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Review Article

Targeting the canonical Wnt/β-catenin pathway in hematological malignancies

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The canonical Wnt/β-catenin pathway plays an important role in different developmental processes through the regulation of stem cell functions. In the activation of the canonical Wnt/β-catenin pathway, β-catenin protein is imported into the nucleus and activates transcription of target genes including *cyclin D1* and *c-myc*. Aberrant activation of the Wnt/β-catenin pathway contributes to carcinogenesis and malignant behaviors, and Wnt signaling is essential for the maintenance of cancer stem cells. The canonical Wnt/β-catenin pathway has been investigated extensively as a target in cancer treatment and several specific inhibitors of this signaling pathway have been identified through high-throughput screening. In this review, the significance of the canonical Wnt/β-catenin pathway in hematological carcinogenesis and screening methods for specific inhibitors are discussed.

Wnt signaling plays important roles in developmental processes and cell growth and differentiation. Three Wnt signaling pathways have been characterized, including the canonical Wnt/β-catenin pathway. Signals of the canonical Wnt/β-catenin pathway are transduced through a member of the Fz receptor family and the LRP5/6 co-receptor to the β-catenin cascade. This pathway regulates cell proliferation and developmental processes. The canonical Wnt/β-catenin pathway is aberrantly activated in cancers, and it has therefore been investigated as a potential therapeutic target for the treatment of cancer. The present review focuses on the role of the canonical Wnt/β-catenin pathway in hematological malignancies and discusses the development of small molecule inhibitors against this canonical pathway.

Canonical Wnt/β-catenin Pathway

The precise signal transduction of the canonical Wnt/β-catenin pathway has been described in several reviews.[1,2] β-Catenin is a multifunctional protein that exists in different subcellular components. A major membrane-bound form of β-catenin interacts with E-cadherin and connects actin filaments through α-catenin to form the cytoskeleton. Membrane-bound β-catenin is released into the cytosol by tyrosine phosphorylation. Cytosolic β-catenin acts as a downstream protein of the canonical Wnt signaling pathway in stimulated cells. In the absence of Wnt proteins, adenomatous polyposis coli, Axin, GSK3β, and casein kinase 1α form the "β-catenin destruction complex". The phosphorylated β-catenin in the β-catenin destruction complex is polyubiquitinated by β-transducin repeat-containing protein, a component of a ubiquitin ligase complex, targeting β-catenin for rapid degradation by the proteasome. Consequently, the transcription of the downstream genes involved in cell-cycle regulation, cell adhesion, and cellular development are repressed. On the other hand, the binding of Wnt proteins to Fz receptors and LRP5/6 co-receptors induces the phosphorylation of Disheveled and prevents GSK3β-dependent phosphorylation of β-catenin. β-Catenin is stabilized in cytoplasm and translocates into the nucleus, where it interacts with TCF/LEF, resulting in activation of the transcription of target genes (Fig. 1).

Wnt/β-catenin Pathway in Hematological Malignancies

Hematopoiesis is a continuous process by which HSCs and HPCs develop into mature hematopoietic cells. Many signaling pathways involved in hematopoiesis have been characterized; among these, the canonical Wnt signaling is essential for the maintenance of HSCs.[3,4] Inhibition of GSK3β, which leads to activation of β-catenin, promotes hematopoiesis. Short-term pretreatment of human HSCs with a GSK3β inhibitor, 6-bromoindirubin 3′-oxime, increased engraftment into immunodeficient

