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Structure-Based Discovery of a Novel Inhibitor Targeting the β-Catenin/Tcf4 Interaction

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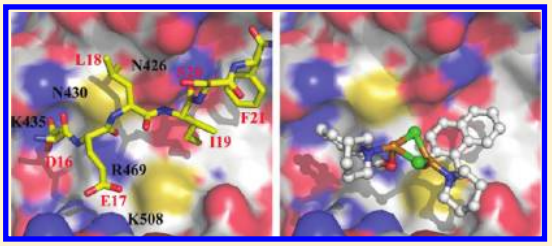
ABSTRACT: Overactivation or overexpression of β-catenin in the Wnt (wingless) signaling pathway plays an important role in tumorigenesis. Interaction of β-catenin with T-cell factor (Tcf) DNA binding proteins is a key step in the activation of the proliferative genes in response to upstream signals of this Wnt/β-catenin pathway. Recently, we identified a new small molecule inhibitor, named BC21 ($C_{32}H_{36}Cl_2Cu_2N_2O_2$), which effectively inhibits the binding of β-catenin with Tcf4-derived peptide and suppresses β-catenin/Tcf4 driven reporter gene activity. This inhibitor decreases the viability of β-catenin overexpressing HCT116 colon cancer cells that harbor the β-catenin mutation, and more significantly, it inhibits the clonogenic activity of these cells. Down-regulation of c-Myc and cyclin D1 expression, the two important effectors of the Wnt/β-catenin signaling, is confirmed by treating HCT116 cells with BC21. This compound represents a new and modifiable potential anticancer candidate that targets β-catenin/Tcf-4 interaction.



The Wnt/β-catenin signaling pathway plays a critical role in cancer cell self-renewal and proliferation.[1,2] Overactivation of the Wnt/β-catenin pathway, resulting from mutations of proteins in this pathway, is common in a wide range of human cancers.[2−4] For example, an abnormally high level of β-catenin has been found in many types of cancers, and this is necessary for tumorigenesis.[2] The overactivation or overexpression of β-catenin is associated with resistance to cancer treatment through enhancement of cancer cell renewal and prevention of apoptosis.[3,4] Inhibitors that specifically target the key regulators in this pathway therefore not only represent a generation of new medicine for treating cancers but also can serve as valuable chemical probes for studying the molecular mechanism underlying the control of cancer cell fate by Wnt/β-catenin signaling.

The cellular location of β-catenin typically is predominantly at cell−cell junctions and at very low levels in both the cytoplasm and nucleus. Excess β-catenin is usually subjected to GSK-3β/APC/axin complex-mediated phosphorylation and subsequent proteasome-mediated degradation.[2] Binding of Wnt ligands to Frizzled (Fzd), a cell surface receptor, activates Disheveled (Dsh), which leads to accumulation of cytoplasmic β-catenin. This accumulation can also be seen in presence of mutations in APC, Axin, and also the β-catenin gene.[5] The elevation of the cytoplasmic β-catenin level results in its translocation to the nucleus and binding to members of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family, stimulating transcription of Wnt target genes, including c-Myc and cyclin D1, that are critical for cell growth, proliferation, and differentiation.[6,7] About 50% of colon cancers contain activating β-catenin mutations at putative GSK-3β phosphorylation sites, which render the β-catenin protein resistant to proteasome-mediated destruction.[8] Recent data indicate that enhanced clonogenic capacity and poor prognosis correlate positively with the constitutively active β-catenin overexpression of β-catenin by acute chronic myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and many solid tumors.[9−12] Thus, disruption of the interaction between β-catenin and Tcfs would be considered as a good strategy for treating cancer cells, particularly CSCs.

The crystalline structures of β-catenin complexed with Tcf3 and Tcf4 have been resolved.[13,14] Both Tcf3 and Tcf4 have similar structures when complexed with β-catenin; both consist of a long irregular head and an α-helix tail. The irregular head forms dense hydrogen bonding contacts with β-catenin, while the α-helix tail binds to β-catenin primarily by hydrophobic interactions. In this study, we used the crystal structure of β-catenin, alone and in complexes with Tcf3/Tcf4, in docking studies and screened a compound library. A small molecule compound was identified that showed potential inhibition of β-catenin/Tcf4 interaction and cancer cell proliferation.

EXPERIMENTAL PROCEDURES

Virtual Screening by Molecular Docking. The diversity set of National Cancer Institute (NCI) open database,[15] consisting

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of 1990 small molecules, was selected for the virtual screening by using Autodock4.[16] Receptor structure solved at 2.5 Å resolution was extracted from crystal structure of the β-catenin/Tcf4 complex (PDB entry 1JPW, chain A) and the N-terminal moiety which are far from site A (Arg151−Asn387) was truncated to facilitate the preprocessing of receptor. The receptor file was converted to PDBQT file, and a 64 × 62 × 54 grid box with a grid spacing of 0.375 Å was defined to cover the whole groove in which site A locates. Side chains of high polar residues around site A (such as Lys435, Arg469, Lys508, Arg515, and Glu571) were set flexible during the docking. Grid maps were then generated by Autogrid4[16] for Autodock simulations. The whole preparation process of receptor files was accomplished with AutoDockTools-1.5.2.[16] Preparation of ligands and docking parameter files were accomplished using scripts of Molecular Graphics Laboratory of Scripps Institute, with all parameters set to default values.

