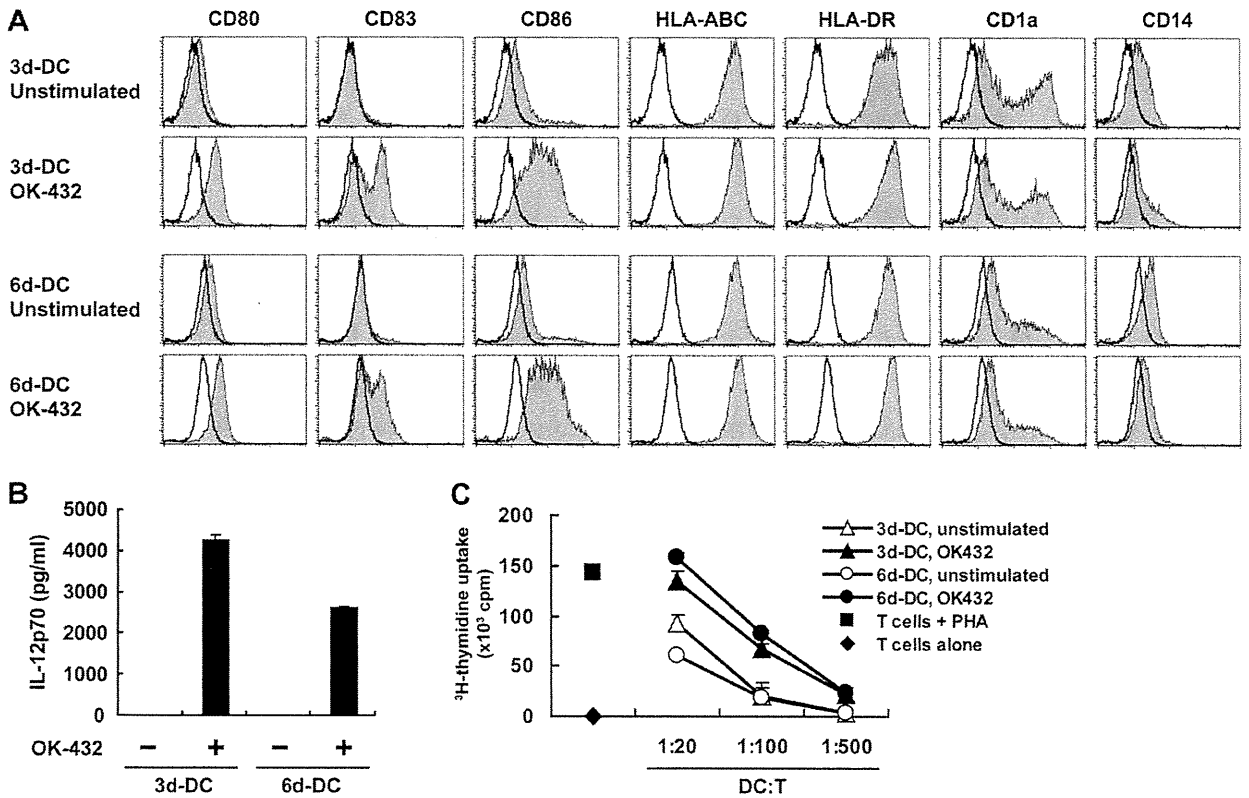
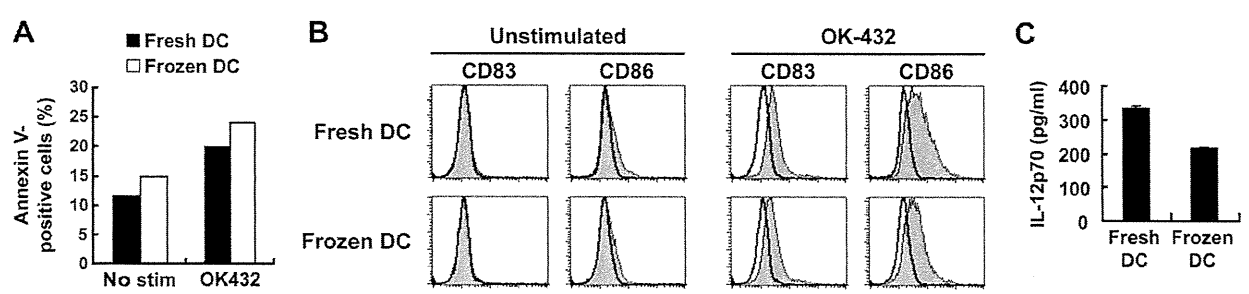
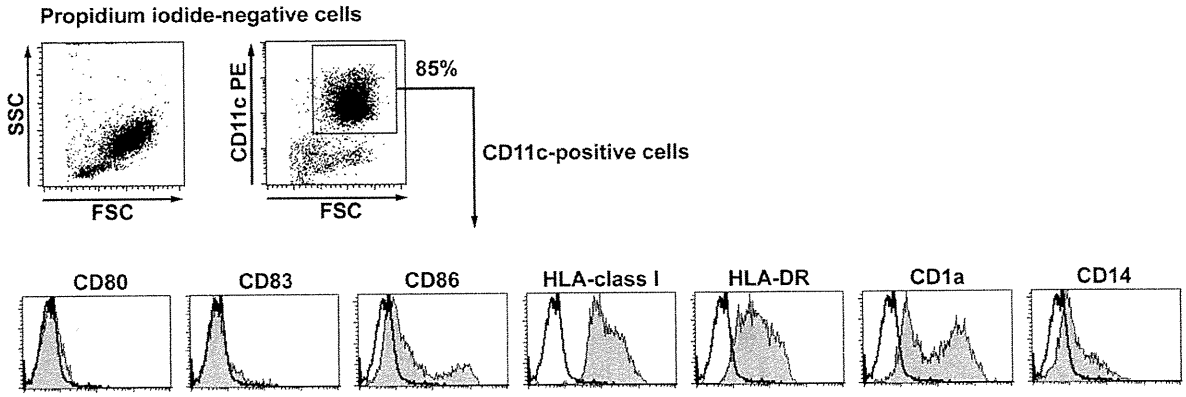


Supplementary Figure E1. 3d-DCs and 6d-DCs have comparable T-cell stimulatory capacity. (**A**) Expressions of surface molecules on DCs. Unstimulated or OK-432-stimulated DCs were analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**) IL-12p70 production by DCs (5×10^5 cells/mL) stimulated with OK-432 (0.1 KE/mL) for 24 hours was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**C**) Proliferation of naive $CD4^+$ T cells stimulated with DCs. Allogeneic naive $CD4^+$ T cells were cocultured with DCs at indicated DC to T-cell ratios. On day 4, 1 Ci of [^{3}H]-thymidine was added. After 16 hours of further incubation, thymidine uptake was counted. Naive $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin as a positive control. Representative data from three experiments are shown.



Supplementary Figure E2. Effects of cryopreservation on immature 3d-DCs. (**A**) Viability of fresh and frozen 3d-DCs after 24 hours of incubation with or without OK-432 (0.1 KE/mL) were evaluated by staining with Annexin-V. Percentages of Annexin-Vpositive cells are indicated. (**B**) Expression of surface molecules on fresh and frozen DCs after 24 hours of incubation with or without OK-432. (**C**) IL-12p70 production by fresh and frozen DCs (5×10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.



Supplementary Figure E3. Expression of surface molecules on DCs for vaccination. Cryopreserved DCs from patients were thawed, stained, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of cells in each quadrant. Representative data from patient no. 1 are shown.

Supplementary Table E1. DC vaccine generation

	At the time of apheresis				
Patient no.	Days after the last CT	PB WBC (/L)	PB Mo (%)	BM LC[a] (%)	Antigen dose (LC:DC)
1	74	4700	7	0.9	1:5
2	31	3000	9	2.0	1:6.5
3	43	3900	15	0[b]	1:6
4	46	4800	16	0.3	1:3.3

CT = chemotherapy; LC = leukemic cells; Mo = monocytes.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Patient 3 was in complete remission at the time of apheresis. The patient subsequently relapsed and became eligible for DC vaccination.

Expert Opinion

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Targeting the Wnt/β-catenin signaling pathway in human cancers

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Introduction: The Wnt/β-catenin signaling pathway plays a pivotal role in the regulation of cell growth, cell development and the differentiation of normal stem cells. Constitutive activation of the Wnt/β-catenin signaling pathway is found in many human cancers, and is thus an attractive target for anti-cancer therapy. Specific inhibitors of this pathway have been keenly researched and developed.

Areas covered: This review discusses the potential of inhibiting the Wnt/β-catenin signaling pathway, as a therapeutic approach for cancer, along with an overview of the development of specific inhibitors.

Expert opinion: Cancer stem cells (CSCs) play a significant role in the development and recurrence of several cancers, and Wnt/β-catenin signaling is important for the proliferation of CSCs. Inhibition of Wnt/β-catenin signaling is therefore a promising treatment approach. Progress has been made in the development of screening methods to identify Wnt/β-catenin signaling inhibitors. Biomarker-based screening is an effective and promising method for the identification of compounds of interest.

