Targeting Wnts at the Source—New Mechanisms, New Biomarkers, New Drugs
Babita Madan and David M. Virshup

Abstract

Wnt signaling is dysregulated in many cancers and is therefore an attractive therapeutic target. The focus of drug development has recently shifted away from downstream inhibitors of β-catenin. Active inhibitors of Wnt secretion and Wnt/receptor interactions have been developed that are now entering clinical trials. Such agents include inhibitors of Wnt secretion, as well as recombinant proteins that minimize Wnt–Frizzled interactions. These new therapies arrive together with the recent insight that cancer-specific upregulation of Wnt receptors at the cell surface regulates cellular sensitivity to Wnts. Loss-of-function mutations in RNF43 or ZNRF3 and gain-of-function chromosome translocations involving RSPO2 and RSPO3 are surprisingly common and markedly increase Wnt/β-catenin signaling in response to secreted Wnts. These mutations may be predictive biomarkers to select patients responsive to newly developed upstream Wnt inhibitors. Mol Cancer Ther; 14(5); 1087–94. ©2015 AACR.

Introduction

Wnts are secreted proteins that are critical for normal embryonic development and homeostasis of select adult tissues. Wnt signaling regulates diverse developmental and homeostatic functions, including proliferation, differentiation, cell polarity, motility, and migration. In humans, there are 19 WNT ligands that couple to multiple receptors and coreceptors to activate various downstream pathways. The most extensively studied Wnt signaling pathway follows from the binding of Wnts to a Frizzled (FZD) and a Lipoprotein receptor-related protein coreceptor, LRPs. A well-studied series of intracellular events cause inhibition of GSK3 and the stabilization of β-catenin (1, 2). This leads to cytoplasmic accumulation and then translocation of β-catenin to the nucleus where it interacts with members of the TCF/LEF family of transcription factors to regulate the cell-type specific expression of target genes (1). Wnts also have important functions independent of β-catenin, for example, by triggering downstream pathways that regulate tissue polarity and cell movement through the activation of RhoA, JNK, and nemo-like kinase signaling cascades (3–5).

Dysregulated Wnt signaling is associated with various pathologic states, including osteoporosis, cancer, vascular diseases, and fibrosis (6). Elevated Wnt signaling promotes tumorigenesis by supporting cellular proliferation, blocking differentiation, driving epithelial mesenchymal transitions, and promoting both stem cell renewal and metastasis (5, 7). Pathologic Wnt signaling can be driven by upstream or downstream events. Targeting the Wnt signaling pathway can be beneficial for diseases with elevated or diminished Wnt activity. Recent findings demonstrate that understanding the mechanism underlying increased Wnt signaling can guide the therapeutic approach.

Downstream Wnt/β-Catenin Pathway Inhibitors

Many downstream components of the Wnt/β-catenin pathway can be mutated in cancers to stabilize β-catenin protein. Well-recognized activating mutations include truncating mutations of APC, found in the majority of colorectal cancers, stabilizing mutations of CTNNB1 (β-catenin) in diverse tumors, and loss-of-function mutations of AXIN1/2 in biliary tract and liver cancers (6). The correlation of β-catenin stabilization with cancer has led to the conclusion that stabilized β-catenin can cause cancer, although in most cases, this has not been rigorously demonstrated (8). Multiple attempts to target elevated downstream Wnt signaling have used small molecules that inhibit β-catenin abundance or its transcriptional activating activity (Fig. 1; refs. 9–12). Screens for downstream inhibitors have provided an important insight that β-catenin stability can be regulated by tankyrase-mediated degradation of AXIN (11, 13). Tankyrase inhibitors increase AXIN abundance and therefore increase β-catenin degradation. Such drugs have been independently developed by several groups, although their oral use may be limited by mechanism-based gut toxicity (14). A more selective approach is use of small molecules to modulate β-catenin’s interaction with transcriptional coactivators such as CREB-binding protein (CREBBP/ CBP). One such small molecule, PRI-724, is in phase I trials for acute myeloid leukemia (clinicaltrials.gov NCT01606579) and advanced solid tumors (clinicaltrials.gov NCT01302405; ref. 15). Inhibitors directed at β-catenin accumulation will, however, not block signaling pathways triggered by β-catenin–independent actions of Wnts.