Reagents. Anti-c-Myc (#9402), anticyclin D1 (DCS6, #2926), and anti-β-actin (#4967) antibodies were purchased from Cell Signaling, Inc. Quercetin was purchased from Sigma-Aldrich. pCMV-SPORT-FL-Tcf4 (Catalog #MHS1010-7508380) was purchased from Open Biosystem. Primers used for amplifying Tcf4 (N-terminal 1−54 amino acids) were synthesized from Invitrogen. Restriction endonuclease and T4 ligase were purchased from New England Lab. Compounds identified by virtual screening were requested from NCI and dissolved in DMSO to 10 mM stock and stored at −20 °C.

Cell Culture. The colon cancer cell line HCT116 was obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Hyclone, Thermal Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

Vector Construction. The expression plasmid containing Tcf4 β-catenin binding domain (CBD, N-terminal 1−54 amino acids) was constructed by amplifying the corresponding DNA fragment by PCR using pCMV-SPORT6-FL-Tcf4 as template and the primers (forward: 5′-CGCGGATCCATGCCGCAGCTGAACG-3′; reverse: 5′-CGCGAATTCTTACGTTTCTGATTCATTG-3′; *BamHI* and *EcoRI* sites are underlined, respectively). Purified PCR fragment was digested with *BamHI* and *EcoRI* and inserted into pGEX-6p-1 vector treated with the same enzymes. The ligation product was transformed into competent DH5α, and single colonies were picked up for characterization.

Protein Expression and Purification. Plasmid pET28a-FL-hβ-catenin (kindly provided by Dr. Wenqing Xu, University of Washington) was used to transform BL21(DE3)star (Invitrogen). Bacteria were induced with 0.5 mM IPTG and then harvested and lysed. The supernatant was loaded onto an affinity column containing Ni-NTA beads for His-tag fusion protein (QIAGEN). Purified protein was concentrated to 6 mg/mL in buffer containing 20 mM Tris-HCl, 100 mM NaCl, 2 mM DTT, and 20% glycerol and stored at −80 °C. The GST-Tcf4-1-54 fusion protein was purified by similar protocol, except that glutathione Sepharoase 4B (GE Healthcare) was used to affinity chromatography and the protein was concentrated to 1 mg/mL.

Luciferase Reporter Gene Assay. HCT116 cells at 1 × 10^4 cells/well were transfected with TOP-FLASH, containing Tcf4 binding sites, or FOP-FLASH harboring mutant Tcf4 binding sites. At 4 h post-transfection, candidate compounds were added to cells. Luciferase activities were determined 24 h after compounds treatment using the Firefly Luciferase Assay Kit (Biotium, Inc. Hayward, CA). Cell viabilities were also determined at this time point as described in the Cell Viability Assay section. The luciferase activities were normalized with cell viability to rule out the nonspecific cytotoxicity. For HEK293 cell, pCDNA3.1-β-catenin was cotransfected into cells with TOP (or FOP) vectors. At 24 h post-transfection, compounds were added to cells. The luciferase activities and cell viabilities were determined as described above. The relative luciferase activity was expressed as follows: for TOP, $(\text{TOP-luciferase activity})_{\text{treatment}}/(\text{TOP-luciferase activity})_{\text{control}} \times 100$; for FOP, $(\text{FOP-luciferase activity})_{\text{treatment}}/(\text{TOP-luciferase activity})_{\text{control}} \times 100$.

Cell Viability Assay. Human colon cancer 116 (HCT116), human embryonic kidney 293 (HEK293), and human umbilical vein endothelial (HUVE) cells were plated at 1 × 10^4/well in 96-well plate and incubated overnight at 37 °C. Cells were treated with different concentrations of selected candidate compounds and incubated for 72 h. Cell viability was measured by a CellTiter-Blue reagent-based assay (Promega).

Clonogenicity Assay. HCT116 cells were plated at 300/well in 6-well plates and incubated overnight at 37 °C. Cells were treated with various concentrations of selected candidate compounds and incubated for 7 days. Colonies containing more than 50 cells were scored. Individual assays were performed with duplicate wells. Relative colony number was calculated as $[(\text{colony number})_{\text{treatment}}/(\text{colony number})_{\text{control}}] \times 100\%$.

Western Blots. HCT116 cells at 1 × 10^6/mL were treated with different concentrations of selected compounds. Cell lysates were prepared after 24 h treatment and subjected to SDS-PAGE and Western blotting with anti-c-Myc or anticyclin D1 antibody (Cell Signaling). The intensities of bands were quantified by Image J. In order to exclude the differences in loading levels, the intensity of c-myc or cyclin D1 was divided by the corresponding intensity of β-actin. The relative intensity expressed as follows: $(\text{intensity})_{\text{treatment}}/(\text{intensity})_{\text{control}} \times 100$.

