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Discovery of chemical probes that suppress Wnt/β-catenin signaling through high-throughput screening

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Abstract

Aberrant activation of the Wnt/β-catenin signaling pathway has been observed in a wide range of human tumors. Deregulation of the pathway is closely linked to various aspects of human carcinogenesis such as cell viability, regulation of cell cycle, epithelial-mesenchymal transition, and maintenance of stemness. In addition, recent studies have disclosed the involvement of Wnt signaling in immune evasion of tumor cells. The accumulation of β-catenin in the nucleus is a common feature of cancer cells carrying defects in the pathway, which leads to the continuous activation of T-cell factor (TCF)/LEF transcription factors. Consequently, a genetic program is switched on, leading to the uncontrolled growth, prolonged survival, and acquisition of mesenchymal phenotype. As β-catenin/TCF serves as a signaling hub for the pathway, β-catenin/TCF-dependent transcriptional activity is a relevant readout of the pathway. To date, a wide variety of synthetic TCF/LEF reporters has been developed, and high-throughput screening (HTS) using these reporters has made significant contributions to the discovery of Wnt inhibitors. Indeed, HTS led to the identification of chemical probes targeting porcupine, a membrane bound O-acyltransferase, and CREB-binding protein, a transcriptional coactivator. This review focuses on various screening strategies for the discovery of Wnt inhibitors and their mode of action to help the creation of new concepts for assay/screening methods.

KEYWORDS

chemical probe, high-throughput screening, reporter assay, TCF/LEF transcription factor, Wnt/β-catenin signaling pathway

1 | INTRODUCTION

The Wnt/β-catenin signaling pathway (also called the canonical Wnt signaling pathway) was originally recognized as an essential pathway for embryonic development and adult tissue homeostasis. Importantly, aberration of Wnt/β-catenin signaling was later found in a wide range of cancers. Recent analysis of genetic alterations using more than 9000 tumors revealed oncogenic pathway signatures in various tumor types.[1] The frequency of activation of the Wnt/β-catenin signaling pathway varies widely, depending on the tumor type and/or subtype, and its high frequency is particularly observed in colorectal tumors. In addition to colorectal cancer, high frequencies of Wnt/β-catenin pathway activation were observed in uterine corpus endometrial carcinoma carrying microsatellite instability

(MSI) and DNA polymerase epsilon (*POLE*) mutation (70%), stomach and esophageal cancer carrying MSI and *POLE* mutation (70%) and diffuse large B-cell lymphoma (70%).[1] Somatic mutations in various components within this pathway, including APC regulator of WNT signaling pathway (*APC*; previous name, adenomatous polyposis coli), β-catenin (*CTNNB1*), transcription factor 7 like 2 (*TCF7L2*), ring finger protein 43 (*RNF43*), R-spondin (*RSPO*), and *AXIN1*, cause its aberrant activation. In line with previous reports,[2,3] *APC* mutations are observed in approximately 50% of colorectal tumors in 2 curated databases of somatic mutations in human cancer (The Catalogue of Somatic Mutations in Cancer [https://cancer.sanger.ac.uk/cosmic] and The cBioPortal for Cancer Genomics [https://www.cbioportal.org/]). A high frequency of mutations in the *CTNNB1* gene has also been found in pituitary (41%), soft tissue (36%), and liver tumors (21%).

In the Wnt/β-catenin signaling pathway, β-catenin is suppressed by a degradation complex consisting of APC, Axin, glycogen synthase kinase-3β, and casein kinase 1α (CK1α). However, dysfunction in any of the components of the complex or activating mutations in β-catenin itself causes abnormal accumulation of β-catenin in the cells. Translocated into the nucleus, β-catenin forms a complex with members of the T-cell factor (TCF) family of DNA-binding proteins, and consequently leads to transcriptional activation of their target genes. These genes, so-called "Wnt target genes", include protoon-cogenes, cell cycle regulators, stem cell markers, and negative feedback regulators of the Wnt pathway.

To date, there have been a number of proof-of-concept studies targeting the Wnt pathway for the treatment of cancer. Using Apc shRNA transgenic mice, Dow et al[4] showed that restoration of Apc could reestablish the control of crypt homeostasis in colorectal hyperproliferative polyps and cancer. The capacity for proliferation and self-renewal of CML cells carrying activated β-catenin was attenuated by the ectopic expression of Axin.[5] β-Catenin knockdown by RNAi significantly suppressed anchorage-independent growth and proliferation of liver cancer cells.[6] In addition, there is a growing body of evidence suggesting that Wnt/β-catenin signaling plays an essential role in the immune system. In metastatic melanoma, activation of the Wnt/β-catenin signaling pathway correlates with T cell exclusion.[7] Consistent with this view, multiomics analysis revealed that colorectal tumors with biallelic loss of the *APC* gene or nuclear accumulation of β-catenin protein were negatively correlated with tumor-infiltrating lymphocytes.[8] These reports suggested that activated Wnt signaling mediates cancer immune evasion and resistance to immunotherapies. Thus, blocking the Wnt pathway is an attractive approach to improve cancer immunotherapy. These data have prompted a search for chemical probes targeting this pathway (hereafter referred to as Wnt inhibitors). High-throughput screening (HTS) using a wide variety of assays has made significant contributions to the discovery of Wnt inhibitors. Indeed, HTS successfully identified small molecule compounds targeting porcupine and CREB-binding protein (CBP), and these compounds have already entered clinical trials. The establishment of a well-designed HTS system is crucial to identify Wnt inhibitor. Here, we comprehensively review Wnt inhibitors, and discuss the strategies involved in their identification.