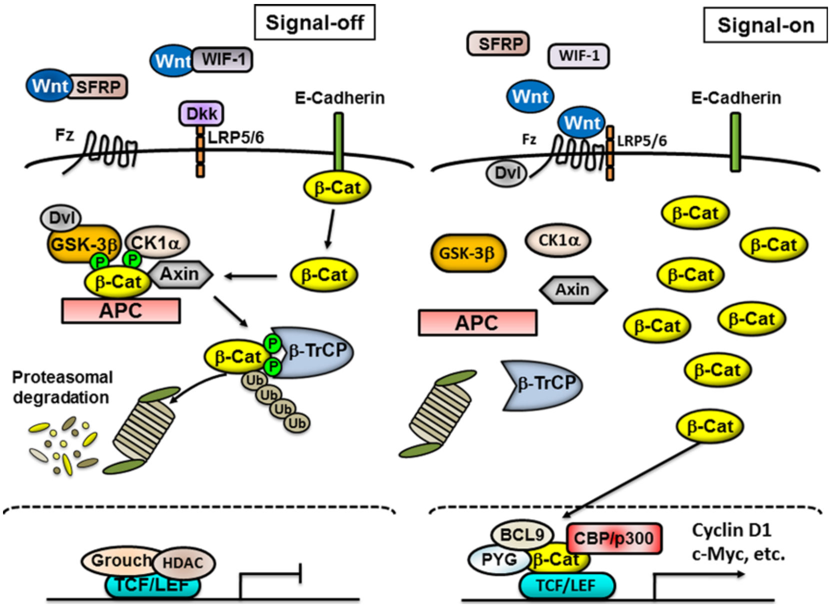


Fig. 1. Wnt/β-catenin signaling pathway. In the absence of Wnt, in the "Wnt signal-off" state (left panel), the β-catenin destruction complex is polyubiquitinated by β-transducin repeat-containing protein (β-TrCP) and phosphorylated β-catenin (β-Cat) is then degraded by the proteasome. In the presence of Wnt, in the "Wnt signal-on" state (right panel), phosphorylation of β-catenin is suppressed and β-catenin escapes from degradation. Free cytoplasmic β-catenin translocates to the nucleus and forms a complex with T-cell factor (TCF)/lymphocyte enhancer factor (LEF). The β-catenin/TCF complex activates the transcription of target genes including *cyclin D1* and *c-myc*. APC, adenomatous polyposis coli; BCL9, B-cell chronic leukocytic leukemia/lymphoma 9; CBP, c-AMP response element binding protein-binding protein; CK1α, casein kinase 1α; Dkk, Dickkopf; Dvl, dishevelled; Fz, Frizzled; HDAC, histone deacetylase; LRP5/6, lipoprotein receptor-related protein 5/6; P, phosphorylation; PYG, pygopus; SFRP, soluble frizzled-related protein; Ub, ubiquitination; WIF-1, Wnt inhibitory factor-1.

mice.[5] The constitutively active form of β-catenin reprogrammed lymphoid and myeloid progenitors to multipotent HPCs.[6] Moreover, HSCs from mice lacking β-catenin are deficient in their long-term maintenance.[7] However, mice expressing stabilized β-catenin in the hematopoietic system showed expansion of HSCs with arrested differentiation, and led to defects in hematopoietic reconstitution. Therefore, the Wnt pathway plays an important role in fine-tuning the regulation of hematopoiesis.[8,9]

The dysregulation of Wnt/β-catenin signaling is associated with the development of hematological malignancies. β-Catenin is aberrantly expressed in patients with AML,[10,11] and high expression of β-catenin is associated with poor prognosis in AML.[12] Normal human CD34$^+$ HPCs overexpress β-catenin compared to mature cells, and β-catenin is downregulated during myeloid differentiation; however, constitutive activation of the Wnt pathway by a retrovirally expressed mutant β-catenin in CD34$^+$ HPCs induces cell proliferation without myelomonocytic differentiation even in myeloid-oriented culture. Wnt pathway components such as *Wnt1*, *Wnt2B*, and *LEF1* mRNA are overexpressed in CD34$^+$ leukemic blast cells from AML patients, and TCF/LEF transcription activities are increased in CD34$^+$ leukemic cells.[13] Acute myelogenous leukemia is frequently associated with somatic mutations of fms-like tyrosine kinase 3 consisting of internal tandem duplications, which occur in approximately 30% of patients with AML and are associated with poor prognosis.[14,15] This mutation induces high β-catenin protein levels and enhances TCF/LEF-dependent transcriptional activity.[16] Moreover, β-catenin and TCF/LEF target genes such as *c-myc* and *cyclin D1* are overexpressed in U937 cells expressing AML-associated transcription products such as AML1-ETO, PML-RARα, and PLZF-RARα.[17] The canonical Wnt pathway plays a role in leukemogenesis.