Upstream Inhibitors of Wnt Production and Function

To inhibit both β-catenin–dependent and –independent signaling, studies have focused on upstream elements of Wnt
signaling, including Wnt production and Wnt ligand–receptor interactions. Wnts secreted from sending cells interact with Frizzleds, LRP5/6 coreceptors, and various other receptors on the surface of receiving cells. Figure 2A illustrates our current understanding of Wnt secretion. Figure 2B illustrates the regulation of cell surface Wnt receptors and the targets of upstream Wnt inhibitors (recently reviewed in ref. 4).

One disorder where it is clinically valuable to increase Wnt signaling is osteoporosis. Sclerostin (SOST) is an osteocyte-specific Wnt inhibitor that interacts with LRP4 as well as LRP5/6 to inhibit excessive bone formation (Fig. 2). SOST and LRP4 loss-of-function mutations cause increased bone mass, a finding that can be mimicked therapeutically for the treatment of osteoporosis by SOST or LRP4 neutralizing antibodies (16, 17).

Monoclonal antibodies and decoy receptors can also block the interaction of Wnts with its receptors. Although early studies examined antibodies directed against Wnts, recent work has focused on inhibition of Wnt receptors. Examples include anti-LRP6 antibodies (18), and the anti-Frizzled antibody vantictumab (OMP-18R5; ref. 19). Vantictumab binds to 5 of the 10 Fzd receptors recognizing an epitope spanning the "cleft" region of Fzd that contains highly conserved residues (19, 20). Vantictumab may effectively target a subset of cancers that are dependent on the function of the appropriate Fzds. A related approach uses a soluble Fzd-based decoy receptor related to the soluble Frizzled-related proteins (sFRP; Fig. 2) that prevents the interaction of Wnts with their primary receptors. One such recombinant protein, Ipafricept (OMP-54F28), has reached clinical trials (21, 22). It is likely that Ipafricept may interact with a subset of the 19 Wnt ligands. Finally, Wnt signaling is markedly stimulated by R-Spondins (RSPO) both physiologically, and pathologically when they are overexpressed due to specific chromosomal translocations (23, 24). Neutralizing antibodies against RSPOs are also being developed (ref. 25; and others). These recombinant proteins and monoclonal antibodies have high specificity, low off-target effects, and infrequent dosing, but share issues of parenteral administration, long half-lives, and slow off-rates.

An alternative approach to blocking Wnt signaling upstream is the use of orally available small molecules that prevent Wnt ligand biogenesis and secretion. All mammalian Wnts require posttranslational acylation. A single essential, nonredundant enzyme, PORCN (the human ortholog of the Drosophila gene porcupine) adds a mono-unsaturated palmitoleate moiety to a serine residue conserved in all mammalian Wnts (26–28). This palmitoleation is essential for both Wnt secretion, and the binding of Wnts to their receptors (Fig. 2A; refs. 13, 29–31).

Thus, inhibition of PORCN blocks the secretion and activity of all human Wnts (32, 33). Small-molecule PORCN inhibitors circumvent some of the limitations of the recombinant protein/monoclonal antibody approaches, and will inhibit both canonical and noncanonical signaling pathways. Several PORCN inhibitors are being developed and have been shown to be effective in cell- and mouse-based models of Wnt-dependent cancers (refs. 30, 34, 35; Madan and colleagues; unpublished results). For all of the upstream Wnt inhibitors, one
important challenge is to select patients who will benefit from their use.

