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Therapeutic window for Wnt-driven cancers: Role of Porcupine inhibitor

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Abstract

Wnt signaling plays a critical role in carcinogenesis; many studies over the last two decades have identified numerous signaling components that have helped to build a molecular framework for the many branches of the Wnt signal transduction pathway. However, the diverse function, integration and specificity of the Wnt signaling are still unclear. The success of Wnt pathway inhibitors has been limited for long-time by the narrow therapeutic window afforded by the requirement for Wnt signaling in normal tissue homeostasis and the lack of predictive biomarkers of response. Porcupine is a membrane bound O-acyltransferase enzyme that is required for and dedicated to palmitoylating Wnt ligands, a necessary step in the process of Wnt ligand secretion. Inhibition of Porcupine blocks Wnt dependent activities, including LRP6 phosphorylation and the expression of Wnt target genes, such as Axin2, which in turn reduces the growth of cancer cells dependent on autocrine or paracrine Wnt signaling. LGK974 is a highly potent, selective and orally bioavailable Porcupine inhibitor and efficacious in multiple tumor models at well-tolerated doses in vivo, including murine and rat mechanistic breast cancer models driven by MMTV–Wnt1, a human head and neck squamous cell carcinoma model (HN30) and RNF43-mutant pancreatic xenograft models. In this review, we will summarize the most recent advances in our understanding of these Wnt signaling pathways and the role of Porcupine in inhibition of Wnt activity.

Key words: Wnt signaling, Porcupine inhibitor, Carcinogenesis, LGK974

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Topic Review

Wnt signaling and clinical trials in human cancers

Studies have shown that the Wnt signaling pathway is involved in many biological processes of cancer cell development, including the initiation, growth, differentiation, metastasis, senescence, and death of cancer cells [1]. β-Catenin is a crucial signaling transducer in Wnt signaling [2]. The β-catenin protein destruction complex composed of adenomatous polyposis coli (APC), casein kinase 1 (CK1), glycogen synthase kinase 3α/β (GSK-3α/β), and AXIN1 tightly controls β-catenin via phosphorylation-mediated proteolysis [3]. In this section, we briefly describe how genetic alterations of Wnt signaling contribute to tumorigenesis and introduce recent clinical trials that have aimed to inhibit Wnt signaling for cancer treatment.

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The β-catenin destruction complex

Colorectal cancer (CRC) is the representative of human cancer caused by Wnt signaling hyperactivation [4-6]. CRC displays a high mutation frequency in *APC* (~70%) [7]. In 1991, *APC* mutation was identified as the cause of hereditary colon cancer syndrome, also called familial adenomatous polyposis [8]. APC forms the β-catenin destruction complex in association with CK1, AXIN1, and GSK-3 and interacts with β-catenin [2]. This protein destruction complex downregulates β-catenin through phosphorylation and ubiquitin-mediated protein degradation [1]. Genetic mutations causing the loss of function of the destruction complex or gain of function of β-catenin lead to nuclear translocation of β-catenin, resulting in T-cell factor (TCF)4/β-catenin-mediated transactivation of Wnt target genes [8]. The Vogelstein group established a multistep tumorigenesis model of CRC. *APC* mutation is an early event that initiates CRC adenoma [6]. CRC progression also requires additional genetic alterations in *KRAS*, *PI3K*, *TGF-β*, *SMAD4*, and *TP53*27. Moreover, epigenetic silencing of negative regulators of Wnt signaling was also frequently found in the absence of *APC* mutations [9]. APC is a multifunctional protein. In addition to its role in β-catenin degradation, APC binds to actin and actin-regulating proteins [5] , which controls the interaction between E-cadherin and α-/β-catenin and various physiological processes, including migration and chromosomal fidelity [4]. Importantly, recent studies revealed that *APC* mutation is insufficient to fully activate Wnt signaling. Furthermore, even if *APC* is mutated, mutant APC still negatively regulates β-catenin to some extent39·40, which will be discussed later.

