Small-molecule antagonists of the oncogenic Tcf/β-catenin protein complex

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Summary

Key molecular lesions in colorectal and other cancers cause β-catenin-dependent transactivation of T cell factor (Tcf)-dependent genes. Disruption of this signal represents an opportunity for rational cancer therapy. To identify compounds that inhibit association between Tcf4 and β-catenin, we screened libraries of natural compounds in a high-throughput assay for immunoenzymatic detection of the protein-protein interaction. Selected compounds disrupt Tcf/β-catenin complexes in several independent in vitro assays and potently antagonize cellular effects of β-catenin-dependent activities, including reporter gene activation, *c-myc* or *cyclin D1* expression, cell proliferation, and duplication of the *Xenopus* embryonic dorsal axis. These compounds thus meet predicted criteria for disrupting Tcf/β-catenin complexes and define a general standard to establish mechanism-based activity of small molecule inhibitors of this pathogenic protein-protein interaction.

Introduction

Each year nearly 150,000 patients are diagnosed with colorectal cancer in the United States alone, and over 55,000 Americans die of this disease (http://seer.cancer.gov). Besides surgical resection, which is rarely curative in advanced disease, current therapy for colon cancer relies on traditional cytotoxic agents with limited effects. These considerations make it important to develop new treatments based on the appreciation of pathogenic molecular lesions. Mutations that drive colorectal cancer are among the best characterized of any human tumor (Kinzler and Vogelstein, 1996; Markowitz et al., 2002) and present opportunities to fashion treatments based on biochemical mechanism. When a similar approach has been applied toward chronic myelogenous leukemia, a disease for which the defining molecular lesion is known, drug development has had a significant impact on patient outcomes (Druker et al., 2001).

The signaling pathway regulated by Wnt ligands, the adenomatous polyposis coli (APC)-axin complex, and β-catenin is invariably disrupted in colorectal tumors and commonly affected by mutation in other cancers (de La Coste et al., 1998; Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997; Satoh et al., 2000). The net result of these disruptions is stabilization of β-catenin, a multifunctional protein whose principal importance in cancers lies in its ability to bind Tcf/LEF family transcription factors to activate gene expression (van Noort and Clevers, 2002). Gene targets of β-catenin-regulated transcription (CRT) include *c-Myc* and *cyclin D1* (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999; van de Wetering et al., 2002), which have plausible or demonstrated roles in malignancy. Because colon cancer most frequently results from loss-of-function mutations in the *APC* gene (Kinzler and Vogelstein, 1996; Sparks et al., 1998), which operates as a negative regulator immediately upstream of β-catenin, few signaling components afford opportunities for pharmacologic intervention. Within this restricted field of candidates, the interaction between β-catenin and Tcf/LEF factors represents a rational and feasible target for drug development. Tcf4 is the factor most prominently ex-

SIGNIFICANCE

Colorectal cancer is a major cause of death in the West. Despite significant advances in appreciation of the underlying molecular mechanisms, current treatments consist exclusively of surgery and conventional chemotherapy, which confer limited benefit. As a basis for future drug development, we sought to target an early event in tumorigenesis, the critical protein-protein interaction between β-catenin and Tcf transcription factors. We have identified a group of small molecules that disrupt the Tcf/β-catenin association, an activity that we confirmed in a series of independent assays. The isolated compounds provide valuable reagents for drug development and for detailed investigation of β-catenin-dependent cellular pathways. Our approach also highlights the feasibility of targeting protein-protein interactions in cancer drug discovery.

pressed in colorectal cancers (Korinek et al., 1997) and also required for gut crypt stem cell differentiation (Korinek et al., 1998). Tcf4/β-catenin complexes determine cellular responses in part by controlling expression of *c-Myc* and *p21*$^{CIP1/WAF1}$ (van de Wetering et al., 2002) and signaling between EphrinB1 and its receptors EphB2 and EphB3 in the gut epithelium (Batlle et al., 2002).

Recent studies reveal that tumors rely throughout their natural history on dysregulation imposed by early molecular lesions (Chin et al., 1999; Felsher and Bishop, 1999; Fisher et al., 2001), and interference with CRT can revert transformed phenotypes in colon cancer. Deletion of the mutant β-*catenin* allele in HCT116 cells abrogates constitutive CRT and reduces cell growth and survival (Chan et al., 2002), whereas overexpression of a dominant inhibitory form of Tcf4 induces G1 cell cycle arrest in Ls174T and DLD-1 cells, regardless of the presence of additional (e.g., *Trp53*) mutations (van de Wetering et al., 2002). Suppression of CRT did not ostensibly affect DLD-1 cell growth in an independent study but restored epithelial cell polarity (Naishiro et al., 2001). Thus, although consequences of dysregulated CRT remain under investigation, disrupting Tcf/β-catenin complexes is likely to convey therapeutic benefit.

Here we report identification of low molecular weight compounds that disrupt Tcf/β-catenin complexes. Using independent assays, we evaluate the specificity of inhibition and establish that two compounds function through the predicted mechanism in vitro and in vivo. The results illustrate an effective strategy to identify and test drugs directed against protein-protein interactions, and they outline criteria for compounds to be regarded as antagonists of distal steps in the Wnt signaling pathway. Further refinements in drug design can build on these studies, possibly in conjunction with insights obtained from the crystal structure of Tcf4/β-catenin (Graham et al., 2001; Poy et al., 2001) and alternative protein complexes.

Results

A screen for small-molecule inhibitors of the Tcf/β-catenin complex

The interaction between Tcf factors and β-catenin requires a minimal N-terminal Tcf fragment and the central domain of 12 armadillo repeats in β-catenin (Graham et al., 2000; Molenaar et al., 1996; Omer et al., 1999). To identify inhibitors of this interaction, we developed a binding assay for high-throughput screening (HTS). Purified β-catenin (amino acids 134–668) was coated on microtiter plates and incubated sequentially with a Tcf4 fragment (residues 8–54) fused to glutathione-*S*-transferase (GST), anti-GST antibody, and alkaline phosphatase (AP)-conjugated secondary antibody (Figure 1A). Compounds that disrupt the Tcf/β-catenin complex thus register reduced AP values relative to the background.

In surface plasmon resonance studies, β-catenin binding was significantly greater in the presence of tethered GST-Tcf4 compared with unrelated GST-fusion proteins (data not shown). Four irrelevant proteins (histidine-tagged SMC1, SMC3 or I-κB kinase, and Hsp70) did not affect Tcf4/β-catenin interaction at concentrations exceeding 100 μg/ml, whereas soluble β-catenin showed dose-dependent inhibition with IC_{50} (concentration giving 50% of the maximal inhibition) ~1 μg/ml or 19 nM (Figure 1B and data not shown), in agreement with previous estimates (Fasolini et al., 2003). Dimethyl sulfoxide (DMSO), the

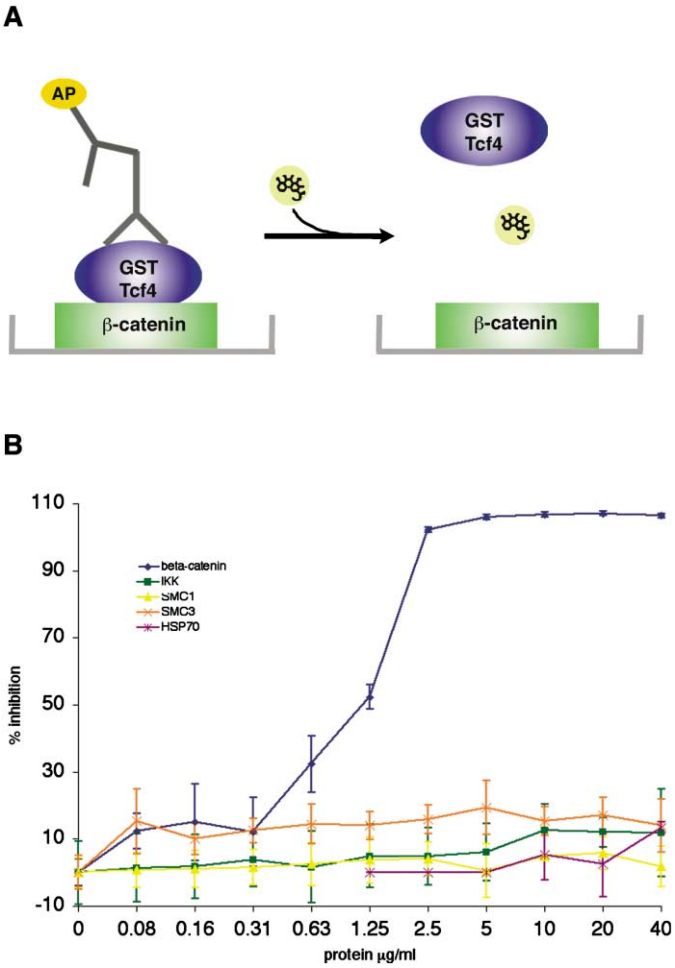

Figure 1. Antagonists of the Tcf4/β-catenin association isolated in a high-throughput screen