2 | DEVELOPMENT OF REPORTER ASSAYS OF THE WNT/β-CATENIN SIGNALING PATHWAY

β-Catenin has been suggested to bind DNA mainly through the TCF/lymphoid enhancer-binding factor (LEF) transcription factors in mammals[9] and *Drosophila*.[10] The first observational evidence linking TCF/LEF directly to Wnt signaling resulted from yeast 2 hybrid screening using TCF1[11] or β-catenin[12] as bait. Another group also reported a physical interaction between β-catenin and LEF1.[13] In addition, these studies disclosed a responsible domain for the interaction, known as a β-catenin binding domain, in the amino terminus of the TCF/LEF protein. Deletion of the β-catenin binding domain produces a dominant negative form (dnTCF), which can outcompete with WT TCF/LEF for binding to the target sites.[14] As dnTCF7L2 abrogated the recruitment of β-catenin to the target chromatin regions in LS174T cells, TCF/LEF factors play a major role in the recruitment of β-catenin, at least in colorectal cancer cells.[9] However, in the absence of TCF/LEF factors, β-catenin is recruited through other transcription factors in HEK293T cells.[15] As TCF/LEF transcription factors act as major end-point mediators of this pathway, their DNA binding motif (Figure 1A) has been used as a faithful reporter for monitoring the Wnt/β-catenin signaling activity.

The TCF/LEF reporter plasmid originally incorporated 7 copies of approximately 30 bp of the *CD3E* enhancer region upstream of a minimal thymidine kinase promoter, and the chloramphenicol acetyltransferase gene (*CAT*) as a reporter (pMW56$_7$). As negative control, a mutant plasmid was prepared in which the WT TCF/LEF-binding motif AACAAAG was replaced by CCGCGGT (pMW56Sac$_7$).[16] TOPFlash, another synthetic TCF/LEF reporter plasmid containing tandemly repeated TCF motifs upstream of a minimal *c-fos* promoter and the versatile luciferase gene as a reporter, and FOPFlash, the negative control plasmid, were constructed (Figure 1B).[17,18] As *c-fos* is a transcriptional target of Wnt/β-catenin signaling[19] and might affect the β-catenin-dependent transactivation,[20] thymidine kinase promoter-driven reporter plasmids have been used for this purpose. SuperTOPFlash, a plasmid with an increased number of TCF motifs, is currently available.[21] Although use of the transgenic TCF reporters to detect Wnt/β-catenin signaling in vivo remains controversial,[22] cell-based assays with TOPFlash or SuperTOPFlash are useful strategies for monitoring the activity of the Wnt signal and the evaluation of chemicals that could affect the activity.

Intriguingly, identification of genes negatively regulated by the β-catenin/TCF complex led to the development of a new reporter plasmid (Figure 1B).[23] This plasmid contains 8 copies of the promoter region of histidine ammonia-lyase (*HAL*) upstream of the luciferase gene. Unlike TOPFlash, the HAL reporter activity is inversely correlated with Wnt/β-catenin signaling activity (Figure 1C). The
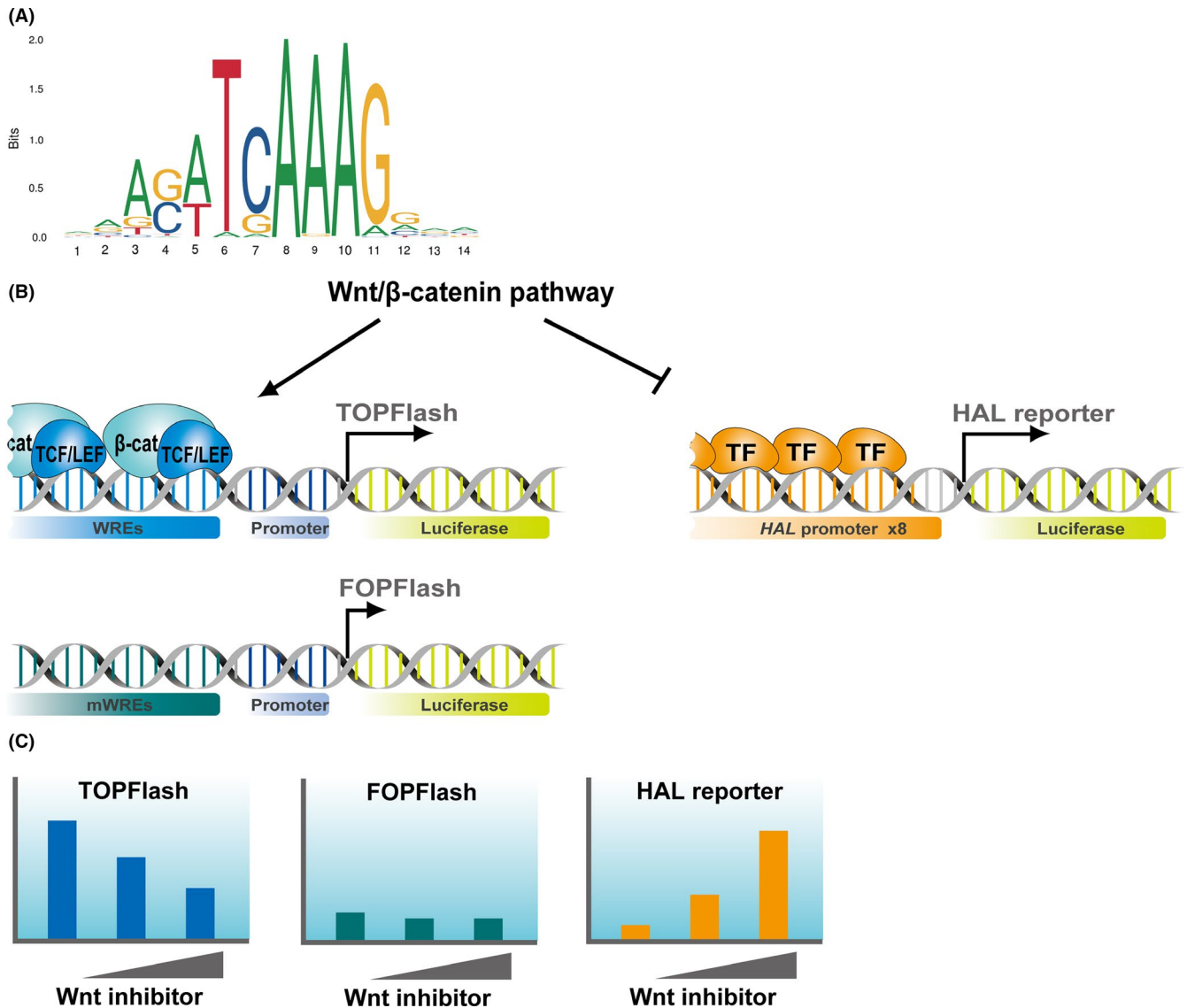
(A)

