT29, SW480 and HCT116) or 96 hours (SNU475 and CCD841Co).

 β -Catenin stability experiments

Mouse L-cells were cultured in DMEM/10% fetal bovine serum and exposed to 7.5 $\mu\text{mol/L}$ BIO, 9 mmol/L LiCl, 50% Wnt-3a conditioned medium (12), or 50 $\mu\text{mol/L}$ MG-132 (Merck Biosciences), and with compound for 6 hours before medium replacement as indicated. β -Catenin was assayed as previously described (13). The following antibodies were used: β -catenin and GSK-3 β (BD Transduction laboratories) and β -actin (Sigma). The β -catenin antibody from BD Transduction Laboratories was used for immunocytochemistry as previously described (14).

For pulse-chase analyses, L-cells were treated throughout with 7.5 $\mu\text{mol/L}$ BIO. Cells were first starved in medium lacking methionine and cysteine for 1 hour then labeled for 1 hour with 5 MBq per 25 cm^2 flask of ^{35}S -labeled methio-

nine/cysteine. Cells were chased with unlabeled medium for the indicated periods. Cells were lysed, and immunoprecipitation was carried out using 2 $\mu\text{g/sample}$ monoclonal β -catenin antibody and 50 μL of Protein G-coated beads (GE Healthcare). After three washes in lysis buffer, bound proteins were eluted in SDS sample buffer, separated by electrophoresis, and analyzed as previously described (15).

Phosphorylated Tau experiment

HEK293 cells were transfected with GSK-3 β and/or Tau-1N4R expression vectors (16). After 48 hours, cells were exposed to 5 $\mu\text{mol/L}$ BIO for 8 hours, after which they were treated with 20 $\mu\text{mol/L}$ CCT031374 and 5 $\mu\text{mol/L}$ BIO for the indicated time periods. Immunoblotting for GSK-3 β and phospho- and nonphospho-Tau antibodies (BT2, AT270, Autogen Bioclear) was carried out as previously described (16). Band peak areas were calculated using ImageJ (Scion). The phospho-Tau peak areas were normalized to the total Tau levels.

Glutathione S-transferase-E-cadherin pull down of β -catenin

The β -catenin binding region of E-cadherin (amino acids 730–882) was fused in frame with the glutathione S-transferase (GST) coding region of pGEX-5X-2 (GE Healthcare). GST-E-cadherin fusion protein was expressed and purified using Glutathione Sepharose 4 Fast Flow beads (GE Healthcare). Ten-centimeter dishes of HEK293 and SW480 cells were treated with CCT031374 and/or BIO and lysed to give a final volume of 1 mL cell extract. Five micrograms of GST-E-cadherin were combined with 500 μL of cell extract and precipitated following addition of glutathione beads. Bound β -catenin was detected by immunoblotting.

Embryonic stem cell quantitative PCR for LEF1

H9 human embryonic stem cells were cultured on feeders using standard culture methods (17). Using a modification of ref. (17), neurogenic embryoid bodies were generated by culturing H9 colony fragments in suspension in neutralizing medium (ADF, Invitrogen 12634010) containing 10 $\mu\text{mol/L}$ SB431542 (TGF- β inhibitor, Tocris) and 10 $\mu\text{mol/L}$ Y-27632 (ROCK inhibitor, Calbiochem) for the first 48 hours. On day 8, medium was supplemented with BIO (5 $\mu\text{mol/L}$) and/or CCT031374 (20 $\mu\text{mol/L}$) for 24 hours. RNA was extracted with Rneasy (Qiagen). cDNA was synthesized with Superscript II Rnase H-RT (Invitrogen). Quantitative PCR reactions were done in DyNAmoHS master mix (New England Biolabs) using the LEF1 primers 5'-accagattcttggcagaagg-3' and 5'-cagaccagcctggataaagc-3'.

Xenopus studies

Xenopus laevis embryos were obtained, degelled, and cultured using standard procedures (18), and staged according to Nieuwkoop and Faber (NF; ref. 19). Test compound or DMSO (control) was added at the 4- to 16-cell stage. Embryos were scored at NF stages 35 to 38. For the animal cap assay, dissected *Xenopus* embryo animal caps were treated with 300 mmol/L LiCl for 10 minutes followed by 2.5 hours

exposure to CCT036477 at the indicated concentrations or with DMSO control (indicated with dash). RNA extraction and reverse transcriptase-PCR conditions were carried out as described (20).

Zebrafish studies

Zebrafish embryos were collected after natural spawning. Embryos were treated from the 16-cell stage until midgastrulation (8 hours postfertilization) with 20 $\mu\text{mol/L}$ of compound CCT036477 in 1% DMSO/fish water or with DMSO equivalent. Control and experimental embryos were imaged when at the postgastrulation somitic stage (48 hours postfertilization).