Keywords: cancer, cancer stem cell, high-throughput screening, Wnt, β-catenin

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1. Introduction

Wnt signaling pathways are important in developmental processes, cell growth and differentiation, and they have been well studied in relation to their role in developmental events [1-4]. Wnt signaling pathways can be divided into three main branches: the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway [1,4]. This review focuses on the Wnt/β-catenin signaling pathway. The control of the Wnt/β-catenin signaling pathway occurs through a variety of mechanisms aimed at preventing its aberrant activation. This signaling pathway has been associated with various type of cancer. The dysregulation of the Wnt/β-catenin signaling pathway was first investigated in a report linking alteration in Wnt/β-catenin signaling to the progression of colon cancer [5,6]. The findings of this study led to the development of molecular targeted therapy using inhibitors of the Wnt pathway. Antibody and siRNA approaches to the inhibition of the Wnt pathway have shown considerable efficacy [7-11], and conventional agents have been reported to contain inhibitors of this signaling pathway [12-16]. To identify small-molecule inhibitors of the Wnt pathway, several different screening techniques are used. In the present work, the potential of Wnt/β-catenin signaling pathway inhibitors as anti-cancer therapies is discussed.

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Article highlights.

- Aberrant activation of the Wnt/β-catenin signaling pathway is associated with various human cancers.
- Wnt/β-catenin signaling activity defines the characteristics of cancer stem cells.
- Inhibition of Wnt/β-catenin signaling is a potent strategy for cancer therapy.
- Progress has been made in the development of screening methods to identify Wnt/β-catenin signaling inhibitors.
- Biomarker-based screening is an effective and promising method for the identification of compounds of interest.

This box summarizes key points contained in the article.

2. Overview of the canonical Wnt/β-catenin signaling pathway

The name Wnt is derived from the Drosophilia segment polarity gene Wingless and mouse proto-oncogene Int-1. Wnt ligands are secreted glycoproteins that have been reported to have 19 isoforms in humans [17], and they bind to the Frizzled (Fz) receptor and the low density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor on the cell membrane, which initiates Wnt signaling. Wnt signaling activity can be downregulated by Wnt inhibitory factor-1 (WIF-1) and soluble frizzled-related proteins (SFRP), which inhibit Wnt signaling by directly binding to Wnt ligands [18,19], and Dickkopf (Dkk) proteins, which act by directly binding to LRP5/6 [20].

In the absence of Wnt signals, adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3β (GSK3β) and casein kinase 1α (CK1α) form a complex called the 'β-catenin destruction complex'. GSK3β and CK1α target serine/threonine residues at the N terminus of β-catenin for phosphorylation [21,22]. Phosphorylated β-catenin is recognized and polyubiquitinated by β-transducin-repeat-containing protein (β-TrCP), a component of a ubiquitin ligase complex [23,24], targeting β-catenin for degradation by the 26S proteasome (Figure 1).

On the other hand, the binding of Wnt ligands to Fz receptors and LRP5/6 coreceptors induces the phosphorylation of Disheveled (Dvl) and prevents GSK3β-dependent phosphorylation of β-catenin. Stabilized β-catenin translocates into the nucleus and interacts with T-cell factor (TCF)/lymphocyte enhancer factor (LEF). In the absence of β-catenin, TCF/LEF, which interacts with Groucho and HDAC, acts as a repressor of the transcription [25,26]. β-catenin displaces Groucho and HDAC from TCF/LEF, and the β-catenin-TCF/LEF complex activates the transcription of target genes. The β-catenin/TCF complex regulates the transcription of a number of genes associated with cell proliferation and apoptosis, as well as the expression of growth factors. Typical β-catenin/TCF target genes that are associated with cell proliferation are c-myc and cyclin D1. The c-myc oncogene regulates cell cycle progression and apoptosis. Cyclin D1 activates cyclin-dependent kinases leading to cell cycle progression. These genes are overexpressed in many human cancers including colorectal cancer [27]. The expression of c-jun and fos-related antigen 1 (fra-1), which are components of the activator protein 1 (AP-1) transcription complex, are also upregulated by Wnt/β-catenin signaling through a TCF-binding site in the promoter. In addition, the expression of the anti-apoptotic protein survivin is induced by β-catenin/TCF-mediated transcription [28]. VEGF plays a critical role in the proliferation of blood vessels and is associated with cancer progression [29]. Because VEGF is a target of the β-catenin/TCF complex [30], activation of the Wnt/β-catenin signaling pathway promotes the invasion and metastasis of cancer cells. In addition to the Wnt target genes mentioned above, an increasing number of Wnt target genes have been reported (see The Wnt homepage: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). Transcriptional regulation mediated by the β-catenin/TCF complex requires a number of co-activators, including histone acetyltransferase p300 and cAMP ressponse element binding protein (CREB)-binding protein (CBP), which interact with the C-terminus of β-catenin to activate β-catenin/TCF-mediated transcription [31], and B-cell chronic lymphocytic leukemia/lymphoma 9 protein (Bcl-9) and Pygopus, which act as co-activators by interacting with the N-terminus of β-catenin [32,33].

3. Wnt signaling in cancers

Aberrant activation of Wnt/β-catenin signaling is observed in many human cancers. Genetic mutations of Wnt signaling pathway components are primarily responsible for this aberrant activation. Genetic mutations of the components of the β-catenin destruction complex are common in human cancers (Table 1). Among them, mutations in the APC gene have been well studied for their role in the dysregulation of Wnt/β-catenin signaling. APC mutations were first found as the cause of familial adenomatous polyposis (FAP) [2]. FAP is an inherited disease characterized by the early onset of hundreds to thousands of colorectal adenomas. If the colon is not removed, FAP patients are at a risk of developing colorectal cancer. Mutations in β-catenin itself are also found in colorectal cancer. Approximately 80 – 90% of sporadic colorectal cancers harbor mutations in the APC gene, whereas β-catenin mutations are detected in 50% of cases without APC mutations, representing less than 10% of all colorectal cancers [34,35]. These mutations cause β-catenin to escape the degradation process and lead to nuclear stabilized β-catenin accumulation. Similarly, overexpression of Wnt ligands or downregulation of Wnt antagonists (Dkk, SFRP and WIF) have been reported in several human cancers. The stimulation of Wnt ligands stabilizes β-catenin and drives the transcription of target genes.

Resistance to chemotherapy and minimal residual disease (MRD) are obstacles to improving survival rates. Cancer stem cells (CSCs) are similar to normal stem cells in many