Potential Toxicities of Wnt Inhibitors

Apart from its role in development, Wnt signaling regulates homeostasis of several adult organs, most notably the intestinal epithelium, hair, and bone (36). Consistent with this, tankyrase inhibitors demonstrated severe, mechanism-based GI toxicity when given orally (14). In contrast with tankyrase inhibitors, PORCN inhibitors and the recombinant Frizzled inhibitory antibodies have not shown significant toxic effects on the intestine or skin, at least in mice, at doses that effectively inhibit cancer proliferation (30, 34, 37). Doses of the PORCN inhibitor Wnt C-59 10-fold higher than the therapeutic dose cause extensive loss of intestinal proliferation (37). The reason for this therapeutic window and the differences in toxicity of these different drugs is unclear, but may be due to redundant pathways, differences in tissue penetration, or dosing schedules.

Wnt signaling plays a critical role in bone metabolism, driving the differentiation and function of osteoblasts (38). Decreased Wnt signaling, both experimentally in mice, and in humans with genetic variants, leads to defects in mineralization, osteoporosis, and fracture risk (38, 39). Not surprisingly, then, vantictumab and iparfrect trials were temporarily halted apparently due to increased bone turnover in the treated patients but restarted with bone loss mitigation strategies (40, 41). Markers of bone turnover such as increase in \( b\)CTX (C-terminal telopeptide \( b\)), BSAP (bone-specific alkaline phosphatase), osteocalcin, and \( P1NP \) (serum type I collagen) may be useful both as pharmacodynamic biomarkers and indicators of toxicity in animal and human studies of PORCN inhibitors.

General Considerations for Predictive Biomarkers for Upstream Inhibitor Therapy in Cancer

Upstream inhibition of Wnt signaling by targeting either PORCN or the interaction of Wnts with their receptors is most likely to be clinically efficacious when the cancer requires continued Wnt ligand activity for proliferation. Conversely, Wnt ligands may not be required when the downstream Wnt pathway is activated due to \( APC, AXIN, GSK3\), or \( \beta\)-catenin mutations. In these cancers, the upstream inhibitors are less likely to be efficacious.

Recent advances in understanding how the Wnt receptors Frizzled and LRP5/6 proteins are regulated, combined with next-generation sequencing of cancer genomes, have identified genetic markers that may predict sensitivity to these upstream Wnt pathway inhibitors.

Genetic Markers

Mutations that sensitize cells to secreted Wnt ligands

Cancers dependent on Wnt signaling are likely to have high levels of Wnt ligands or Fzd receptors. Genetic mutations that make cancers more sensitive to Wnts by increasing Fzd abundance have recently been identified, and may be excellent predictive biomarkers. These genes, listed in Table 1, can constitute the first-line tests for enrichment of patients in clinical trials of upstream Wnt inhibitors.
RNFL and ZNRF3 loss-of-function mutations

Inactivating mutations in the structurally related integral membrane E3 ubiquitin ligases RNFL and ZNRF3 cause increased cell surface Wnt receptors and hence sensitize cells to Wnts (Fig. 1; Table 1). RNFL and ZNRF3 negatively regulate Wnt signaling by promoting ubiquitination, internalization, and degradation of the Wnt receptors Frizzled and LRP5/6 (42, 43). Recent studies (ref. 44; Madan and colleagues; unpublished data) have demonstrated that the proliferation of pancreatic cancer cell lines and patient-derived xenografts with inactivating RNFL mutations is inhibited by PORCN inhibitors, including LGK974, Wnt-C59, and ETC-159. Hence, RNFL and ZNRF3 mutations, by sensitizing cells to Wnts, may also be predictive of response to both PORCN inhibitors and Frizzled antibodies. Mutations in RNFL are surprisingly common, occurring in 18% to 27% of endometrial cancers, 3% to 5% of pancreatic cancers, 21% ovarian mucinous carcinomas, and 38.5% liver fluke associated cholangiocarcinomas (45–48). Notably, up to 18% of colorectal cancers have RNFL mutations, often associated with mismatch repair deficient, microsatellite instability (MSI) cancers (46). These mutations are mutually exclusive with APC mutations, consistent with their ability to activate Wnt/β-catenin signaling. ZNRF3 mutations are found in adrenocortical and endometrial cancers (Table 1). The MSI-associated mutations show a strong predilection for a G/C tract resulting in a frameshift at G659, and to a lesser extent at R117. RNFL loss-of-function mutations are found in other MSI+ cancers, including endometrial and gastric cancers (36, 49–51). Whether PORCN inhibitors are effective in MSI+ cancers remains to be tested.