Aberrant Wnt pathway activation is associated with the pathogenesis of lymphoid malignancies. In normal hematopoiesis, LEF1 plays a crucial role in the development of B and T cells.[18,19] LEF1 is overexpressed in lymphoid malignancies including ALL,[20] CLL,[21] and malignant lymphoma.[22] In B-cell progenitor ALL cell lines and primary B-ALL cells, the Wnt/β-catenin pathway is activated by the overexpression of Wnt genes including *WNT2B*, *WNT5A*, *WNT10B*, and *WNT16B*, and also the Wnt receptors *FZD7* and *FZD8*. Wnt3A stimulates the proliferation and survival of these cells.[23] Furthermore, overexpression of *LEF-1* mRNA reveals a predictor of poor prognosis in patients with adult B-precursor ALL.[20] These observations indicate that the canonical Wnt signaling pathway plays a role in the pathogenesis of B-ALL.

B-cell CLL is characterized by the accumulation of mature and functionally incompetent B cells. The canonical Wnt pathway-related genes and proteins are overexpressed in CLL and β-catenin signaling inhibition decreases cell survival.[24,25] Pharmacological inhibition of GSK-3β promotes β-catenin-mediated transcription, and Wnt/β-catenin inhibition by an analog of a non-steroidal anti-inflammatory drug induces apoptosis of CLL cells.[25] Multiple myeloma is a neoplastic disorder of plasma cells. Multiple myeloma cell lines and primary MM cells overexpress β-catenin,[26,27] and soluble Wnt proteins increase β-catenin protein levels and β-catenin/TCF transcription.[26,28] Therefore, the canonical Wnt pathway is considered a therapeutic target for the treatment of MM.[26,27,29,30] In addition to B cell malignancies, the Wnt/β-catenin signaling cascade is required for thymopoiesis.[31,32] β-Catenin stabilization inhibits the developmental transition from double-positive to single-positive thymocytes and induces T-ALL independently of Notch signaling.[33]

Wnt/β-catenin Pathway in Leukemic Stem Cells

The Wnt pathway plays an important role in the maintenance of adult somatic stem cells.[34] The R-spondin/leucine-rich repeat containing, G-protein-coupled receptor 5 signaling maintains intestinal stem cells through the Wnt pathway.[35] The activation of the Wnt/β-catenin pathway by orphan nuclear receptor tailless stimulates the proliferation and the self-renewality of neural stem cells.[36] In addition to the maintenance of these somatic stem cells, the Wnt/β-catenin pathway is essential for the maintenance of HSCs, as discussed in the previous section.

The Wnt/β-catenin pathway also contributes to the development of LSCs. Wang *et al.*[37] produced leukemias in mice by overexpressing HOXA9 and a HOX coactivator, MEIS1a, or the MLL-AF9 fusion protein in HSCs and non-self-renewal GMPs. In the absence of the activated Wnt pathway, AML developed in transformed HSC-transplanted mice; however, in the presence of the constitutively activated β-catenin protein, the transformed GMPs induced AML and reduced the survival of transplanted mice, indicating that the activation of Wnt/β-catenin signaling produces LSCs from either HSCs or more differentiated GMPs.

Chronic myelogenous leukemia is a clonal myeloproliferative disorder of HSC origin caused by the constitutive activation of the BCR–ABL 1 tyrosine kinase. The development of TKIs such as imatinib, dasatinib, and bosutinib has dramatically improved the prognosis of CML patients.[38,39] However, TKIs cannot eradicate CML stem cells because CML stem cells are insensitive to TKIs.[40–42] Activation of the Wnt/β-catenin pathway was detected in samples from patients with CML in blastic crisis. Additionally, appropriate activation of the Wnt signaling pathway in GMPs confers self-renewal capacity, suggesting that aberrant Wnt pathway activation results in the acquisition of CML stem cell features by leukemic GMPs in the blastic phase of CML.[42] These observations were confirmed in murine studies. Mice transplanted with BCR–ABL-transfected HSCs from β-catenin knockout mice show a significant delay in the onset of CML, and loss of β-catenin impairs the self-renewal capacity of CML stem cells.[7] Taken together, these findings indicate that the Wnt/β-catenin pathway is involved in the maintenance of LSCs and is therefore a promising target for the development of therapies against LSCs, as reviewed previously.[43]

Epigenetic Dysregulation of the Wnt/β-catenin Pathway in Hematological Malignancies