Results

Optimization and outcome of high-throughput screen

To identify novel small-molecule regulators of the Wnt signaling pathway, a HEK293-based reporter cell line (7df3) was generated to allow the inducible induction of TCF-dependent transcription. The rationale for this approach was twofold. First, inducible induction of the Wnt pathway was predicted to minimize positive and negative feedback from transcriptional targets, thereby simplifying the process of target deconvolution. Second, the cell line did not require Wnt signaling for proliferation, thereby allowing the rapid distinction of nonspecific growth inhibitors and compounds acting specifically on the Wnt pathway.

A bicistronic reporter coding for firefly luciferase and GFP under the control of a promoter containing four canonical TCF binding sites and the Wnt-responsive region from the *Xenopus Xnr3* promoter (ref. 21; Fig. 1B) was introduced into HEK293 cells that already contained an integrated Disheveled-estrogen receptor fusion (Dvl2-ER; ref. 11). Previous studies showed that estradiol induction of Dvl2 activity raised β -catenin levels within 30 minutes in these cells (11). Clones that showed tight regulation of TCF-dependent transcription in response to estradiol and the GSK-3 inhibitor LiCl (Fig. 1C) were identified by FACS sorting.

Assays in a 384-well format showed reproducible 14-fold and 10,389-fold induction with estradiol and LiCl, respectively, with low coefficients of variation (%CV) and high Z' factors (estradiol, 0.59; LiCl 0.89; Fig. 1C). Importantly, the cell line also showed robust behavior with multiple cellular passages over more than 2 years. For the primary screen, 63,040 chemically diverse compounds were assayed by sequential addition of compound (20 $\mu\text{mol/L}$ in 0.2% DMSO) and estradiol 24 hours before luciferase assay. Data from a subset of 384-well plates from the primary assay (Fig. 1D) illustrate the range of responses to compound addition. The 1,902 compounds that showed >50% inhibition were re-assayed ($n = 2$) and counterscreened to exclude direct inhibitors of luciferase enzyme activity and to remove acutely cytotoxic compounds. At this stage, 306 molecules were selected for subsequent analysis (Fig. 2A).

Hit triage and mechanistic deconvolution assays

To identify molecules that specifically blocked TCF-dependent transcription, compounds with a greater than 5:1 ratio of ac-

tivity against a TCF-luciferase versus a TK-renilla luciferase reporter were selected following cotransfection with a Dvl-2-ER expression plasmid in HEK293 cells. Thirty-seven compounds showed a greater than 5:1 specificity ratio together with low toxicity and high TCF activity, as originally detected in the 7df3 reporter line (Fig. 2A). All 37 hits showed activity against TCF-dependent transcription that was induced by treatment of HEK293 cells with the GSK-3 inhibitor BIO (22).