(B)

(C)

FIGURE 1 A, Position frequency matrix of the T-cell factor (TCF) motif was obtained from the JASPAR database (http://jaspar.gener eg.net). B, TOPFlash consists of tandemly repeated TCF motifs (Wnt response elements [WREs]) upstream of a minimal promoter that drives luciferase gene expression. FOPFlash has mutated motifs (mWREs) and is used to normalize the TOPFlash activity. HAL reporter was developed as a luciferase reporter driven by 8 copies of the promoter of histidine ammonia-lyase (*HAL*). Transcription factor (TF) that regulates the activity of *HAL* promoter is under investigation. C, These reporter plasmids were designed for monitoring the activity of Wnt/β-catenin pathway in cultured cells. When the pathway is inhibited, TOPFlash and HAL reporter activities are decreased and increased, respectively

combination of HAL reporter and TOPFlash plasmids could serve as an effective screening system for the discovery of new Wnt inhibitors.

3 | APPLICATION OF REPORTER ASSAY FOR HTS

Reporter assays using synthetic promoters containing multiple copies of a responsive element have been applied for HTS of small molecules and natural compounds affecting transcription and/or cellular signaling pathways. Because sensitivity, specificity, robustness, technical simplicity, and cost effectiveness are required for HTS, various strategies have been devised to meet the conditions.

The Z′-factor has been widely accepted for quality control of the HTS assay.[24] An acceptable assay for HTS usually requires a Z′-value more than 0.5. Reportedly, the Z′-factor of a luciferase assay using HEK293 cells stably expressing TCF reporter and LiCl for the activation of the reporter was as high as 0.89.[25] Regarding the luciferase assay using fly cell-optimized TCF reporter and *Drosophila* imaginal disc-derived clone 8 cells, the Z′-factor was 0.77.[26] In the reciprocal assay, the Z′-factors for TOPFlash and the HAL promoter luciferase assays were 0.69 and 0.79, respectively.[23]

Bioluminescent assays have been used in HTS as a major strategy, due to their high sensitivity, broad linearity, and robustness to chemicals.[27] Firefly luciferase (*Photinus pyralis*) and *Renilla* luciferase (*Renilla reniformis*) are commonly used as reporter genes. Firefly luciferase is

an enzyme of 61 kDa that catalyzes oxidation of a substrate (luciferin) in the presence of ATP and O_2. This chemical reaction results in an emission of a yellow-green light with a spectral maximum of 560 nm.[27] *Renilla* luciferase, a 36 kDa enzyme, is often used as an internal control in the dual luciferase format. Genetically engineered luciferase genes have improved assay sensitivity by increasing intensity of the luminescent signal and enhanced response dynamics by reducing expression lifetime. NanoLuc luciferase has emerged as a potential alternative to firefly/*Renilla* luciferase for reporter gene assay because it showed an approximately 100-fold greater activity than that of firefly or *Renilla* luciferase.[28] Importantly, since NanoLuc is a relatively small protein (19 kDa) it could have less effect on nonspecific chemical binding.

4 | CELL-BASED REPORTER ASSAY FOR SCREENING COMPOUNDS IN LIBRARIES

To date, cell-based HTS with TCF/LEF reporter plasmids have been frequently utilized for the identification of Wnt inhibitors. One of the greatest advantages in using secreted alkaline phosphate (SEAP) as a reporter is that there is no need to lyse the cells to measure its levels, because it is secreted directly into the culture medium. For example, screening of a library of 11 600 compounds using a SEAP reporter driven by Wnt response elements (WREs) identified FH535 and FH615 that suppressed Wnt/β-catenin signaling.[29] However, we might struggle with a high background in the assay because mammalian cells have endogenous alkaline phosphatase activity.