Epigenetic abnormalities play an important role in carcinogenesis. DNA methylation abnormalities have been investigated in relation to the canonical Wnt pathway in hematological malignancies. DNA methylation usually occurs in the region of "CpG islands" and involves the addition of a methyl group to the carbon-5 position of the cytosine ring in the CpG dinucleotide catalyzed by DNA methyltransferase. CpG island methylation is associated with gene silencing and aberrant CpG island methylation (hypermethylation) is observed in many cancers. Abnormal methylation of Wnt antagonists including *SFRPs, DKKs,* and *WIF-1* is detected in several types of hematological malignancies,[44–48] and is associated with decreased survival in patients with ALL and AML.[45,46] Moreover, hypermethylation of Wnt inhibitors is associated with genetic aberrations including class II mutations such as *AML1/RUNX1*, *MLL/PTD*, *PML/RARα*, and *ASXL1*.[44]

Dysregulation of the Wnt/β-catenin Pathway Through the Bone Marrow Microenvironment

The BM microenvironment supports hematopoiesis, and the BM niche regulates the proliferation and differentiation of HSCs and hematopoietic progenitors through various mechanisms (cell-to-cell contact or humoral factors). Among these mechanisms, canonical Wnt/β-catenin signaling in BM mesenchymal cells is dispensable for hematopoiesis.[49–51] Similar to normal hematopoiesis, the BM microenvironment has a significant effect on Wnt/β-catenin signaling. In a coculture system using human BM stromal cells and CML cells, adhesion of CML cells to MSCs through N-cadherin induced β-catenin nuclear translocation and transcriptional activities, resulting in the protection of CML $CD34^+/CD38^-$ progenitors from TKI treatment.[52] Acute lymphoblastic leukemia cell lines cocultured with MSCs are also protected from the effects of anticancer drugs. Mesenchymal stromal cells express Wnt ligands, especially Wnt3 and Wnt5A, and ALL cells cocultured with MSCs express LEF1 and cyclin-D1-binding protein 1, which explains the resistance of ALL cells to anticancer agents. Wnt/β-catenin signaling in the BM microenvironment also plays a role in the pathogenesis of leukemias.

The BM is hypoxic, particularly at the epiphysis.[53] Normal HSCs reside in this hypoxic epiphyseal region "niche", and HSCs are protected from DNA damage induced by reactive oxygen species.[53,54] In previous studies, we showed that CML cells engrafted in the BM survive and proliferate in the severely hypoxic environment and these hypoxia-adapted leukemic cells are resistant to TKIs and acquire stem cell-like characters.[55,56] These cells express β-catenin at much higher levels than CML cells cultured under normoxic conditions, and the novel Wnt/β-catenin signaling inhibitor AV-65 (discussed later) suppresses the proliferation of these CML stem cell-like cells.[56] These observations suggest that the Wnt/β-catenin signaling pathway plays a role in the maintenance of CML stem cells and that inhibition of the Wnt pathway may eradicate CML stem cells.

Small Molecule Inhibitors of the Wnt/β-catenin Pathway

Strategies to inhibit Wnt/β-catenin signaling have been researched for their potential in the treatment of cancers. Small molecule compounds have been developed extensively as therapeutic agents because of their ability to target intracellular proteins.[57,58] Small molecule screening, which is critical for the identification and development of effective compounds, is