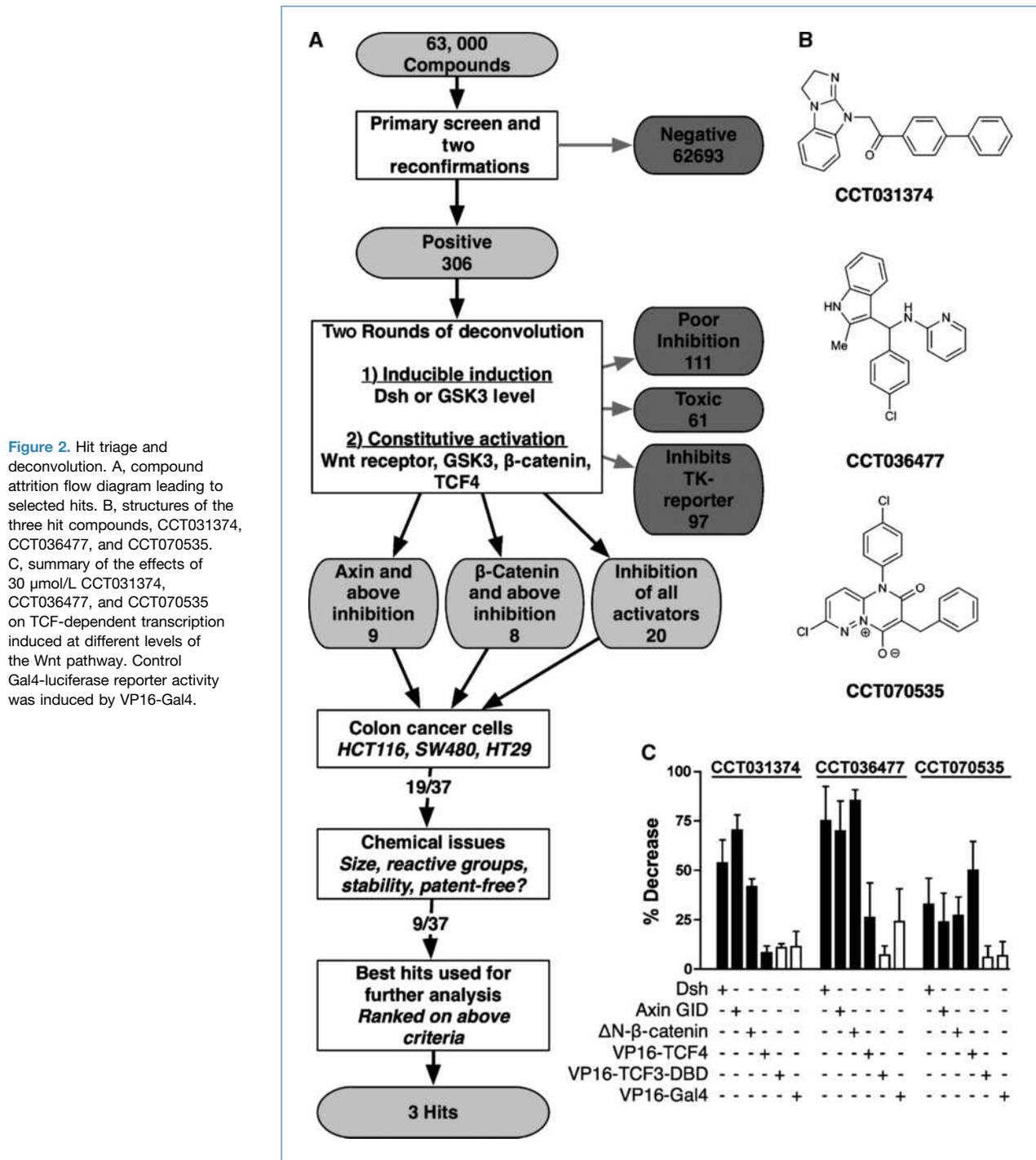
To identify the point in the canonical Wnt signaling pathway at which the compounds acted, the pathway was activated at distinct levels by expression of activating cDNAs (see Fig. 1A). At the receptor level, TCF-dependent transcription was induced by expression of a dominantly active NH_2 -terminal deletion of the LRP6 coreceptor, $\Delta\text{N-LRP6}$ (23). Activation at the Disheveled level involved expression of Dvl-2. At the GSK-3 level, transcription was activated by expression of the GSK-3 binding domain of Axin (Axin-GID), which acts to titrate GSK-3 from the endogenous Axin protein (24). A nondegradable, β -catenin with an NH_2 -terminal deletion ($\Delta\text{N-89-}\beta$ -catenin; ref. 25) activated transcription downstream of the β -catenin turnover complex, and a TCF4-VP16 fusion protein was used to probe compound activity at the level of the nuclear transcription factor. All 37 compounds blocked activation by Axin-GID: 9 of these failed to block $\Delta\text{N-}\beta$ -catenin or TCF4-VP16 (i.e., they act at the level of Axin), a further 8 blocked $\Delta\text{N-}\beta$ -catenin but failed to block TCF4-VP16 (i.e., they act at the level of β -catenin), whereas 20 compounds blocked all three activators (Fig. 2A). Results from a subset of the deconvolution assays are shown for three compounds (Fig. 2B and C).

Hit selection

Selective antiproliferative activity for human tumor cell lines with Wnt pathway-activating oncogenic β -catenin, *APC*, or *AXIN* deletions [HT29 (26), SW480 (27), HCT116 (28), and SNU475 (29)], compared with a nontransformed epithelial cell line, was used as a criterion in combination with chemical tractability to focus on a subset of nine and subsequently three compounds for further analysis (Table 1; Fig. 2B; Supplementary Fig. S1). The set of three compounds (CCT070535, CCT036477, and CCT031374; see Fig. 2B) was selected based on a combination of the following criteria: metabolic stability (compound stability in mouse liver microsomes; Supplementary Fig. S2), low growth-inhibitory activity in nontumor control cells, promoter specificity (TCF versus TK), and the availability of commercially available analogues. A key feature in this selection was the clarity of the deconvolution response to different Wnt pathway activators because unambiguous activity suggested that the mechanism of compound action could be tracked in subsequent assays (Fig. 2C). All three compounds blocked HCT116 human colon cancer cell proliferation by inducing apoptosis as shown by caspase-3 activity assays (Supplementary Fig. S3), but CCT031374 induced almost twice as much caspase activity than the other compounds.