An alternative approach is the use of fluorescent proteins as a reporter. Waaler et al[30] prepared HEK293 cells stably expressing GFP reporter under the control of a synthetic TCF-responsive promoter. They screened 37 000 compounds using the reporter cells after activation with Wnt3a-conditioned medium and identified 77 compounds as primary hits by image analysis. Subsequent analysis identified 2 potent inhibitors, namely JW67 and JW74. Fluorescence-based reporter gene assays are cost effective because the addition of substrate is not required for its activity. However, these assays tend to have higher backgrounds, leading to the low signal-to-background ratio. Details about the property of biological reporters are described elsewhere.[31]
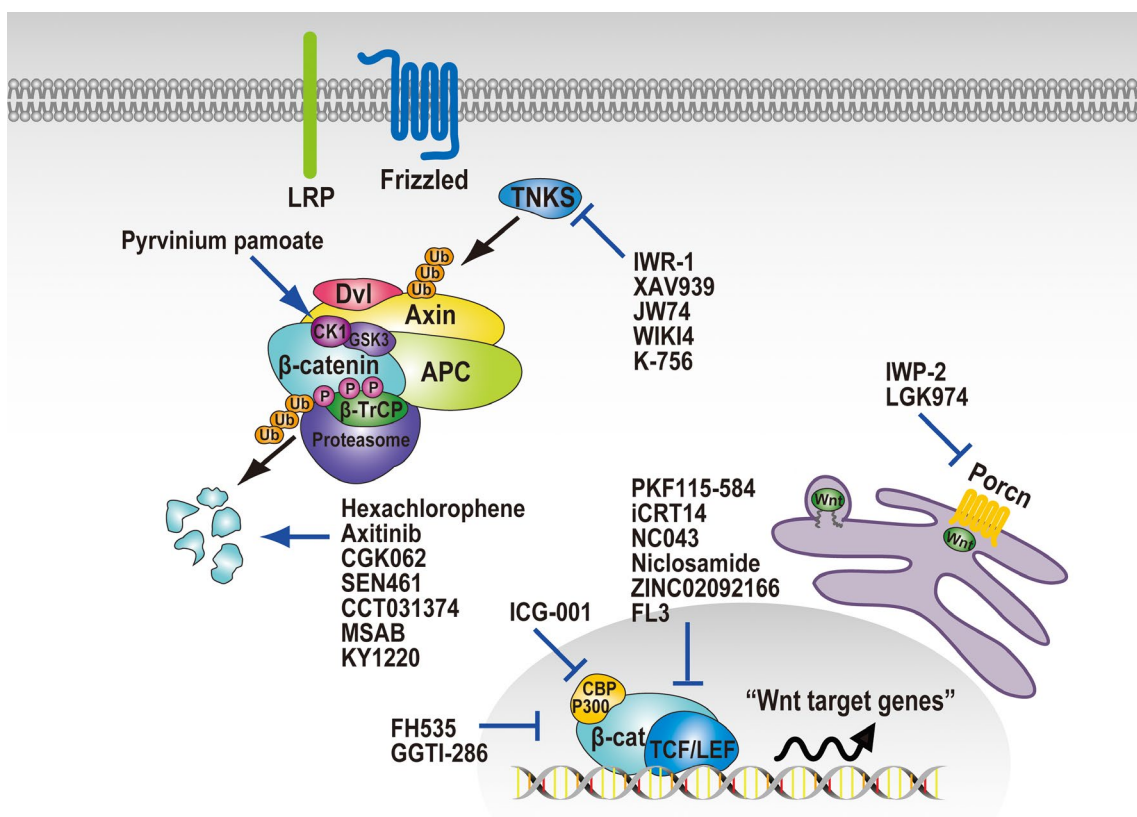

FIGURE 2 Pharmacological manipulation of the activity of Wnt/β-catenin signaling pathway. High-throughput screening identified chemical probes that target tankyrase (TNKS), porcupine (Porcn), casein kinase 1α (CK1α), β-catenin-TCF interaction, transcriptional co-activators of TCF, and β-catenin degradation (see Table 1 for more details and references). β-TrCP, β-transducin repeat containing E3 ubiquitin protein ligase; APC, APC regulator of WNT signaling pathway (adenomatous polyposis coli); CBP, CREB-binding protein; CK1, casein kinase-1; Dvl, Dishevelled segment polarity protein; GSK-3, glycogen synthase kinase-3; LEF, lymphoid enhancer binding factor; LRP, LDL receptor related protein; TCF7L2, transcription factor 7 like 2; TNKS, tankyrase

5 | WNT INHIBITORS DISCOVERED BY HTS

Over the past 2 decades, a wide range of HTS systems have been developed and applied for the screening of Wnt inhibitors. The inhibitors found in HTS are divided into 6 groups according to their mode of action: (i) inhibition of tankyrase (TNKS); (ii) inhibition of porcupine; (iii) activation of CK1α; (iv) inhibition of the β-catenin-TCF interaction; (v) inhibition of transcriptional co-activators; and (vi) induction of β-catenin degradation (Figure 2). Their chemical structures, assay methods, compound libraries, and four physicochemical parameters of the Lipinski's rule of five (RO5)[32] are listed in Table 1. The RO5 is a rule of thumb to evaluate drug-likeness, and defines 4 parameter ranges (molecular weight, 500 or less; calculated octanol/water partition coefficient value, 5 or less; H-bond donors, 5 or less; and H-bond acceptors, 10 or less).