A: Schematic illustration of the Tcf4/β-catenin ELISA. A β-catenin fragment (residues 134–668) adsorbed to ELISA plates was exposed to GST-Tcf4 (residues 8–54). Bound protein was detected with goat anti-GST antibody, alkaline phosphatase (AP)-coupled anti-goat IgG, and AP substrate. Molecules that disrupt the Tcf4/β-catenin complex yield reduced AP signals.

B: Inhibition of Tcf4/β-catenin association, monitored as described in the Experimental Procedures, in the presence of various concentrations of recombinant fusion proteins: SMC1 (yellow triangle), SMC3 (ochre ×), HSP70 (violet asterisk), IkB kinase (green square), or β-catenin (blue diamond). The percent inhibition of β-catenin binding is expressed as a function of the concentration of soluble protein used as competitor. Only β-catenin behaved competitively in this context.

solvent for the chemical libraries, did not interfere with the assay (data not shown). Antagonism of Tcf4 binding by representative groups of chemicals showed a normal distribution, with 13% standard deviation (SD). These results support our use of a nonhomogeneous, microplate-based binding assay to screen chemical libraries; we chose inhibition >3 SD of mean binding as the criterion for activity.

We screened approximately 7000 purified natural compounds from proprietary and public collections, initially at 10 μM concentrations. Eight compounds displayed reproducible, dose-dependent inhibition of the Tcf4/β-catenin interaction with $IC_{50} < 10$ μM, and their structures and purity were confirmed by

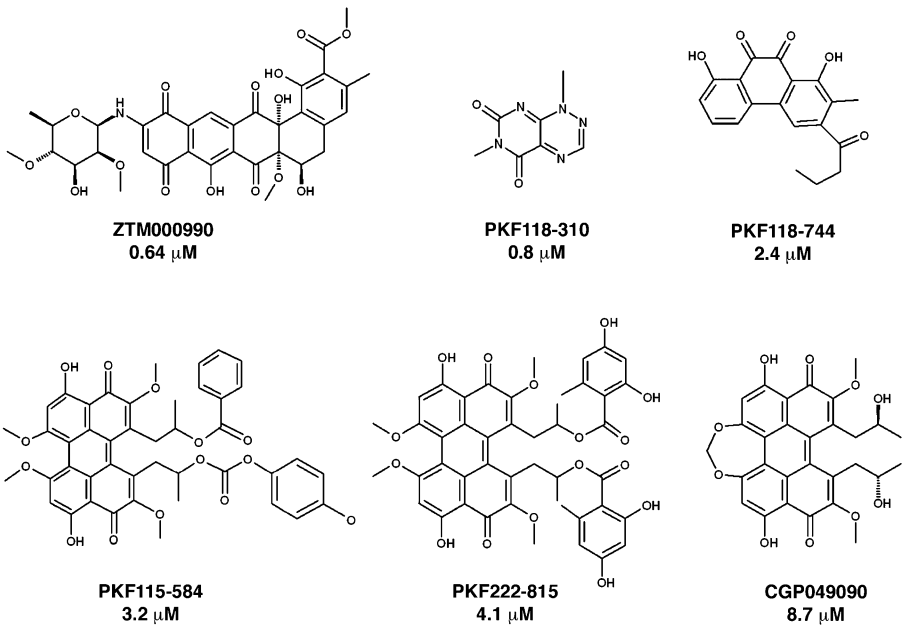

Figure 2. Chemical structures of inhibitors of the Tcf4/β-catenin interaction

The activity of six compounds in the ELISA, measured in confirmatory assays following the HTS, is expressed as the concentration (μM) of compound required to inhibit the Tcf4/β-catenin association by 50% (IC_{50}). Structures of two compounds that we did not characterize extensively and of two compounds used in specificity studies are shown in Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/1/91/DC1.

high-performance liquid chromatography and high-resolution mass spectrometry (Figure 2 and Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/1/91/DC1). This group of compounds shows diverse structural properties, although several of them share polyhydroxylated planar features; the stereochemistry, where shown, is taken from published studies (Takeda et al., 1988). Six compounds are of microbial origin: PKF115-584, CGP049090, and PKF222-815 were isolated from fungal organisms, whereas PKF118-744, PKF118-310, and ZTM000990 originated in Actinomycete strains. NPDDG39.024 was isolated from the marine sponge *Fascaplysinopsis reticulata* (Jimenez et al., 1991), and NPDDG1.024 derives from National Cancer Institute collections (NSC#618650-F/1) without a listed source organism. We identified no additional active molecules among 45,000 synthetic compounds from Novartis archival collections.

Individual compounds isolated in a single HTS are likely to vary in tests of function and no single test may provide definitive evidence of specific biological effects. We evaluated the activities of six candidate compounds (Figure 2) in antagonizing β-catenin-dependent processes using a range of independent assays.

Independent confirmation of disrupted Tcf/β-catenin complexes

We incubated protein extracts from HCT116 colon cancer cells with compounds identified in the HTS and used a GST-Tcf4 fusion protein to isolate β-catenin by affinity chromatography (Figure 3A). Three compounds (PKF115-584, CGP049090, and PKF222-815) eliminated binding and two others (PKF118-310 and NPDDG39.024) interfered measurably with β-catenin precipitation. Active compounds showed dose-dependent inhibition, with IC_{50} between 10 μM and 30 μM (Figure 3B). These data confirm the HTS results and reveal subtle differences in the efficiencies with which individual compounds score in the two biochemical assays. These differences may reflect interaction of the compounds and/or the Tcf/β-catenin complex with other proteins in the cell extracts or differences in target susceptibility between endogenous β-catenin and the shorter fragment used in the HTS. We also examined the effects of three potent compounds on two other protein interactions, CyclinA-E2F1 (Shirodkar et al., 1992) and Bcl-xL/Bax (Yang et al., 1995). Absence of inhibition of either complex (Figure 3B and data not shown), other than a trivial decrease in CyclinA precipitation at the highest concentration of PKF115-584, suggests that these chemicals do not disrupt protein interactions indiscriminately.

We used electrophoretic mobility shift assays (EMSA) in independent biochemical tests of five compounds. EMSA poses a challenge in colorectal cancer cells, where the abundance of Tcf factors and β-catenin restricts resolution of individual DNA-protein complexes (Korinek et al., 1997; Morin et al., 1997). Although we could detect dose-dependent effects of small molecules despite this limitation, protein targets were revealed more clearly in transfected epithelial cells. Nuclear extracts from COS-7 cells transfected with Tcf4 and Flag epitope-tagged β-catenin retarded gel migration of a Tcf-consensus DNA probe, as expected, and four compounds completely blocked appearance of the Tcf4/β-catenin complex bound to DNA (Figure 3C). PKF118-744 and PKF118-310 did so selectively, whereas PKF222-815 and PKF115-584 interfered with the faster migrating complex between Tcf4 and DNA. The latter effect is independent of β-catenin, as it occurred when cells were transfected only with Tcf4 or with a Tcf4 construct deleted for 53 N-terminal residues (data not shown). Whereas the results with PKF222-815 and PKF115-584 are hence less informative with respect to higher-order Tcf complexes, they may suggest that these compounds interact with Tcf4. Inhibition of β-catenin:Tcf4:DNA complexes by CGP049090, which is active in other biochemical tests (Figure 3A), was consistent but incomplete.