Real-Time PCR. HCT116 cells at 1 × 10^6/mL were treated with different concentrations of BC21 for 24 or 5 h. Total RNA was isolated using a RNeasy Kit (Qiagen). The cDNA was synthesized with a high-capacity cDNA reverse transcription Kit (Invitrogen). Real-time PCR was performed on an Applied Biosystems Stepone Plus system using SYBR-green Mastermix (SABiosciences). The threshold cycle (C_T) values were normalized to β-actin internal reference. The primers used in real-time PCR were as follows: β-actin, forward 5′-AGAAAATCTGGCACCACACC-3′; reverse 5′-AGAGGCGTACAGGGATAGCA-3′; c-myc, forward 5′-TCAAGAGGCGAACACACAAC-3′; reverse 5′-GGCCTTTTCATTGTTTTCCA-3′; cyclin D1, forward 5′-AACTACCTGGACCGCTTCCT-3′; reverse 5′-GGGGATGGTCTCCTTCATCT-3′.

Fluorescence Polarization (FP)-Based Competitive Binding Assay. The effect of candidate compounds on β-catenin/Tcf4 binding was tested using a FP-based assay developed for that purpose (unpublished data). In the competitive binding experiments, 20 nM tracer (FITC labeled Tcf4 peptide, sequence is not shown) and 1.5 μM β-catenin were mixed and incubated with/without competitor in 1 × PBS (containing 0.01% Triton X-100) to produce a final volume of 100 μL. The reaction was incubated at room temperature for 3 h to reach equilibrium.[17] Polarization values were read using the "synergy 2" reader (BioTek) at excitation and emission wavelengths of 485/20 and 528/20 nm, respectively. The relative binding was calculated as $(mP_T - mP_f)/(mP_C - mP_f) \times 100$, where mP_C = control (without inhibitor), mP_f = free tracer (without β-catenin and inhibitor), and mP_T = treatment (treated with inhibitors).

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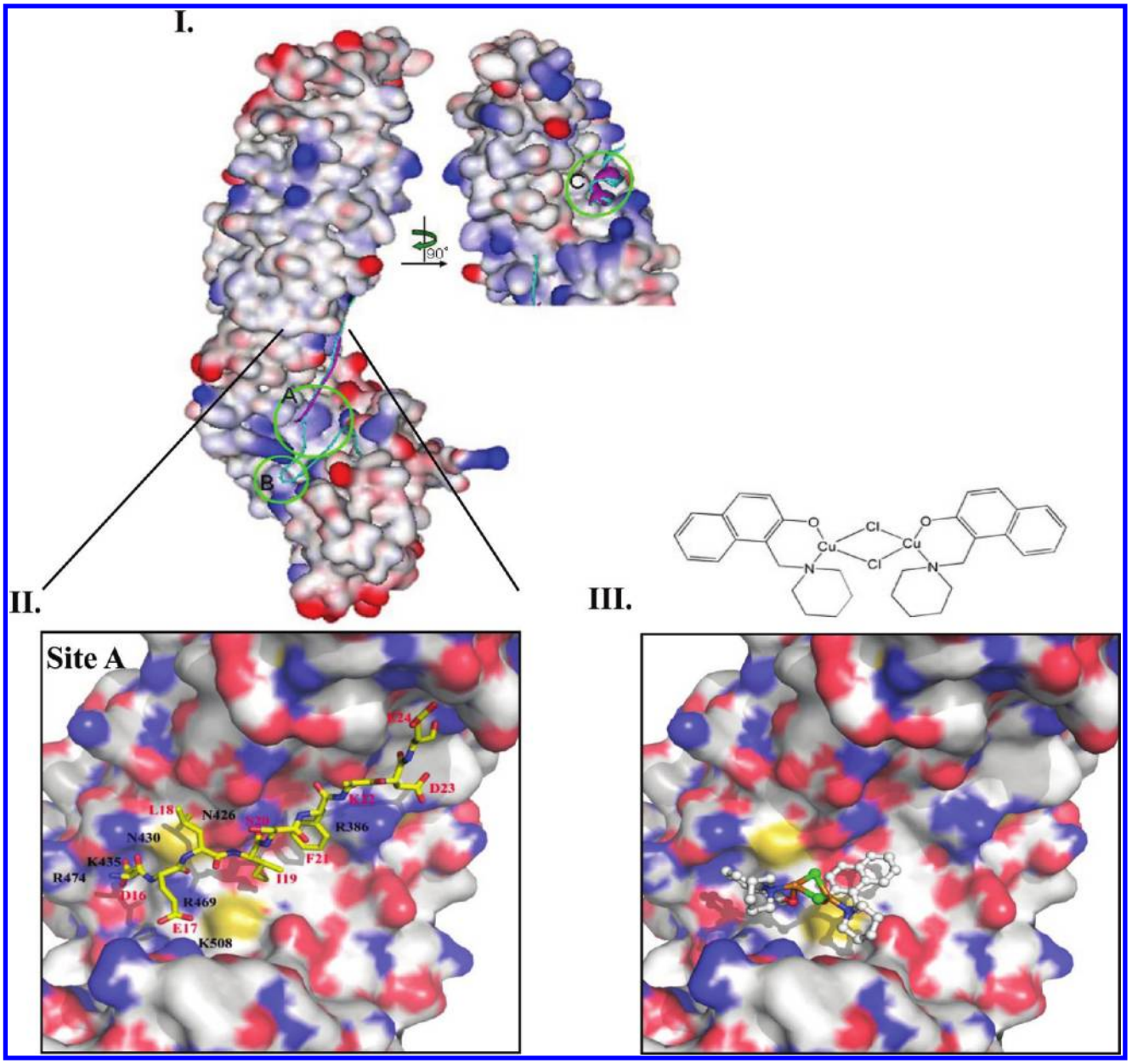