R-Spondin translocations and gene amplification

RSPOs are a family of four genes, RSP01-4, that enhance Wnt signaling (ref. 52, reviewed in ref. 53). Their mechanism of action has recently been elucidated, and importantly, cancer-associated gain-of-function mutations have been identified. RSPO proteins can be thought of as an independent volume control on Wnt signaling. RSPOs normally inhibit the activity of RNF43 and ZNRF3 by sequestering them into a heterotrimeric complex with PORCN (46, 49–51). Whether PORCN inhibitors are effective in MSI+ cancers remains to be tested.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of mutations</th>
<th>Primary tissues % Mutated</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNFL</td>
<td>Loss-of-function: frameshift, indels, and nonsense mutations (esp. at R117 and G659 in MSI tumors) cause increased levels of Frizzleds on the cell surface.</td>
<td>Biliary tract 38.5% (48)</td>
<td>(ref. 44; Madan and colleagues; unpublished data)</td>
</tr>
<tr>
<td>ZNRF3</td>
<td>Loss-of-function: frameshift, indels and nonsense mutations may lead to increased levels of Frizzleds on the cell surface.</td>
<td>Endometrium 3.8% a</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>RSPOs</td>
<td>E1F3E(e1)–RSPO2 fusion, PTPRK(e1)–RSPO3(e2) PTPRK(e7)–RSPO3(e2) fusion leading to increased levels of RSPOs, consequently preventing clearance of Frizzleds from the cell surface.</td>
<td>Colon cancer 10% (23, 24)</td>
<td>(ref. 44; Madan and colleagues; unpublished data)</td>
</tr>
</tbody>
</table>

aCOSMIC (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).
bCbioportal (http://www.cbioportal.org/public-portal/).
found that fuse upstream signal sequences onto the amino-terminus of the RSPO genes in about 10% of colon cancers and to varying frequencies in head and neck, esophageal, and ovarian cancers (23, 24, 56). These fusion products markedly stimulate Wnt/β-catenin signaling. As with the RNF43 mutations, these fusions are mutually exclusive with APC mutations. There are also subsets of ovarian (25%), prostate (10%), and breast (10%) cancers with R-spondin2 gene amplifications. The tumors with elevated expression of RSPO either due to translocations or due to amplifications could also potentially respond to PORCN inhibitors, anti-Fzd antibodies, or anti-RSPO antibodies (19, 25). Thus, RSPO2/3 gain-of-function mutations, together with RNF43/ZNRF3 loss-of-function mutations, molecularly define a population of patients with pathologically increased cellular sensitivity to Wnts, who may benefit from new upstream Wnt inhibitors.

The mutually exclusive nature of RNF43 or RSPO translocations on one hand, and APC mutations on the other, could in a simple world be interpreted as the need to activate β-catenin one way or another. However, increased Wnt activity will also activate β-catenin–independent pathways, and in fact, some Wnts can actually block β-catenin signaling. Conversely, APC mutations do much more than simply elevate β-catenin protein abundance. Hence, it remains to be tested whether upstream activating mutations in RNF43 and RSPO in fact work in part or wholly through β-catenin or whether they are oncogenic via β-catenin–independent mechanisms.