None of the active agents blocked additional complexes detected on the Tcf-consensus probe (Figure 3C), implying a degree of specificity in their action. To extend this specificity,

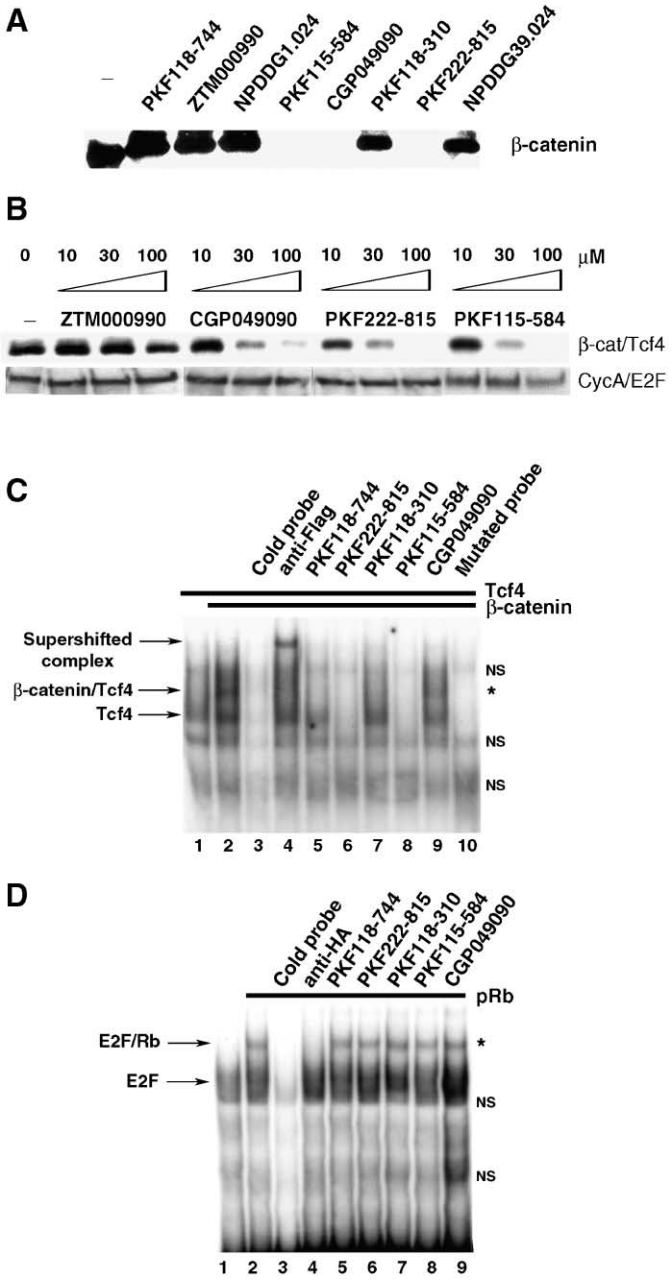

Figure 3. Compounds isolated in the HTS inhibit Tcf4/β-catenin interaction in independent assays

A: Precipitation of cellular β-catenin by a GST-tethered Tcf4 fragment. HCT116 cell extracts were incubated in the absence (−) or presence (100 μM) of the indicated compounds and recombinant GST-Tcf4 fusion protein. Precipitated β-catenin levels were assessed by SDS-PAGE and immunoblotting. Many ELISA-active compounds attenuated the Tcf4/β-catenin interaction, whereas PKF115-584, CGP049090, and PKF222-815 abrogated binding totally.

B: Inhibition of the interaction with GST-Tcf4 is dose dependent. Increasing concentrations (10, 30, and 100 μM) of the indicated compounds inhibited the Tcf4/β-catenin interaction with increasing efficiency. Vehicle-treated sample (−) provides a control and little inhibition was observed for other protein-protein interactions tested under identical conditions, including that between Cyclin A and the E2F1 transcription factor.

C: Electrophoretic mobility shift assay (EMSA) with COS-7 cell extracts expressing full-length Tcf4 and Flag epitope-tagged β-catenin. 2.5 μg of nuclear extract expressing Tcf4, alone (lane 1) or mixed with 1.5 μg of nuclear extract expressing exogenous β-catenin (lanes 2–10), were incubated with radiolabeled Tcf-consensus oligonucleotide (lanes 1–10) or mutated (lane 11) probes. All samples contained 1% DMSO. Nonspecific DNA-protein associations (NS) are revealed through the pattern seen with the mutated probe (lane 11) or in the presence of excess (100×) unlabeled probe (lane 3), and a supershifted complex is seen with inclusion of anti-Flag antibody in the reaction (lane 4). This latter signal defines the Tcf4/β-catenin complex indicated by an arrow to the left and * to the right.

D: Control EMSA with COS cell extracts expressing HA epitope-tagged retinoblastoma protein pRb, showing binding to a radiolabeled probe specific for E2F-family proteins, which are expressed endogenously. Experimental conditions and labeling of lanes and complexes is as indicated in **B**.

we studied effects of the five compounds on other interactions. Association of pRb-E2F (Figure 3D) and PPARγ-RXR (data not shown) complexes with their cognate DNAs was unaffected. Compounds identified in the HTS thus disrupt formation of Tcf/β-catenin complexes with demonstrable specificity.

Inhibition of biological markers of Tcf4/β-catenin transactivation

To investigate biologic activity of these compounds, we performed CRT reporter gene assays in HCT116 cells. Because cytotoxicity was evident over 72 hr of compound treatment (see below), we measured reporter gene activation at 24 hr. Moreover, we interpreted CRT results strictly in relation to the activity of a Tcf-independent β-galactosidase reporter, which served as an internal control for both DNA transfection efficiency and cell viability and was not impaired by any test compound. As expected, transient transfection of a firefly luciferase reporter regulated by three tandem Tcf-consensus binding sites yielded 60-fold induction relative to a control reporter mutated at each Tcf binding site (data not shown). Compounds isolated in the HTS inhibited reporter gene activation significantly (Figure 4A), whereas six independent control compounds, including some that resemble the test compounds in chemical structure, did not affect luciferase expression (Figure 4A and data not shown). Inhibition of the reporter gene by the active compounds was dose dependent (Figure 4B). These results imply that compounds isolated in the HTS interfere with the targeted signaling pathway.

To extend these studies on expression of genes known to be regulated naturally by Tcf4 and β-catenin in human cells, we monitored levels of the CRT-responsive proteins Cyclin D1 and c-Myc (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999; van de Wetering et al., 2002). We treated HCT116 cells with the compounds and assessed target expression levels by immunoblot analysis (Figure 4B). PKF115-584 and CGP049090 abrogated expression of both targets, whereas ZTM000990, PKF118-310, and NPDDG1.024 reduced levels of either c-Myc or cyclin D1; PKF118-744 did not affect either target (Figure 4C). No compound affected Cyclin E levels significantly, suggesting a selective effect on β-catenin targets. Although HCT116 cells carry the β-*catenin* ΔS45 mutation, which prevents protein degradation (Liu et al., 2002; Morin et al., 1997), conceivably the active compounds could activate some alternative pathway to destroy β-catenin. As evidence against this possibility, and consistent with a selective effect, test compounds did not reduce cellular β-catenin levels (Figure 4C).