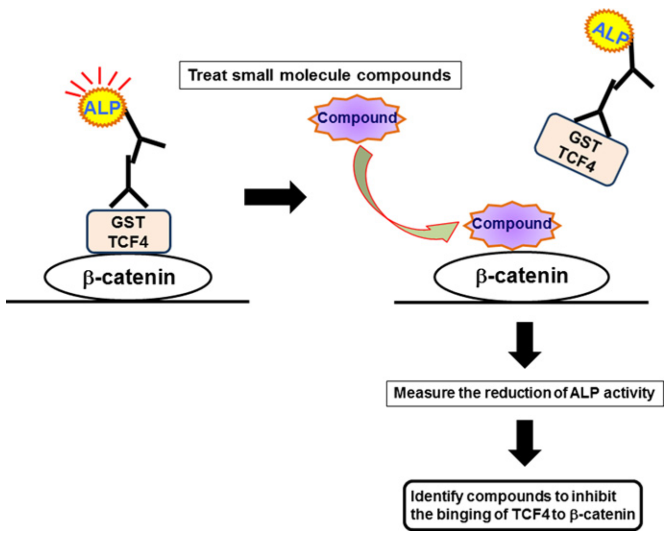
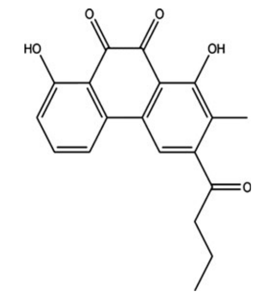
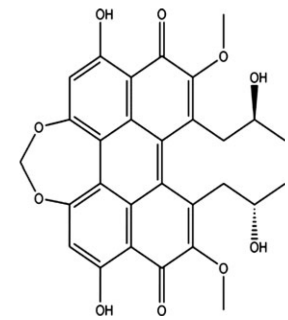
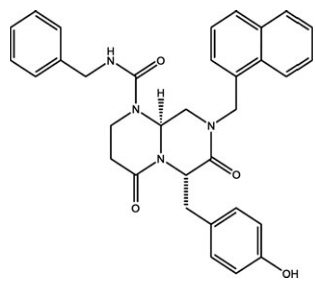
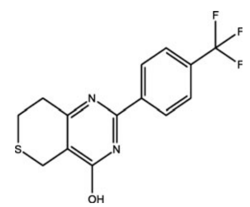
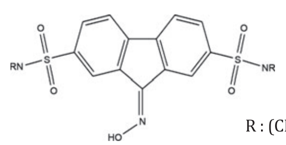


Fig. 2. Schematic representation of protein–protein interaction-based assay screening. β-Catenin attached onto plates was exposed to glutathione-S-transferase (GST)-fused T-cell factor 4 (TCF4). Anti-GST antibody and an alkaline phosphatase (AP)-conjugated secondary antibody were added to the plate. After the addition of compounds, disruption of the β-catenin/TCF complex was measured by the reduction of AP signals. In addition to compounds, *in silico* synthesized peptides are also used for screening. ALP, alkaline phosphatase.

performed by three methods. The first approach is based on protein–protein interactions. Lepourcelet *et al.*[59] established an HTS method for the identification of inhibitors of β-catenin ⁄TCF complex formation. His group developed a binding assay by attaching purified β-catenin, including the TCF binding site, onto a plate (Fig. 2). Approximately 7000 purified natural compounds were screened and six compounds were identified as inhibitors, among which two fungal derivatives, namely PKF115-584 and CGP049090 (Table 1), were effective antagonists of the β-catenin⁄TCF complex. These compounds have been shown to be effective against hematological malignancies *in vitro* and *in vivo*.[26,60,61]

The second approach is cell-based reporter assay screening. Wnt⁄β-catenin signaling activity can be assessed using the TOPFlash reporter that contains TCF⁄LEF binding sites upstream of the luciferase ORF. Luciferase activity in reporter

Table 1. Recent examples of Wnt⁄β-catenin inhibitors

Inhibitor	Screening method	Chemical structure	Hematological malignancies	References
PKF115-584	Protein–protein interaction		Acute myelogenous leukemia Multiple myeloma Chronic lymphocytic leukemia	(26,59–61)
CGP049090	Protein-protein interaction		Acute myelogenous leukemia Chronic lymphocytic leukemia	(26,59–61)
ICG-001	Cell-based reporter assay		Acute lymphoblastic leukemia Chronic myelogenous leukemia	(64–66)
XAV939	Cell-based reporter assay		Acute lymphoblastic leukemia	(62,63)
AV-65	Biomarker-based	 R : (CH2)x	Multiple myeloma Acute myelogenous leukemia Chronic myelogenous leukemia	(30,56,70,71)