6 | TANKYRASE INHIBITORS

Using a cell-based SuperTOPFlash assay, Chen et al[33] screened a ~200 000 synthetic chemical library to identify Wnt inhibitors. Secondary tests were carried out to select specific Wnt inhibitors from the first hit compounds, such as dose-dependent test (cytotoxicity), firefly/Gaussia luciferase assays (firefly luciferase inhibitor/exocytosis), and Notch/Hedgehog reporter assays (stem cell-associated signal transduction pathways). This screening strategy identified 5 inhibitors of Wnt response (IWR) compounds that abrogated destruction of Axin proteins. Axin is an essential scaffold protein required for assembly of the β-catenin destruction complex. Degradation of Axin is controlled through its poly-ADP-ribosylation (PARsylation) by TNKS.[34] Subsequently, IWR compounds turned out to be TNKS inhibitors (Figure 3A). Soon after, Huang et al[34] discovered another Axin stabilizer, XAV939 (Figure 3B), that directly binds TNKS and inhibits its PARsylation activity. An additional phenotype-based assay using zebrafish fin corroborated the inhibitory effect of Wnt activity by IWR and XAV939. Other TNKS inhibitors such as JW74,[30] WIKI4,[35] and K-756[36] have also been found by cell-based HTS.

7 | PORCUPINE INHIBITORS

One of the possible therapeutic strategies for targeting Wnt-driven cancers is to block the production of Wnt ligands. Porcupine (PORCN), a membrane-bound O-acyltransferase, was found to catalyze the palmitoylation of Wnt ligands, which is an essential step in the processing of Wnt into active ligands.[37] IWP-2, one of the inhibitors of Wnt production (IWPs), directly binds to PORCN, and inhibits the function of PORCN.[33] Another potent PORCN inhibitor, LGK974, was discovered by the screening of ~2 400 000 compounds using a TCF/LEF reporter assay, where Wnt3a-secreting cells were cocultured with mouse Leydig TM3 cells expressing SuperTOPFlash for the activation of the Wnt pathway.[38] An in vivo study reported that LGK974 diminished/eradicated tumors carrying RSPO fusions from the intestinal mucosa without effects on normal intestinal crypts.[39] In addition, LGK974 prevented proliferation and induced differentiation of RNF43-mutant pancreatic adenocarcinoma in xenograft models.[40] Currently, a phase I clinical trial (Clinicaltrials.gov ID NCT01351103) of LGK974 for patients with malignancies of histological origin carrying genetic alterations upstream in the Wnt signaling pathway (eg, RNF43 mutation or RSPO fusion) is underway.
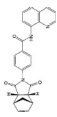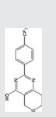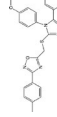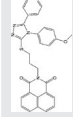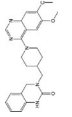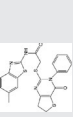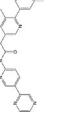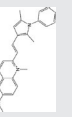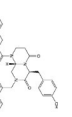
8 | CASEIN KINASE-1α ACTIVATOR

To explore chemical probes that inhibit the turnover of Axin and promote the degradation of β-catenin, Thorne et al[41] developed an assay system using extract of Xenopus eggs expressing β-catenin-firefly luciferase and Axin-Renilla luciferase fusion proteins mixed with a soluble form of LRP6 for the activation of the Wnt signaling. This assay was designed to analyze the reciprocal stability of β-catenin and Axin, thus Wnt inhibitors should decrease firefly luciferase (β-catenin) and increase Renilla luciferase (Axin). As Xenopus egg extracts are transcriptionally and translationally inactive, hit compounds are expected to modulate Wnt signaling through post-translational events. In addition, compounds that target energy metabolism (reduce both reporter activities) and general protein degradation (increase both reporter activities) would be avoided from the first hits. By the screening of an FDA-approved drug library, they identified pyrvinium pamoate, a CK1α activator, previously used in the treatment of pinworm infection. However, this effect was not confirmed by other studies.[42] Details about cytotoxic effects and molecular targets of pyrvinium pamoate are described elsewhere (http://www.oncm.org/v03p0001.htm).

## 9 | INHIBITORS OF INTERACTION BETWEEN β-CATENIN AND TCF
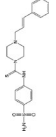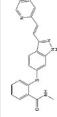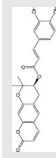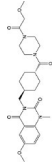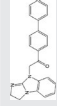
Activating mutations in β-catenin are frequently observed in several types of cancer, such as hepatocellular carcinoma,[43] where CTNNB1 mutations disrupt the phosphorylation and degradation of the β-catenin protein. In this case, inhibition of the upstream components is unable to induce the degradation of β-catenin. Thus, inhibition of the interaction of TCF/LEF with β-catenin or the coactivators is a rational and straightforward approach. To identify small molecules that disrupt the interaction, Lepourcelet et al[44] developed ELISA-based HTS using β-catenin and GST-fused TCF7L2 recombinant proteins. Consequently, they obtained 6 hits including PKF115-584 and CGP049090 from a library of 7000 natural compounds. Two other groups independently adopted AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay), a method for detecting intramolecular binding, for the discovery of inhibitors of the β-catenin-TCF interaction, and discovered LF3 and ZINC02092116.[45,46]