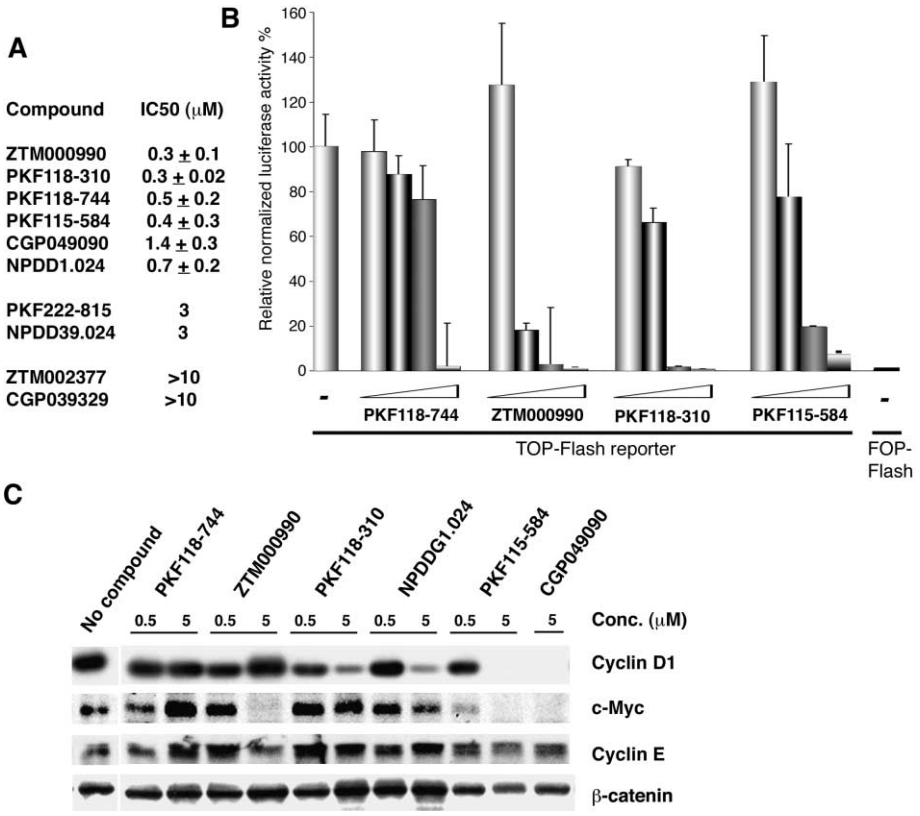

Figure 4. Specific inhibition of molecular correlates of CRT

A and B: Inhibition of β-catenin-dependent transactivation of a Tcf-dependent luciferase reporter gene. HCT116 cells were cotransfected with plasmid encoding *E. coli* β-galactosidase (a control for transfection efficiency) and either the TOP-FLASH or FOP-FLASH reporters. Cells were incubated with different concentrations (0.125–10 μM) of the compounds and harvested at 24 hr to measure luciferase and β-galactosidase activities. Reporter gene activation is expressed in terms of relative light units (RLU) detected in TOP-FLASH- or FOP-FLASH-transfected cells and normalized for β-galactosidase activity. Results represent ≥3 independent experiments with each test compound and are shown in **A**. Panel **B** shows that CRT inhibition was dose dependent. β-galactosidase activity was not reduced by compound exposure, excluding cytotoxicity as a trivial explanation for the results.

C: Compounds selectively inhibit the endogenous Tcf/β-catenin target genes *cyclin D1* and *c-Myc*. HCT116 cells were incubated for 24 hr with the chemical compounds (0.5 or 5 μM) and cyclin D1, c-Myc, cyclin E, and β-catenin protein levels were determined by immunoblotting.

Validation of inhibitor activity in vivo

Early in *Xenopus* development, β-catenin accumulates on the prospective dorsal side (Larabell et al., 1997); accurate formation of a dorsal body axis requires nuclear β-catenin and activation of Tcf/β-catenin target genes such as *Siamois* (Brannon et al., 1997). Introducing β-*catenin* mRNA on the future ventral side activates the Wnt pathway ectopically and induces a secondary body axis (Figure 5B, compare β-catenin-injected tadpoles with untreated siblings). This developmental pathway thus provides a rigorous means to test biologic effects of compounds that might target Tcf/β-catenin compexes.

200 pg of ventrally expressed β-*catenin* mRNA induced dorsal axis duplication in ~80% of embryos (Figure 5A). PKF222-815 and PKF118-744 had little or no effect, respectively, on this result (data not shown), even at doses that caused excessive embryo death. In contrast, co-injection of β-*catenin* mRNA with 0.5 pmol PKF118-310, 3.5 pmol PKF115-584, or 0.25 pmol CGP049090 abrogated axis duplication almost completely, with no contribution from the vehicle, DMSO (Figure 5A). Except for a modest increase in embryo death rates with CGP049090, toxic effects at these doses were not significant (Figure 5A), and each compound restored normal tadpole development in most embryos (Figure 5B). The three compounds also failed to alter normal development through tadpole stages in the absence of ectopic β-*catenin* (data not shown), which in part likely reflects limited cytoplasmic diffusion of injected molecules (Drummond et al., 1985).

To exclude an effect resulting from interference with translation of injected transcripts, we introduced each active compound together with mRNA encoding the green fluorescent protein. Embryo fluorescence was the same with injection of the compounds as without (Figure 5C). To establish the specificity of inhibited β-catenin activity, we co-injected each compound with *siamois* mRNA, a target and immediate downstream effector of Wnt signaling in *Xenopus* (Brannon et al., 1997). Siamois's ability to induce a secondary dorsal axis was not affected by any of the three compounds (Figures 5D and 5E). The active compounds thus disrupt axis duplication by interfering with β-catenin at a step proximal to *Siamois* activation and achieve their effects with minimal developmental toxicity.

Tests for antiproliferative and Tcf-specific activities

Our results highlight the likely specific activity of two or three compounds in disrupting Tcf/β-catenin complexes to predictable biologic effect. To profile cytotoxicity of the eight compounds isolated in our HTS, we assessed their effects on proliferation of HCT116 (mutant β-*catenin*, wild-type *APC*) and HT29 (wild-type β-*catenin*, mutant *APC*) colon cancer cells (Ilyas et al., 1997; Sparks et al., 1998). Six compounds inhibited cell proliferation with IC_{50} 0.2–2 μM in both cell lines; PKF222-815 and NPDDG39.024 were less potent (Table 1). Compared to only 1.3% of the natural compounds from this collection that induce apoptosis in HCT116 cells in vitro (Lee et al., 2003), the finding that so many compounds in this small group are cytotoxic is highly significant. Six other compounds derived from the same sources (e.g., ZTM002377 and CGP039329, structures shown in Supplemental Figure S1 at http://www.cancercell.org/content/full/5/1/91/DC1) did not inhibit growth of either cell line.

If the active compounds interfere with Tcf/β-catenin complexes in cells, they may be expected to show greater cytotoxic-