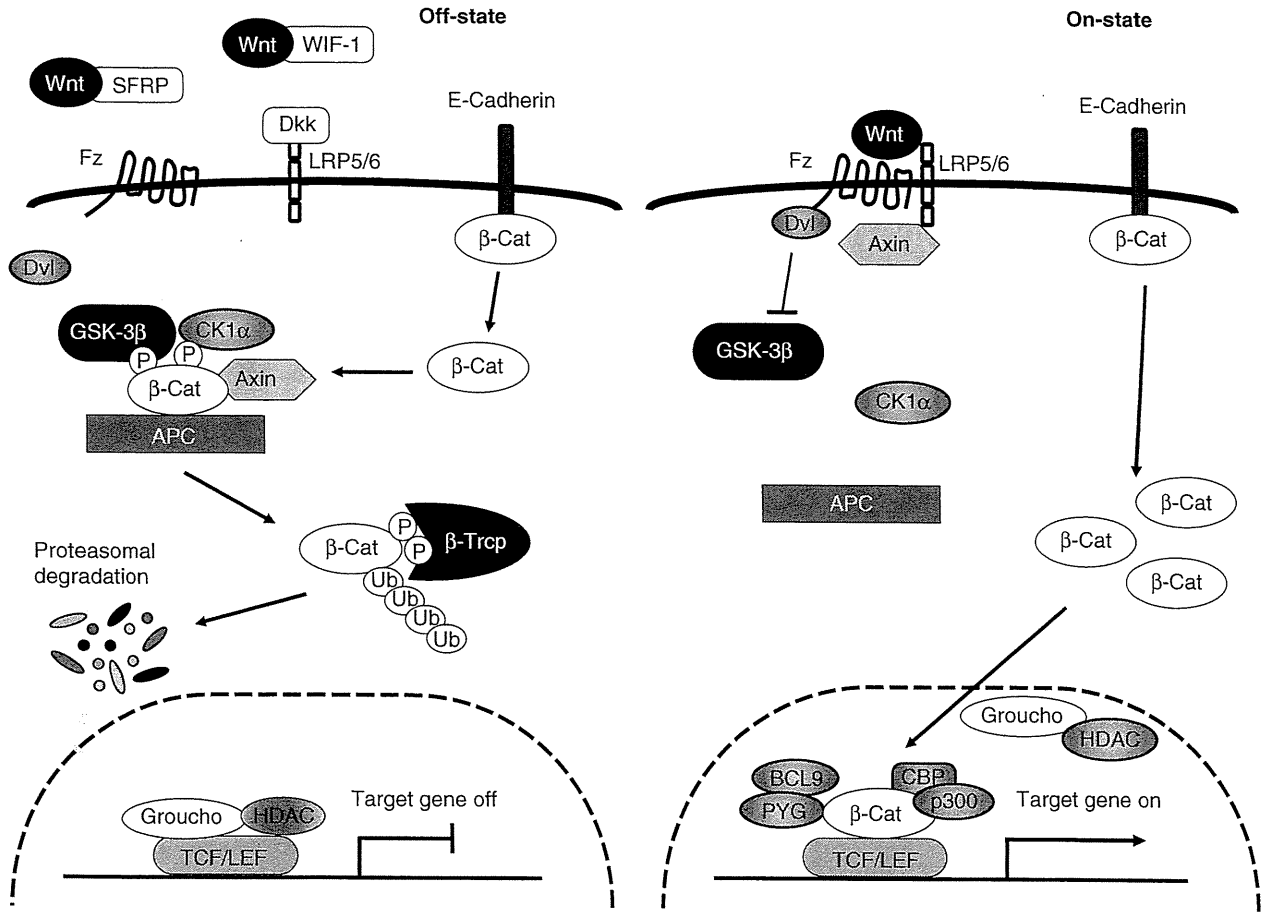


Figure 1. The Wnt/β-catenin signaling pathway. The majority of β-catenin (β-Cat) is localized to the cell membrane, where it binds to the cytoplasmic domain of E-cadherin, a cell adhesion molecule. A small part of free β-catenin exists in the cytoplasmic pool. Soluble frizzled-related proteins (SFRP) and Wnt inhibitory factor-1 (WIF-1) inhibit binding of Wnt ligands to frizzled (Fz) receptors. Dickkopf (Dkk) interacts with low density lipoprotein receptor-related protein 5/6 (LRP5/6) to inhibit binding of Wnt ligands. In the absence of Wnt/β-catenin signaling, "Wnt off" state (Left panel), free cytoplasmic β-catenin is phosphorylated by a complex formed by casein kinase 1α (CK1α), glycogen synthase kinase-3β (GSK3β), adenomatous polyposis coli (APC), and Axin. Phosphorylated β-catenin is recognized by β-transducin repeat-containing protein (β-TrCP) and subsequently ubiquitinated, which targets it for degradation by the proteasome. Interactions between the Wnt ligand and the Fz/LRP receptor trigger Wnt signal transduction, as seen in the 'Wnt on' state (Right panel). Phosphorylation of β-catenin is suppressed and β-catenin escapes from the degradation. Free cytoplasmic β-catenin translocates to the nucleus and forms a complex with TCF/LEF. The β-catenin-TCF complex activates the transcription of target genes, such as cyclin D1 and c-myc.

Bcl-9: B-cell chronic lymphocytic leukemia/lymphoma 9 protein; CBP: cAMP response element binding protein (CREB)-binding protein; Dvl: Disheveled; LEF: Lymphocyte enhancer factor; P: Phosphorylation; PYG: Pygopus; Ub: Ubiquitin.

respects and they possess the capacity to generate progeny cells and self-renewal abilities, resulting in relapse and chemo-resistance [36,37]. Wnt/β-catenin signaling is activated in CSCs as well as in normal stem cells. The Wnt/β-catenin signaling pathway plays a crucial role in the maintenance of CSCs in many cancers, including colon, skin and breast cancer and leukemia [38-42]. Vermeulen *et al.* demonstrated the heterogeneity of Wnt signaling activity levels in a colon CSC population using the TCF/LEF reporter driving the expression of GFP [39]. These authors revealed that cells with high Wnt signaling activity possess clonogenicity. Moreover, colon cancer cells in which Wnt signaling is activated at high levels were found to be located close to stromal myofibroblasts. These observations suggest that the microenvironment regulates Wnt signaling activity in CSCs.

Several studies suggest that the epithelial-to-mesenchymal transition (EMT) is an important process in tumor progression [43,44]. EMT is the process by which epitherial cells lose their epithelial characteristics and acquire mesenchymal properties. EMT allows cancer cells to detach from the tumor mass and disseminate to other tissues [43,45]. Recent reports indicate that the accumulation of nuclear β-catenin is required for EMT, although it is not sufficient to induce EMT [46,47].

Table 1. Dysregulation of Wnt/β-catenin signaling pathway in cancer.

Gene	Alteration	Type of cancer	Ref.
Wnt ligands	Increased expression	Colon cancer	[95,96]
		Breast cancer	[62]
		Head/neck cancer	[97]
		Lung cancer (NSCLC)	[61]
		Gastric cancer	[98]
		Melanoma	[99]
		Prostate cancer	[100]
Frizzled receptors	Increased expression	Colon cancer	[95,101]
		Gastric cancer	[102]
		Synovial sarcoma	[103]
SFRP	CpG methylation and reduced expression	Colon cancer	[58,104,105]
		Breast cancer	[106,107]
		Gastric cancer	[102,108,109]
		Lung cancer	[110,111]
		Leukemia	[112]
		Bladder cancer	[113]
DKK	CpG methylation and reduced expression	Colon cancer	[56,114]
		Gastric cancer	[115]
		Breast cancer	[106]
WIF	CpG methylation and reduced expression	Breast cancer	[116,117]
		Bladder cancer	[117]
		Proatate cancer	[117]
		Lung cancer	[117,118]
APC	Inactivating mutation	Colon cancer	[119-121]
		Barret's esophagus	[122]
β-catenin	Activating mutation	Colon cancer	[123,124]
		Gastric cancer	[125,126]
		Hepatocellular cancer	[127,128]
		Hepatoblastoma	[128]
		Ovarian cancer	[129]
		Wilm's tumor	[130]
Axin	Inactivating mutation	Hepatocellular cancer	[128]
		Colon cancer	[131]

APC: Adenomatous polyposis coli; Dkk: Dickkopf; SFRP: Soluble frizzled-related proteins; WIF-1: Wnt inhibitory factor-1.

4. Wnt/β-catenin signaling pathway inhibitors for cancer therapy

Strategies to inhibit the Wnt/β-catenin signaling pathway have been eagerly developed for the treatment of cancers. Wnt/β-catenin signaling pathway inhibitors are classified roughly into two groups: biologic inhibitors and small-molecule compounds. Biologic inhibitors include monoclonal antibodies and recombinant nucleic acids (siRNA). Small-molecule compounds fall into two categories: conventional agents and novel agents. Compounds with inhibitory effects on the Wnt/β-catenin signaling pathway are contained in certain existing agents, such as NSAIDs and imatinib. The identification of novel compounds is done using various screening methods.

4.1 NSAIDs

NSAIDs are primarily used to treat inflammation, pain and fever. Epidemiological and clinical studies have shown that NSAIDs possess the ability to lower the risk of developing various type of cancers [48,49]. NSAIDs were also found to prevent the development of polyps and reduce tumor tissue in FAP patients [50]. Because COX-2 is highly expressed in various cancers [51], and it is also correlated with cancer progression, the effect of NSAIDs in preventing cancer progression is thought to be mediated by a direct inhibition of COX2. In addition, several studies indicate that NSAIDs inhibit Wnt/β-catenin signaling, which results in the prevention of cancer progression [12-14]. These results demonstrate a direct correlation between COX-2 and the Wnt/β-catenin signaling pathway.

Castellone *et al.* demonstrated the connection between COX-2 and Wnt signaling by showing that COX-2-derived prostaglandin E2 (PGE2) decreased phosphorylated β-catenin and increased nuclear β-catenin in colon cancer cell lines [52]. PGE2 released GSK3β from the destruction complex via the EP2 receptor, concurrently with the phosphorylation and inactivation of GSK3β through the PI3K/Akt signaling pathway, which was stimulated by PGE2.

However, some NSAIDs that do not possess COX2 inhibitory activity retain potent inhibitory effects on the Wnt/β-catenin signaling pathway in cancer cells, implying that the inhibition of this signaling pathway by NSAIDs results not only from COX2 inhibition but can also be mediated by other mechanisms [53,54]. Tuynman *et al.* described a mechanism of inhibition of Wnt/β-catenin signaling by NSAIDs [55]. Analysis of the activity of cellular kinases revealed that celecoxib impairs the auto-phosphorylation of the tyrosine kinase receptor c-Met, which results in the inhibition of Wnt/β-catenin signaling. The phosphorylation of c-Met leads to the activation of PI3K signaling and subsequent inactivation of GSK-3β. Consequently, dephosphorylation of c-Met by celecoxib induces a decrease of GSK-3β activity and the subsequent degradation of β-catenin.

4.2 Restoration of SFRP, WIF, and Dkk function

The decreased expression of secreted antagonists such as SFRP, WIF-1 and Dkk is observed in some cancers. SFRP and WIF-1 inhibit binding of the Wnt ligand and Fz receptor by binding directly to the Wnt ligand, while Dkk inhibits the Wnt signaling pathway by binding to LRP5/6. In some cancer cells, methylation of the promoter CpG island leads to a decrease in the expression of these Wnt antagonists. Efficient gene transfer of these antagonists has been evaluated as a therapeutic approach. Several reports showed that overexpression of Dkks inhibits cell growth in colon and lung cancer cells [56,57]. Moreover, the restoration of the function of SFRPs attenuated Wnt/β-catenin signaling in colon cancer cells, even in the presence of downstream mutations [58]. Similarly, the restoration of WIF-1 function attenuated Wnt signaling and induced apoptosis in colon cancer cells

harboring downstream mutations [59]. These reports indicated that restoration of the function of secreted antagonists might be a potent therapeutic approach to the treatment of certain tumors, despite the presence of downstream mutations.