TABLE 1 Inhibitors of Wnt/β-catenin signaling discovered by high-throughput screening (HTS)

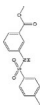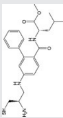| Chemical probe	HTS assay	Cells (condition)	Library	Mode of action	Parameters of RO5	Structure	References
IWR-1	Luciferase reporter (SuperTOPFlash)	Mouse L cells (Wnt3a-CM)	198 080 (UTSouthwestern chemical library)	Tankyrase inhibition	MWT = 409.45 CLog P = 2.522 H-bond donors = 1 H-bond acceptors = 4		33
XAV939	Luciferase reporter (SuperTOPFlash)	HEK293	N/A	Tankyrase inhibition	MWT = 312.31 CLog P = 3.82964 H-bond donors = 1 H-bond acceptors = 3		34
JW74	Fluorescence imaging (SuperTOP-d1EGFP)	HEK293 (Wnt3a-CM)	37 000	Tankyrase inhibition	MWT = 456.52 CLog P = 4.05445 H-bond donors = 0 H-bond acceptors = 6		30
WIKI4	Luciferase reporter (BAR, TCF reporter)	A375 (Wnt3a-CM)	6492 (KINASet library, Chembridge)	Tankyrase inhibition	MWT = 521.60 CLog P = 5.21344 H-bond donors = 0 H-bond acceptors = 6		35
K-756	Luciferase reporter (TCF reporter)	DLD-1	N/A	Tankyrase inhibition	MWT = 433.51 CLog P = 3.4062 H-bond donors = 1 H-bond acceptors = 5		36
IWP-2	Luciferase reporter (SuperTOPFlash)	Mouse L cells (Wnt3a-CM)	198 080 (UTSouthwestern chemical library)	Porcupine inhibition	MWT = 466.59 CLog P = 4.94517 H-bond donors = 1 H-bond acceptors = 4		33
LGK974	Luciferase reporter (SuperTOPFlash)	TM3 cells (co-cultured with L-cell Wnt3A)	~2 400 000	Porcupine inhibition	MWT = 396.45 CLog P = 1.77295 H-bond donors = 1 H-bond acceptors = 6		38
Pyrvinium pamoate	Luciferase reporter (β-catenin-Fluc/Axin-RLuc fusion proteins)	Xenopus egg extracts (LRP6ICD)	FDA-approved drug library etc	Activation of CK1α	MWT = 382.53 CLog P = 2.78575 H-bond donors = 0 H-bond acceptors = 0		41
ICG-001	Luciferase reporter (TOPFlash)	SW480	5000	Inhibition of β-catenin-CBP interaction	MWT = 548.64 CLog P = 6.13165 H-bond donors = 2 H-bond acceptors = 4		54
PKF115-584	ELISA (β-catenin/ GST-TCF4 recombinant proteins)	N/A	7000 (natural compounds library)	Inhibition of β-catenin-TCF interaction	MWT = 790.77 CLog P = 6.87757 H-bond donors = 3 H-bond acceptors = 11		44

(Continues)

TABLE 1 (Continued)

