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C644-0303, a small-molecule inhibitor of the Wnt/β-catenin pathway, suppresses colorectal cancer growth

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Abstract

The Wnt/β-catenin signaling pathway plays an important role in tissue homeostasis, and its malignant activation is closely related to the occurrence and development of many cancers, especially colorectal cancer with adenomatous polyposis coli (APC) and CTNNB1 mutations. By applying a TCF/lymphoid-enhancing factor (LEF) luciferase reporter system, the high-throughput screening of 18 840 small-molecule compounds was performed. A novel scaffold compound, C644-0303, was identified as a Wnt/β-catenin signaling inhibitor and exhibited antitumor efficacy. It inhibited both constitutive and ligand activated Wnt signals and its downstream gene expression. Functional studies showed that C644-0303 causes cell cycle arrest, induces apoptosis, and inhibits cancer cell migration. Moreover, transcription factor array indicated that C644-0303 could suppress various tumor-promoting transcription factor activities in addition to Wnt/β-catenin. Finally, C644-0303 suppressed tumor spheroidization in a 3-dimensional cell culture model and inhibited xenograft tumor growth in mice. In conclusion, we report a novel structural small molecular inhibitor targeting the Wnt/β-catenin signaling pathway that has therapeutic potential for colorectal cancer treatment.

KEYWORDS
antitumor, colorectal cancer, high-throughput screening, small molecular inhibitor, Wnt/β-catenin signaling

1 | INTRODUCTION

The Wnt/β-catenin signaling pathway plays a critical role in regulating cell proliferation, differentiation, migration, and survival. Its aberrant activation is associated with multiple tumors such as colorectal cancer (CRC), melanoma, and hepatocellular carcinoma.[1-4]

Under resting conditions, β-catenin is continuously degraded through ubiquitination due to the phosphorylation by a destruction complex containing scaffold protein Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and casein kinase 1α (CK1α). Once the secreted Wnt ligands bind to cell surface receptor frizzled (FZD) and co-receptor LDL-receptor-related protein

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Yu Yan and Yidan Zhang contributed equally to this work.

(LRP), the destruction complex is inactivated through recruiting Disheveled (Dvl) polymers to the plasma membrane and β-catenin is dephosphorylated, stabilized, and eventually transfers into the nucleus. The β-catenin forms an active complex with T cell-specific transcription factor (TCF)/lymphoid-enhancing factor (LEF) to regulate target gene transcription such as Cyclin D1, c-Myc, and Axin2.[5]

CRC is one of the most common and deadly cancer types in both men and women, with an annual incidence rate increase of 2% in adults under age 55.[6] Through large-scale genome sequencing, mutation of several key genes, such as APC and β-catenin, were identified to constitutively an activate Wnt signaling pathway, closely related to CRC occurrence and development.[7] Therefore, targeting Wnt signaling might be an efficient therapeutic strategy for CRC treatment. In addition, current clinical CRC treatment is mainly surgical resection combined with traditional radiotherapy and chemotherapy, while targeted treatment is relatively scarce. In the past decade, several small-molecule compounds have been reported to downregulate Wnt signaling.[8-11] Compounds targeting β-catenin or β-catenin/complex exhibit antitumor effects in mutant CRC, furthering confirming the therapeutic potential of Wnt signaling inhibitors for CRC.[12,13] Our research aimed to discover new scaffold compounds that target the Wnt signaling pathway to inhibit APC or CTNNB1 mutated CRC, providing lead compounds for later drug development.

In current study, a TCF/LEF luciferase reporter-based drug screening system was developed and 18 840 small-molecule compounds were screened. C644-0303 was identified as a Wnt/β-catenin signaling inhibitor. It shows potent antitumor effects on Wnt-dependent CRCs both in 3D organoids in vitro and in xenograft tumor models in vivo. Our studies identified a lead compound with a previously unreported scaffold structure to efficiently inhibit Wnt signaling and mutant CRCs growth, providing novel chemical molecules for further development of drug-like compounds to treat CRC.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Antibodies against β-catenin (D10A8), non-phospho active β-catenin (Ser45) (D2U8Y), non-phospho (active) β-catenin (Ser33/37/Thr41) (D13A1), phospho-β-catenin (Ser33/37/Thr41), phospho-β-catenin (Ser552) (D8E11), phospho-β-catenin (Ser675) (D2F1), GSK-3β (3D10), phospho-GSK-3β (Ser9) (D85E12), Akt (pan) (C67E7), phospho-Akt (Ser473) (D9E), Histone H3 (D1H2), cyclin D1, β-actin (13E5), E-cadherin (24E10), Axin2 (76G6), c-Myc (D3N8F), N-cadherin (D4R1H), Vimentin (D21H3), and Snail (C15D3) were from Cell Signaling Technology, α-tubulin (B-7) from Santa Cruz, and GAPDH from Kang Chen. Bioactive compound libraries, diversity libraries, clinical compound libraries, natural compound libraries, and approved drug libraries were purchased from Target Mol.[14] LF3 (Cat. No. HY-101486) IWR-1 (Cat. No. HY-12238) was acquired from MedChemExpress. The Urea Assay Kit (C013-2-1), Creatinine (Cr) Assay Kit (C011-2-1) and Alanine Aminotransferase Assay Kit

(C009-2-1) were from Nanjing Jiancheng Bioengineering Institute. The bicinchoninic acid (BCA) Assay Kit was from Solarbio.

2.2 | Cell culture

Cell lines L Wnt-3A, HEK293T, HCT-116, HT-29, DLD-1, LS-174T, NCI-H460, MCF-7 were obtained from the American Type Culture Collection and human normal colon epithelial cell line CCD-841CoN from BLUEFCELL. Media were supplemented with 10% FBS, penicillin (100 IU/mL) and streptomycin (100 mg/mL), and the cells were cultured in an incubator containing 5% CO_2 at 37°C.

2.3 | Luciferase reporter assay

HCT-116 cells were stably transfected with TCF/LEF luciferase reporter plasmid (Genomeditech, GM-021042) (HCT-116-Luc). Transfection was performed with lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol and positive clones were selected with 500 μg/mL G418 (Sigma). Reporter plasmid FOPFlash (Beyotime, D2503) served as a negative control. Reporter cells (3000/well) were seeded into white 96-well plates and cultured for 24 h. Cells were then treated with compounds for 24 h and luciferase assay reagent (Promega) was added to each well. The fluorescence value was read using a SpectraMax® L microplate reader.

2.4 | Cell viability assay

Cells (3000/well) were seeded into 96-well plates and incubated overnight. Then the cells were treated with either vehicle or compounds at the indicated concentrations. After 72 h, resazurin (Sigma) was added and cells were incubated for another 4 h. The absorbance was measured using a SpectraMax® i3 microplate reader at 544 nm/595 nm (excitation/emission) wavelengths.

2.5 | Real-time PCR measurement

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen). Reverse transcription was performed with PrimeScript™ RT reagent kit (Roche). The cDNA samples were amplified using SYBR Green (Roche) in the StepOne Plus Real-Time PCR System (Applied Biosystems). Specific gene expression levels were determined as previously described and normalized to GAPDH or β-actin.[15] The primer sequences for real-time PCR are listed in Table S1.