Alteration of β -catenin stability

To assess whether the compounds altered β -catenin levels or localization, mouse L-cells were treated with each



compound together with the GSK-3 inhibitor, BIO, to block β -catenin degradation. Following 8 hours of incubation, control BIO-treated cells showed a strong increase in total β -catenin levels that was associated with raised nuclear and cytosolic pools as determined by immunocytochemistry (Fig. 3A and B). Of the top nine compounds, only CCT031374 prevented BIO-induced accumulation of β -catenin (Fig. 3A).

The blockade of β -catenin accumulation by CCT031374 was accompanied by a reduction in both nuclear and cytosolic β -catenin pools (Fig. 3B; Supplementary Figs. S4 and S5). In U2OS GFP- β -catenin human osteosarcoma cells (Bioimage, Thermo-Fisher), addition of CCT031374 induced formation of GFP- β -catenin aggregates (Supplementary Fig. S4B) possibly due to sequestration by subcellular organelles, a phenotype

Table 1. Inhibition of 7dF3 reporter activity (IC₅₀) and growth inhibition (GI₅₀) of hit compounds

Compound	Reporter IC ₅₀ (μmol/L)	HT29 (APC mutant) GI ₅₀ (μmol/L)	HCT116 (oncogenic β-catenin) GI ₅₀ (μmol/L)	SW480 (APC mutant) GI ₅₀ (μmol/L)	SNU475 (Axin mutant) GI ₅₀ (μmol/L)	CCD841Co (control) GI ₅₀ (μmol/L)
CCT007812	6.2	11.0	11.1	46.7	32.0	44*
CCT014939	20.5	>100	>100	>100	28.5	44*
CCT020435	14.1	10.0	12.5	9.1	ND	44*
CCT028492	2.7	8.0	14.1	11.7	ND	ND
CCT031374	6.1	11.5	13.9	13.2	9.6	44*
CCT036098	21.5	>100	31.9	27.9	17.3	44*
CCT036477	4.6	17.9	17.7	33.3	ND	100*
CCT038407	5.3	3.0	2.3	2.5	21.1	44*
CCT070535	5.1	17.6	11.1	11.8	13.4	44*

NOTE: HT29 (26), SW480 (27), and HCT116 (28) are colon carcinoma cell lines. SNU475 is a hepatocellular carcinoma cell line (29). CCD841Co is a colonic epithelial cell line whose use as a control has been described previously (5).

Abbreviation: ND, not done.

*No growth inhibition at the highest concentration tested (indicated).

that was occasionally observed with endogenous β-catenin in mouse L-cells (Supplementary Fig. S4C). By contrast, CCT036477 did not alter β-catenin levels but blocked transcription at the β-catenin level, although not by blocking the interaction of β-catenin with the histone acetyltransferases CBP or p300 (Supplementary Fig. S6). Compound CCT070535, which blocked TCF-dependent transcription at the TCF level, did not alter BIO-induced levels of β-catenin, but increased the levels of nuclear β-catenin (Supplementary Fig. S4C).

Exploratory studies with CCT031374 series compounds

The IC₅₀ for CCT031374 activity in blocking BIO-induced β-catenin stabilization in L-cells was similar to that determined in the TCF-reporter cell line and transient assays (Fig. 3B; Supplementary Fig. S3A), suggesting that the compound may interfere with TCF-dependent transcription by blocking the function of β-catenin. The activity of several CCT031374 analogues showed both TCF-reporter and BIO/β-catenin L-cell inhibition (Fig. 3D). The biphenyl-keto-methyl analogues (compounds 7, 8, 9, and 10 in Fig. 3C) retained potency in the reporter cell assay comparable with that of CCT031374 (compound 1 in Fig. 3D), but compounds 9 and 10 were significantly less potent in the L-cell β-catenin stabilization assay (Fig. 3D). Compounds 9 and 10 have low permeability in PAMPA assays (Supplementary Fig. S7), which may confound the interpretation of compound activity across different cell types. The remaining compounds (2–6 in Fig. 3C) are less lipophilic close analogues of CCT031374 with equivalent or lower potency (higher IC₅₀ values) in the reporter and mouse L-cell assays. Because of the lipophilic basic nature of the compound series, compounds 11 and 12 (Fig. 3C), with a minimal core scaffold of similar pKa and lipophilicity, were tested as controls. Compounds 11 and 12 did not destabilize β-catenin in mouse L-cells, a finding con-

sistent with the hypothesis that CCT031374 activity on β-catenin levels is not solely due to a nonspecific effect driven by the highly lipophilic and basic compounds.