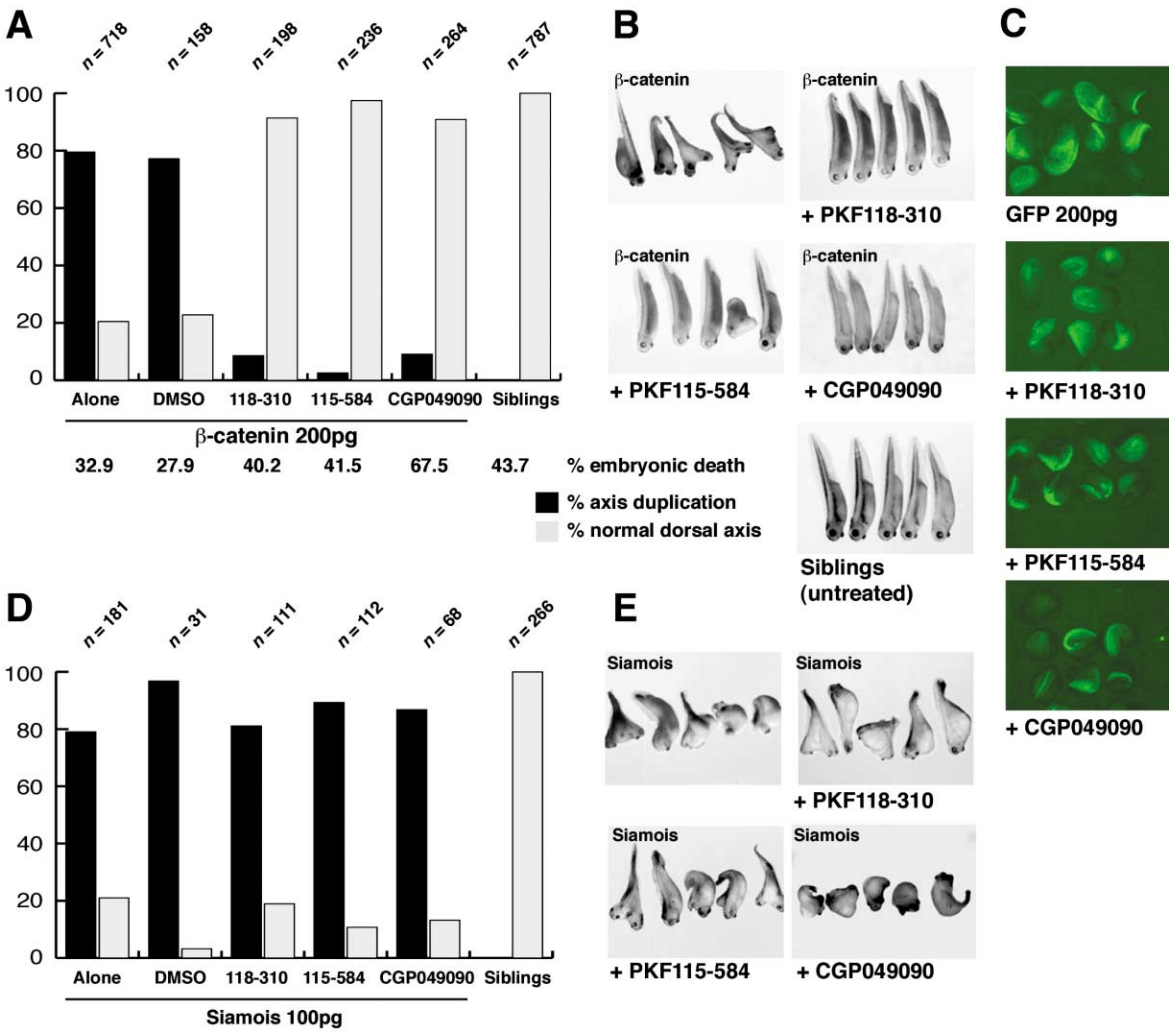

Figure 5. Compounds inhibit β-catenin-induced axis duplication in *Xenopus* embryos

A: 200 pg β-*catenin* mRNA was injected with selected compounds, vehicle (DMSO), or alone, into the equatorial region of one ventral blastomere of 4-cell stage *Xenopus* embryos, and duplicated axes were scored at Nieuwkoop-Faber stage 39. Injection of β-catenin mRNA induces an ectopic body axis, which was blocked by co-injection of PKF118-310 (0.5 pmol/embryo), PKF115-584 (3.5 pmol/embryo), or CGP049090 (0.25 pmol/embryo). The histogram shows the percent of embryos with normal (gray bars) or duplicated (black bars) axes; injection of DMSO (up to 7% in the injection solution) had no effect on axis duplication. Results are pooled from several independent experiments, which gave consistent results, and total numbers of scored embryos (*n*) are indicated, as is the fraction of embryos that failed to survive to stage 39.

B: Pictures of representative embryos from each pool injected with β-*catenin* mRNA.

C: mRNA encoding the green fluorescent protein (GFP) was injected in the same manner, with or without drugs, and 8–14 embryos, representing all the samples in a given experiment, were photographed under UV light 30 hr later.

D: 100 pg of *Siamois* mRNA was injected, with or without candidate compounds, as above, and induction of ectopic dorsal axes was not inhibited by co-injection of PKF118-310, PKF115-584, or CGP049090. The percent of embryos with normal (gray bars) or duplicated (black bars) axes is depicted.

E: Pictures of representative embryos from each pool injected with *Siamois* mRNA.

ity toward carcinomas of the colon than those originating in other sites. Although excessive β-catenin signaling is suspected to contribute to tumorigenesis in tissues other than the intestine (Chesire et al., 2002; Lin et al., 2000; Mulholland et al., 2002), we compared the activity of six compounds toward HCT116 cells with that against PC-3 and DU-145 prostate cancer cells. Cytotoxicity from three compounds was nonselective, whereas PKF222-815, PKF115-584, and CGP049090 showed 3- to 8-fold lower IC_{50} for colon cancer cells (Table 1). The latter two molecules also showed >2-fold higher IC_{100} toward both prostate-derived lines than against HCT116 cells (data not shown). We therefore tested their activity against HCT116 subclones carrying deletion of either the wild-type or mutant β-*catenin* allele (Chan et al., 2002); growth defects in clones lacking constitutive CRT are seen only at low cell densities, which implies a limited role for β-catenin in cell replication, at least as measured in conventional assays. Parental HCT116 cells and the KO58 derivative bearing the constitutively active β-*catenin* allele responded similarly to treatment with PKF115-584, whereas DO20 cells with a wild-type β-*catenin* allele appeared more sensitive (Figure 6A). All three clones showed similar responses to CGP049090 and compound-induced apoptosis

Table 1. Compound effects on growth of colon and prostate cancer cell lines

Compound	IC_{100} in MTS assay HCT116	IC_{50} in MTS assay HCT116	HT29	PC-3	DU-145	IC_{50} ratio PC3:HCT116
ZTM000990	<0.15	<0.15	0.15 ± 0.01	0.26 ± 0.06	0.20 ± 0.05	>1
PKF118-310	0.35 ± 0.12	0.24 ± 0.09	0.22 ± 0.05	0.30 ± 0.11	0.17 ± 0.01	~1
PKF118-744	0.78 ± 0.44	0.65 ± 0.28	0.50 ± 0.10	1.20 ± 0.50	0.42 ± 0.14	1.8
PKF115-584	2.86 ± 0.98	0.40 ± 0.05	2.00 ± 0.16	3.40 ± 0.80	2.88 ± 1.60	8.5
CGP049090	1.20 ± 0.16	0.64 ± 0.20	0.80 ± 0.20	1.70 ± 0.20	0.92 ± 0.13	2.6
NPDDG1.024	NT	0.70	0.60	NT	NT	
PKF222-815	3.60 ± 0.60	1.20 ± 0.50	NT	7.10 ± 1.40	3.16 ± 0.80	5.9
NPDDG39.024	NT	1.20 ± 0.10	4.20	NT	NT	
ZTM002377	NT	>10	9	NT	NT	
CGP039329	NT	>30	>30	NT	NT	

MTS proliferation assays were performed on two colon (HCT116 and HT29) and two prostate (PC-3 and DU-145) cancer cell lines 72 hr after adding compounds. Results were obtained in triplicate in each of 5 experiments and are expressed as the average compound concentration (±SD) that gives 50% of the maximum inhibition (IC_{50}) or the concentration that returns A_{490} to the level present at $t = 0$, before addition of compounds (IC_{100}). NT, not tested

with both compounds, as judged by nuclear condensation (DAPI staining not shown).

β-catenin is essential for many cellular processes. Associations with membrane bound E-cadherin and with APC mediate homotypic cell adhesion and β-catenin stability, respectively, through domains that overlap with Tcf binding armadillo repeats (Graham et al., 2000; Huber and Weis, 2001); this overlap presents a potential obstacle in developing selective drugs (Huber and Weis, 2001). To evaluate specificity of the active chemicals, we determined their effect on the presence of E-cadherin and APC in β-catenin immunoprecipitates from HCT116 and HT29 colon cancer cells, respectively. Although three compounds (PKF222-815, PKF115-584, and CGP049090) reduced the efficiency of β-catenin immunoprecipitation (see lower relative intensities of both the immunoglobulin and β-catenin bands in Figure 6B), the E-cadherin:β-catenin ratio remained constant (Figure 6C), whereas these compounds eliminated the interaction with APC. These results highlight the challenge in selective antagonism of Tcf/β-catenin complexes and suggest that rational drug design may need to exploit the known subtle differences between β-catenin's interactions with Tcf and with proteins like APC (Eklof Spink et al., 2001).