2.6 | Western blotting

Cells were lysed in cell lysis buffer (Cell Signaling Technology, Cat. No. 9803) using protease and phosphatase inhibitor cocktails (Roche,
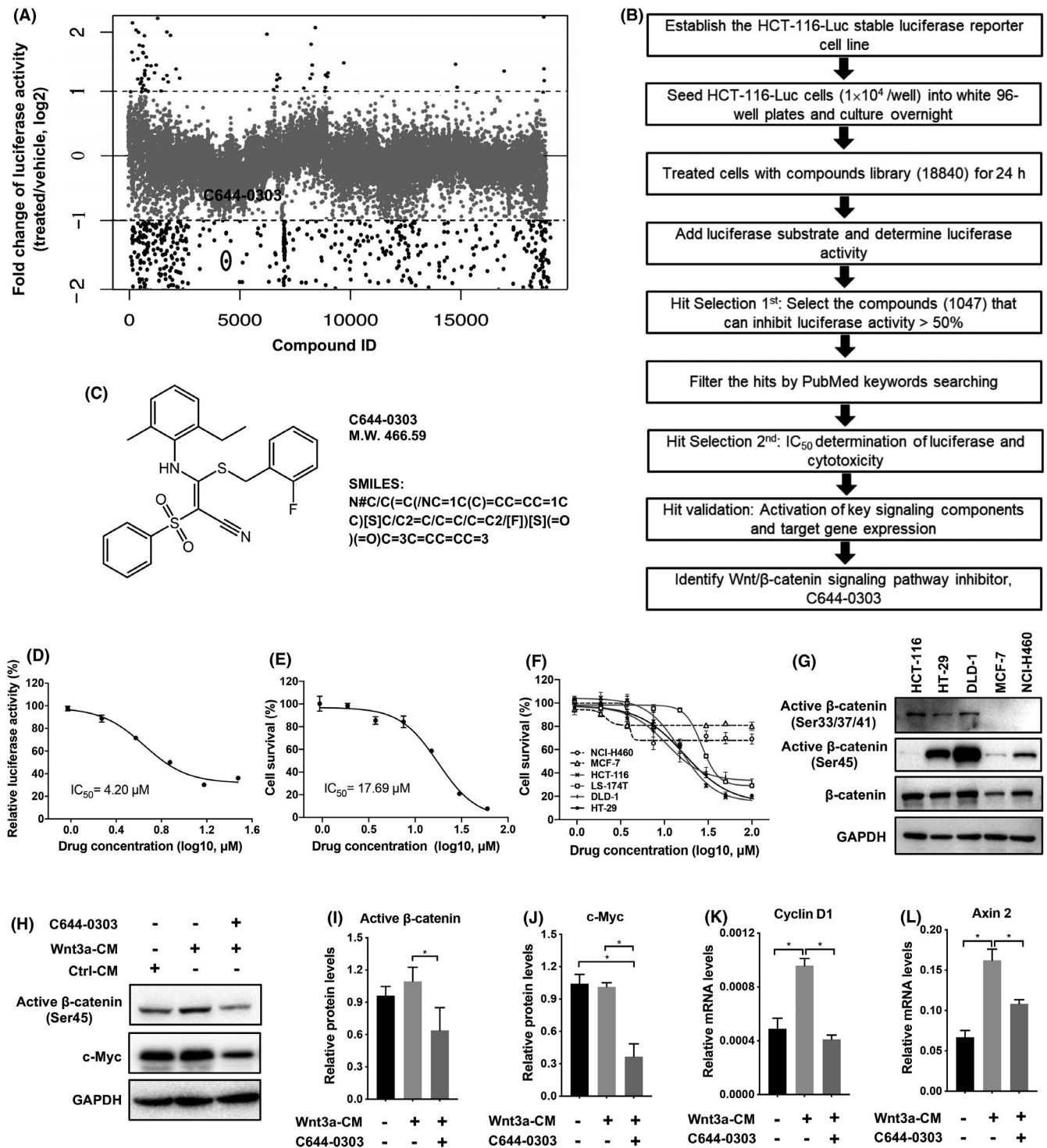
FIGURE 1 C644-0303 was identified as an inhibitor of Wnt/β-catenin signaling by a TCF/LEF-based luciferase drug screening system. A, HCT-116-Luc cells were treated with 18 840 drugs (20 μmol/L) respectively, and the luciferase activity was detected 24 h later. B, Flowchart of high-throughput drug screening. C, Chemical structure and SMILES of C644-0303. The effects of C644-0303 on TCF/LEF luciferase activity for 24 h (D), and cell viability for 72 h (E), in HCT-116-Luc cells were measured. F, Cell viability upon C644-0303 treatment at the indicated concentrations for 72 h. G, The basal level of β-catenin, active β-catenin (Ser33/37/Thr41), and active β-catenin (Ser45) in the indicated cells were analyzed by western blot. H, HEK293T cells were treated with a control-conditioned medium (Ctrl-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of DMSO or C644-0303 (20 μmol/L) for 18 h, followed using western blot analysis. Levels of active β-catenin (Ser45) (I), and c-Myc (J), were quantified using ImageJ software. Wnt3a-CM stimulated HEK293T cells were treated with C644-0303 (20 μmol/L) for 8 h. The mRNA levels of Cyclin D1 (K), and Axin2 (L) were detected using real-time PCR. Error bars indicate means ± SEM. *$P < .05$, significant, one-way ANOVA

Cat. No. 04693132001/4906837001) and total protein (20 μg) was analyzed as previously described.[15] For cytoplasmic and nuclear extracts, the Nuclear Protein Extraction Kit (Solarbio, R0050) was used.

2.7 | Scratch assay

When cells had spread to 6-well plates, cell surfaces were scratched with a 200-μL pipette tip. Cells were washed with sterile PBS and cultured with compounds in 1% FBS medium. The scratch widths were observed under a Zeiss Primovert inverted microscope and quantified using ImageJ software after 0, 24, and 48 h.

2.8 | Flow cytometry analysis

Cells were seeded overnight and treated with C644-0303 for 24 h. Cells were detached, fixed, and stained using the Cell Cycle Staining Kit (Lianke Bio). Cells were harvested and stained using the Annexin V-FITC Apoptosis Kit (Invitrogen) after 48 h treatment by C644-0303 or IWR-1. Then the collected cells were analyzed using a BD FACSAria™ III flow cytometer. Data were processed using FlowJo software.

2.9 | 3D cell culture model

3D cell culture was performed as described previously.[16-18] Briefly, 25 000 cells/well (embedded system) and 40 000 cells/well (on-top system) were seeded into 24-well plates. Cells were treated with C644-0303 for 5 or 6 d, photographed every 2 d and quantified with ImageJ software.

2.10 | Transcription factors (TF) activation assay

A TF Activation Profiling Plate Array II (Signosis, Cat. No. FA-1002) was used for monitoring the activation of 96 different TFs simultaneously from 1 sample. HCT-116 cells were treated with C644-0303 (20 μmol/L) for 18 h. Then nucleoproteins were extracted and the TF

TABLE 1 IC_{50} of C644-0303 in different cell lines

Cell lines	Mutant	IC_{50} (μmol/L)
HCT-116	β-Catenin	15.31 ± 1.99
LS-174T	β-Catenin	26.70 ± 1.82
HT-29	APC	16.91 ± 3.22
DLD-1	APC	10.51 ± 1.65
NCI-H460	Wild type	>100
MCF-7	Wild type	>100

Activation assay were performed according to the manufacturer's instructions.

2.11 | Animals

All of the animal procedures were approved by the Committee of Experimental Animals of School of Medicine and Pharmacy, Ocean University of China (OUCSMP-20200702). Here, 6-wk-old female BALB/c-nu/nu mice were purchased from GemPharmatech (Nanjing, China).

2.12 | Immunohistochemistry

Tumor samples were fixed in 4% paraformaldehyde (Servicebio) and embedded in paraffin (FFPE). Immunohistochemistry staining for c-Myc and Ki67 (c-Myc, 1:1600; Ki67, 1:500) was performed as previously described.[19] All slides were mounted and observed under ×200 magnification under a microscope (Nikon, Japan) and images were quantified using ImageJ software.