Figure 1. Complex of β-catenin with Tcfs and predicted binding model for BC21. (I) Tcf3 (cyan) and Tcf4 (magenta) are structurally similar when complexed with β-catenin; both consist of a long, irregular head and α-helix tail. Three "hot spots" (A, B, and C) on β-catenin were critical for Tcf4 binding. Site A is sufficiently large and deep for a small molecule to bind and consisted of a number of polar residues (Lys435, Arg469, Lys508, Arg515, Glu571, etc.). In the present study, site A was chosen as a binding pocket for the identification of small molecules that could disrupt β-catenin/Tcfs interaction. (II) The detailed interaction of β-catenin/Tcf4 at site A. Key residues for the interaction in Tcf4 and β-catenin are labeled in red or black, respectively. (III) The predicted binding position for BC21. The Tcf4 peptide is shown in yellow. BC21 is shown as a ball–stick model. Gray balls represent carbon atoms, blue balls represent nitrogen atoms, red balls represent oxygen atoms, green balls represent chloride atoms, and brown balls represent copper atoms. The pictures were generated by Autodock and PyMol.

Statistical Analysis. The statistical analysis was performed using the paired Student's *t*-test. Two-sided *P* values of <0.05 were considered statistically significant. Average values were expressed as mean ± SD.

RESULTS

Identify β-Catenin and Tcf4 Interaction-Targeted Lead Inhibitor BC21 by Virtual Screen. We analyzed the interactions of β-catenin and Tcf4 in the crystalline structures (Figure 1) based on the proposed hot spots by Fasolini et al.[18] Three "hot spots" (sites A, B, and C) on the β-catenin were critical for the binding of Tcf4. We chose site A as our pharmacophore for virtual screening because it is sufficiently large and deep for a small molecule to bind. This hot spot consists of a number of polar residues, including Lys435, Arg469, Lys508, Arg515, and Glu571. These polar residues are critical for binding of β-catenin with Tcf4 as well as for β-catenin-mediated proliferative signaling. Asp16 of Tcf4 formed a salt bridge with Lys435 with a distance of 2.5 Å and three hydrogen bonds with Asn430 on site A,[13,19] while Glu17 of Tcf4, which was adjacent to Asp16, formed a salt bridge with Lys508 on site A. In addition, the Ile19 and Phe21 of Tcf4 side chain packed together into a cleft, which is lined by Cys466 and Pro463 residues and the Arg386 aliphatic portion

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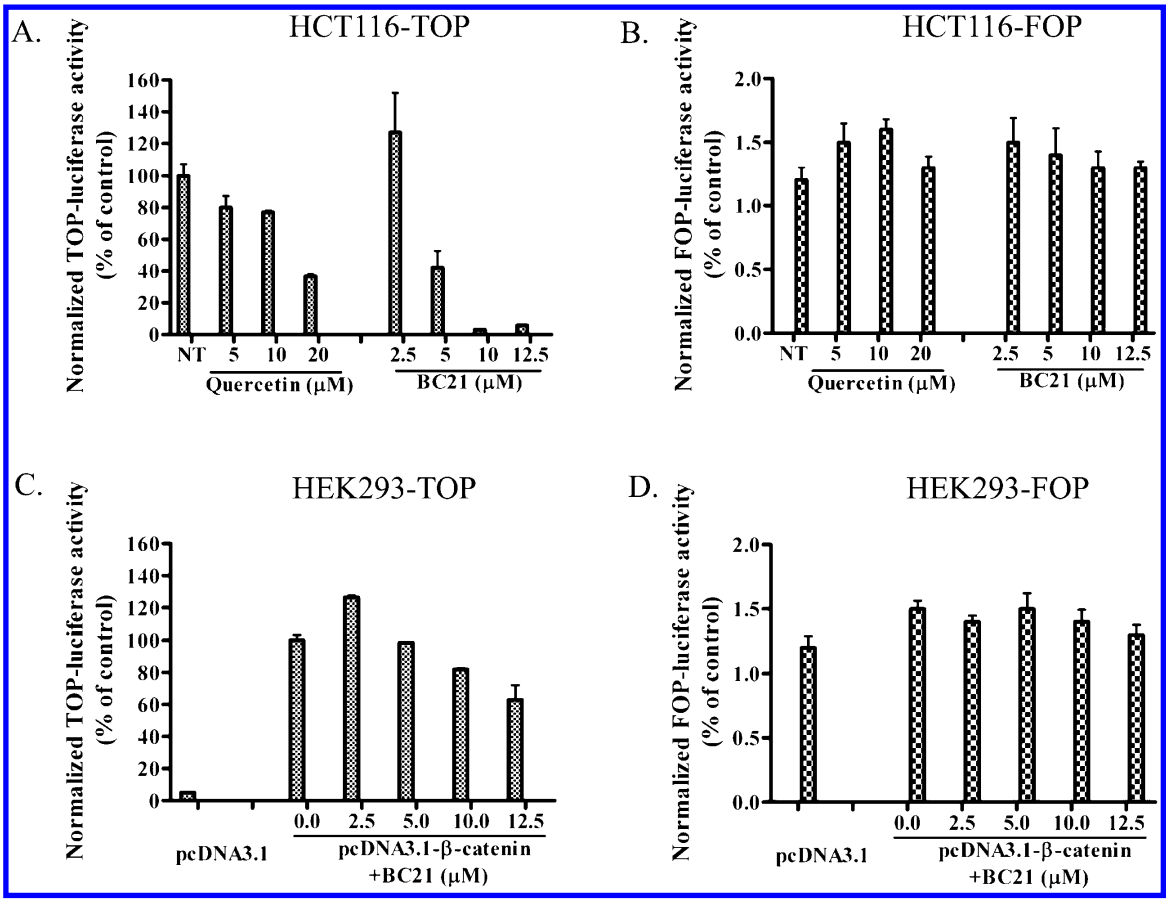