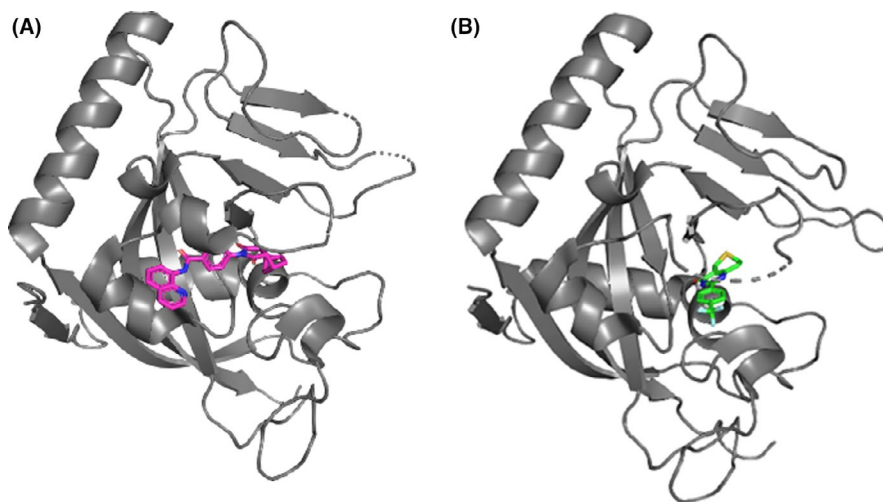| Chemical probe	HTS assay	Cells (condition)	Library	Mode of action	Parameters of RO5	Structure	References
iCRT14	Luciferase reporter (TCF reporter)	*Drosophila* Cl8 cells (dAxin-dsRNA)	14 977 (ICCB-Longwood collection, Harvard)	Inhibition of β-catenin-TCF interaction	MWT = 375.45 CLog P = 5.70349 H-bond donors = 0 H-bond acceptors = 3		26
NC043	Luciferase reporter (TOPFlash)	HEK293T (Wnt1 overexpression, Wnt3a-CM, or LiCl)	4000	Indirect inhibition of β-catenin-TCF interaction	MWT = 330.42 CLog P = 1.2362 H-bond donors = 1 H-bond acceptors = 3		48
Niclosamide	Luciferase reporter (S1004A4 promoter reporter)	HCT116	1280 (LOPAC Sigma-Aldrich)	Inhibition of formation of β-catenin/TCF complex	MWT = 327.12 CLog P = 4.34465 H-bond donors = 2 H-bond acceptors = 2		47
ZINC02092166	Fluorescence polarization and AlphaScreen assays (β-catenin/fluorescein-TCF4 recombinant proteins)	N/A	2093 (Sigma-Aldrich, Pfizer, NCI etc)	Inhibition of β-catenin-TCF interaction	MWT = 349.27 CLog P = 3.91846 H-bond donors = 2 H-bond acceptors = 8		45
LF3	AlphaScreen assay (GST-β-catenin/His-TCF4 recombinant proteins)	N/A	16 671 (WDI compounds, ChemBioNet)	Inhibition of β-catenin-TCF interaction	MWT = 416.56 CLog P = 2.582 H-bond donors = 2 H-bond acceptors = 4		46
Hexachlorophene	Luciferase reporter (TOPFlash)	HEK293 expressing hFz-1 (Wnt3a-CM)	960 (Genesis Plus Collection, MicroSource Discovery)	β-Catenin degradation through SIAH-1 induction	MWT = 406.89 CLog P = 7.02708 H-bond donors = 2 H-bond acceptors = 2		56
Axitinib	Luciferase reporter (SuperTOPFlash)	HEK293FT (GSK-3 inhibitor, 6BIO)	460 (FDA-approved drug library)	β-Catenin degradation through SHPRH stabilization	MWT = 386.47 CLog P = 3.3269 H-bond donors = 3 H-bond acceptors = 4		58
CGK062	Luciferase reporter (TOPFlash)	HEK293 expressing hFz-1 (Wnt3a-CM)	800	β-Catenin degradation through PKCα activation	MWT = 408.41 CLog P = 3.75537 H-bond donors = 2 H-bond acceptors = 5		59
SEN461	Luciferase reporter (TCF reporter)	DBTRG.05MG	16 000 (Siena Biotech internal compounds collection)	β-Catenin degradation through Axin stabilization	MWT = 486.57 CLog P = 2.0554 H-bond donors = 0 H-bond acceptors = 6		60
CCT031374	Luciferase reporter (TCF reporter)	HEK293 expressing Dvl2-ER (Disheveled-estrogen receptor fusion)	63 040 (The Cancer Research UK Center for Cancer Therapeutics compound library)	β-Catenin degradation + unknown mechanism	MWT = 353.43 CLog P = 4.5174 H-bond donors = 0 H-bond acceptors = 2		25

(Continues)

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TABLE 1 (Continued)

Chemical probe	HTS assay	Cells (condition)	Library	Mode of action	Parameters of RO5	Structure	References
MSAB	Luciferase reporter (TOPFlash)	HCT116	22 000 (Chembridge and Broad Institute)	β-Catenin binding and degradation	MWT = 305.35 CLog P = 3.405 H-bond donors = 1 H-bond acceptors = 3		61
KY1220	Luciferase reporter (TOPFlash)	HEK293 (Wnt3a-CM)	~3599 (Chemdiv and Sigma LOPAC 1280)	Axin binding and β-catenin degradation	MWT = 314.32 CLog P = 1.60552 H-bond donors = 2 H-bond acceptors = 2		62
FH535	SEAP reporter (TOPFlash)	HepG2	11 600 (DIVERSet collection, ChemBridge)	Repression of β-catenin recruitment	MWT = 361.19 CLog P = 3.95088 H-bond donors = 1 H-bond acceptors = 2		29
GGTI-286	Eye phenotype	Zebrafish embryos (GSK-3 inhibitor, 6BIO)	282 (SCADS inhibitor kit)	Inhibition of nuclear accumulation of β-catenin	MWT = 429.58 CLog P = 3.4042 H-bond donors = 3 H-bond acceptors = 3		63
Brefeldin A	Luciferase reporter (TOPFlash and HAL promoter reporter)	HepG2	361 (SCADS inhibitor kit)	Repression of β-catenin mRNA	MWT = 280.36 CLog P = .538999 H-bond donors = 2 H-bond acceptors = 3		23

Note: Chemical structures were drawn using ChemDraw. The molecular weights (MWT), calculated octanol/water partition coefficient (CLog P) values, and number of hydrogen bond donors and acceptors were calculated using ChemDraw or Molecular Operating Environment (MOE).

BAR, β-catenin-activated reporter; CBP, CREB-binding protein; CK1α, casein kinase-1α; GSK-3, glycogen synthase kinase-3; N/A, not available; PKCα, protein kinase Cα; SCADS, Screening Committee of Anticancer Drugs; SEAP, secreted alkaline phosphate; TCF, T-cell factor.