2.13 | Statistical analysis

Statistical significance of differences between indicated samples was determined using unpaired Student t test, one-way, or two-way ANOVA using GraphPad Prism 8 software. A *P-value < .05 was considered to be statistically significant.

3 | RESULTS

3.1 | C644-0303 was identified as a small-molecule inhibitor of the Wnt/β-catenin signaling pathway

To identify compounds that could effectively inhibit Wnt signaling, we constructed a luciferase reporter-based drug screening system using the human colon cancer cell line HCT-116 with constitutive Wnt signaling activation due to CTNNB1 heterozygous Ser45 deficiency.[20] In total, 18 840 small molecules from various compound libraries[14] were screened for the perturbation of TCF/LEF luciferase activities at 20 μmol/L post 24 h treatment using the established system (Figure 1A,B). The 1047 compounds exhibited more than 50% inhibition activity compared with vehicle at 24 h, compared with 78% inhibition activity of the reported β-catenin/TCF4 antagonist LF3 (20 μmol/L).[13] Results were filtered using the PubMed database to omit previously reported compounds related to Wnt/β-catenin signaling, the remaining compounds were further selected through IC_{50} determination of luciferase and cytotoxicity, as well as the activation of key signaling components and target gene expression. C644-0303 in a mini scaffold library, namely (2E)-3-[(2-ethyl-6-methylphenyl)
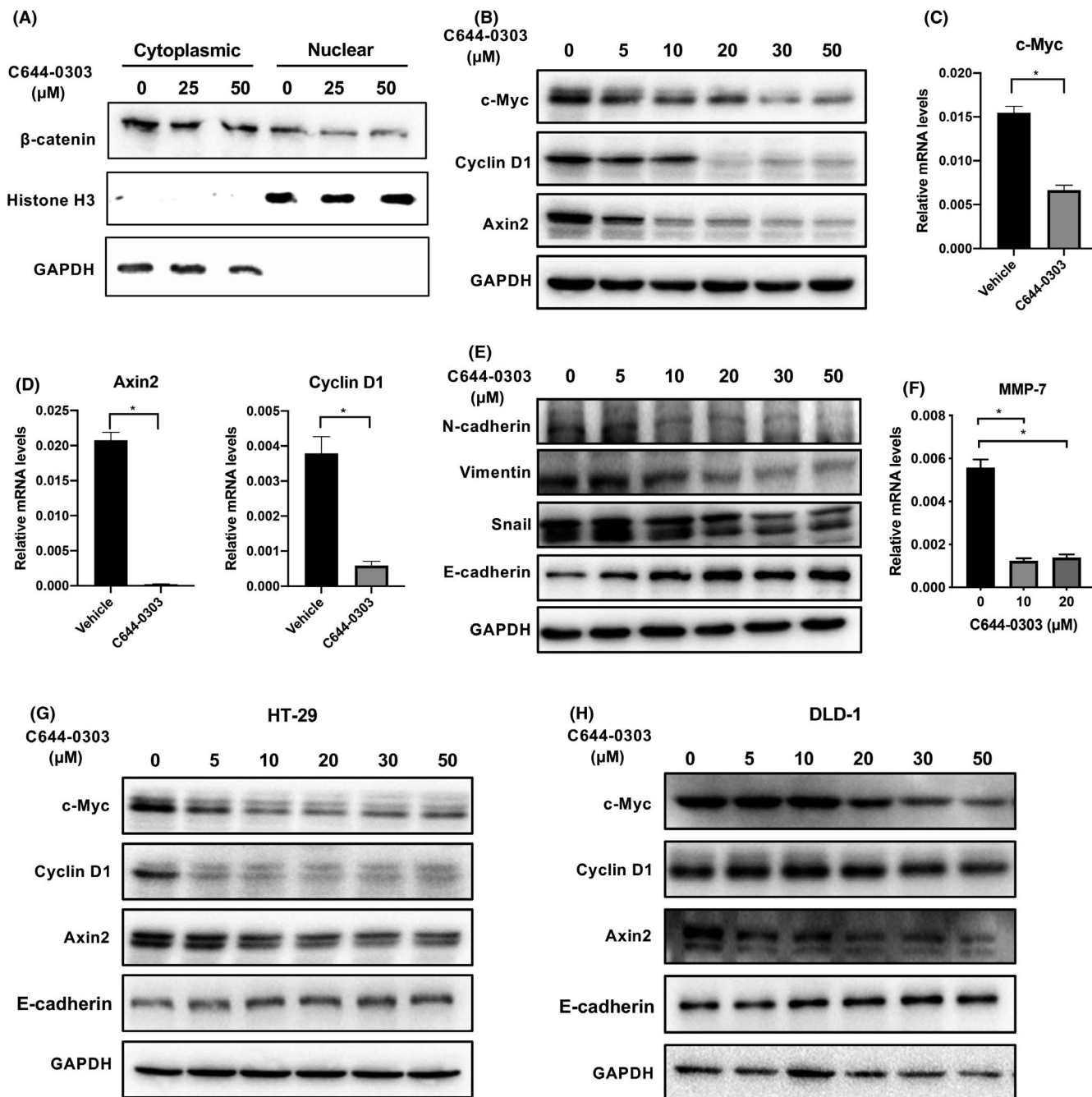
FIGURE 2 C644-0303 inhibits β-catenin activity and its downstream target gene expression. HCT-116 cells were treated with C644-0303 for 18 h. A, The cytoplasmic and nuclear protein were separated and analyzed using western blot. GAPDH and Histone H3 serve as a loading control of cytoplasmic and nuclear respectively. B, F, Whole-cell lysates were analyzed using western blot. C-E, HCT-116 cells were treated with C644-0303 (20 μmol/L) for 18 h. The mRNA levels of c-Myc (C), Axin2 (D) and Cyclin D1 (E) were determined using real-time PCR. G, HCT-116 cells were treated with C644-0303 for 24 h. The mRNA levels of MMP-7 were determined using real-time PCR. HT-29 (H) and DLD-1 (I) cells were treated with C644-0303 for 18 h, whole-cell lysates were analyzed using western blot. Error bars indicate means ± SEM. *$P < .05$, significant, Student t test for (C-E), one-way ANOVA for (G)

amino]-3-[(2-fluorobenzyl)sulfanyl]-2-(phenylsulfonyl)acrylonitrile, was identified as a Wnt signaling inhibitor with a novel structure not reported in previous publications (Figure 1C). The drug-likeness prediction by SwissADME tool[21] showed that the compound obeyed the Lipinski rule of five[22] (below 5 hydrogen bond donors, 10 hydrogen bond acceptors and 500 molecular

weight) with 1 violation (MLOGP > 4.15), confirming its drug-like capability. C644-0303 efficiently inhibited luciferase activity of TCF/LEF (IC$_{50}$, 4.20 μmol/L) but not negative control FOPFlash (Figures 1D and S1A). Consistently, C644-0303 potently impeded the cell growth of HCT-116 (IC$_{50}$, 17.69 μmol/L) but not a human normal colon epithelial cell line CCD-841CoN[23] (IC$_{50}$,

53.33 µmol/L) (Figures 1E and S1B) while the IC$_{50}$ of known Wnt signaling inhibitor IWR-1[24] is 60.77 µmol/L for HCT-116 and more than 100 µmol/L for CCD-841CoN (Figure S1C,D). These data suggested that C644-0303 could target the Wnt signaling pathway in mutant CRC with strong inhibitory potency, while its potential toxicity to normal colon cells could be further optimized.