Figure 2. BC21 can inhibit the transcriptional activity of β-catenin/Tcf4 in HCT116 cells and HEK293 cells. (A) HCT116 cells were transfected with a reporter gene (luciferase) harboring the Tcf4 binding site (TOP) or a mutant Tcf4 binding site (FOP). At 4 h post-transfection, candidate compounds were added to the cells. Luciferase activities were determined 24 h post-transfection. BC21 can effectively reduce the TOP-luciferase activity at a concentration of 10 μM, without significantly affecting FOP-luciferase activity. Quercetin was used as a positive control. (B) HEK293 cells were co-transfected with TOP (or FOP) and pcDNA3.1-β-catenin. 24 h post-transfection, BC21 were added to cells. Luciferase activities were determined 24 h after drug treatment.

of β-catenin.[20] We used structure-based virtual screening to identify a new class of small molecule BC21 among 200 top-ranked compounds in a 1990 compound library. In the case of BC21, hydrophobic interactions contributed to blocking of the binding of Tcf4 and β-catenin. The estimated binding free energy between BC21 and β-catenin given by Autodock 4 is −7.07 kcal/mol, and predicted K_i = 6.55 μM. Half of the BC21 molecule located at the same position where the Ile19 and Phe21 residues of Tcf4 were found and showed very good hydrophobic interactions with the β-catenin hydrophobic cleft, especially with Pro463 ring. The other half of the BC21 molecule occupied the position at Asp16, and this also contributed to blocking of the β-catenin/Tcf4 interaction.

BC21 Inhibits the β-Catenin/Tcf4 Transcriptional Activity. We investigated the biological activity of 100 lead compounds using a cell-based TOP/FOP assay for testing the β-catenin/Tcf4 signaling. HCT116 cells were transfected with a reporter gene (luciferase) harboring the Tcf4 binding site (TOP) or a mutant Tcf4 binding site (FOP). At 4 h post-transfection, candidate compounds (20 μM) were added to the cells. Luciferase activities were determined 24 h post-transfection. Compounds capable of passing through the cell membrane and specifically inhibiting the Wnt/β-catenin signaling would reduce TOP-luciferase activity, without reducing FOP-luciferase activity. Figure 2 shows that the lead compound, BC21 ($C_{32}H_{36}Cl_2Cu_2N_2O_2$, MW = 679, NSC109268), at concentration of 10 μM, effectively reduced the β-catenin/Tcf4 driven TOP-luciferase activity in HCT116 cells, without significantly affecting FOP-luciferase activity (Figure 2A,B). BC21 also exhibited an inhibitory effect on TOP-luciferase activity in HEK293 cells that transiently expressed high levels of β-catenin (Figure 2C,D) Quercetin is a flavonoid that has recently been shown to have ability to inhibit β-catenin/Tcf4 pathway;[21] it was used here as a positive control.

BC21 Decreases the Viability and Reduces the Colony Forming Activity of β-Catenin Overexpressing Colon Cancer Carcinoma Cells. We examined the cell death and clonogenic cell death effects of this compound on HCT116 cells using CellTiter-Blue Cell Viability and colony-formation assays. BC21 effectively induced HCT116 cell death in a dose-dependent manner; over 80% of the cells died after a 72 h treatment with 30 μM BC21 (Figure 3). At concentrations of 5 and 10 μM, BC21 was significantly blocked more than 80 and 100% of the colony-forming activity of HCT116 cells, respectively (Figure 4). However, at 3−15 μM, BC21 exhibited no cytotoxic effects on normal HEK293 and HUVEC cells. These effects on cell viability and clonogenic cancer cell death were stronger for BC21 than for quercetin, $p < 0.05$. These results indicated that the interruption of β-catenin/Tcf4-mediated