Wnt signalling transduction is tightly regulated at the level of the ligand–receptor interaction. This is achieved by titration of the ligands and/or of the receptors. Ligand availability can be modulated by the production of secreted FZD-related proteins (SFRPs). SFRPs are secreted molecules with no direct signalling activity, but they possess a Wnt-binding domain through which they sequester extracellular Wnts [10]. Another way to modulate Wnt signalling is to alter the level and/or availability of the receptors or co-receptors. The four secreted Dickkopf (DKK) proteins are a well-studied class of molecules that act in this way. In the Wnt cascade, DKKs act by binding to the FZD co-receptors LRP5/6, thereby inhibiting the binding of the Wnts [9]. Three of the DKK proteins (DKK1, 2 and 4) appear to be specific for the Wnt pathway and act by binding to LRP5/6 [11]. Interestingly, DKK2 and DKK4 can act as either activators or as repressors of the pathway, depending on the abundance of the cofactor Kremen 2 [2]. In contrast to the three other members of the DKK family, DKK3 acts in the TGF-β signalling cascade [7]. In addition to above-mentioned mechanisms, there is a variety of other transmembrane or secreted inhibitors with various modes of action, such as WIF, WISE/SOST, CERBERUS, IGFBP, TIKI1, SHISA, WAIF1 and APCDD1 [12]. Besides LRP proteins, there are other receptor-co-receptor pairs such as Ryk, which can enhance Wnt signalling [8]. Additionally, there are ancillary receptor complexes, which regulate the levels of available Wnt receptors. Most prominent among them are LGR4/5/6. Those proteins came to fame as Wnt target genes expressed in the intestine and were found to mark various stem cell populations [7]. Later on, it was revealed that they greatly

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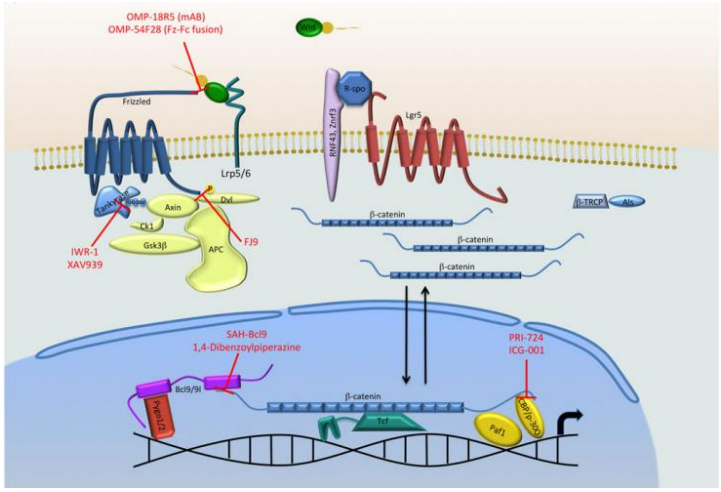
increase Wnt signal transduction when they are bound by the extracellular R-spondin. They act by inhibiting the ubiquitination of FZD receptors and their subsequent degradation by ZNRF3 and RNF43 [13]. The diversity of mechanisms by which Wnt signalling is initiated by the Wnt-FZD receptor interaction is regulated is both a bane and a boon. There are many potential targets, but their diversity also means that redundancy could affect the efficacy of any intervention. Mammalian genomes encode for 19 different Wnt molecules, which can bind to 10 different Frizzled (FZD) receptors [14]. FZDs belong to the family of seven-pass transmembrane GPCRs. When bound by Wnt proteins on their extracellular cysteine rich domain, they activate the cytosolic protein Dishevelled to transduce the signal inside the cell [4]. Several independent Wnt signalling cascades are activated in response to Wnts binding to their cognate receptors. The best studied and perhaps the most important is the β-catenin-dependent signalling cascade, mediated by β-catenin. The β-catenin-dependent cascade is of foremost importance for normal development and tissue homeostasis. When deregulated, it causes the initiation and progression of a myriad of different tumor types. Besides the β-catenin-mediated cascade, there are other β-catenin-independent outputs, such as the planar cell polarity and the Wnt/Ca2+ signalling pathway. The nature of the pathway transduced depends on the receptors/co- receptors present [15]. To transduce the β-catenin-dependent signal, FZD proteins bind the co-receptors low-density lipoprotein 5 (LRP5) or LRP6. Why in each specific context a particular Wnt/receptor combination activates one cascade or another is not entirely clear. However, some Wnts are thought to be preferentially β-catenin dependent (e.g. Wnt3a) or independent (e.g. Wnt5a). Wnt5a normally binds to FZD receptors and Ror/Ryk instead of Lrp5/6 and activates, among others, JNK signalling (Yamanaka, 2002). β-Catenin- independent signalling is often associated with the regulation of cell adhesion, migration and polarity ([16]. Furthermore, it is also thought to suppress β-catenin-dependent signalling [17]. The β-catenin-independent cascade has received increasing attention in recent years due to its role in melanoma formation and metastasis [18].