To test the specificity of C644-0303 to the Wnt/β-catenin signaling pathway, we selected 6 cell lines that could be classified into 2 groups: Wnt-dependent cells (HCT-116, LS-174T, HT-29, DLD-1)[25,26] and Wnt-independent cells (NCI-H460, MCF-7)[12,13] for a growth inhibition assay (Figure 1F). C644-0303 efficiently inhibited proliferation of Wnt-dependent cancer cell lines (IC$_{50}$, 10-25 µmol/L) with
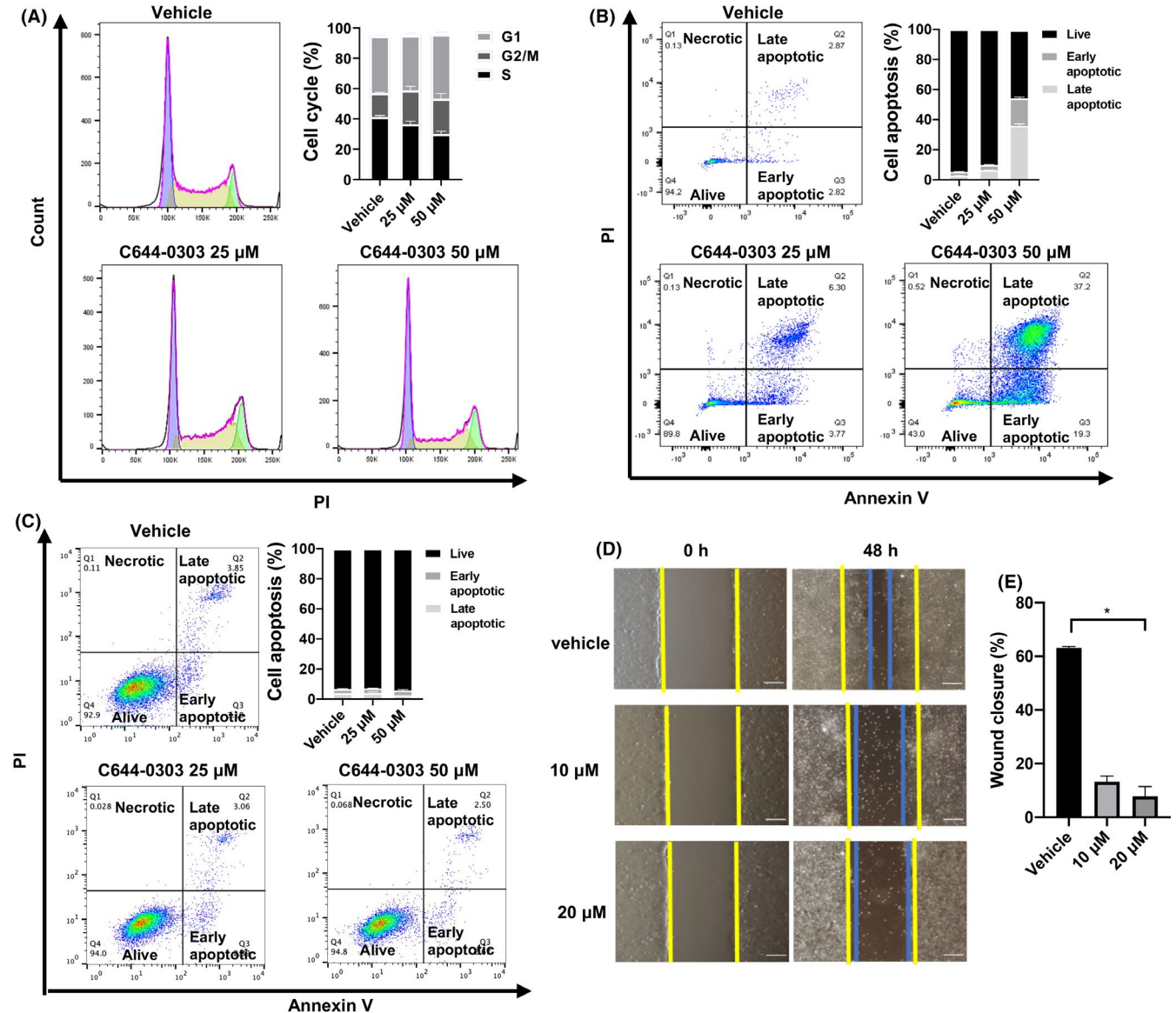

FIGURE 3 C644-0303 causes cell cycle arrest, induces apoptosis, and inhibits cancer cell migration. HCT-116 cells were treated with C644-0303 for 24 h and then processed for cell cycle analysis (A) and for 48 h for apoptosis analysis using Annexin V-FITC/PI assay (B). C, CCD-841CoN cells were treated with C644-0303 for 48 h, and cell apoptosis rate was analyzed. HCT-116 cells were scratched before treatment with C644-0303 for 48 h. The widths of the scratches were observed under a Zeiss Primovert inverted microscope (D) and quantified using ImageJ software (E). Scale bars, 200 µm. Error bars indicate means ± SEM. *P < .05, significant, one-way ANOVA