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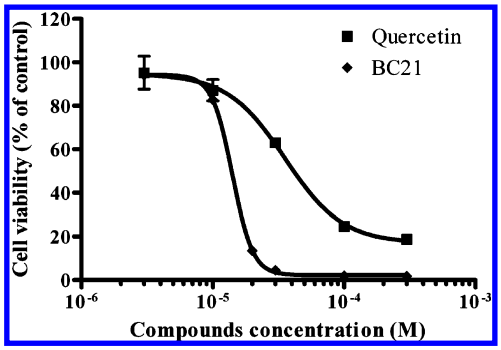

Figure 3. Effect of BC21 on the viability in HCT116 cells. The HCT116 cells were treated with the indicated concentration of each compound. The cell viability was determined by CellTiter-Blue assays (Promega) after a 72 h treatment. The IC_{50} for quercetin and BC21 was 35 and 15 μM, respectively.

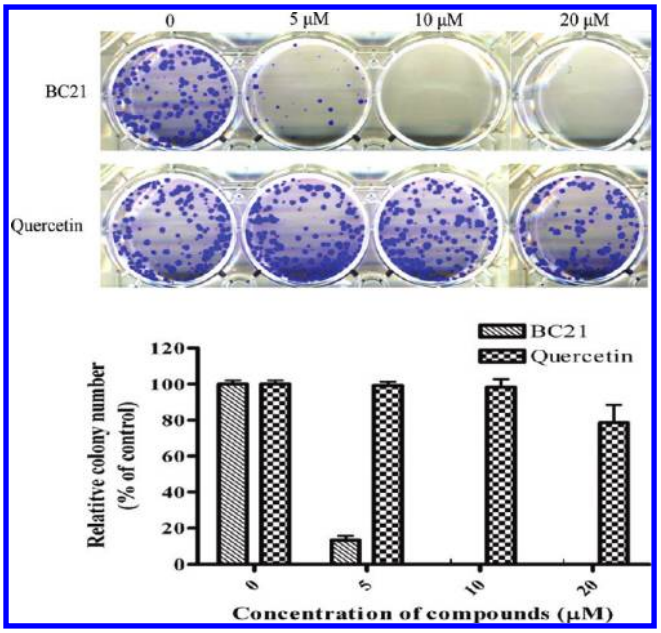

Figure 4. Effect of BC21 on HCT116 colony forming activity. The HCT116 cells were treated with the indicated concentrations of BC21 or quercetin and incubated for 7 days at 37 °C in a humidified atmosphere containing 5% CO_2. Colonies consisting of more than 50 cells were scored (left panel). Relative colony number was calculated as $[(\text{colony number})_{\text{treatment}}/(\text{colony number})_{\text{control}}] \times 100\%$ and is shown in the right panel.

signaling pathway caused by BC21 resulted in the induction of HCT116 cell death, particularly clonogenic cell death.

BC21 Down-Regulates the Expression of Wnt/β-Catenin Target Genes. Cyclin D1 and c-Myc are two important target genes of the Wnt/β-catenin signaling pathway that are up-regulated in many cancer cells including HCT116 carcinoma cells. After a 24 h treatment with 20 and 40 μM BC21, the expressions of c-myc (Figure 5A) and cyclin D1 (Figure 5B), respectively, were significantly reduced to levels comparable to those seen with 50 μM quercetin. We also examined the mRNA levels for c-myc and cyclin D1 after treated with BC21. As shown in Figure 6, after 5 h treatment at 40 μM, the cyclin D1 mRNA amount was significantly reduced. The c-myc mRNA levels were decreased at both 20 and 40 μM treatments. Most RNA were degraded after treating with 40 μM BC21 for 24 h