cells stably expressing TOPFlash indicates β-catenin/TCF transcriptional activity. This assay is used to screen small molecule libraries for inhibitors of the Wnt/β-catenin signaling pathway (Fig. 3). Huang *et al.*[62] identified XAV939 (Table 1) as a Wnt/β-catenin pathway inhibitor using the TOPFlash reporter assay and showed that this synthetic compound inhibits tankyrase1 and tankyrase2, leading to the stabilization of Axin and the degradation of β-catenin. Tankyrases promote the ubiquitination of Axin, possibly through poly-ADP-ribosylation. XAV939 inhibits poly-ADP-ribosylation by binding tightly to the poly-(ADP-ribose) polymerase domain of tankyrases, and was shown to reduce stroma-mediated drug resistance in ALL cells through this mechanism.[63] Emami *et al.*[64] screened a small molecule library of 5000 compounds using a cell-based reporter assay system and identified a small molecule, ICG-001, based on its ability to downregulate the expression of β-catenin/TCF target genes. c-AMP response element binding protein-binding protein is a transcriptional co-activator that binds to the C-terminal region of β-catenin, modulating its stability through protein acetylation. ICG-001 (Table 1) binds CBP (but not p300) and competes for binding to β-catenin, resulting in the inhibition of colon cancer cell proliferation. Recently, this unique ICG-001 compound was shown to eliminate drug-resistant clones in ALL[65] as well as CML stem cell-like cells under hypoxic conditions.[66] PRI-724 was developed as a second generation CBP/β-catenin antagonist, and the clinical trial (phase I) of PRI-724 in advanced solid tumors was carried out (NCT01302405). The results of this clinical trial revealed that PRI-724 has an acceptable toxicity.[67] The following clinical trials in subjects

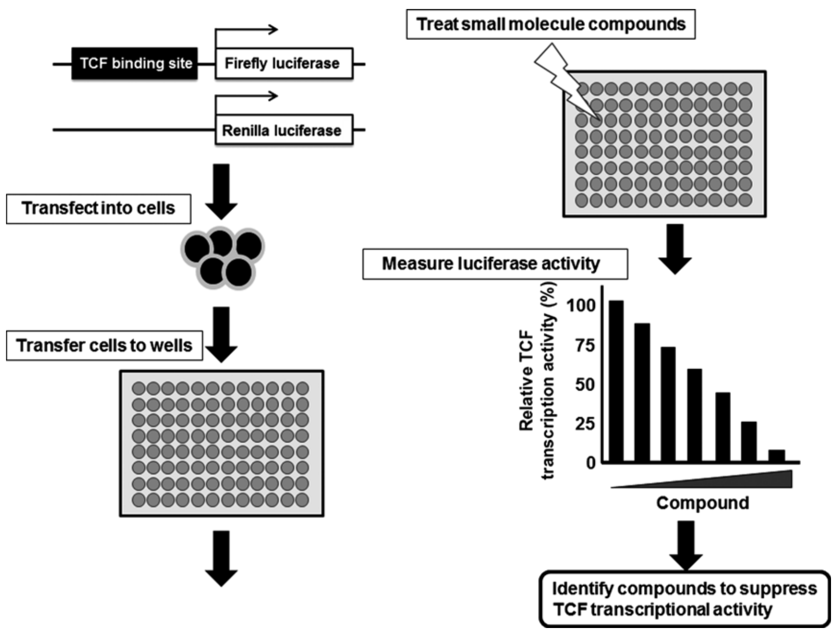


Fig. 3. Schematic representation of cell-based reporter assay screening. A dual-luciferase assay system is used. Wnt/β-catenin signaling activity can be assessed using the TOPFlash reporter that contains T-cell factor (TCF)/lymphocyte enhancer factor binding sites upstream of the luciferase ORF. Firefly luciferase is expressed in response to β-catenin/TCF transcriptional activity. Renilla luciferase is constitutively expressed and used as a control.