FIGURE 4 C644-0303 potently inhibits TCF/LEF activity and modulates β-catenin activity associated phosphorylation. A, Schematic diagram of Transcription Factor (TF) Activation Profiling Plate Array II. B, Nuclear extracts of HCT-116 cells incubated with 20 µmol/L C644-0303 for 18 h were processed as in (A). Scaled fold change of relative light units (RLU) of TFs in C644-0303 vs vehicle treated cells was determined. C, Heatmap of the normalized RLU of Top20 TFs. D, Graphical summary of tumor-associated functions of Top20 TFs using Ingenuity Pathways Analysis (IPA). Wnt3a-CM stimulated HEK293T cells were treated with C644-0303 (20 µmol/L) for 8 h in (E) and 18 h in (F), and analyzed using western blot
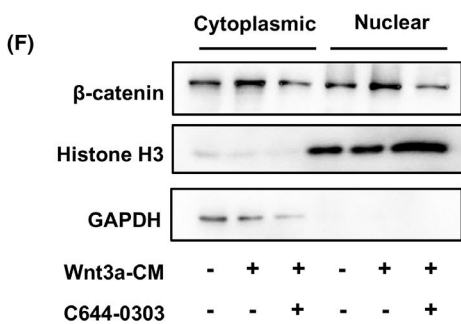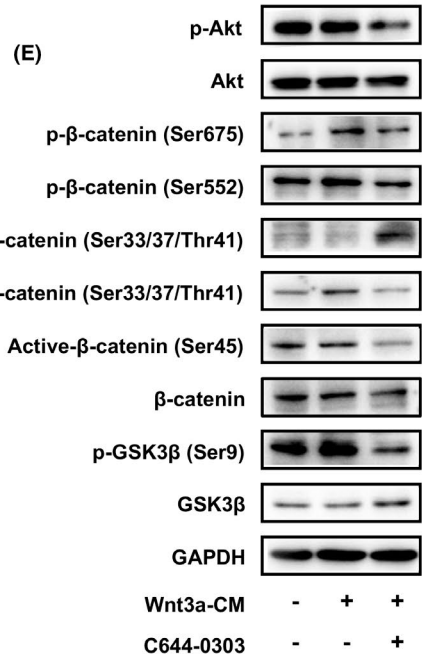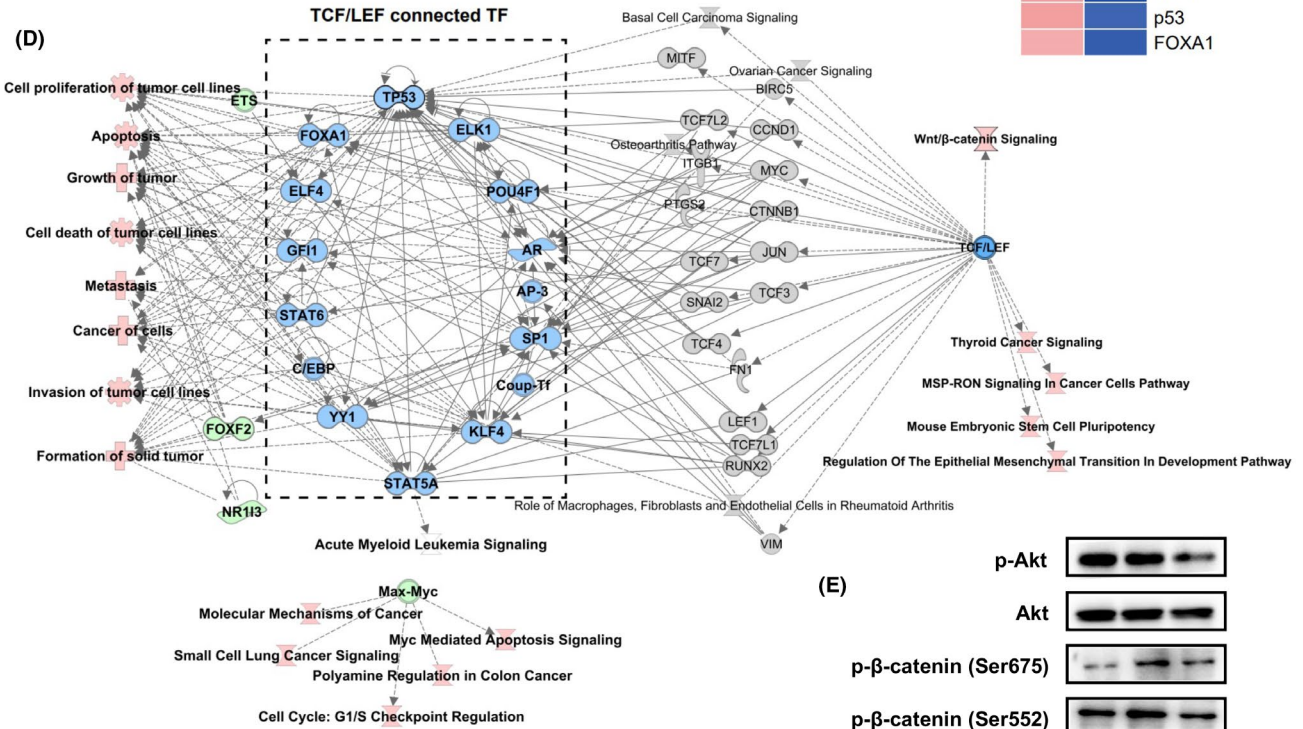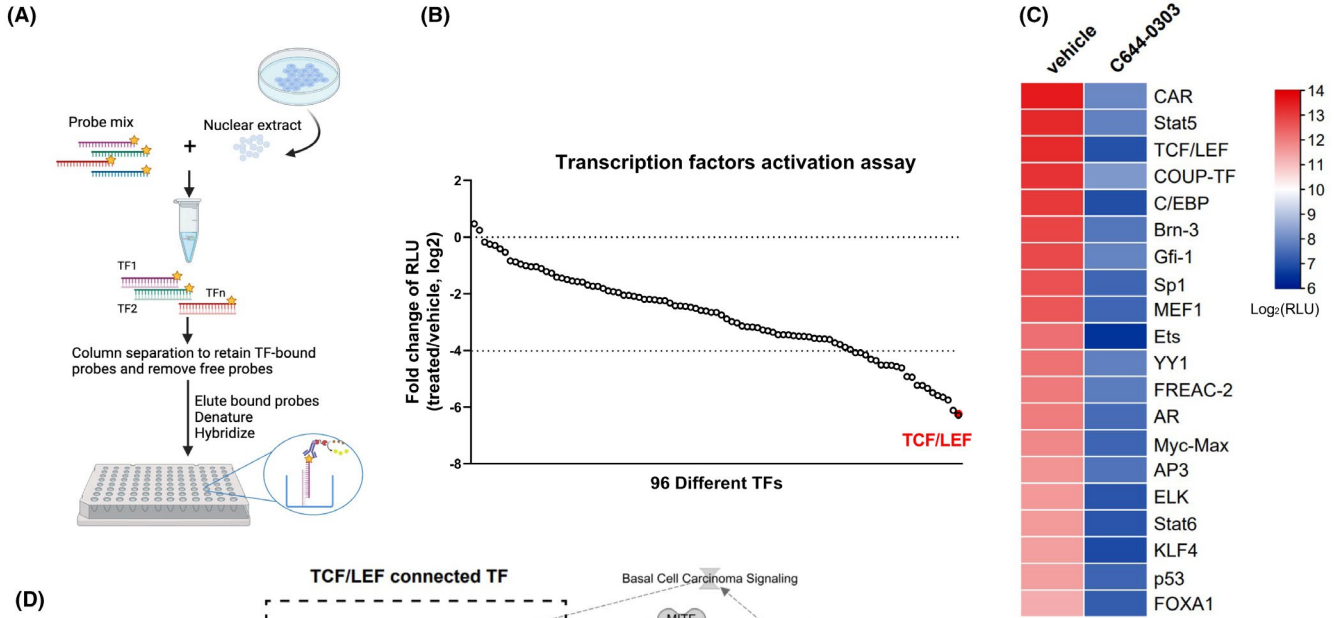
little effect on the viability of Wnt-independent cancer cell lines (IC_{50} > 100 μmol/L) (Table 1), which is consistent with their Wnt/β-catenin signaling activation levels (Figure 1G).

Next, C644-0303 inhibition efficiency on ligand-dependent Wnt signal activation was tested in Wnt3a stimulated HEK293T cells. C644-0303 inhibited Wnt3a-conditioned medium[27] (Wnt3a-CM)