4.3 Monoclonal antibodies

With regard to biological safety and specificity of treatment, antibody-based therapies provide an excellent therapeutic approach. Because small-molecule compounds can diffuse into the cells, they can affect intracellular targets. On the other hand, antibodies cannot cross the cell membrane and therefore do not target intracellular proteins, thus providing a safer treatment modality. Certain autocrine Wnt ligands contribute to cell proliferation and survival in various tumors [60,61]. Antibody-based therapies targeting Wnt ligand–Fz receptor binding at the cell surface can be effective in the treatment of certain cancers.

Upregulation of the Wnt-1 ligand (WNT-1) has been reported in various human cancers [9,62]. The activation of the Wnt/β-catenin signaling pathway by WNT-1 was found to correlate with tumor proliferation, and WNT-1 was therefore used as a direct target of treatment, as demonstrated by blocking WNT-1 signaling using an anti-WNT-1 monoclonal antibody, which induced apoptosis in WNT-1-overexpressing tumor cells, and suppressed tumor growth in mice [7-9]. The advantage of this treatment approach is that the anti-WNT-1 antibody has minimal effects in cells that do not express WNT-1 or express it at low levels, and it is therefore effective only in WNT-1-overexpressing cells, showing the specificity and selectivity of this treatment strategy.

4.4 siRNA

siRNA is an attractive therapeutic approach based on the induction of sequence-specific gene silencing [63]. In colon cancer cell lines, β-catenin siRNA reduced β-catenin expression and β-catenin/TCF-mediated transcription. In addition, β-catenin siRNA inhibited tumor growth in a SW480 xenograft model [11]. Moreover, work from our group demonstrated that β-catenin siRNA inhibited the growth of multiple myeloma tumors in a xenograft model [10]. Multiple myeloma (MM) is a human hematological malignancy characterized by the clonal expansion of plasma cells in bone marrow. β-catenin is overexpressed in MM cells compared with normal plasma cells, although the mechanisms underlying β-catenin overexpression are not clear. A recent study reported that β-catenin overexpression is caused by epigenetic dysregulation of the Wnt/β-catenin signaling pathway; this study also found that Wnt antagonists (WIF1, Dkk3, APC, SFRP1, SFRP2, SFRP4 and SFRP5) are methylated in MM cell lines and primary MM cells [64]. This dysfunction of Wnt antagonists induced the activation of the Wnt/β-catenin signaling pathway. siRNA-mediated blockade of Wnt/β-catenin signaling resulted in the inhibition of the progression of MM [10]. Because siRNAs induce gene-specific silencing, this treatment does not affect other signaling pathway or cause non-specific effects. β-catenin siRNA specifically inhibits β-catenin expression and therefore only suppresses activities downstream of β-catenin. However, problems associated with this treatment approach that have not yet been resolved include the inability of intravenously-administered siRNA to reach target tissues because siRNA is unstable in the bloodstream. If the stability of siRNA in the blood can be improved and a better drug delivery system can be designed, siRNA-based therapeutic approaches could become effective in the treatment of cancers.

β-catenin is a component of cadherin junctions and acts as a bridge connecting E-cadherin to actin filaments. The downregulation of β-catenin should therefore impair E-cadherin-dependent cell–cell adhesion. siRNAs should be designed to specifically target tumor cells to minimize the damage to normal adherent cells. This could be achieved through the conjugation of siRNA to an antibody against a tumor-expressed antigen [65].

4.5 Imatinib

The function of β-catenin is regulated by phosphorylation of different serine and threonine residues, namely S31, S33, T41 and S45, which are associated with its degradation. The phosphorylation of tyrosine residues plays a different role from serine/threonine phosphorylation and is important in determining the localization of β-catenin at the cell membrane, where it co-localizes with E-cadherin and interacts with β-catenin. Tyrosine 654 of β-catenin plays a role in the binding to E-cadherin and the phosphorylation of tyrosine 654 decreases the binding. Similarly tyrosine 142 is necessary for the interaction of β-catenin with α-catenin and the phosphorylation of tyrosine 142 decreases this interaction. These tyrosine residues are phosphorylated by specific tyrosine kinases: tyrosine 654 by EGFR and Src [66-68], and tyrosine 142 by Fyn, Fer and Met [66,69]. The phosphorylation of these tyrosine residues impairs E-cadherin–mediated cell adhesion and induces the activation of Wnt signaling by increasing cytoplasmic β-catenin.

Imatinib is a tyrosine kinase inhibitor used for the treatment of chronic myeloid leukemia (CML) [70,71]. Imatinib has been shown to inhibit the Wnt/β-catenin signaling pathway in colon cancer and thyroid cancer cell lines [15,16] through a mechanism involving the indirect inhibition of the tyrosine-phosphorylation of β-catenin, which caused a decrease in the accumulation of β-catenin in the nucleus.

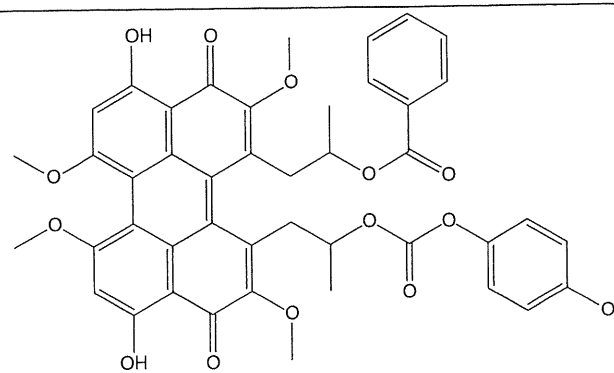
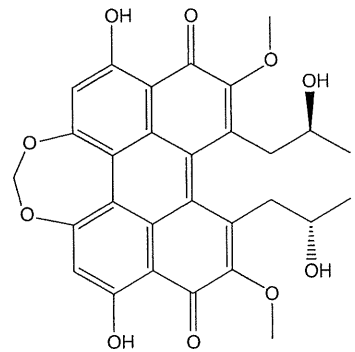
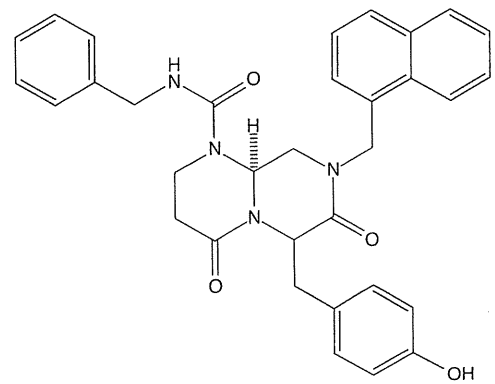
4.6 Small-molecule compounds

Small-molecule compounds have been developed extensively as therapeutic agents (Table 2) mainly due to their ability to target intracellular proteins. The screening of small-molecule compounds is crucial to their development. In the following sections, the small-molecule inhibitors that have been identified and the screening methods used are described in detail.

4.6.1 The screening of protein–protein interactions

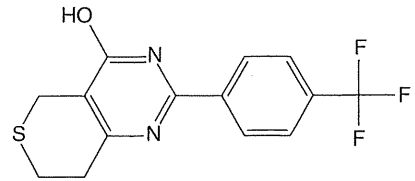
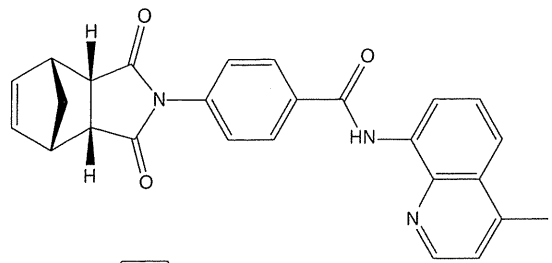
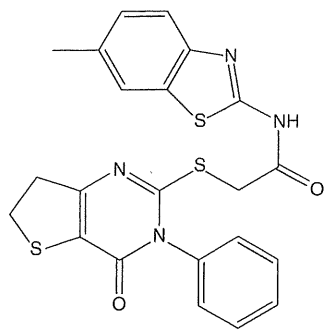
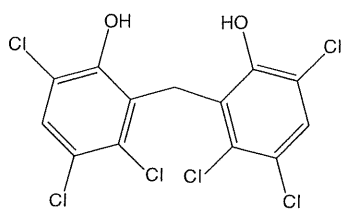
The identification of small-molecule inhibitors based on the interaction between the components of a signaling pathway is an effective method in Wnt/β-catenin signaling due to the fact that this pathway is well characterized.

Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway.