He et al., 1997; van Amerongen et al‹.2008).

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Figure 1.

The β-catenin-dependent Wnt signalling cascade in the ON-state. Upon binding of the Wnts to the receptors of the FZD family and the co- receptors LRP5/6, Dishevelled (Dvl) is recruited to the membrane, thus disassembling the destruction complex consisting of Axin, GSK3β, APC and CK1, preventing phosphorylation and thus protecting β-catenin from proteasomal degradation. This allows β-catenin to accumulate and translocate to the nucleus to initiate target gene transcription. Tankyrases can further increase the signal by marking Axin for degradation. Furthermore, when ZNRF3 and RNF43 are bound by R-spondin and LGR5 and, therefore, unable to target FZD receptors for degradation, Wnt signalling is enhanced in the Wnt-ON state. A further step protecting β-catenin from degradation is the inhibition of E3 ubiquitin ligases such as βTRCP by Armless, at least in D. melanogaster. A selection of Wnt-pathway inhibitors currently used in research are shown in red; red bars indicate the interaction they inhibit.

Targeting the interaction between β-catenin and its C-terminal cofactors – a difficult case

Various screens have been conducted in order to find suitable inhibitors of β-catenin's interaction with C-terminal cofactors like CBP and p300. Even though some of these screens yielded efficacious inhibitors, none of them seem to specifically inhibit the interaction with β-catenin. ICG-001, which does inhibit Wnt signalling, generally interferes with CBP's activity and does not inhibit the binding of CBP to β- catenin. Interestingly, ICG-001 does not inhibit the very closely related p300. Since the inhibitor is effective in colon cancer mouse xenograft models, there may be a tissue-specific requirement for CBP in the colon [19]. However, because ICG-001 inhibits CBP, which is part of the general transcriptional machinery, administering this compound could result in severe side effects. Several phase 1 clinical trials are currently being conducted to study the efficacy and side effects of this inhibitor in patients.

The Wnt/β-catenin transcriptional pathway is executed by N- and C- terminal co-activators

β-catenin facilitates transcription by recruiting several N- and C- terminal binding co-activators. The factors directly binding the N-terminus of β-catenin are Bcl9 and Bcl9l (the two mammalian paralogues of the Drosophila Legless); they in turn recruit Pygopus (Pygo1 and 2 in mammals). Bcl9/9l and Pygo are thought to form a 'chain of adaptors' extending from β-catenin. The simple model arising from Drosophila is that Legless and Pygo are essential for the Wnt transcriptional output [20] In mammals, although also required for a maximal Wnt output, the relative importance of Bcl9/9l and Pygo seems to be context-dependent. In the mouse loss of function mutations in these genes do not replicate loss of β-catenin-dependent Wnt signalling (i.e. by mutations in β-catenin). For example, β-catenin signalling mutants die at E6.5, whereas Bcl9/9l knockout (KO) animals die at E10.5, while Pygo KO animals survive at least to E13.5. Moreover, recent work has demonstrated that the Pygo-Bcl9 complex can also act independently of β-catenin [9]. We therefore speculate that the role of Bcl9 as well as Pygo is to act as a booster of the signal and facilitate transcription of specific target genes in a subset of cells with active Wnt signalling. As described later, the context-dependent requirement of the so-called N-terminal