induced β-catenin activation, and its downstream targets c-Myc, cyclin D1 and Axin2 expression levels (Figures 1H-L and S1E,F). In summary, C644-0303 could selectively inhibit Wnt signaling, but had little effect on Wnt-independent cells. Therefore, C644-0303 was regarded as a potential Wnt/β-catenin inhibitor for in-depth exploration.
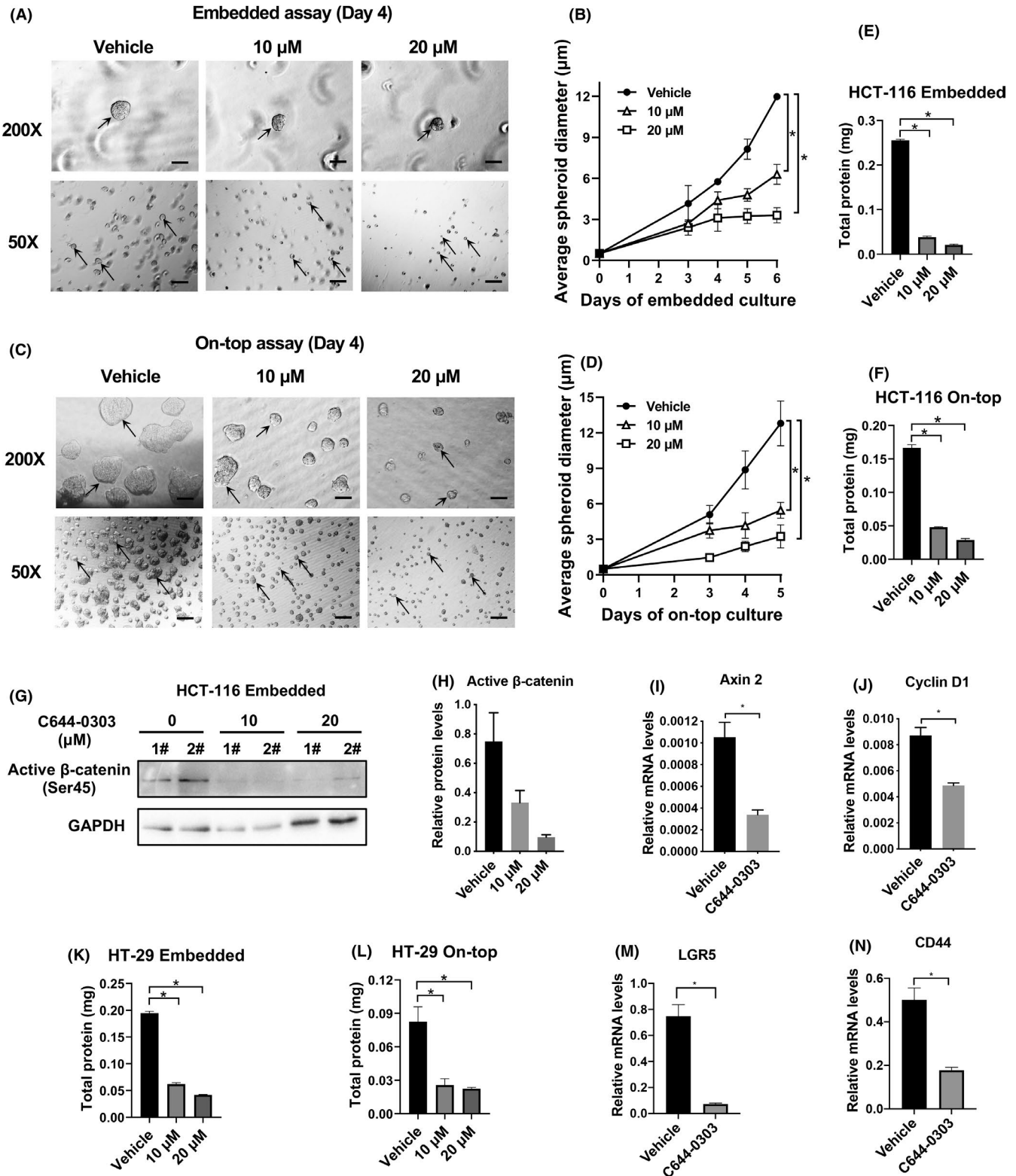
3.2 | C644-0303 inhibits β-catenin activity and endogenous Wnt target genes' expression

To understand the inhibition of Wnt/β-catenin signaling pathway by C644-0303, we examined the activation of β-catenin and downstream gene expression. In HCT-116 cells, nuclear β-catenin was decreased 18 h post C644-0303 treatment (Figure 2A), as well as nuclear translocation associated β-catenin phosphorylation at Ser675[28] (Figure S2A). Non-phosphorylated β-catenin at Ser33/37/ Thr41 and Ser45, which suppress β-catenin ubiquitination and degradation, were inhibited upon C644-0303 treatment (Figure S2A,B). In addition, C644-0303 strongly downregulated the protein and mRNA levels of Wnt target genes associated with cell growth and survival (Axin2, Cyclin D1, and c-Myc)[29] in HCT-116 cells (Figure 2B-E). Epithelial-mesenchymal transition (EMT) of cancer cells is known to be tightly regulated by the Wnt signaling pathway.[30] C644-0303 could decrease the expression of tumor pro-metastatic mesenchymal markers N-cadherin, Vimentin, Snail and matrix metallopeptidase MMP-7,[31] while increasing the expression of anti-metastatic epithelial marker E-cadherin (Figure 2F,G). Similarly, C644-0303 could also inhibit Wnt signaling in APC mutant CRC cell lines HT-29 and DLD-1 (Figures 2H,I and S2C,D). Therefore, C644-0303 blocked β-catenin activation and Wnt target gene expression in both CTNNB1 and APC mutant Wnt-dependent CRC cells.

3.3 | C644-0303 causes cell cycle arrest, induces apoptosis, and inhibits cancer cell migration

Cell cycle regulation is the key to control cell proliferation and closely related to the Wnt signaling pathway.[32] Wnt downstream targets such as Cyclin D1 and c-Myc are involved in phase transitions of the cell cycle.[33] Consistent with decreased expression of Cyclin D1 and c-Myc, cell cycle arrest induced by C644-0303 was observed (Figure 3A) and cell abundance in S phase was decreased after C644-0303 treatment (vehicle: 41.25%; 25 μmol/L: 36.53%; 50 μmol/L: 30%).

The Wnt/β-catenin signaling pathway also regulates cell apoptosis through a variety of mechanisms.[34] C644-0303 induced apoptosis of HCT-116 cells in a dose-dependent manner (Figure 3B). Specifically, 25 μmol/L C644-0303 induced 3.8% early apoptosis rate (Annexin V$^+$/PI$^-$) and 6.3% late apoptosis rate (Annexin V$^+$/PI$^+$), while at 50 μmol/L, 19.3% early apoptosis and 37.2% late apoptosis

were observed, which was higher than IWR-1 under the same concentrations (Figure S3A,B). Neither C644-0303 nor IWR-1 induced obvious cell apoptosis in normal colon CCD-841CoN cells at the tested concentrations (Figures 3C and S3A,C).

The Wnt signaling pathway was also reported to be vital in cell migration.[35-37] Consistent with the EMT marker changes post C644-0303 treatment, the motility ability of HCT-116 cells in wound-healing experiments was significantly inhibited 24 and 48 h post treatment in a dose-dependent manner, which was stronger than IWR-1 (Figures 3D,E and S3D,E). Similar results were observed in HT-29 (Figure S3D,F). These data suggested that C644-0303 could exert antitumor effects via cell cycle arrest, apoptosis induction, and EMT perturbation in CRC cancer cells.

3.4 | C644-0303 impairs TCF/LEF activity and phosphorylation mediated β-catenin activity

To get an overview of potential TFs influenced by C644-0303,[38,39] a cell nuclear extract was incubated with a 96 TF probe mixture and the TF activities were determined after treatment with C644-0303 (Figure 4A). Here, 20 out of 96 TFs exhibited high inhibition activities (cut-off: log2(RLU) < −4) (Figure 4B). Among them, Wnt signaling-mediated TCF/LEF transcriptional activity in the nucleus was mostly inhibited (solid red dot), which further supported C644-0303 as a potent Wnt signaling pathway inhibitor. In addition, we observed relatively strong downregulation of other tumor-associated TFs (Figure 4C) such as Myc-Max,[40] androgen receptor (AR),[41] signal transducer and activator of transcription 5 (STAT5),[42] and Ets1.[43] Using Ingenuity Pathway Analysis (IPA),[44] the previously reported crosstalk between the Top20 downregulated TFs associated with disease and function qre summarized (Figure 4D). TCF/LEF might regulate another 15 TFs directly (blue) and 3 TFs indirectly (ETS, FOXF2 and NR1I3 in green), suggesting the possible Wnt-dependent inhibition of these TF activities. Most of these TFs are functionally related to tumor progression such as cell cycle, apoptosis and metastasis (red). The dual inhibition of Max/Myc and TCF/LEF activity might result from the inhibition of shared upstream kinases such as GSK3.[45,46] These data indicated that C644-0303 may perform antitumor effects through inhibiting multiple tumor-promoting signals, possibly in a Wnt/β-catenin-dependent manner.