Name	Structure	Action	Ref.
PKF115 – 584		Prevention of β-catenin–TCF interaction	[74-77]
CGP049090		Prevention of β-catenin–TCF interaction	[74,76,77]
ICG-001		Prevention of β-catenin–CBP interaction	[79]

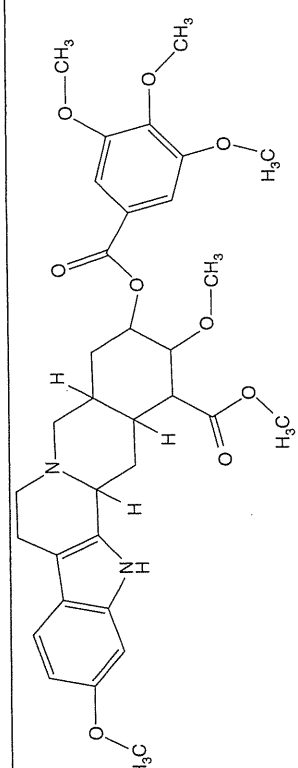
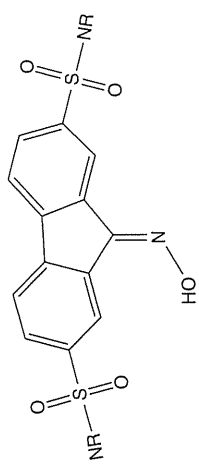
CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.

Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway (continued).

Name	Structure	Action	Ref.
XAV939		Upregulation of axin protein levels by tankyrase inhibition	[80]
IWR-2		Stabilization of axin	[81]
IWP-2		Prevention of Wnt ligand secretion by Porcn inhibition	[81]
Hexachlorophene		Upregulation of ubiquitin ligase Siah-1	[82]

CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.

Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway (continued).

Name	Structure	Action	Ref.
Isoreserpine		Upregulation of ubiquitin ligase Siah-1	[83]
LC-363		Degradation of β-catenin by ubiquitin-dependent proteolysis	[84,85]

CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.

The TCF/LEF family (TCF-1, TCF-3, TCF-4 and LEF-1) transcription factors are characterized by a DNA-binding high mobility group (HMG) box. In the absence of β-catenin, TCFs function as target gene transcriptional repressors by occupying the promoter. Stabilized β-catenin forms a complex with TCFs and converts them into transcriptional activators. The targeting of gene transcription by β-catenin/TCF requires co-activators such as CBP/p300 and brahma homolog 1 (Brg1), which interact with the C-terminus of β-catenin, and Bcl9/Pygopus, which interacts with its N-terminus. Lepourcelet *et al.* [72]described the interaction between β-catenin and TCF factors. Disruption of β-catenin/TCF complex formation blocks the expression of the Wnt/β-catenin signaling target genes. The N-terminal peptide of TCF binds to the groove formed by the armadillo repeats of β-catenin [73,74]. A binding assay to identify β-catenin/TCF complex inhibitors by high-throughput screening (HTS) was developed by attaching purified β-catenin, including the TCF binding site, onto a plate. Glutathione-S-transferase (GST)-fused TCF, anti-GST antibody, and an alkaline phosphatase (AP)-conjugated secondary antibody were then added to the plate, and disruption of the β-catenin/TCF complex was measured by the reduction in AP signals. After approximately 7000 purified natural compounds were screened, 6 compounds were identified as inhibitors. Biochemical assays and the Xenopus axis duplication assay were performed to validate the function of the inhibitors. Two fungal derivatives, namely PKF115-584 and CGP049090, were found to be effective antagonists of the β-catenin/TCF complex [72].

Sukhdeo *et al.* investigated the effects of PKF115-584 on human MM cells [75]. PKF115-584 blocks β-catenin/TCF transcriptional activity and induces cytotoxicity in MM cell lines and primary cells from MM patients *in vitro*. PKF115-584 treatment inhibits the growth of human MM cells *in vivo* and prolongs the survival of MM-cell-bearing mice. Similarly, Wei *et al.* evaluated the activity of the three inhibitors (PKF118-310, PKF115-584- and CGP049090) on human hepatocellular carcinoma (HCC) cells [76]. These inhibitors displayed dose-dependent cytotoxicity *in vitro* and suppressed tumor growth in xenograft models. A recent study showing that PKF115-584 and CGP049090 have growth inhibitory effects on human chronic lymphocytic leukemia cells was presented at the American Society of Hematology (ASH) meeting in 2009 [77].

4.6.2 *Cell-based reporter assay screening*

Wnt/β-catenin signaling activity can be assessed using the TOPFLASH reporter which contains TCF/LEF binding sites upstream of the luciferase open reading frame [78]. The reporter cells that stably harbor TOPFLASH show luciferase activity as β-catenin/TCF transcriptional activity. This assay has been used to screen small-molecule libraries for inhibitors of the Wnt/β-catenin signaling pathway (**Figure 2**).

Emami *et al.* identified a small molecule ICG-001, that showed activity in down-regulating the expression of β-catenin/TCF target genes [79]. These authors screened a small molecule library of 5000 compounds using a cell-based

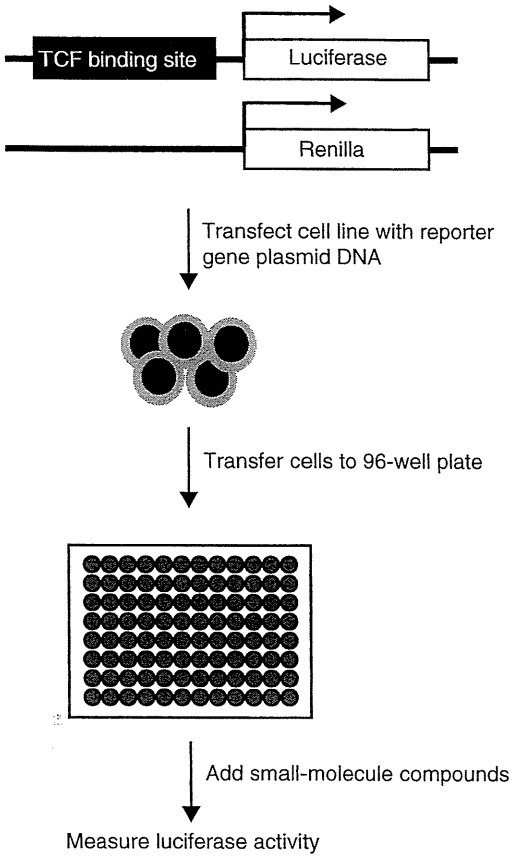


Figure 2. Cell-based reporter assay screening. A dual-luciferase system is used in this assay. Firefly luciferase is expressed in response to β-catenin/T cell factor (TCF) transcriptional activity. Renilla luciferase is expressed constantly.

reporter assay system. CBP is a transcriptional coactivator that binds to the C-terminal region of β-catenin and regulates its stability through acetylation. Emami *et al.* demonstrated that ICG-001 binds the CBP protein and competes for binding to β-catenin. The anti-apoptotic gene survivin and cyclin D1 were also downregulated by ICG-001 in colon cancer cell lines. ICG-001 increased caspase activity in colon cancer cell lines but not in normal colonic epithelial cells. In an *in vivo* study, ICG-001 reduced the number of intestinal polyps in Min micee, which carry a nonsense mutation in one APC allele. Moreover, ICG-001 reduced the tumor volume in a xenograft model of the colon cancer cell line SW620.

XAV939 is a synthetic compound that was first identified as a Wnt/β-catenin pathway inhibitor in a HTS using HEK293 cells stably harboring the Wnt reporter Super(8×) TOPFLASH [80]. XAV939 inhibits tankyrase1 and tankyrase2, and as a result, stabilizes Axin and subsequently degrades β-catenin. Tankyrases promote the ubiquitination of Axin, possibly through poly-ADP-ribosylation (PARsylation). XAV939 inhibits PARsylation by binding tightly to the poly-(ADP-ribose) polymerase (PARP) domain of tankyrases. XAV939 is a potent inhibitor of Wnt/β-catenin signaling through the inhibition of tankyrases.

Chen *et al.* identified potent inhibitors of Wnt/β-catenin signaling by multiple stage screening [81]. A 200,000-compound library was screened using mouse L cells stably harboring the Wnt reporter Super-(8×)TOPFLASH and a Wnt3A expression vector. After confirmation of the inhibitory activity, the compounds were categorized into two groups according to their site of action within the Wnt/β-catenin signaling pathway: inhibitors of Wnt production (IWPs) and inhibitors of Wnt response (IWRs). IWPs inhibit the secretion of the Wnt ligand through binding to porcupine (Porcn). Porcn is a member of the membrane-bound O-acyltransferase (MBOAT) family and it plays an important role in Wnt ligand secretion by adding a palmitoyl group to Wnt ligand proteins in the endoplasmic reticulum. Biochemical analysis revealed that IWRs bind directly to Axin and stabilize it, which leads to β-catenin degradation.