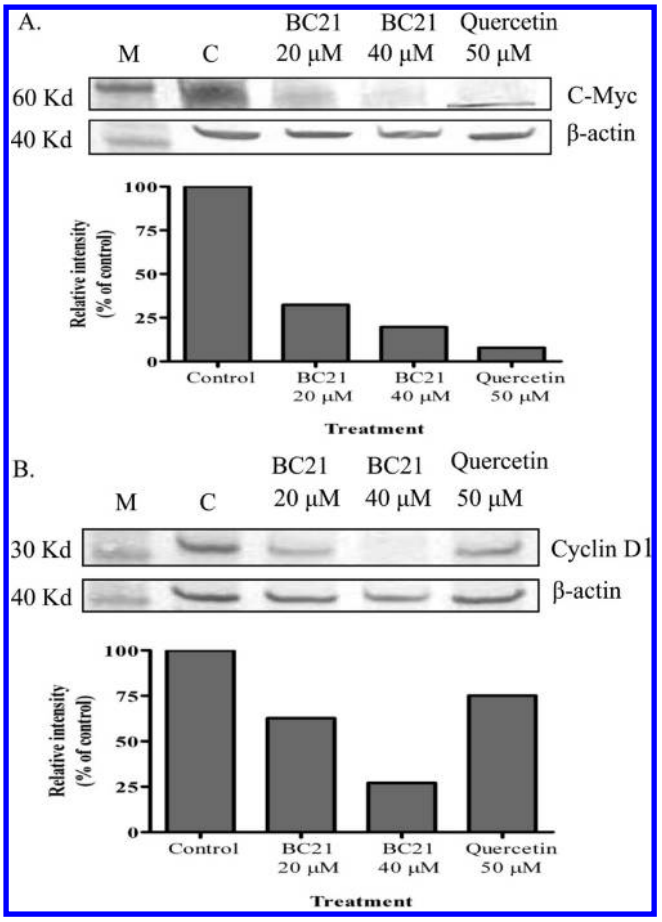

Figure 5. Effect of BC21 on the expression of the Wnt/β-catenin target gene. The HCT116 cells were treated with the indicated concentrations of BC21 or quercetin. Cell lysates were prepared after 24 h treatment and subjected to Western blotting with anti-c-myc (A) or with anticyclin D1 (B) antibody. Anti-β-actin antibody was used as a loading control. The intensity of each band was quantified by Image J and normalized against a nontreatment control. The result was representative of three independent experiments.

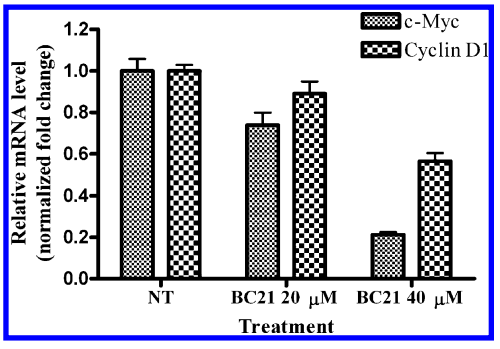

Figure 6. Effect of BC21 on the mRNA level of the Wnt/β-catenin target gene. The HCT116 cells were treated with the indicated concentrations of BC21. Total RNA was prepared after 24 h (for the 20 μM treatment) or 5 h (for the 40 μM treatment). The cDNA was synthesized and quantified by real-time PCR and normalized against a nontreatment control.

due to the high cytotoxicity. So the data at this time point and concentration were not determined.

BC21 Interferes the Binding between β-Catenin and Tcf4 Peptide. We determined the direct inhibitory effects of

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BC21 on interaction between β-catenin and Tcf4 by developing a new cell-free FP-based competitive binding assay. BC21 was able to inhibit the binding between β-catenin and FITC-labeled Tcf4 probe with an IC_{50} value of 5 μM (Figure 7).

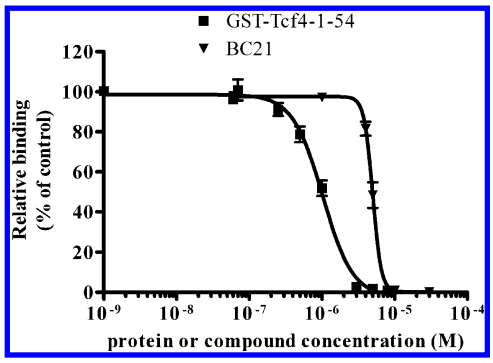

Figure 7. Competitive binding assay for GST-Tcf4-1-54 and BC21. A mixture of 1.5 μM β-catenin and 20 nM tracer (FITC labeled Tcf4 peptide) was incubated in the absence or presence of indicated concentrations of competitor. Fluorescence polarization values were recorded after 3 h. The relative binding was calculated as $(mP_T - mP_f)/(mP_C - mP_f) \times 100$.

The β-catenin binding domain of Tcf4 (GST-Tcf4-1-54) was used as a positive control, which has an IC_{50} of 1 μM.

DISCUSSION

Current cancer treatments, such as chemo- and radiotherapies, are successful at destroying bulk cancer cells but are ineffective at eliminating self-renewing cancer stem cells (CSCs). The Wnt/β-catenin pathway is one of the important signaling pathways that are critical for CSC renewal and proliferation. Targeted disruption of the interaction between β-catenin and Tcf DNA binding proteins, which is a key step involved in Wnt/β-catenin-mediated proliferative signaling,[18,22] would be a valuable strategy as a tumor therapy. In the present studies, we discovered a new class of small molecule inhibitor of β-catenin−Tcf4 interaction. It not only effectively inhibits the binding between β-catenin and Tcf4 derived peptide as well as the β-catenin-Tcf4 reporter TOP gene activity but also causes apoptosis and clonogenic cell death for β-catenin overexpressing HCT116 colon cancer cells.