To further characterize the mechanism of action of CCT031374, a series of time-course analyses were done (Fig. 4A; Supplementary Fig. S8A,B). Although there seemed to be a transient increase of β-catenin (Fig. 4A), this was due to change of medium as it was also seen in the control analysis (Supplementary Fig. S8A). When the β-catenin band peak areas were normalized to that of loading control (Supplementary Fig. S8B), addition of CCT031374 was seen to reduce levels of BIO-stabilized β-catenin within 60 minutes. Pulse-chase analyses showed that CCT031374 increased β-catenin degradation, within as little as 10 minutes of compound addition (Fig. 4A). By contrast, β-catenin stabilized with the proteasome inhibitor MG132 was not degraded upon addition of CCT031374, suggesting that proteasomal degradation may act downstream of CCT031374 in lowering β-catenin levels (Fig. 4A). Surprisingly, β-catenin stabilized by treatment of cells with soluble Wnt-3A and LiCl (9 mmol/L) was resistant to degradation induced by CCT031374 (Supplementary Fig. S9). Direct comparison was not straightforward because Wnt-3A and LiCl induction resulted in significantly lower levels of β-catenin than with BIO induction (Fig. 4A).

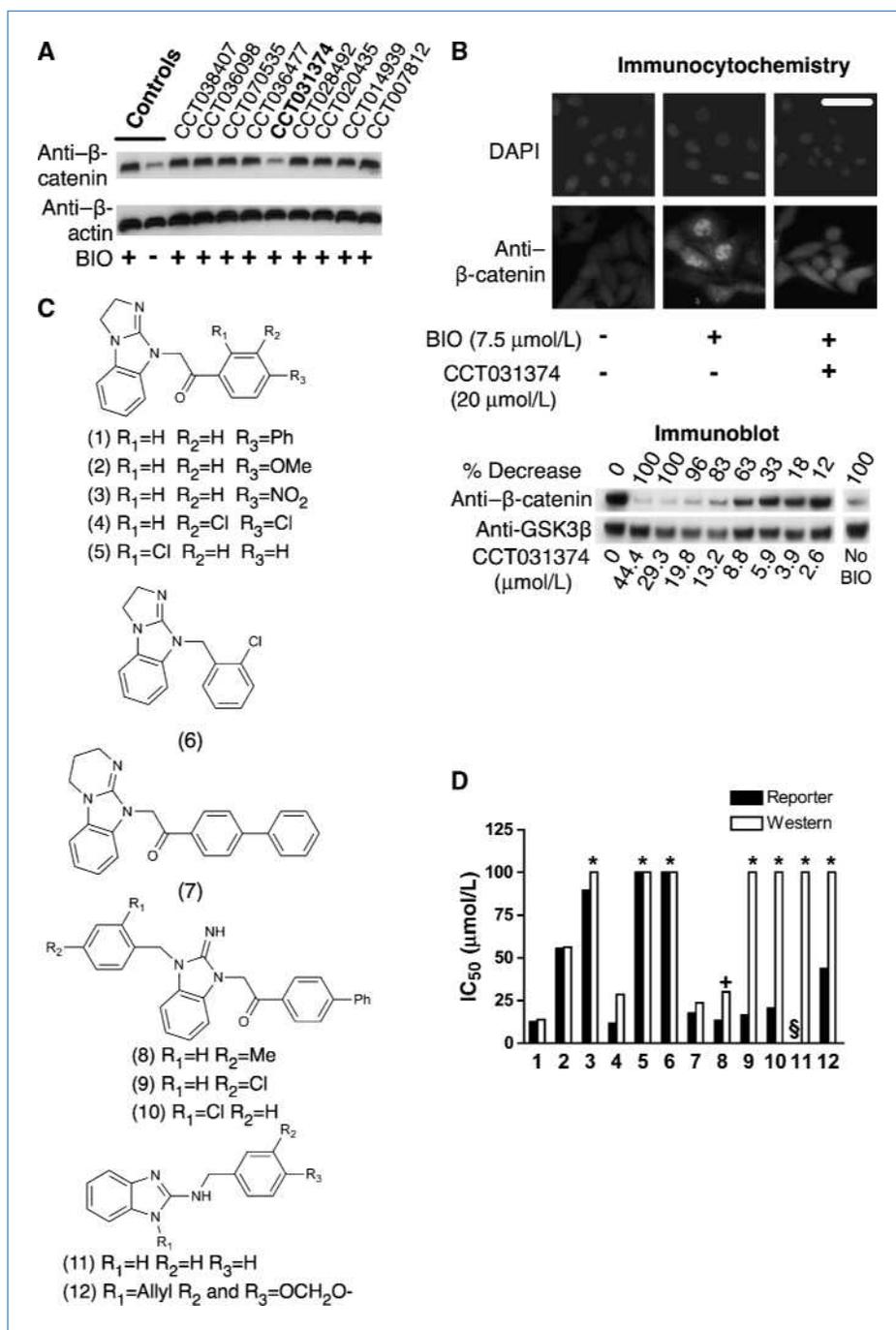
To determine whether CCT031374 reversed the action of BIO against other GSK-3 targets, an assay for GSK-3-dependent Tau phosphorylation was used (Fig. 4B). As expected, BIO was able to reduce GSK-3-dependent Tau phosphorylation on Thr-181, a well-characterized GSK-3 phosphorylation site (30). The addition of CCT031374 largely failed to reduce the BIO block of Tau phosphorylation, although there was a minor reversal of BIO action after 2 hours of treatment. This low-level “response” to CCT031374 occurred much later than the effect of CCT031374 on β-catenin levels, suggesting that the action against β-catenin was a