Wnt signaling activation could be modulated via the multiple phosphorylation sites of β-catenin to interfere with nuclear TCF/LEF activity.[28,47,48]
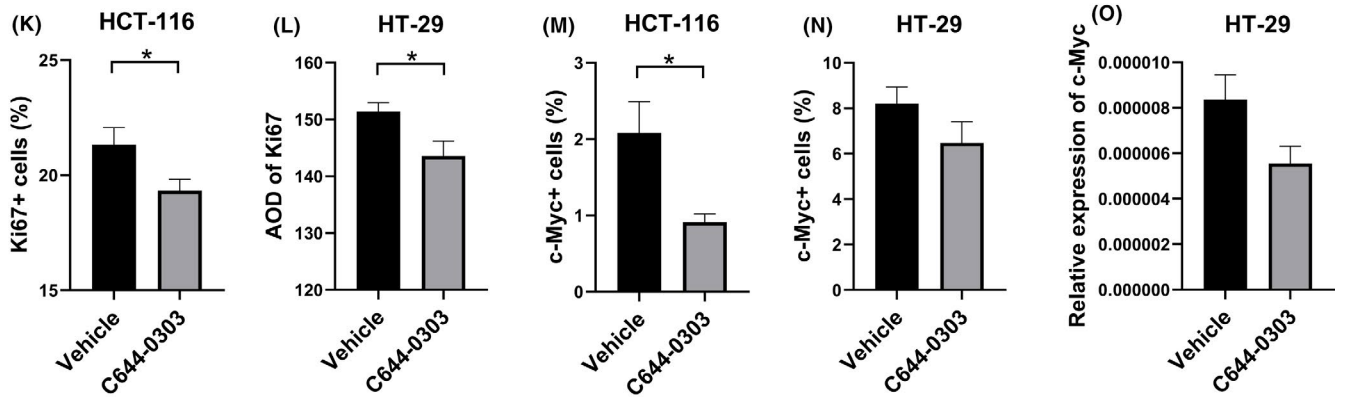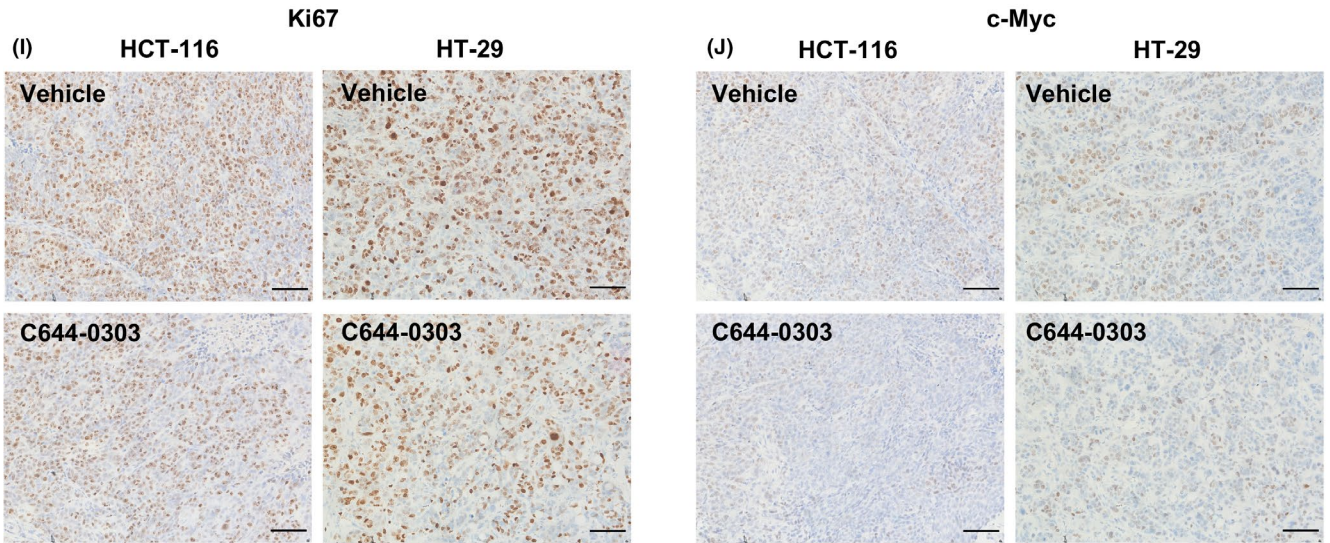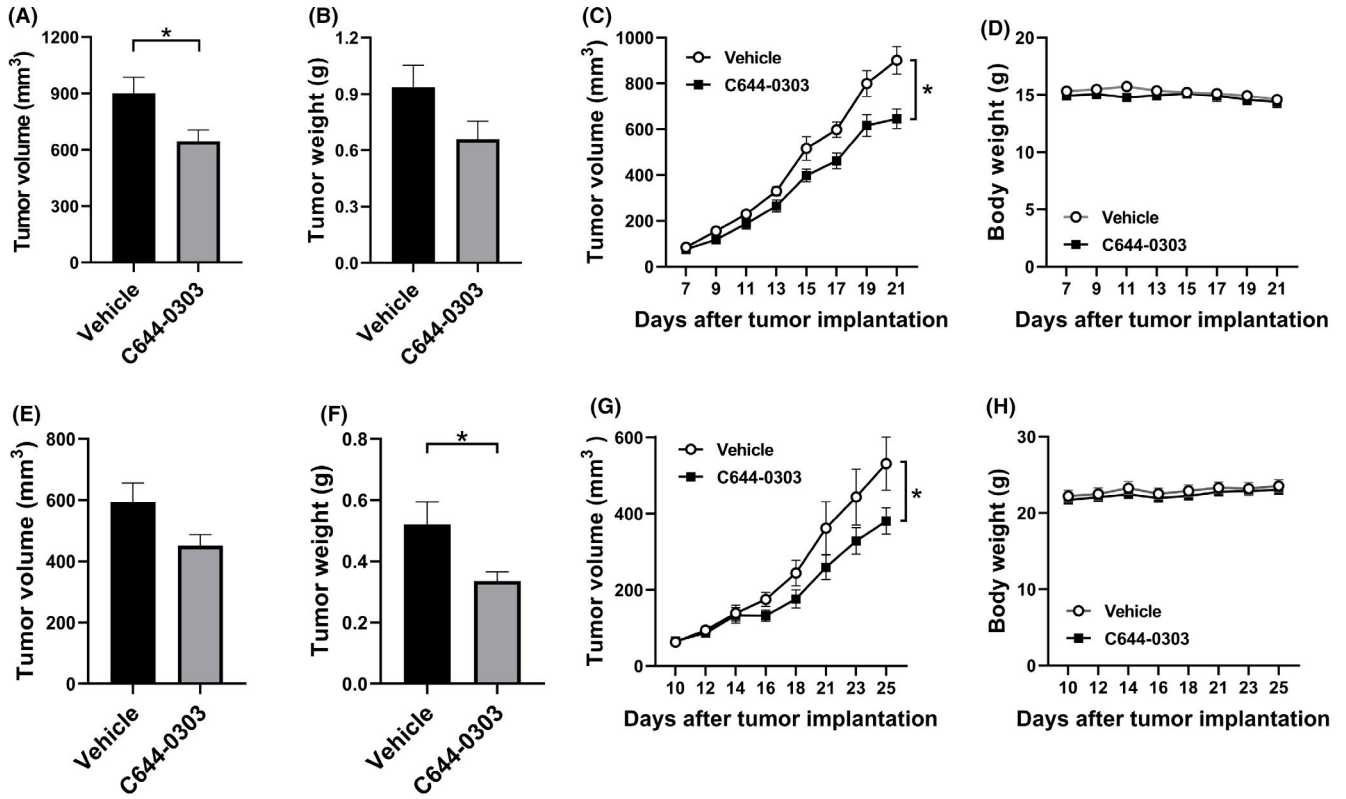
Next, we determined the phosphorylation status of β-catenin at Ser552 and Ser675, which are involved in β-catenin translocation from cell contacts into the cytosol and nucleus, and subsequent transcriptional activity, and Ser33/37/Thr41 and Ser45, which participate in β-catenin ubiquitination and degradation, and their upstream kinases AKT and GSK-3 (Figure 4E). As expected, the level of p-β-catenin (Ser552) and its corresponding upstream kinase p-AKT (Ser473), as well as p-β-catenin (Ser675), could be reduced by C644-0303, leading to the inhibition of β-catenin nuclear translocation. Consistently, nuclear β-catenin was reduced after C644-0303 treatment (Figure 4F). Moreover, C644-0303 impaired the phosphorylation of GSK-3 at Ser9 and subsequently increased the phosphorylation of downstream target β-catenin at Ser33/37/Thr41 (Figure 4E). The level of active-β-catenin (Ser45) was also downregulated after C644-0303 treatment. In conclusion, C644-0303 could inhibit Wnt/β-catenin signaling in multiple ways.