Discussion

Molecular pathways of tumorigenesis present opportunities for targeted therapy. This is illustrated well by the utility of Abl kinase or epidermal growth factor receptor antagonists in treating chronic myeloid leukemia and breast cancer, respectively (Druker et al., 2001; Pegram et al., 1998). The mutations implicated in colon cancer pathogenesis (Kinzler and Vogelstein, 1996) confer constitutive CRT and high nuclear β-catenin levels (Anderson et al., 2002; Korinek et al., 1997; Morin et al., 1997). Previous attempts to exploit these properties through gene targeting or antisense strategies (Green et al., 2001; Kwong et al., 2002) validate the seminal role of β-catenin in colon cancer, but their promise is limited by fundamental barriers to gene-based treatments. Small molecules that target individual components distally in the APC pathway could bypass these limitations, and β-catenin's interaction with Tcf4 represents one such target.

Small molecules are commonly considered ill-suited to disrupt the typically large and hydrophobic interface between interacting proteins (Cochran, 2001; Toogood, 2002). However, critical cellular functions rely on protein associations, and neglecting this reality in drug discovery is needlessly limiting. Drugs with established value in treating cancer or other diseases are known to target essential protein-protein interactions, including the vinca alkaloid inhibitors of tubulin dimerization (Owellen et al., 1976). Recent studies show that other protein-protein interactions are also vulnerable to small-molecule interference (Berg et al., 2002; Chen et al., 2002) and define allosteric interactions between drug candidates and their target proteins (Arkin et al., 2003; Baba et al., 1999). Protein associations commonly rely on a few key residues and the Tcf/β-catenin complex is no exception, as revealed by crystal structure and in the deleterious consequences of single point mutations (Fasolini et al., 2003; Graham et al., 2000, 2001; Knapp et al., 2001; Omer et al., 1999; Poy et al., 2001). Small molecule antagonists hence need to interfere mainly with critical amino acid contacts, and modest disruption of the binding equilibrium could have significant outcomes.

We therefore sought to identify natural products that disrupt interactions between Tcfs and β-catenin. To confirm that isolated compounds function as predicted, we tested their ability to inhibit CRT in vitro and in vivo. As expected, six lead compounds function differently in various secondary assays, albeit with a correlation between their activities in tests of protein interaction, cell proliferation, and CRT (Table 2). Two fungal derivatives (PKF115-854 and CGP049090) fulfill nearly every tested prediction, including disruption of Tcf/β-catenin complexes in vitro and inhibition of colon cancer cell proliferation, CRT, and β-catenin-mediated axis duplication in *Xenopus* embryos. These compounds are close analogs of each other and their effects may occur through the common core structure. This structure does, however, raise the possibility of their aggregating in solution, which can generate false positive results in chemical screens (McGovern et al., 2002). By nuclear magnetic resonance imaging, these molecules did not self-aggregate in several aqueous buffers but did so in solutions containing 0.1% Nonidet-P40 detergent (data not shown). While this may account in part for the effects we observed in affinity chromatography studies (Figure 3), molecular aggregates cannot be implicated in our

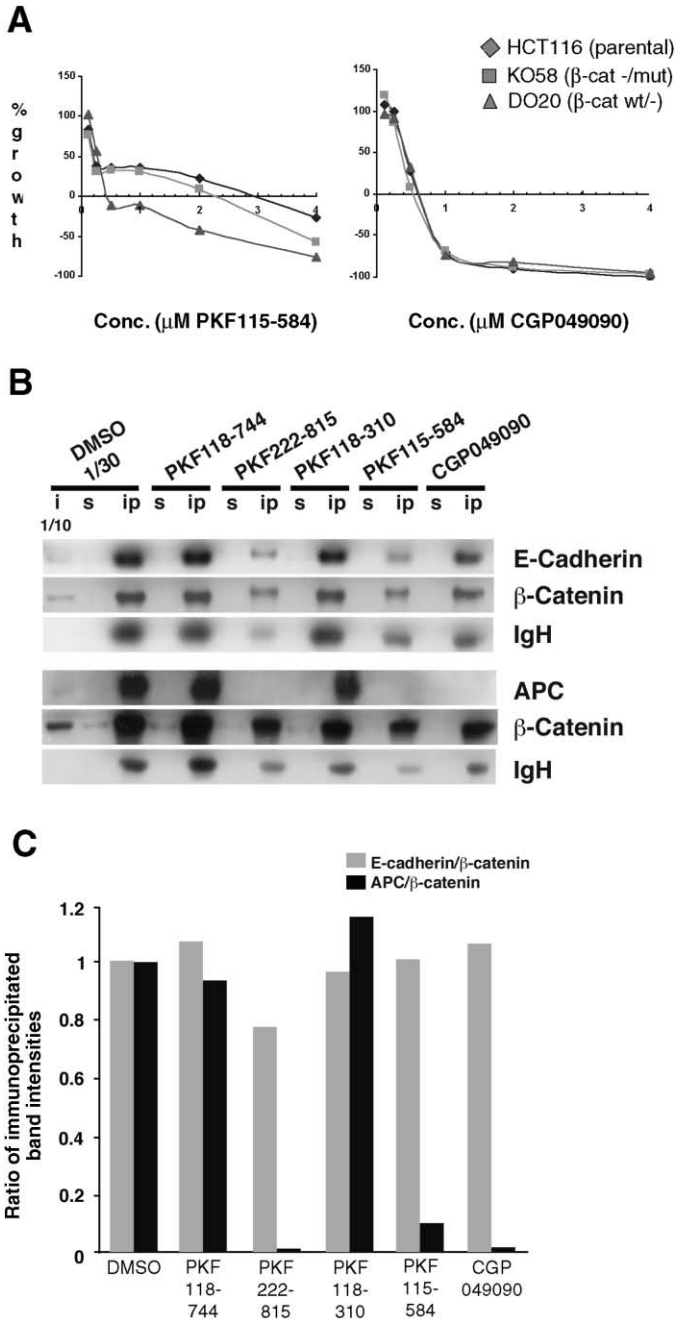

Figure 6. Activity of compounds against colon cancer cells and other β-catenin interactions

A: Concentration curves for cytotoxicity (MTS) of PKF115-584 (left) and CGP049090 (right) toward parental HCT116 colon cancer cells and its independent hemizygous derivatives DO20 (which retains the wild-type β-catenin allele) and KO58 (which retains the mutant β-catenin allele). A_{490} measurements at 72 hr are reported relative to vehicle (DMSO)-treated cells.

B and C: Compound effects on β-catenin's interaction with proteins other than Tcf4. Compounds were added to whole-cell lysates of HCT116 or HT29 cells to detect E-cadherin or APC, respectively, by immunoblotting of β-catenin immunoprecipitates (i.p.) or the remaining supernatant (s). i = 1/10 of the input cell lysate. Three compounds consistently reduced efficiency of β-catenin immunoprecipitation, as judged by the corresponding decrease in abundance of immunoglobulin heavy (IgH) chain. When the results are normalized for this effect (**C**), they indicate that E-cadherin complexes are preserved, whereas these compounds interfere with the APC/β-catenin interaction.

results with nonaggregating buffers in embryos, cells, and nuclear extracts. Moreover, these compounds passed rigorous tests for biochemical specificity, and the weight of evidence points to effects occurring through the targeted mechanism of β-catenin-protein interactions.