Park *et al.* identified hexachlorophene as a compound that decreased the level of intercellular β-catenin using cell-based reporter assay screening [82]. Hexachlorophene is able to degrade β-catenin by a mechanism independent of β-TrCP-mediated ubiquitination. Two types of ubiquitin ligases target β-catenin for destruction: the ubiquitin ligase β-TrCP recognizes GSK-3β-phosphorylated β-catenin, and the ubiquitin ligase seven in absentia homolog (Siah) targets β-catenin for degradation independently of its phosphorylation. Siah-1 expression is induced by p53, and Siah-2 is implicated in the regulation of the response to hypoxia. Siah forms a complex with Siah-interacting protein (SIP), S-phase kinase-associated protein 1 (Skp1) and Ebi, and interacts with the β-catenin/APC complex to induce β-catenin degradation. Hexachlorophene treatment caused an increase in the levels of Siah-1 mRNA. This conclusion indicated that the effect of hexachlorophene on β-catenin occurred through the Siah/APC pathway. Similar to hexachlorophene, isoreserpine, which was identified using the cell-based TOPFLASH reporter assay, was found to up-regulate Siah-1 and promote β-catenin degradation [83].

4.6.3 *Biomarker-based screening*

Biomarker-based screening is a new screening method based on transcriptional profiling. Transcriptional activity can correlate with the specific state of a disease. The application of transcriptional profiling to entire genomes is difficult due to cost and time requirements. However transcriptional profiling can be used in HTS when the cellular state can be monitored through the expression of selected genes. Transcriptional profiling techniques have progressed in terms of scale, cost, and ease of use. Biomarker-based screening focuses on specific transcriptional activities to identify the compounds of interest. In addition, transcriptional profiling enables the comparison of results and offers good reproducibility.

Avalon Pharmaceuticals (MD, USA) assessed the transcriptional response of a colon cancer cell line to treatment with β-catenin siRNA using full-genome microarray analysis [84]. In this study, nine biomarkers were selected for their potential as indicators for cancer therapy. A library of 90,000 individual compounds was screened to identify compounds that showed a similar expression pattern to the siRNA, and the compound LC-363 was detected based on its ability to mimic the effect of β-catenin knockdown (**Figure 3**). Interestingly, indomethacin and calphostin C, which are different type of Wnt/β-catenin pathway inhibitors, showed a similar expression pattern than that of the siRNA. The effect of LC-363 in promoting the degradation of β-catenin and inhibiting β-catenin/TCF transcriptional activity was validated in MM cells [85]. LC-363 decreased the expression of c-myc, cyclin D1 and survivin, which resulted in the inhibition of MM cell proliferation through the apoptotic pathway. LC-363 treatment prolonged the survival of MM-bearing mice. A clinical study with this compound series in solid and hematopoietic malignancies will be carried out in the future. These results demonstrated the effectiveness of biomarker-based screening in the identification of compounds of interest.

5. Conclusion

The effect of the aberrant activation of Wnt/β-catenin signaling in promoting tumor progression has been studied extensively. Because the inhibition of Wnt/β-catenin signaling activity is an effective approach to the treatment of human cancers, therapeutic agents that target for the protein components of the Wnt/β-catenin signaling pathway are being developed.

The identification of Wnt/β-catenin signaling inhibitors can be approached by different types of screening methods, including reporter assay screening for β-catenin/TCF-mediated transcriptional activity and binding assays for β-catenin-coactivators. Inhibitors that were identified using these methods were shown to have growth inhibitory effects on tumor cells *in vitro*. These inhibitors that target the Wnt/β-catenin signaling pathway need to be evaluated for their effects on other signaling pathways and on normal stem cells. Because the relationship between the components of the Wnt signaling pathway and those of other signaling pathways are not clear, inhibition of Wnt signaling might affect other pathways and therefore result in unexpected adverse effects.

The Wnt/β-catenin signaling pathway is important for not only for the developmental process but also for the maintenance of stem cells [86,87]. The potential effect of Wnt/β-catenin signaling inhibitors on normal somatic stem cell homeostasis and tissue maintenance therefore needs to be carefully evaluated.

Biomarker-based screening uses several parameters derived from full-genome microarray analysis to identify compounds of interest. LC-363 was identified by a screening study that used a biomarker driven approach, in which multiple parameters were designed from β-catenin siRNA [84]. LC-363 was identified and validated as a potential Wnt/β-catenin signaling pathway inhibitor. This result clearly shows that biomarker-based screening is a very effective method for the identification of compounds of interest.

6. Expert opinion

CSCs show different characteristics than bulk cancer cells. CSCs are resistant to conventional drugs and are associated with recurrent cancer and MRD [36,88]. The elimination of CSCs is therefore important for the successful treatment of cancer. Activation of Wnt/β-catenin signaling is found in several CSCs [38-42], which suggests that the Wnt/β-catenin signaling pathway is a potential therapeutic target in CSCs. However, the maintenance and proliferation of normal stem cells [86,89], including hematopoietic stem cells, are dependent of Wnt/β-catenin signaling. In addition, Wnt/β-catenin signaling is involved in bone formation through the regulation of osteoblast differentiation [90,91]. The possibility of adverse effects caused by the inhibitors of this pathway in normal stem cells and normal tissues is an issue of concern. Still, targeting Wnt/β-catenin signaling is an attractive possibility for cancer therapy because Wnt/β-catenin signaling is not only associated with CSCs, but also with EMT-mediated metastasis, and is therefore of prognostic value in cancer. Recent reports indicate that the accumulation of nuclear β-catenin is associated with EMT, which results in cell migration and metastasis. The inhibition of Wnt/β-catenin signaling is therefore a potential strategy for the prevention of cell migration and metastasis through its effect on EMT. The prognostic relevance of β-catenin in some cancers has been reported, and elevated β-catenin levels indicate a possible adverse prognosis for cancer patients [92-94]. As discussed above, CSCs play a significant role in the development and recurrence of several cancers, and Wnt/β-catenin signaling is important for the proliferation of CSCs. Inhibition of Wnt/β-catenin signaling is therefore a promising approach to the treatment of cancers.

The dosing and duration of the application of Wnt/β-catenin signaling inhibitors needs to be carefully determined to prevent adverse effects. In addition, the dependence of tumor growth on Wnt/β-catenin signaling varies according to the type of cancer; therefore, a Wnt/β-catenin-signaling-pathway inhibitor will be more effective in the treatment of cancers that are heavily dependent on the Wnt/β-catenin signaling pathway. In the treatment of cancers in which growth is associated with other signaling pathways, combination therapy consisting of Wnt/β-catenin-signaling-pathway inhibitors and other agents could improve the treatment efficacy. Wnt/β-catenin-signaling-pathway inhibitors possess anti-cancer properties and represent a promising approach for the treatment of cancer patients.

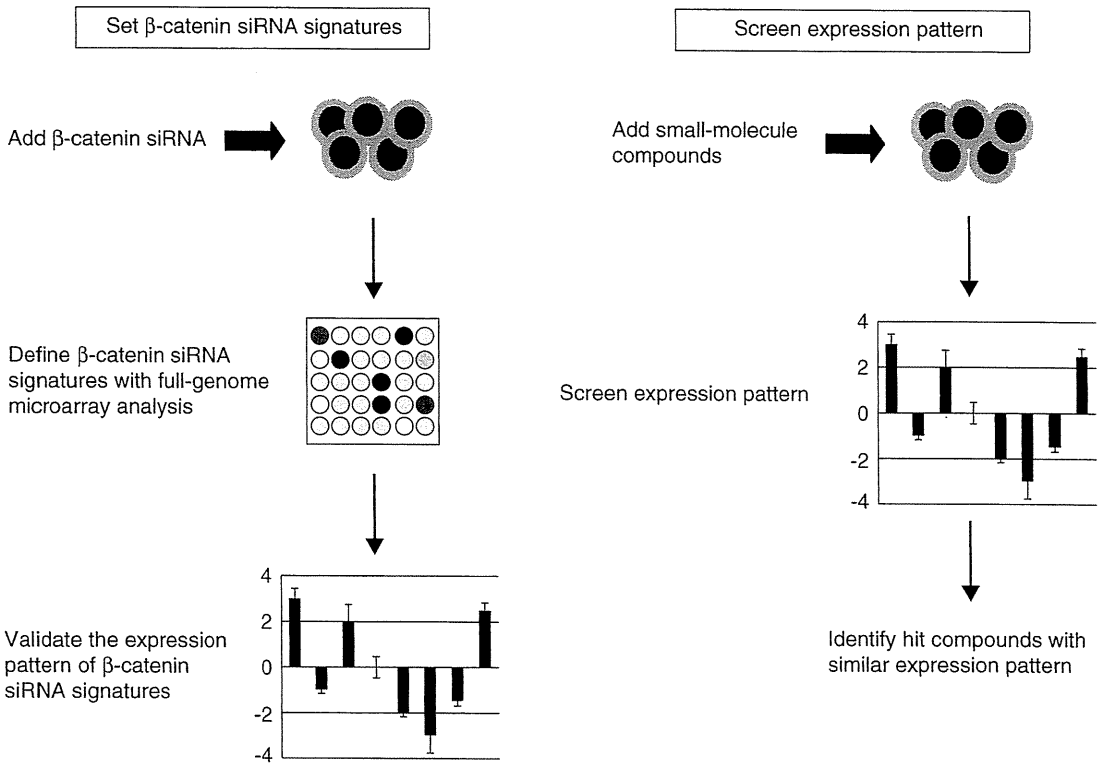


Figure 3. Biomarker-based screening. This assay proceeds in two steps: the first step consists of setting up the β-catenin siRNA signatures. The second step involves screening for compounds with similar expression patterns.