3.5 | C644-0303 inhibits the spheroidization and growth of CRC cells

Tumor spheroids can better mimic tumor status in vivo and predicted the drug response more accurately.[49,50] We explored C644-0303 antitumor efficiency in 2 different 3-dimensional (3D) culture systems. The embedded culture system more realistically restored the 3D culture environment, while the on-top culture system was more conducive for observation and imaging.[17,18] Therefore, the combination of these 2 cultivation methods could more accurately verify the antitumor effect of C644-0303.

In both embedded and on-top systems, C644-0303 inhibited spheroid-forming of HCT-116 cells (Figure 5A-D). The diameters of cell spheroids were tracked (Figure 5A,C) and measured (Figure 5B,D), which objectively presented the growth progression of the tumor spheres. C644-0303 treatment continuously inhibited spheroid enlargement.

At the end of treatment, the extracted whole-cell protein was quantified and C644-0303 treatment significantly decreased total protein content, further verifying the spheroid growth inhibition (Figure 5E,F). Active β-catenin, as well as the expression of downstream targets Axin2 and Cyclin D1, were also inhibited in the 3D system (Figure 5G-J). The similar inhibitory effects of C644-0303 were obtained in 3D-cultured HT-29 cells (Figure 5K,L).

The renewal and proliferation of cancer stem cells (CSCs) were tightly regulated by aberrant Wnt/β-catenin signaling.[51] Interestingly, CSC signature genes *CD44*[52,53] and *LGR5*[54,55] were significantly reduced in C644-0303-treated HCT-116 cells (Figure 5M,N), indicating

that C644-0303 may have an inhibitory effect on Wnt-dependent CSCs.

3.6 | C644-0303 inhibits the growth of CRC xenograft tumors

We further tested the antitumor effects of C644-0303 on HCT-116 and HT-29 xenograft tumors in vivo. As shown in Figure 6A-C, C644-0303 could retard the growth of HCT-116 xenograft tumors, as did that of HT-29 (Figure 6E-G). No obvious loss of body weight (Figure 6D,H) and organ damage (Figure S4A-D) were observed, indicating that all treatments had no or relatively low toxicity.

To test the effect of C644-0303 on the malignant proliferation of tumor cells in vivo, Ki67 staining of xenograft tumors was performed. We observed that in C644-0303-treated HCT-116 and HT-29 tumors, the expression level of Ki67 decreased (Figure 6I), as did the percentage of Ki67$^+$ cells or average optical density (AOD) (Figure 6K,L). Moreover, the percentage of c-Myc$^+$ cells was measured (Figure 6J). Consistent with the in vitro results, c-Myc was significantly reduced in HCT-116 xenograft tumors treated with C644-0303 (Figure 6M). The reducing trend of c-Myc$^+$ cell percentage and c-Myc mRNA levels was also observed in C644-0303-treated HT-29 xenograft tumors (Figure 6N,O). These results indicated that C644-0303 could also inhibit the growth of Wnt-dependent tumors in vivo.

4 | DISCUSSION

Wnt/β-catenin signaling is one of the key cascades regulating development and stemness, and its malignant activation is tightly associated with various types of tumors. There are high-frequency mutations of Wnt signaling components, especially in CRC with APC or CTNNB1 mutations.[7] Drug development targeting Wnt signaling in CRC with constitutive Wnt activation due to these mutations provides a valuable therapeutic strategy for antitumor treatment.[12,13] In the current study, we identified a new scaffold compound, C644-0303, as a Wnt signaling inhibitor. C644-0303 decreased β-catenin activation, TCF/LEF activity, and downstream target gene expression. It also induced cell cycle arrest and apoptosis, suppressed tumor growth in 2D and 3D in vitro, and reduced tumor burden in a xenograft tumor model in vivo. Taken together, our study identified a novel scaffold molecule C644-0303 as a lead compound for the inhibition of Wnt signaling at multiple levels.

Numerous attempts have been made to develop Wnt signaling inhibitors. For example, porcupine inhibitors such as LGK974, ETC-1922159, CGX1321[56-58]; CK1α activators pyrvinium[59]; Dvl/FZD interaction inhibitors FJ9, NSC668036[60,61]; tankyrase inhibitors G007-LK, AZ1366, K-756;[62-64] etc. Among them, several compounds are under clinical trials for the treatment of gastrointestinal tumors (NCT03507998), squamous cell carcinoma (NCT02649530) and malignancies dependent on Wnt ligands (NCT01351103). However, the inhibitors targeting upstream of Wnt pathway are unlikely to inhibit APC or CTNNB1 mutant Wnt signaling and are not suitable as therapeutic agents for most CRCs.[65] For example, LGK974 targeting palmitoylation, which is necessary to activate and secrete Wnt ligands, is limited to treat Wnt-dependent cancers with CTNNB1 or APC mutations.[66] Therefore, it is valuable to develop a Wnt signaling reporter system in CTNNB1 or APC mutant CRCs and conduct a high-throughput drug screening.

Wnt signaling inhibitors developed for CRC treatment mainly target the stabilization of the "destruction complex," β-catenin/TCF interactions, or transcriptional co-activators such CBP and p300.[67] In our study, C644-0303 strongly downregulated TCF/LEF transcriptional activity as well as Wnt3a stimulated β-catenin activities (Ser552/675 for translocation and Ser45/33/37/Thr41 for degradation). In both β-catenin Ser45 deficient or APC mutant CRC cells, C644-0303 could potently inhibit β-catenin target gene expression and subsequently impede cancer cell growth and metastasis. These data suggested that C644-0303 might act upstream of β-catenin or both upstream and downstream of β-catenin, but this needs to be further investigated. Considering other C644-0303 downregulated cancer-related TFs in addition to TCF/LEF, such as STAT5, AR, and Myc-Max, which all contributed to tumor progression,[40,68-70] C644-0303 may play antitumor roles by inhibiting a variety of tumor-promoting signals. As communication and compensation among different signaling pathways happens in most circumstances,[71,72] the inhibition of TCF/LEF activity might account for the non-TCF/LEF signaling inhibition, which is also implied by IPA. The downregulation of these TFs activity is most likely to be Wnt dependent. However, whether these signal pathways are directly inhibited by C644-0303 remains to be verified.

New scaffold compounds are the cornerstone of drug discovery.[73,74] We retrieved the structurally similar compounds of C644-0303 through the SciFinder database and 77 analogs (score ≥80) were identified. No publications regarding the biological activities of C644-0303 and its analogs have been found. Whether these isomers possess anti-Wnt signaling capabilities to suppress tumor growth as C644-0303 remains to be determined, and the druggable potential of these compounds can be explored in future studies. Moreover, structural modifications can be implemented based on the C644-0303 backbone to improve biological activities while reducing any unfavorable side effects.

In conclusion, scaffold compound C644-0303 was identified as a Wnt signaling inhibitor from a high-throughput screening. C644-0303 hindered ligand-stimulated and mutation-driven activation of Wnt signaling, and inhibited Wnt-dependent tumor growth in vitro and in vivo. C644-0303 provides a novel scaffold structure for further chemical optimization to enhance its drug-like properties.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.