The molecular mechanisms by which the isolated compounds interfere with the targeted interaction are unclear. In the future, it will be important to map their docking sites within β-catenin and/or Tcf, a goal in which we have met with limited success to date. Meanwhile, the observation that three compounds also interfere with APC/β-catenin complexes suggests that their actions are mediated through β-catenin. Two compounds that disrupt the Tcf4/β-catenin complex (PKF115-584 and PKF222-815) also prevent association of Tcf proteins with DNA. Possibly, these compounds interact additionally with DNA, as occurs with many small molecules, or modulate Tcf4 allosterically and less specifically. PKF115-584, whose structure corresponds to that of calphostin C, is known to inhibit protein kinase C (Kobayashi et al., 1989), but the role of PKC in colon cancer is unclear. Expression of the α isoform is reduced in colon cancer cells, hinting at the possibility of a tumor suppressor function (Assert et al., 1999), whereas PKCβII expression increases early in experimental colon tumors in rodents and PKCβII transgenic mice show colonic epithelial hyperproliferation (Gokmen-Polar et al., 2001; Murray et al., 1999). PKC also inhibits GSK3-β (Cook et al., 1996), the enzyme that targets β-catenin for degradation (Rubinfeld et al., 1996; Yost et al., 1996). Indeed, calphostin C promotes GSK3-β effects and impairs Wnt-induced accumulation of β-catenin (Chen et al., 2000), although other PKC antagonists exert opposite effects (Orford et al., 1997). Whereas inhibition of PKC could potentially contribute to PKF115-584 activity, our biochemical and *Xenopus* data clearly point to independent effects on Tcf/β-catenin complexes; a combination of the two properties may explain the high potency of this compound in some assays. Conversely, our results offer an alternative interpretation to the previous finding of calphostin effects on Wnt signaling (Chen et al., 2000), which may have resulted from disrupted Tcf/β-catenin complexes. Various inhibitors of the β-catenin pathway could thus be useful in studying CRT in normal cellular processes, including cell growth and replication.

Differences in cell growth between HCT116 cells bearing deletions of different β-*catenin* alleles are apparent only at low cell densities (Chan et al., 2002), and alternative means of suppressing CRT affect growth of the DLD-1 cell line differently (Naishiro et al., 2001; van de Wetering et al., 2002). Conventional cytotoxicity assessments may hence provide limited perspective on the potential roles of new cancer treatments. The cytotoxicity of compounds isolated in our HTS correlates with the degree to which they attenuate CRT, and the most promising candidates kill colon cancer cells more efficiently than prostate cancer cells in vitro. By contrast, they show little difference in cytotoxicity toward HCT116 derivatives with single β-*catenin* alleles. Thus, whereas effects on protein complexes, endogenous CRT targets, and embryogenesis seem to occur through β-catenin, cell death may have another basis. The viability of β-*catenin* Δ*S45*-deleted HCT116 cells is itself surprising and may reflect CRT-independent cell growth that resists β-catenin interference. Alternatively, our compounds may show more pronounced or specific effects in sensitizing CRT-positive cells to cytotoxic agents.

Table 2. Summary of biochemical and biologic activities of five compounds studied extensively

Compound	ELISA	GST	EMSA	MTS	RGA	c-Myc	Cyclin D1	Axis
PKF115-584	+	++	**?**	++	++	++	++	++
PKF118-310	++	+	++	++	++	-	+	++
CGP049090	+	++	+	++	+	++	++	++
PKF118-744	++	-	++	++	++	-	-	-
PKF222-815	+	++	**?**	+	+	NT	NT	±

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GST, precipitation by glutiathone-*S*-transferase-tethered Tcf4; EMSA, electrophoretic mobility shift assay; MTS, cell proliferation assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; RGA, luciferase reporter gene activation.

Activities are rated relative to other tested compounds (NT, not tested) according to the following scheme for ++ and + ratings, respectively: $IC_{50} < 3$ μM or ≥ 3 μM in the ELISA, $IC_{50} < 1$ μM or ≥ 1 μM in one or both cell lines in the cell proliferation (MTS) and reporter gene (RGA) assays, inhibition achieved with 0.5 μM compound or only at the 5 μM concentration for the endogenous CRT targets c-Myc and cyclin D1, and >80% or 50%–80% inhibition of axis duplication in *Xenopus* embryos.

β-catenin interacts with several proteins, including E-cadherin, APC, and γ-catenin, so that antagonists of the Tcf interaction should be chosen to avoid disrupting its other essential functions. CGP049090 and PKF 115-584 antagonize β-catenin effects in vivo with limited toxicity, as *Xenopus* embryos develop into grossly normal swimming tadpoles, although this observation likely reflects restricted diffusion of injected molecules (Drummond et al., 1985). Indeed, these compounds also disrupt cellular β-catenin/APC complexes, supporting the speculation that pharmacologic interference with β-catenin complexes may be inherently unselective (Eklof Spink et al., 2001; Graham et al., 2000). However, crystallographic studies do reveal structural differences among alternative β-catenin complexes (Eklof Spink et al., 2001; Graham et al., 2000), and it may yet be possible to identify antagonists specific for Tcf interactions. Our study demonstrates that some small molecules can disrupt these complexes with biologic effects. Additional, focused screens and structural refinement could build on these results to develop new treatments for colorectal and other cancers with constitutive CRT.

Experimental procedures

Protein purification and surface plasmon resonance

GST-Tcf4 and untagged human β-catenin were prepared as described previously (Poy et al., 2001) to estimated purities >90%. For specificity studies, 6XHis-SMC1, 6XHis-SMC3, 6XHis-IκB kinase, and untagged Hsc70 were purified from baculovirus-infected Sf9 cells and diluted in Dulbecco's PBS (GIBCO-BRL). CM-5 sensor chips (Biacore Inc., Piscataway, NJ) were coated with goat anti-GST antibody (Amersham Biosciences) using the Biacore BR-1002-23a kit for GST fusion protein capture. Following injection of either control or GST-Tcf4 (5 μl at 20 μg/ml) onto separate quadrants of the sensor chip, β-catenin (5 μl at 160 μg/ml) was introduced to assess specificity of protein-protein interactions.

Compound libraries and high-throughput ELISA

Approximately 6,000 purified natural products and 45,000 synthetic compounds originated in Novartis collections, and 1,000 pure natural products were provided through the Natural Product Collaborative Drug Discovery Group (NPCDDG). Nunc-Immuno MaxiSorp Plates (Nunclon) were coated with 5 μg/ml purified β-catenin fragment in phosphate-buffered saline (PBS); nonspecific binding was assessed in uncoated wells. Plates were washed 8 times with TBST (50 mM Tris [pH 8.0], 0.15 M NaCl, 0.05% Tween-20), blocked for 2 hr at 4°C with SuperBlock (Pierce), washed with TBST, incubated for 2 hr at 4°C with 1 μg/ml purified GST-Tcf4 in the presence of 10 μM compounds diluted in assay buffer (50 mM Tris [pH 8.0], 0.15 M NaCl, 0.05% Tween-20, 2% bovine serum albumin) with a final concentration of 1% DMSO, followed by 8 washes in TBST. To detect bound GST-Tcf4, plates were incubated sequentially with goat anti-GST antibody (Amersham Biosciences) for 2 hr and alkaline phosphatase (AP)-conjugated anti-goat IgG (Bio-Rad) overnight at 4°C with 8 washes in TBST in between and afterward. Samples were treated for 5 min with 600 μg/ml fluorescent AP substrate Attophos (JBL Scientific) in 11 mM $MgCl_2$ (pH 8.8), 4.8 mM diethanolamine, and enzyme activity was assessed in a fluorescent plate reader (450 nm excitation, 580 nm emission). Compound effects are expressed as the concentration required to inhibit 50% (IC_{50}) of GST-Tcf4 binding to β-catenin-coated wells.