Cell-based approaches have also discovered small molecule compounds iCRT, NC043, and niclosamide that target the β-catenin-TCF interaction.[26,47,48] For example, niclosamide was identified by HTS with 1280 pharmacologically active compounds in HCT116 cells expressing luciferase reporter driven by the promoter of the *S1004A4* gene, a transcriptional target of the Wnt/β-catenin pathway.[47] Gonsalves et al identified iCRT3, iCRT4, and iCRT14 by screening 14 977 compounds using fly cell-optimized TCF reporter and *Drosophila* imaginal disc-derived clone 8 cells that had silenced dAxin expression for the activation of the reporter.[26] According to crystallographic analysis, the TCF7L2-binding region on β-catenin overlaps with the binding regions for APC and E-cadherin[49,50]; this might become a potential obstacle to develop selective Wnt inhibitor. Nevertheless, further characterization of iCRTs revealed that these compounds inhibited β-catenin-TCF interaction, whereas they had little or no effect on β-catenin-E-cadherin or β-catenin-α-catenin interaction.[26] In contrast, PKF222-815, PKF115-584, and CGP049090 blocked the interaction of β-catenin with TCF7L2, and unfavorably blocked its interaction with APC.[44] Increasing the specificity of inhibitors targeting the interaction of β-catenin and TCF remains a major challenge.

10 | INHIBITORS OF TRANSCRIPTIONAL COACTIVATORS OF β-CATENIN

Targeting the coactivators of β-catenin-dependent transcription is another strategy for the suppression of aberrant Wnt signaling. Molecular studies have identified a number of coactivators that interact with β-catenin (Wnt homepage, http://web.stanford.edu/group/nusselab/cgi-bin/wnt/protein_interactions).[51-53] High-throughput screening using TOPFlash assay in *APC*-mutated SW480 colorectal cancer cells identified ICG-001 that targets CBP, an interacting protein of β-catenin.[54] Intriguingly, treatment with ICG-001 suppressed β-catenin/TCF-mediated transcription of survivin in HCT116 cells, suggesting that CBP inhibitor can

block Wnt/β-catenin signaling. In this regard, B-cell lymphoma 9 (BCL9) and BCL9-like (B9L) might also be targets of the nuclear coactivator. Carnosic acid that blocked the binding of β-catenin to BCL9 resulted in the inhibition of β-catenin-dependent transcription in colorectal cancer cells.[55]

11 | SMALL MOLECULES THAT PROMOTE β-CATENIN DEGRADATION

Enzymes such as ubiquitin ligases are associated with β-catenin degradation, and can be an attractive target of small molecule compound. Park et al screened 960 bioactive compounds using TOPFlash luciferase assay, and discovered hexachlorophene that promotes β-catenin degradation through induction of SIAH-1, an E3 ubiquitin ligase.[56] Recently, another E3 ubiquitin ligase, SHPRH that controls β-catenin stability, was also found to be targeted by a small molecule compound. Screening of an FDA-approved drug library using SuperTOPFlash luciferase assay led to the discovery of axitinib, a known inhibitor of multireceptor tyrosine kinases, especially vascular endothelial growth factor receptors,[57] that stabilize SHPRH protein, thereby increasing the degradation of β-catenin.[58] Another screening of 800 compounds using TOPFlash assay identified CGK062 that promotes protein kinase Cα-mediated phosphorylation of β-catenin at Ser33/Ser37.[59] De Robertis et al[60] identified SEN461 using TCF/LEF reporter with an increased number of WRE and DBTRG.05MG human glioma cells. Although SEN461 enhanced the degradation of β-catenin through the stabilization of Axin, it showed limited effect on auto-PARsylation and stabilization of TNKS compared with a TNKS inhibitor, XAV939, suggesting that TNKS are not the pharmacological target of SEN461. In addition, the HTS using a cell-based TOPFlash assay identified a set of small molecules including MSAB[61] and KY1220.[62] It was reported that MSAB and KY1220 physically interact with the Armadillo repeat region of β-catenin and the regulator of G-protein signaling domain of Axin, respectively. These interactions might promote the formation of β-catenin destruction complex.

12 | SMALL MOLECULES THAT AFFECT β-CATENIN FUNCTION OR EXPRESSION

Development of novel screening strategies has discovered different types of Wnt inhibitors. Nishiya et al[63] explored compounds that suppress the chemically induced eyeless phenotype in zebrafish embryos. This phenotypic screening resulted in the discovery of GGTI-286, and they found that GGTI-286 reduces nuclear translocation of β-catenin through the inhibition of geranylgeranyltransferase (GGTase). It has been reported that GGTase catalyzes the addition of the geranylgeranyl group to Rac1 protein that is required for its membrane association and biological activity.[64] Rac1 activates JNK2 that in turn phosphorylates β-catenin at Ser191 and regulates its nuclear translocation,[65] suggesting that the reduced nuclear translocation of β-catenin by GGTI-286 might be associated with its interference with Rac1 protein. A reciprocal reporter assay using TOPFlash coupled with HAL reporter led to a decrease in the number of false positives, and identified brefeldin A (BFA), a fungal metabolite, that suppresses the expression of β-catenin.[23] Although BFA was reported to inhibit protein secretion by blocking transport from the endoplasmic reticulum to the Golgi,[66] the precise mechanism(s) underlying the reduction in β-catenin by BFA needs further investigation.

13 | CONCLUSIONS

The discovery of Wnt inhibitors should provide potential benefits for cancer therapy. Although systematic unbiased screenings of the pathway have helped the identification of Wnt inhibitors, much of the underlying mechanisms remain to be elucidated. The target identification of small molecules from unbiased HTS is a challenge to be resolved.

DISCLOSURE

The authors have no conflict of interest.

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