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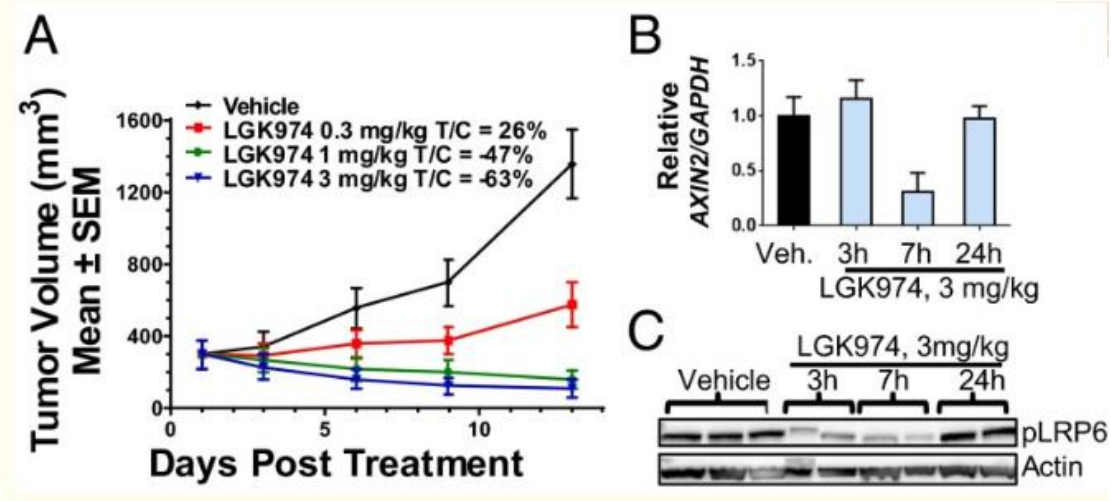
chain of adaptors for facilitating the Wnt transcriptional output presents an exciting therapeutic target.

LGK974 Induces Tumor Regression at a Well-Tolerated Dose in a Wnt-Driven Murine Tumor Model.

Given the pivotal role of Wnt signaling in tissue stem cell renewal, achieving an acceptable therapeutic index with targeted inhibition of Wnt signaling poses an important clinical challenge [7]. The discovery of the potent, selective, and orally bioavailable PORCN inhibitor LGK974 enabled us to investigate this issue using a well-established Wnt-dependent murine breast tumor model, the mouse mammary tumor virus (MMTV)-driven Wnt1 model [10].

In a murine MMTV-Wnt1 tumor model using s.c. implanted tumor fragments derived from MMTV-Wnt1 transgenic mice, LGK974 exhibited strong dose-dependent efficacy when administered daily (Fig. 2*A*). Briefly, changes in tumor volume for each of the treated (T) and control (C) groups were measured and used to calculate growth delay as expressed by the T/C ratio. A dose of 0.3 mg/kg LGK974 led to tumor growth delay (T/C: 26%), whereas a dose of 1 or 3 mg/kg induced very significant tumor regression (T/C: −47% or −63%, respectively) on day 13 of treatment. As shown in Fig. S2*A*, the regimen was well-tolerated without significant body weight loss in the mice. Similar efficacy was observed with LGK974 in a murine MMTV-Wnt3 model (Fig. S2*B*).



Proc Natl Acad Sci U S A. 2013 Dec 10; 110(50): 20224–20229. Published online 2013 Nov 25. doi: 10.1073/pnas.1314239110 Medical Sciences [21].

Figure 2.

LGK974 inhibits Wnt signaling in vivo and induces tumor regression in a mechanistic MMTV-Wnt1 tumor model. (*A*) LGK974 showed a strong efficacy in a Wnt tumor model (MMTV-Wnt) in nude mice. Spontaneous tumors from the MMTV-Wnt1 transgenic mice were implanted in nude mice. LGK974 was dosed at 0.3, 1.0, and 3.0 mg/kg per day for 13 d. LGK974 induced robust tumor regression at 1.0- and 3.0-mg/kg doses. (*B*) A PD study was performed in the murine MMTV-Wnt tumor model. LGK974 significantly inhibited *AXIN2* expression 7 h after the last dose, and the effect diminished 24 h after the dose. (*C*) In the same PD study, pLRP6 expression level showed a very similar pattern to the PD response of *AXIN2*. LGK974 inhibitory effect peaked at 7 h after the last dose and diminished 24 h after the last dose.

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Given the key role of Wnt signaling in cancer, targeting this pathway has been an attractive therapeutic approach. However, success has been limited because of the lack of effective therapeutic agents for targets in the Wnt pathway and the lack of a defined patient population that would be sensitive to a Wnt inhibitor. Herein, this study describes a cellular high-throughput screen for small molecules that block Wnt secretion. This effort led to the discovery of LGK974, a potent and specific PORCN inhibitor. LGK974 potently inhibits Wnt signaling in vitro and in vivo and has strong efficacy in tumor models in vivo. The above study also showed that head and neck squamous cell carcinoma cell lines with LoF mutations in the Notch signaling pathway have a high response rate to LGK974. These findings provide a path forward to target Wnt-driven cancer through the inhibition of PORCN.