Plasmids and cell lines

hTcf4 and β-catenin expression constructs, TOP-FLASH and FOP-FLASH reporters, and the β-galactosidase control plasmid used in luciferase reporter gene assays were purchased from Upstate Biotechnology (Lake Placid, NY). Flag epitope-tagged β-catenin cDNA in the *Xenopus* expression vector pCS was kindly provided by Dr. Xi He (Children's Hospital, Boston, MA), and GFP cDNA was subcloned in the same vector. The Tcf4 expression construct used in gel retardation assays contains the open reading frame of mTcf4B (Lee et al., 1999) cloned into the pCMV vector (Stratagene). HCT-116, HT-29, and SW480 human colon cancer cells were obtained from the American Type Culture Collection (ATCC) and maintained in McCoy's, RPMI 1640, or Dulbecco's Modified Eagle medium (Life Technologies), respectively, each supplemented with 10% fetal bovine serum (FBS). PC-3 and DU-145 prostate cancer cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS. HCT116-derived DO20 and KO58 cell lines were generously provided by Drs. Ken Kinzler and Bert Vogelstein (Johns Hopkins University).

Precipitation of cellular proteins and immunoblot analysis

GST-Tcf4, GST-E2F1(1-171), and GST-Bcl-xL(135-775) fusion proteins were purified from *E. coli* BL21 lysates by affinity chromatography with glutathione-sepharose followed either by cation-exchange chromatography or dialysis in PBS. HCT116 lysates (125 μg protein in buffer A containing 50 mM Tris [pH 8.0], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10% glycerol, 0.1 mM sodium vanadate, 1 mM aprotinin, 1 mM leupeptin, 50 mM NaF, 1 mM phenylmethyl sulfonyl fluoride) were incubated with various concentrations of compounds and 5 μg GST-fusion proteins overnight at 4°C. Samples were immobilized on glutathione-sepharose A beads (Amersham Pharmacia) for 2 hr at 4°C, washed 3 times in buffer B containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 1 mM EDTA, and the retained material was eluted in SDS-PAGE buffer and resolved in 8% polyacrylamide gels for immunoblot analysis. Cyclin A (H432) and Bax (N20) antibodies (Ab) were from Santa Cruz Laboratories and β-catenin Ab (C19220) was from Transduction Laboratories. Separately, HCT116 cells were seeded at 10^6 cells/plate, cultured overnight at 37°C, treated with compounds (0.5–5 μM) for 24 hr, and washed. Cells were lysed in buffer A and 50 μg protein was resolved by SDS-PAGE. Relative protein levels were judged by immunoblot using β-catenin, cyclin D1 (SC753, Santa Cruz), c-Myc (Ab1, Oncogene Research Products), or cyclin E (SC247, Santa Cruz) Ab and enzymatic chemiluminescence (Amersham Pharmacia). Finally, HT29 and HCT116 whole-cell extracts prepared in buffer C (140 mM NaCl, 20 mM Tris [pH 8], 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol) were precleared

with protein G beads (Amersham Pharmacia), incubated with DMSO or compounds at 30 μM for 4 hr at 4°C, and treated with 4 μg mouse β-catenin Ab (BD Biosciences) at 4°C overnight, followed by 20 μl protein G slurry and 3 washes in buffer C. Precipitates were resolved on Tris-acetate gels (GIBCO) and immunoblotted with E-cadherin (BD Biosciences) and APC (Oncogene Research Products) Ab. Band intensities were quantified by densitometry (Molecular Dynamics) in the linear range of film exposure.

Electrophoretic mobility shift assay

COS-7 cells were transfected with Flag epitope-tagged β-catenin or mTcf4B or HA epitope-tagged pRb over 5 hr using Lipofectamine reagent (Invitrogen) and nuclear extracts were prepared 36 hr later, as described (Andrews and Faller, 1991). Radiolabeled double-strand DNA oligomers (CCCTTTGATCT TACC, containing a consensus Tcf binding site (underlined), or CCC TTTGGCCTTACC, mutated to abrogate Tcf binding) were used as probes (Korinek et al., 1997). The probe sequence for control E2F binding is published (Cobrinik et al., 1993). Binding was performed in 30 μl buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, 4 mg/l poly-deoxyinosine-deoxycytidine and 10% glycerol). Tcf4 (2.5 μg) and β-catenin (1.5 μg) nuclear extracts were individually incubated in 15 μl binding buffer with 1% DMSO or 30 μM concentrations of test compounds for 30 min at ambient temperature, then pooled and incubated for 30 min before adding the probe. Nonspecific binding is revealed by incubation with the mutant Tcf probe. Relevant complexes were identified by a final 20 min incubation with Ab against Tcf4 (Upstate Biotechnology), β-catenin (Transduction Laboratories), or Flag (M2, Sigma) or HA (12CA5, Roche) epitopes. Complexes were resolved in 3.5% native polyacrylamide gels.

Cell proliferation assay

Cultured lines were seeded in 96-well plates at 3×10^3 cells/well, maintained overnight at 37°C, and incubated in the presence of test compounds at various concentrations. Cell viability and density were monitored after 72 hr using a freshly prepared mixture of 1 part phenazine methosulfate (PMS, Sigma) solution (0.92 mg/ml) and 19 parts 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) solution (2 mg/ml). Cells were incubated in 10 μl of this solution at 37°C for 3 hr and A_{490} was measured. The effect of each compound is expressed as the concentration required to reduce A_{490} by 50% (IC_{50}) relative to vehicle-treated cells or to return A_{490} to the level present at $t = 0$, before addition of compounds (IC_{100}).

Luciferase reporter gene assay

HCT116 cells were transfected with the luciferase reporter constructs TOP-FLASH, containing 3 Tcf consensus binding sites upstream of firefly luciferase cDNA, or FOP-FLASH, a plasmid with mutated Tcf binding sites (Upstate Biotechnology). 10^4 cells were transfected using Fugene6 reagent (Roche) with 16.5 μg reporter construct and 8.25 μg β-galactosidase reporter plasmid and incubated with various concentrations of selected compounds at 37°C. After 24 hr, cells were lysed in 25 μl luciferase cell culture lysis buffer (Promega). 5 μl cell lysate was incubated with 50 μl 2× β-Gal assay buffer and 45 μl reporter lysis buffer (Promega) at 37°C for 2 hr, and A_{420} was measured in a microplate spectrophotometer to determine β-galactosidase activity. The remaining cell lysate was mixed with 100 μl luciferin (Promega) and the light output determined in a Luminoskan Ascent luminometer (Labsystems). Results are expressed as the mean (±SEM) of normalized ratios of luciferase and β-galactosidase measurements for each triplicate set. Reporter activity in compound-treated cells is expressed as the percentage of mock (vehicle)-treated samples.

***Xenopus* experiments**

Capped mRNAs were synthesized in vitro using linearized plasmid templates and the mMESSAGE mMACHINE kit (Ambion, Austin, TX). *Xenopus* embryos were collected, fertilized, cultured, and staged as described previously (Shoichet et al., 2000). 4.6 nl β-*catenin* or *Siamois* mRNA (200 pg and 100 pg, respectively) was injected in the equatorial region of one prospective ventral blastomere at the 4-cell stage with or without test compounds. Embryos were incubated at 19°C until untreated sibling tadpoles reached Nieuwkoop-Faber stage 39, fixed overnight in 0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4.7H_2O$, 3.7% formaldehyde, and stored in ethanol before scoring for presence of ectopic dorsal axes. Coinjection of test compounds with GFP mRNA was performed similarly and fluorescence was assessed after 36 hr at stage 25.

Acknowledgments

We thank Karen Tenney, William Gerwick, and Amy Wright for supporting NPCDDG chemical collections, which are funded in part by grant CA52955 from the National Institutes of Health; Xi He, Jim DeCaprio, and Jim Rhee for plasmids; Jeremy Green, Barbara Laureys, Tim Ramsey, and Anthony Romer for advice and technical assistance; Greg Paris for drawing chemical structures; Florence Poy and Michael Eck for recombinant proteins; Ken Kinzler and Bert Vogelstein for modified HCT116 cell lines; Jianling Wang and Xiaolu Zhang for turbidity and NMR studies, respectively; and Myles Brown, David Livingston, and Tim Ramsey for comments on the manuscript. R.A.S. is a Scholar of the Leukemia and Lymphoma Society.

Received: May 30, 2003
Revised: October 8, 2003
Accepted: October 29, 2003
Published: January 19, 2004

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