strong, immediate response to compound action (Fig. 4B). Consistent with the Tau phosphorylation data, CCT031374 did not inhibit recombinant GSK-3 in a kinase assay (Supplementary Fig. S10). In addition, CCT031374 had no activity in biochemical assays for PP1, PP2A, and tyrosine phosphatase enzyme activity (Supplementary Fig. S11).

As CCT031374 blocked the growth of a number of cancer cell lines, including SW480 colon cancer cells (Table 1), it was anticipated that the compound would reduce free β -catenin

levels within these cells. Surprisingly, a pull-down assay with GST-E-cadherin to detect free β -catenin levels found that these were not altered by CCT031374 (Fig. 4C). By contrast, CCT031374 blocked free β -catenin levels in BIO-stimulated HEK293 cells (Fig. 4C). Despite the lack of effect on β -catenin levels, CCT031374 reduced TCF-dependent transcription in SW480 cells (Fig. 4D). Similarly, CCT031374 did not alter levels of free β -catenin resulting from overexpression of Δ N- β -catenin or Axin-GID in HEK293 cells (Supplementary

Figure 3. Prevention of β -catenin stabilization by CCT031374. A, β -catenin abundance in Western blots of lysates from mouse L-cells treated for 6 h with 7.5 μ mol/L BIO and 20 μ mol/L of the indicated hit compounds. B, decreased abundance of β -catenin in immunostained mouse L-cells and in Western blots of lysates from mouse L-cells treated with indicated concentrations of CCT031374 and 7.5 μ mol/L BIO for 8 h. Decreases of relative β -catenin/ β -actin ratios in the Western blot were normalized to BIO (100%) and control (0%). Bar, 30 μ m. C, structures of analogues of CCT031374. CCT031374 is compound 1. EN300-05350 (compound 11) is a structurally unrelated control of similar lipophilicity, pKa, and H-bonding. D, IC_{50} values from reporter cell assays and Western blots of lysates from mouse L-cell using CCT031374 and its analogues. *, no effect at the maximum concentration used; +, compound 8 was toxic to mouse L-cells at this concentration (30 μ mol/L); §, assay not done.