Wnt/β-catenin signaling in CRC

A single layer of epithelial cells lines the lumen of the mammalian small intestine and colon, forming invaginations, termed crypts. The intestinal stem cells (ISCs) drive a massive renewal process to replenish the loss of differentiated intestinal epithelial cells at the crypt base [19]. Wnt/β-catenin pathway activity is highest at the base of the crypt [9]. Suppression of Wnt signaling leads to both suspended proliferation and ISC deficiency, resulting in ablation of the intestinal epithelium [2]. On the contrary, the number of ISCs is increased by the potentiation of Wnt signaling [21]. This shows that Wnt signaling plays a crucial role in ISC self-renewal and proliferation during homeostasis.

The progression of CRC from normal colonic epithelium to a malignant phenotype often develops over a period of more than 10 years [22], accompanied by numerous genetic changes closely related to the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway is an evolutionarily conserved and unique signaling pathway that regulates gene expression and cell invasion, migration, proliferation, and differentiation in the initiation and progression of CRC [23]. The APC gene, as the main cause of familial adenomatous polyposis (FAP) syndrome, has been found to be up to 80% mutated in sporadic CRCs [24]. In the majority of cases, these mutations occur in the APC gene, but additional mutations may occur in genes such as β-catenin or Axin [21]. Of the known Wnt signaling cascades, at least one protein of the Wnt/β-catenin signaling pathway is mutated in more than 94% of CRC cases [25]. The development of these mutations is thought to be an early event and the primary driving force in early-stage CRC. When APC is absent or dysregulated, β-catenin accumulates to high levels, translocates to the nucleus, and associates with TCF/LEF, leading to its binding to DNA and subsequent transcription of genes associated with CRC development [26]. Activation can be further enhanced by tumor suppressor proteins, such as cytoplasmic Dickkopf [24] and sFRP [35], and by modifying the expression of non-canonical Wnt signaling members [20]. As negatively regulated proteins of canonical Wnt signaling, sFRPs bind to Wnt in the extracellular matrix and prevent binding to bona fide Frizzled (FZD) receptors; Dickkopf can bind to Wnt-activated lipoprotein receptor-related protein and promote its internalization.

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Targeting Wnt/β-catenin signaling in CRC therapy

As with many solid tumors, the only fully curative treatment for CRC is surgery. However, many CRC patients are asymptomatic until the disease has progressed to a stage that precludes curative surgery. These patients may be treated with a combination of chemotherapy, radiotherapy, and biotherapy.

The use of the therapeutic antibodies cetuximab or bevacizumab in combination chemotherapy, such as FOLFIRI, XELOX/CAPOX, FOLFOX, and FOLFOXIRI, has been shown to increase survival. Preliminary results indicate that combination therapy will be required to effectively treat most malignancies, including CRC. Several phase I–II trials are underway in which a Wnt antagonist or modulator is used in combination with chemotherapy agents (https://clinicaltrials.gov/). CRC therapeutics currently under investigation that involve Wnt/β-catenin signaling include natural compounds, existing drugs, small molecules, and biological agents [25].

APC mutations in CRC are a long-standing challenge. CK1α kinase activator and TNKS enzyme inhibitors appear to selectively block β-catenin activity, even in APC mutation mice. These have great potential in CRC, although research into these types of inhibitors is still at an early stage. BBI608, another potential inhibitor, in combination with chemotherapy and other targeted drugs, showed great potential in advanced CRC. The results of a clinical trial of BBI608 conducted 2013 were reported at the ASCO meeting. Studies on inhibitors of Wnt/β-catenin signaling in CRC stem cells are at very early stages and still have a long way to go. In summary, advances in understanding Wnt/β-catenin signaling mechanisms and the development of new technologies have facilitated the discovery of drugs that may provide a foundation for innovative therapeutic approaches to the treatment of CRC. Although most drugs are still at a very early stage of development, the significance of this pathway in CRC provides the ability to benefit from these new therapies [26-30].

Competing interests

The authors declare that they have no competing interests.

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