Loss-of-function mutations in Notch ligands

Notch expression suppresses Wnt signaling in keratinocytes and skin tissues (57, 58). Inhibition of Notch signaling increases the expression of Wnt ligands, leading to increased Wnt signaling (59). Consistent with this, loss-of-function mutations in Notch1 in head and neck squamous cell carcinoma cell lines sensitize them to PORCN inhibitors (34). Loss-of-function mutations in Notch1 are found in diverse epithelial cancers. Notch mutations may predict Wnt dependency and sensitivity to upstream inhibitors, but confirmatory studies are needed (60).

Mutations that may sensitize cancer cells to secreted Wnt ligands

Liu and colleagues noted that approximately one of three of head and neck cancer cell lines responded to PORCN inhibition in vitro, and performed exome sequencing on a subset of those lines (34). They found a number of mutations that correlated with PORCN inhibitor sensitivity. These genes are candidate predictive biomarkers, although the associations are not yet validated and the mechanistic connection is obscure. It is also possible that these tumors harbor unidentified RSPO translocations (24).

Two genes associated with PORCN inhibitor sensitivity in head and neck cancer cell lines, were FAT1 and FAM58A. FAT1 is a protocadherin that can interact with β-catenin and sequester it to the cell membrane. Inactivation of FAT1 leads to aberrant Wnt/β-catenin signaling in multiple types of cancer (61). In addition to head and neck squamous cell carcinomas, FAT1 mutations are frequently observed in central nervous system, colorectal, and ovarian cancers. FAM58A family (family with sequence similarity 58, member A) encodes cyclin M, which regulates CDK10 and interacts with SALL1 (62, 63). Mutations in FAM58A cause an X-linked dominant disorder characterized by syndactyly, thanatophoric, anogenital, and renal malformations. Mutations in FAM58A are found in 5% of diffuse large B-cell lymphomas. As of yet, there is no evidence that mutations in these genes will sensitize cells to PORCN or other upstream Wnt inhibitors.

Upstream inhibitors may be less effective in cancers with downstream Wnt/β-catenin pathway mutations

Mutations in pathway components downstream of the plasma membrane can activate Wnt/β-catenin signaling. Such downstream mutations may render cells insensitive to upstream PORCN and Wnt inhibition. These mutations increase β-catenin signaling primarily by preventing its phosphorylation or degradation. These include loss-of-function mutations in AXIN and APC that impair their ability to serve as scaffolding proteins for the β-catenin degradation complex. Somatic and inherited mutations in APC leading to prematurely truncated protein are most frequently found in colorectal cancers (49). Mutations in β-catenin altering or deleting the GSK3 phosphorylation sites (Ser33, Ser37, and Thr41) are reported in various cancers, including pancreatic, gastric, liver, and endometrial cancers (64). These mutations prevent β-catenin degradation, leading to enhanced LEF/TCF-mediated transcription. Mutations in WTX, another scaffolding protein and part of the β-catenin degradation complex, are also reported in colon cancers and prevent β-catenin degradation (65).

Although mutations in APC and β-catenin are common in colon cancer, these cancers still produce Wnt ligands that incrementally activate β-catenin (66, 67). Thus, it will be interesting to test whether upstream Wnt inhibitors regulate the growth of cancers with downstream β-catenin activating mutations.

Gene Expression Markers

Wnt/β-catenin pathway activation turns on diverse target genes that have been extensively investigated in multiple gene expression profiling studies. If gene expression data are available from individual cancers, it may be potentially useful in predicting Wnt pathway activation. However, despite years of study, there is no robust, clinically useful gene expression signature that predicts Wnt pathway activation across tissues. AXIN2 is the most broadly used Wnt/β-catenin target gene and high expression of AXIN2 is routinely used as a measure of elevated canonical Wnt signaling in the research laboratory. Assessment of AXIN2 expression in clinical samples would require mRNA analysis, as reliable monoclonal antibodies are not available. Importantly, these assays will have only limited ability to guide the decision of whether to use an upstream Wnt inhibitor, because activation of Wnt target genes will not differentiate between upstream or downstream mutations in the Wnt/β-catenin pathway.