Target specificity is regarded as an important feature of a good inhibitor. An inhibitor that non-specifically suppresses other signaling activity is considered inadequate, even if it can suppress Wnt/β-catenin signaling activity. Generally, target specificity of hit compounds is confirmed by microarray analysis after the primary screening. If the primary screening considers the specificity and *in vivo* effects, it is possible to identify hit compounds at a more advanced stage for clinical application. Biomarker-based screening is therefore the preferred method of drug discovery. In the future, agents identified by these new screening methods will be ready for testing in clinical applications.

Declaration of interest

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Allograft inflammatory factor-1 is overexpressed and induces fibroblast chemotaxis in the skin of sclerodermatous GVHD in a murine model

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ABSTRACT

Allograft inflammatory factor (AIF)-1 has been identified in chronic rejection of rat cardiac allografts and is thought to be involved in the immune response. We previously showed that AIF-1 was strongly expressed in synovial tissues in rheumatoid arthritis and that rAIF-1 increased the IL-6 production of synoviocytes and peripheral blood mononuclear cells. Recently, the expression of AIF-1 has been reported in systemic sclerosis (SSc) tissues, whose clinical features and histopathology are similar to those of chronic graft-vs-host disease (GVHD). To clarify the pathogenic mechanism of fibrosis, we examined the expression and function of AIF in sclerodermatous (Scl) GVHD mice. We demonstrated that immunoreactive AIF-1 and IL-6 were significantly expressed in infiltrating mononuclear cells and fibroblasts in thickened skin of Scl GVHD mice compared with control. The immunohistochemical findings were confirmed by Western blot analysis. Wound healing assay also revealed that rAIF-1 increased the migration of normal human dermal fibroblasts (NHDF) directly, but cell growth assay did not show that rAIF-1 increased the proliferation of them. These findings suggest that AIF-1, which can induce the migration of fibroblasts and the production of IL-6 in affected skin tissues, is an important molecule promoting fibrosis in GVHD. Although the biological function of AIF-1 has not been completely elucidated, AIF-1 can induce IL-6 secretion on mononuclear cells and fibroblast chemotaxis. AIF-1 may accordingly provide an attractive new target for antifibrotic therapy in SSc as well as Scl GVHD.

1. Introduction

Allograft inflammatory factor (AIF)-1 is an IFN-γ-inducible, Ca^{2+}-binding EF-hand protein that is encoded within the HLA class III genomic region in the direct vicinity of TNF-α [1,2]. AIF-1 was originally identified and cloned from rat cardiac allografts undergoing chronic rejection [3]. AIF-1 expression has been documented in various human tissues and cells such as macrophage cell lines [4], peritoneal macrophages, spleen, peripheral blood leukocytes, and thymus [5], although its detailed physiological functions remain unclear. We previously proved that AIF-1 is strongly expressed in infiltrating mononuclear cells and synovial fibroblasts in rheumatoid arthritis (RA) compared with osteoarthritis (OA). In addition, AIF-1 induced the proliferation of cultured synovial cells in a dose-dependent manner and increased the IL-6 production of synoviocytes and peripheral blood mononuclear cells (PBMCs) [6]. Moreover, the expression of AIF-1 has been reported in systemic sclerosis (SSc) tissues [7]. The frequency of the AIF-1 rs2269475 TT genotype that results in a tryptophan to arginine amino acid substitution is significantly high in the patients with RA and SSc, and is associated with an increased risk of their development [8–10].

SSc is a chronic autoimmune disease characterized by fibrosis of skin and major organs such as lung, heart, gastrointestinal tract and widespread blood vessels. Progressive substitution of tissue structure by collagen-rich extra cellular matrix induces functional impairment of affected organs. The etiology is probably associated

Abbreviations: AIF-1, allograft inflammatory factor-1; BMT, bone marrow transplantation; GVHD, graft-vs-host disease; NHDF, normal human dermal fibroblasts; PDGF, platelet-derived growth factor; RA, rheumatoid arthritis; SSc, systemic sclerosis; Scl, sclerodermatous.

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with environmental and occupational exposure to organic solvents [11], genetic background, vascular damage, disorders of autoimmunity, collagen metabolism, cell growth factor and cytokines like connective tissue growth factor (CTGF) and TGF-β [12,13]. These factors are intricately intertwined and induce multiple clinical manifestations, but have not been elucidated in detail yet. In cells and tissues of various diseases including SSc, AIF-1 is expressed in T cells [14,15], macrophages, and endothelial cells [16,17] and may promote the expression of adhesion molecules that mediate specific homing into affected tissues [7]. Another recent study in vitro indicated that AIF-1 enhances the activation of T cells, which increase chemotaxis and induces a profibrotic phenotype [15,14], but the T cells are forcedly transfected with the vector expressing AIF-1. Thus, AIF-1 is thought to play a fundamental role in several cell types involved in chronic immunological inflammatory processes.

SSc resembles graft-vs-host disease (GVHD) both in its clinical features and histopathology [18]. For example, chronic GVHD has SSc like clinical features such as a skin fibrosis. Investigation of the fibrosing process in GVHD may help to elucidate the pathogenesis of fibrosis in SSc as well. The occurrence and extent of tissue fibrosis are thought to be influenced by minor histocompatibility mismatches and radiation exposure before transplant. These factors cause various immune responses of graft failure in bone marrow transplantation [19,20], but the detailed mechanism of fibrosis is still unclear. The phenotypic features in Scl GVHD get expressed about 3 weeks after transplant and are characterized by loss of dermal fat, thickening of skin, infiltration of numerous mononuclear cells and fibroblasts, and acceleration of collagen synthesis [19,21]. Clarification of the immunological mechanisms underlying skin fibrosis in GVHD will also shed light on the pathogenesis of SSc. However, no reports are available on the involvement of AIF-1 in GVHD. Furthermore, the pathophysiologic significance of AIF-1 in skin fibrosis has not been elucidated. This prompted us to examine the expression of AIF-1 and its function in fibrosis by using this Scl GVHD model [19] that recapitulates important features of SSc.

2. Materials and methods

2.1. Bone marrow transplantation

Six to 8-week-old male B10.D2 mice (H-2^d, Oriental Bio Service) were used as donors and 6–8-week-old female BALB/c mice (H-2^d, SRL) as recipients, for bone marrow transplantation to Scl GVHD with a standard method using spleen cells as the source of mature T cells. BALB/c mice were irradiated with 7.5 Gy. About 6 h later, recipient mice were injected via the tail vein with male donor bone marrow (4×10^6/mouse) and spleen cells (1×10^7/mouse) suspended in PBS. The control group consisted of female BALB/c recipient mice that received male B10.D2 bone marrow cells, namely a T cell depleted-bone marrow transplantation (TCD-BMT) group. Transplanted mice were maintained in sterile cages and supplied with autoclaved food. We performed the experiments five times, with five animals per group (Scl GVHD or TCD-BMT) examined in each experiment. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

2.2. Collection of tissue and preparation of normal human dermal fibroblasts

Allogeneic BMT mice had significantly lower body weights than TCD-BMT mice from day 10 after BMT and extensive thickened skin (data not shown). Five transplanted animals per group were sacrificed by cervical dislocation on day 21 after BMT. Day 21 after BMT was chosen because it is the earliest time point when Scl GVHD reliably develops in mice receiving allogeneic BMT. Skin was depilated and harvested for immunostaining and Western blotting. Normal human dermal fibroblasts (NHDF) were obtained from Sanko Junyaku Co., Ltd [22,23] and cultured in fibroblast basal medium with human fibroblast growth factor-B (1 μg/ml), insulin (5 mg/ml), 0.1% gentamicin/amphotericin-B, and 10% fetal bovine serum in a humidified incubator at 37 °C in the presence of 5% CO_2. We actually used NHDF after three or four passages in cell culture.

2.3. Peptide synthesis and preparation of anti-human AIF-1_{53-71} and AIF-$1_{113-129}$ Abs

Two synthetic peptides, which corresponded to residues 53–71 and 113–129 of human AIF-1 (AIF-1_{53-71} and AIF-$1_{113-129}$, respectively) as deduced from the nucleotide sequence of the human AIF-1 gene, were obtained with an additional cysteine residue at the N terminus (Biologica). Following purification by reversed phase HPLC, the synthetic peptide (purity >90%) was coupled to keyhole limpet hemocyanin with N-(ε-maleimidocaproyloxy) succinimide (Sigma–Aldrich). The carrier-conjugated peptide was then emulsified with Freund's complete adjuvant (Difco Laboratories) and injected s.c. (0.5 mg/injection) into rabbits. The rabbits were immunized six times at 10-day intervals. Blood samples were collected 10 days after the last injection, and the specific Ab in the sera was purified using an AIF-1 peptide-coupled cyanogen bromide-activated Sepharose affinity column. The Abs reacted with protein from abdominal adipose tissue and PBMC that is identical to the molecular size of purified recombinant human AIF-1.