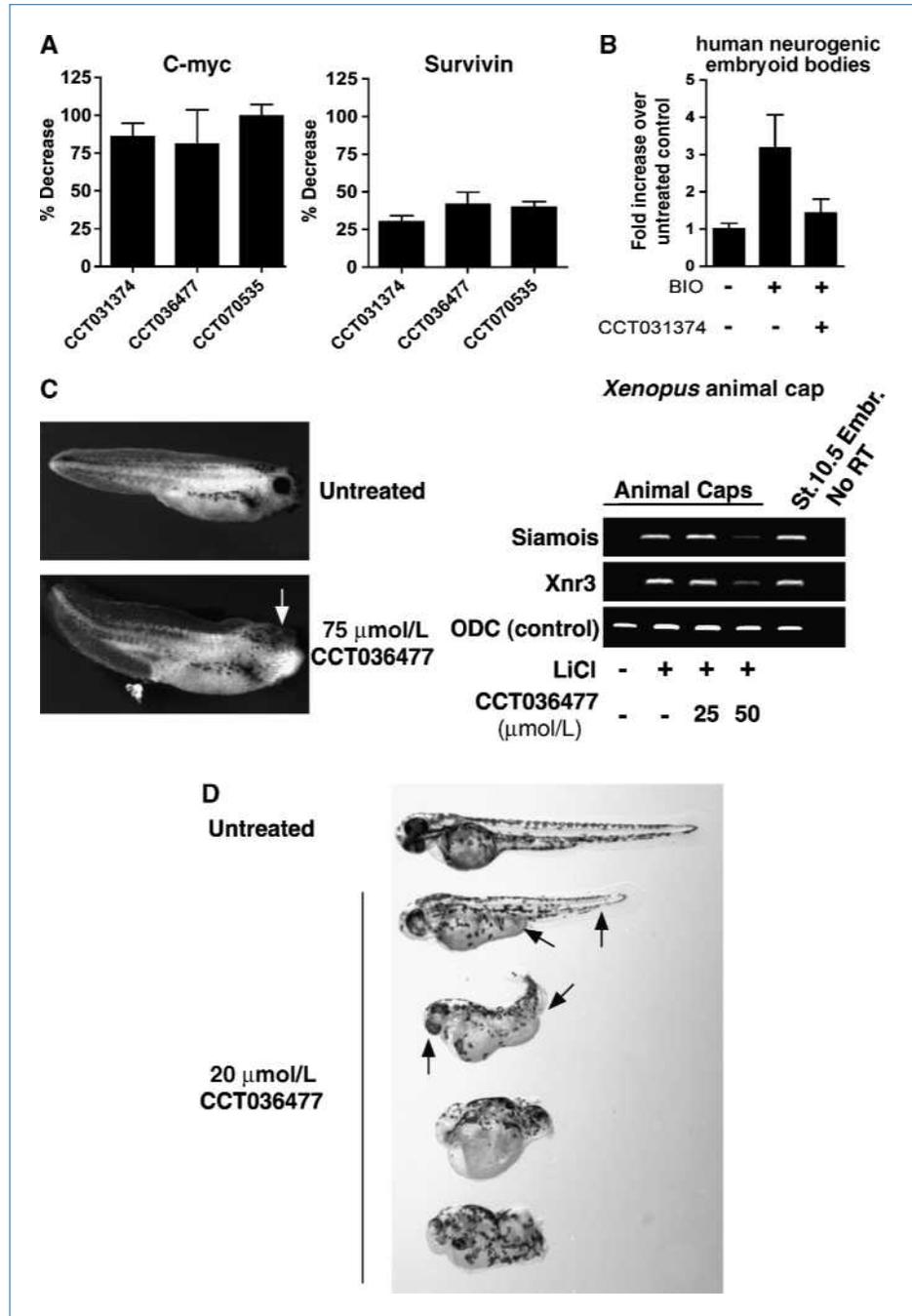
embryos at the 16-cell stage also induced axis defects, leading to phenotypes (Fig. 5D) that have been associated with alterations to Wnt signaling (32).

Discussion

In the current study, we showed that a highly sensitive Wnt reporter cell line can be used to identify small-molecule inhibitors of Wnt signaling. The cell-based screen described here was similar to previous studies by Emami and collea-

gues (5), Huang and colleagues (7), and Chen and colleagues (3) in that it relied on the identification of compounds that blocked the activity of an integrated TCF-luciferase reporter. However, the present study used a reporter cell line that had an inactive basal TCF-reporter that could be induced through the activation of a Dishevelled-estrogen receptor fusion protein following addition of estrogen. The advantage of this strategy is that Wnt signaling can be transiently induced and that the cell response to inhibition of the pathway is less likely to be dependent on complex positive and negative

Figure 5. Effect of compounds on Wnt target gene expression and development. **A**, HEK293 cells were transfected with *CMYC*-luc or *SURVIVIN*-luc promoter-reporter constructs together with Axin-GID inducer and exposed to 40 $\mu\text{mol/L}$ compound for 24 h. Reporter activity was normalized to expression from a cotransfected CMV-lacZ reporter. **B**, CCT031374, at 20 $\mu\text{mol/L}$, repressed BIO-induced *LEF1* expression in human neurogenic embryoid bodies exposed to 3 $\mu\text{mol/L}$ BIO and CCT031374 for 24 h. The relative abundance of *LEF1* was normalized to *ACTB*. CCT036477 decreased *Siamois* and *Xnr3* expression in *Xenopus* animal cap assays. Animal caps were treated with 0.3 mol/L LiCl for 10 min, then incubated in medium with or without CCT036477 for 3 h. RNA from NF stage 10.5 embryos provided a second positive control. **C**, CCT036477 inhibited the development of *Xenopus* anterior head structures such as the eye (indicated with an arrow) and the cement gland. Embryos were exposed to DMSO or 75 $\mu\text{mol/L}$ CCT036477 from the 4- to 16-cell stage to NF stage 38. **D**, CCT036477 induced head and tail patterning defects (indicated with arrows) in zebrafish embryos. Zebrafish embryos were exposed to 1% DMSO or 20 $\mu\text{mol/L}$ CCT036477 from fertilization until midgastrulation. A range of mild to severe head patterning and tail patterning phenotypes were seen.



feedback pathways commonly found in cancer cell lines (33–35). Furthermore, cell lines whose growth is dependent on the Wnt pathway have been shown to undergo apoptosis (as seen for CCT031374, CCT036477, and CCT070535; Supplementary Fig. S3) following Wnt pathway inhibition, and this can be difficult to distinguish from nonspecific cell killing during high-throughput screening.