Increase in the expression of various genes such as NDK1, RNF43, ZNRF3, TNFRSF19, SPS, BMP7, LGR4/5, APCDD1, and NOTUM correlates with AXIN2 expression, consistent with other evidence that these are Wnt/β-catenin target genes (ref. 43; A. Alok, Z. Lei, DMV, manuscript in preparation). Increase in the expression of RSPOs, either due to amplification or translocations, as noted above, may also predict enhanced Wnt signaling. With the development of sensitive monoclonal antibodies and recent advances in techniques such as RNAscope (68) that allow for reliable quantification of gene expression on formalin-fixed tissues, profiling of expression of these genes might be useful for patient selection. Thus, it may be possible to develop panels that assess expression of a combination of these
genes and gene products to predict sensitivity to upstream Wnt inhibitors.

**Genes Whose Decreased Expression Suggests Increased Wnt Signaling at the Membrane**

There are multiple secreted Wnt antagonists that may normally dampen Wnt signaling but are epigenetically silenced in various cancers (66, 69). Most of the reported antagonists function by preventing the interaction of Wnts with their receptors LRP5/6 or Fzds. Although epigenetic silencing may be a challenge to monitor clinically, it provides an additional mechanism to sensitise cancers to Wnts, and hence to upstream Wnt inhibitors.

The best-known Wnt antagonists are the Dickkopf (DKK) and the sFRP families. DKKs are secreted glycoproteins that bind to LRP5/6. DKK1/2 interacts with LRP5/6, whereas DKK4 interacts with LRP6. Epigenetic silencing of DKK1 and other DKK family members is described in colon cancers (reviewed in ref. 70). The sFRPs interact with Wnts via the Wnt-binding cysteine rich domain of the Frizzleds and can activate or antagonize Wnt signaling depending on their concentration (71, 72). Epigenetic silencing of sFRP promoters by hypermethylation is seen in colorectal and breast cancers (66, 69). Wnt inhibitory factor-1, another secreted Wnt inhibitor, is silenced in several tumor types (73–75). Profiling the expression of Wnt antagonists or epigenetic marks associated with their silencing, while challenging in clinical practice, could be useful to predict sensitivity of tumors to upstream Wnt inhibitors.

**Protein Markers**

Increased abundance of the Wnt cell surface receptors Fzds is likely to be seen in cancers with mutant RNF43 or overexpressed RSPO2/3. It may be possible to develop this as a predictor of responsiveness to PORCN and other upstream pathway inhibitors. IHC tools to assess the expression of Wnt antagonists or epigenetic marks associated with their silencing, while challenging in clinical practice, could be useful to identify the patients with cancer who may benefit from Wnt pathway inhibitors.

**Conclusions**

Recent studies have revealed further complexity in the Wnt/β-catenin pathway. Posttranslational modifications of WNTs and AXIN have been identified and can be targeted to benefit patients with elevated Wnt signaling. Accumulating data demonstrating the β-catenin–independent role of Wnts combined with the limitations of the current downstream pathway inhibitors have led to development of drugs that inhibit PORCN and target Wnt secretion and antibodies and decoy receptors that prevent the interaction of Wnts with their receptors. A variety of tools may assist in selecting patients who can benefit from these new Wnt pathway inhibitors. Most notably, recent studies show that Wnt receptor abundance is regulated by ubiquitination and internalization via an RSPO/LGR5/RNF43 axis. Large-scale sequencing efforts have identified common loss-of-function mutations in RNF43/ZNRF3 and gain-of-function translocations in RSPO2/3 that can be clinically utilized to guide the selection of the patients who may respond to upstream Wnt pathway inhibitors. IHC tools to assess the mutational activation of Wnt signaling at the membrane may also be useful to identify the patients with cancer who may benefit from Wnt pathway inhibitors.

**Disclosure of Potential Conflicts of Interest**

B. Madan and D.M. Virshup are co-inventors on patents on PORCN inhibitors. D.M. Virshup is a consultant/advisory board member for Experimental Therapeutics Center Singapore.

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