2.4. Expression of rAIF-1 and preparation of anti-rAIF-1 Ab

Human AIF-1 cDNA was amplified from human peripheral lymphocyte cDNA (BD Clontech, California, USA) using PCR. The forward and reverse primers were 5′ -GTG GAT CCA TGA GCC AAA CCA GGG ATT T-3′ (containing *Bam*HI site) and 5′-CAC TCG AGT CAG ATA GGG CTT TCT TGG CT-3′ (containing *Xho*I site), respectively. To express AIF-1 as a GST fusion protein, the DNA fragment obtained was inserted in the *Bam*HI/*Xho*I sites of pGEX-4 (Amersham Biosciences) in frame. The fusion protein was purified with a GST purification system (Amersham Biosciences) and affinity chromatography with anti-AIF-$1_{113-129}$ Ab. To investigate the effect of AIF-1 on the cell proliferation and cytokine induction, rAIF-1 was treated with detoxi-gel endotoxin removing gel (Pierce). Endotoxin detection was performed using *Limulus* amoebocyte lysate analysis (Wako Pure Chemical), and treated AIF protein was confirmed to contain <0.1 ng/μg of endotoxin. AIF protein we synthesized was named AIF-5 according to a new nomenclature of the AIF family of proteins [24]. This AIF splice variants IRT-1, G1, BART-1 are encoded in the same region of the BAT2 gene on chromosome 6 [5]. Anti-rAIF-1 antiserum was raised in a similar manner as anti-synthetic peptide Ab by injecting 50 mg of human rAIF-1 into a rabbit. The human rAIF-1 Ab IgG fraction was prepared by chromatography on a human rAIF-1-coupled cyanogen bromide-activated Sepharose affinity column and biotinylated with 5-(N-succinimidyloxycarbonyl)pentyl D-biotinamide (Dojindo Chemical).

2.5. Immunohistochemical analysis of AIF-1 expression in skin

Immunohistochemical staining was performed using the avidin–biotin peroxidase complex system. Skin tissue specimens were preserved in 10% buffered formalin and embedded in paraf-

fin. Specimens were serially sectioned onto microscope slides at a thickness of 4 μm and then deparaffinized. Tissue sections were immersed for 45 min in 0.3% peroxide in methanol to block endogenous peroxidase activity. Nonspecific binding sites were saturated by exposure to 0.2% BSA and normal goat serum diluted 1/66.7 in PBS (pH 7.4) for 20 min. Primary Abs against human AIF-$1_{113-129}$ (1/800 dilution in PBS), IL-6 (1/50 dilution in PBS) (Santa Cruz), collagen I (1/200 dilution in PBS) (Abcam) and control normal rabbit serum (Dako) were applied to tissue sections and incubated overnight at 4 °C. The slides were then washed with PBS for 10 min. Biotinylated goat anti-rabbit IgG (Vector Laboratories) in 10 ml of PBS was applied to tissue sections, and the slides were incubated at room temperature for 30 min. They were then washed with PBS for 10 min, followed by incubation with prepared avidin DH-biotinylated peroxidase complex (Vector Laboratories) for 45 min. After washing with PBS for 10 min, color was developed by immersing the sections in peroxidase substrate solution containing 0.05% (w/v) 3,3′-diaminobenzidine tetrahydorochloride (Vector Laboratories) and 0.01% H_2O_2 in 0.05 mol/L Tris-buffered saline (pH 7.2) for 5 min. Positive staining was indicated by brownish black deposits, and counterstaining was performed with hematoxylin. Specific Ab staining was always compared with corresponding isotype control-stained sections on the same slide.

For each of the tissue specimens from Scl GVHD and TCD-BMT mice, the extent and intensity of staining with anti-AIF-$1_{113-129}$, IL-6, and collagen I Abs in mononuclear cells and fibroblasts in the skin were graded on a scale of 0–3+ by two blinded observers on two separate occasions using coded slides as previously described [6]. A 3+ grade implies maximally intense staining, whereas 0 implies no staining.

2.6. Western blot analysis

Western blot analysis was performed as previously described [25]. Briefly, skin specimens were homogenized on ice by sonication and solubilized in lysis buffer (PBS, pH 7.4, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.6 mM PMSF). Thirty micrograms of each total protein extract from skin tissues were electrophorised in 4–20% gradient gel SDS-PAGE. Following electrotransfer to a polyvinylidine difluoride membrane. The membranes were saturated with 5% (wt/vol) nonfat dry milk in TBST (25 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.8, 140 mM NaCl, 0.1% [vol/vol] Tween 20) and then incubated overnight with anti-rAIF-1 Ab (1/500 diluted by Can Get Signal [26,27]; Toyobo Life Science) and anti-IL-6 Ab (Santa Cruz; 1/500 dilution). The membranes were washed thoroughly with TBST and incubated with an HRP-linked anti-rabbit IgG (Amersham Biosciences, 1/1000 diluted with Can Get Signal) for 1 h. After washing, immunoreactivity was detected by ECL using ECL Plus Western blotting detection reagents (Amersham Biosciences). Images were obtained by exposure to Hyperfilm (Amersham Pharmacia Biotech) for 5 min.

2.7. Wound healing assay

NHDF were seeded in 92 mm dishes at a concentration of 3×10^5 cells/dish in a volume of 10 ml of Fibroblast Cell Medium (Sanko Junyaku Co., Ltd) consisting of Fibroblast Cell Basal Medium and the following growth supplements: human fibroblast growth factor-B, 0.5 ml; insulin, 0.5 ml; FBS, 10 ml; GA-1000, 0.5 ml and cultured to confluence. Confluent monolayer NHDF in these dishes were scratched with 200 μl tip (0 h), and then cultured [28] in the presence of 0.2% FBS in combination with 100 ng/ml of Lipopolysaccharide (LPS) or 1–100 ng/ml of AIF-1 with or without anti-AIF-1 Ab for 24 h. At 0 h and 24 h, the scratched monolayer cultures were photographed using an inverted microscope. Quantification was done by measuring the number of pixels in each wound closure area using Adobe Photoshop [28]. Data are expressed as percentage wound closure relative to the wound closure area in the control medium.

2.8. Cell growth assay

To evaluate the effect of AIF-1 on cell growth of NHDF, NHDF were inoculated into 96 well plates (1.5×10^3 cells/well) and incubated in Fibroblast Cell Medium. Twenty-four hours later, the medium were changed to in the presence of 0.2% FBS in combination with 100 ng/ml of LPS or 1-100 ng/ml of AIF-1 with or without anti-AIF-1 Ab for 72 h. The medium of positive control group remained as it was for following 72 h. Then the cell viability was estimated by WST-1 assay (Takara Bio Inc.) by measuring the absorbance at 450 nm in multiwell plate reader EMax (Molecular Devices, Japan). Each measurement was determined in six separate experiments, and the results are presented as a percentage of the value for the control cultures.

2.9. ELISA

We measured IL-6 and platelet-derived growth factor (PDGF) in the supernatant of cultured NHDF stimulated by LPS or various concentrations of AIF-1 with or without anti-AIF-1 Ab using commercial ELISA kits (IL-6 ELISA kit; eBioscience, PDGF ELISA kit; R&D Systems) according to the manufacturer's instructions. The supernatant used in the experiments was the same as that obtained at the wound healing assay (details described in the paragraph on the wound healing assay). In brief, microtiter plates (Corning Costar 9018) were coated with pre-titrated capture Ab overnight at 4 °C. After washing, nonspecific binding sites in each well were blocked with assay diluent included in each kit. Standard solution (4–500 pg/ml) and supernatant samples were added to the wells, and the plate was incubated at room temperature for 1 h. After washing, pre-titrated avidin-HRP was added to each well and incubated at room temperature for 30 min. After final washing, substrate solution was added to each well and allowed to react at room temperature for 15 min. The reaction was stopped by the addition of 1 M phosphoric acid, after which OD values at 450 nm were read with an ELISA plate reader. The detection limit of the assay was 4 pg/ml.

2.10. Statistical analysis

We conducted experiments to reveal the cutaneous cytokine expression in murine Scl GVHD five times. Five mice per group and per time point were examined. The results from Scl GVHD mice were compared each time with those from TCD-BMT mice according to the same protocols of irradiation and transplantation. The graded scores of the extent and intensity of immunostaining with anti-AIF-1 and IL-6 Ab between Scl GVHD and TCD-BMT groups were analyzed by the Mann–Whitney *U* test. We repeated each in vitro experiment five times. Differences in induction of IL-6 in the cultured NHDF supernatant after rAIF-1 stimulation between the six groups were analyzed by ANOVA followed by Bonferroni/Dunn's multiple comparison test. The results of the percentage wound closure of NHDF and WST-1 assay were also analyzed by ANOVA followed by Bonferroni/Dunn's multiple comparison test. $p < 0.05$ was considered significantly different.