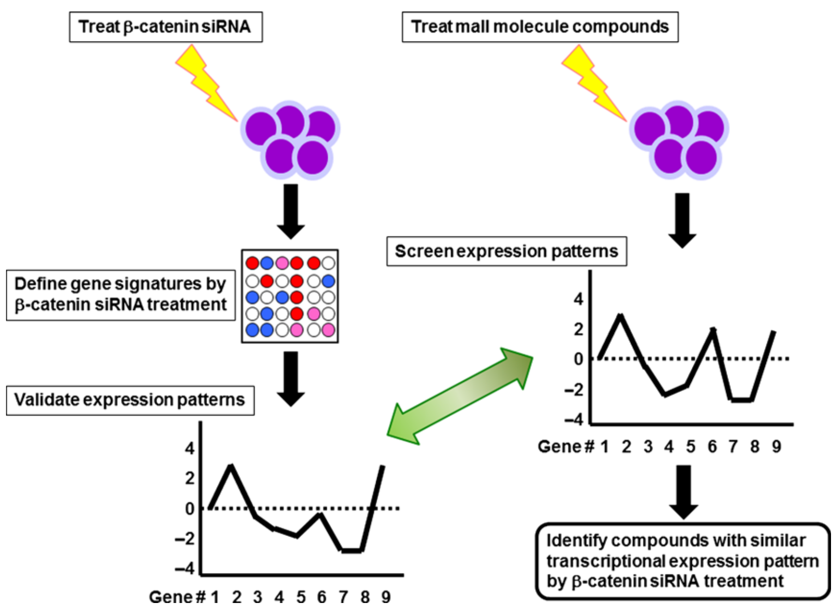


Fig. 4. Schematic representation of biomarker-based screening. This assay proceeds in two steps: (i) setting up gene signatures through β-catenin siRNA treatment; and (ii) screening for compounds with similar expression patterns.

with AML and CML are underway (NCT01606579). Moreover, Kida *et al*. and Ma *et al*. clearly demonstrated that ICG-001 inhibited the CBP-associated gene transcription.[64,68] Interestingly, the transcriptional coactivator CBP, not p300, is essential for HSC self-renewality.[69] Considering these observations, specific CBP/β-catenin inhibitors such as ICG-001 and PRI-724 can eliminate LSCs, and these compounds are expected to cure hematological malignancies.

The third method is biomarker-based screening, which is a new HTS method based on transcriptional profiling. Transcriptional activity can correlate with the specific state of a disease. Whole genome transcriptional profiling is costly and time consuming; however, transcriptional profiling using HTS is possible when the cellular state can be monitored through the expression of selected genes. Advances in transcriptional profiling techniques have improved the scale, cost, and ease of use of this method. Biomarker-based screening focuses on specific transcriptional activities to identify compounds of interest. In addition, transcriptional profiling enables the comparison of results and offers good reproducibility.

Bol and Ebner[70] examined the transcriptional response of a colon cancer cell line to β-catenin siRNA using full-genome microarray analysis (Fig. 4), and selected nine biomarkers for their potential as indicators of the response to cancer therapy. To identify compounds showing a similar expression pattern to that of the siRNA, a library of 90 000 individual compounds was screened, resulting in the identification of AV-65, an anthraquinone oxime compound (Table 1) capable of mimicking β-catenin knockdown. The effect of AV-65 on promoting the degradation of β-catenin and inhibiting β-catenin/TCF transcriptional activity was validated in MM cells. AV-65 induces the degradation of β-catenin by promoting β-TrCP-mediated ubiqutination, and downregulates the expression of c-myc, cyclin D1, and survivin, leading to the inhibition of MM cell proliferation. Moreover, AV-65 treatment prolongs the survival of MM-bearing mice, making it an attractive agent against MM.[29] AV-65 inhibits the proliferation of imatinib-resistant CML cells with the T315I mutation and stem-like characteristics.[56] BC2059, a derivative of AV-65, inhibited the proliferation of AML cells by disrupting the canonical Wnt/β-catenin pathway.[71]

Conclusion

Aberrant activation of canonical Wnt/β-catenin signaling plays a role in carcinogenesis and the progression of hematological malignancies; therefore, the inhibition of Wnt/β-catenin signaling is an effective approach to the treatment of hematological malignancies. Advances in screening methodology have enabled the identification of Wnt/β-catenin signaling inhibitors, and the efficacy of these compounds has been established in preclinical and clinical investigations.

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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
BM	bone marrow
CBP	c-AMP response element binding protein-binding protein
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
Fz	Frizzled
GMP	granulocyte/macrophage progenitors
GSK3β	glycogen synthase kinase-3β
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
HTS	high-throughput screening
LEF	lymphocyte enhancer factor
LRP5/6	low-density lipoprotein receptor-related protein 5/6
LSC	leukemic stem cell
MM	multiple myeloma
MSC	mesenchymal stromal cell
TCF	T-cell factor
TKI	tyrosine kinase inhibitor

Disclosure statement

The authors have no conflict of interest.

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