Multiple compounds were identified in the primary screen that showed specificity for the TCF-reporter when compared with control promoters. A hit triage/deconvolution cascade was designed to identify the most promising candidates among these compounds for further study. These assays included growth-inhibitory activity against tumor cell lines with activated Wnt signaling. However, a key criterion was the “clarity” of the deconvolution response such that reproducible, mechanistically consistent readouts were identified, which allowed the logical pursuit of the mechanism of action of the compound to distinct points in the Wnt pathway. From a total of 63,040 compounds, 9 compounds that operated at either the β -catenin or TCF levels of the pathway were selected for further investigation. Downstream assays focused on 2 compounds that operated at the β -catenin level and 1 that operated at the TCF level.

CCT031374 acted at the β -catenin level based on the observation that it blocked TCF-dependent transcription induced by a stabilized form of β -catenin (NH₂-terminal deletion of 89 amino acids; ref. 36), but not by a constitutively active TCF-VP16 fusion protein. Consistent with this mechanism, CCT031374 increased the degradation of endogenous wild-type β -catenin in mouse L-cells that had been stabilized by the GSK-3 inhibitor BIO. CCT031374 did not increase β -catenin NH₂-terminal phosphorylation in the presence of BIO-treated cells (data not shown), suggesting that a pathway independent of phospho- β -catenin binding to β -TRCP induced increased degradation. A number of alternative β -catenin degradation routes have been described. These include pathways involving APC and the Siah1 ubiquitin ligase (37, 38), the presenilin/PKA complex (39), and the protease calpain (40). CCT031374 failed to induce β -catenin degradation products characteristic of calpain-induced proteolysis (ref. 40; data not shown), suggesting that distinct novel pathways are involved in its mechanism of action.

Surprisingly, the block of TCF-dependent transcription in SW480 colon cancer cells by CCT031374 was not accompanied by a corresponding reduction in β -catenin protein levels (Fig. 4F). Moreover, levels of transfected stabilized β -catenin (Δ N-89- β -catenin) were not reduced in HEK293 cells treated with CCT031374 (Supplementary Fig. S12), although the compound blocked TCF-dependent transcription in these cells (Fig. 2B). Taken together, these data argue that the degradation of β -catenin may not be necessary for the blocking effect

of CCT031374 on TCF-dependent transcription in all cellular contexts, suggesting that CCT031374 operates through more than one mechanism. A similar correlation between β -catenin stability and TCF-dependent transcription was observed in studies of the prolyl oligopeptidase Pin-1. Pin-1 activity correlated with both β -catenin stability and TCF-dependent transcription in HeLa cells by modulating its interaction with APC (41). By contrast, in PTEN-deficient LNCaP cells, Pin-1 altered TCF-dependent transcription without altering β -catenin levels by blocking β -catenin interactions with the androgen receptor (42). CCT031374 may similarly function through molecular pathways that can be coupled to protein degradation in selected contexts.

Of the three compound series studied, only CCT036477 showed clear activity *in vivo* where it blocked development of zebrafish and *Xenopus* embryos and expression of Wnt target genes. CCT031374 and CCT070535 did not show clear responses in these systems, and it is currently unclear whether the compounds reached the required concentration to block signaling in these *in vivo* models or whether their lack of *in vivo* activity reflects a specificity for cells with oncogenically activated Wnt signaling. Overall, these studies have identified novel small molecules that target distinct levels of Wnt signal transduction pathway. Further studies with these compounds (see Supplementary Fig. S13) could lead to the identification of molecular targets of therapeutic benefit in multiple tumor types with activated Wnt signaling, and further investigations will be published in due course.

Disclosure of Potential Conflicts of Interest

T. Dale: commercial research grant, Merck Serono. Authors affiliated to the Cancer Research UK Centre for Cancer Therapeutics are employees of the Institute of Cancer Research, which has a commercial interest in inhibitors of the Wnt pathway and has a funded research collaboration with Merck Serono, from which authors may benefit from a rewards to inventors scheme. The other authors disclosed no potential conflicts of interest.

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