β-Catenin has a superhelix structure and bears a long and shallow groove, while Tcf3 and Tcf4 bind to this groove as a linear structure. The binding interfaces of β-catenin and Tcfs are very large which create difficulty in finding a small molecule that can compete with Tcfs by occupying the whole binding pocket. However, "hot spots" exist on the protein surface; that is, small regions that are essential for maintenance of the binding affinity of protein−protein interactions.[23,24] Fasolini et al. defined the hot spots for Tcf4 in β-catenin-binding by performing an alanine scanning of all Tcf4 residues on the binding interface.[18] They found that, in addition to Asp16, which is the most important residue for binding, alanine replacement of Asp11 and three hydrophobic residues on the α-helix (Leu41, Val44, and Leu48) also results in significant decrease in the binding constants. Another research group showed that Lys435 and Lys508 were also critical for β-catenin binding to Tcf4.[20] Therefore, blocking a hot spot using a small molecule resulted in disruption of the protein−protein interactions. In fact, some small molecules have been reported to show inhibitory effects on this interaction.[21,25−27] However, most of these compounds are natural products extracted from plant or fungi, and their chemical modification is difficult; this limits the capacity for improving their potency and selectivity. BC21, the small molecule we identified in this study, is a potent candidate inhibitor for β-catenin/Tcf4 interaction. This small molecule is an organic compound and, as far as we know, is different from other published inhibitors in its chemical structure. Previous studies showed that the hydrophobic interactions between Tcf4 Ile19 and Phe21 with β-catenin had ∼60% functional contributions.[20] This means that blocking the Tcf4 Ile19−Phe21 interaction with β-catenin is critical. BC21 is located at the β-catenin hydrophobic cleft just where it supposedly would interact with Tcf4 Ile19 and Phe21. In addition, BC21 occupied another important binding site and blocked the Tcf4 Asp16 from forming a hydrogen bond with β-catenin Lys435, which is a key Tcf4/β-catenin interaction. One naphthalene ring of BC21 undergoes hydrophobic interactions with the side chains of Pro463 and Cys466, and the other naphthalene ring also undergoes hydrophobic interactions with the fatty carbon chains of Lys508 and Arg469. Therefore, although BC21 binds part of the Tcf4 binding pocket of the β-catenin, it is capable of hindering the Tcf4 binding, thereby disrupting the β-catenin/Tcf4 interactions. Our biological data demonstrated this inhibitory activity of BC21.

A high level of β-catenin has been found in many cancers, especially colon cancer.[28,29] We investigated the biological activities of our identified compound using a human colon cancer cell line HCT116, which expresses elevated mutant β-catenin. In addition, in order to determine the specific effects of inhibitors on Wnt/β-catenin pathway, in addition to the primary HCT116 colon cancer cell line, we employed HEK293 cells by transfecting them with plasmid pcDNA3.1-β-catenin (wild-type). This caused them to express β-catenin and specifically activate the Wnt/β-catenin signaling pathway for the measurement of the TOP/FOP luciferase activity of the reporter gene in this pathway. BC21 showed dose-dependent inhibitory effects on the β-catenin/Tcf4 driven luciferase activity in both primary HCT116 and pcDNA3.1-β-catenin transfecred HEK293 cell lines (Figure 2). The less inhibitory effect of BC21 on TOP luciferase activity in HEK293 cells compared to the effect seen in HCT116 cells may be caused by higher transient expression level of β-catenin in HEK293 cells. When we next examined the effects of BC21 on cell viability and clonogenic activity of HCT116 cells, we found that BC21 effectively decreased the HCT116 cell viability and colony forming activities (Figures 3 and 4). At 5 μM, BC21 was able to block more than 80% of the colony-forming activity of HCT116 cells (Figure 4). However, 5 μM BC21 exhibited no cytotoxic effects on normal HEK293 and HUVEC cells. This indicates that BC21 represents a new anticolon cancer candidate which possesses potential β-catenin/Tcf4 specific antiproliferative activity. We further examined its effects on c-Myc and cyclin D1, which are Wnt/β-catenin signal target genes,[2] and as expected, BC21 reduced the expression of these two important genes.[4] The 5 μM IC_{50} value of BC21 in the colony forming assay (Figure 4) was lower than the 15 μM IC_{50} value of BC21 in the cell viability assay (Figure 3) but was consistent with the concentration in the competitive binding assay (Figure 7) and luciferase reporter assay in HCT116 cells (Figure 1A). Possibly, BC21 at lower concentrations exerts its antiproliferation activity mainly by blocking Wnt/β-catenin pathway, whereas at higher concentrations, other pathways might be affected which result in

cytotoxicity. For example, at concentrations above 50 μM, BC21 has been reported to inhibit protein phosphatase 2C and proteasome and to induce apoptosis in human cancer cells.[30,31]

In summary, we demonstrated that BC21 represents a new class of small molecule inhibitors of β-catenin/Tcf4 interaction and signaling. It interrupts the direct binding of β-catenin to Tcf4 and down-regulates the expression and activity of Wnt/β-catenin target genes and gene products. This results in the induction of HCT116 cell death, particularly clonogenic cell death. Further modification and optimization of the BC21 chemical structure would be worth pursuing in order to generate new analogues with improved potency and selectivity against β-catenin/Tcf4 interactions.

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ABBREVIATIONS

Wnt, wingless; Tcf/Lef, T cell factor/lymphoid enhancer factor; GSK-3β, glycogen synthase kinase-3β; APC, axin/adenomatous polyposis coli tumor suppressor protein; Fzd, frizzled; Dsh, disheveled; CSC, cancer stem cell; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; FP, fluorescence polarization; FITC, fluorescein isothiocyanate.

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