[Frontiers in Bioscience, Landmark, 22, 137-156, January 1, 2017]

Molecular strategies for modulating wnt signaling

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1. ABSTRACT

The importance of the Wnt signaling cascade in the fields of developmental biology, regenerative medicine, cancer genetics, and neurobiology has fueled a wide search for potent pharmacological agents capable of controlling Wnt signaling. Numerous fields of study have lent assistance to this endeavor, yielding both natural and synthetic compounds that are capable of inducing or inhibiting Wnt at multiple stages within the pathway. Further, there is a large of body research which has investigated endogenous Wnt inducers and inhibitors, namely the secreted Wnts, Dickkof proteins (Dkks), secreted Frizzled-Related Proteins (sFRPs), and Wnt Inhibitory Factor-1 (WIF-1), along with others which may act via indirect means to stimulate or inhibit Wnt (e.g. the Smads, bone morphogenetic proteins, and Hedgehog proteins). This review will summarize the research surrounding currently available small molecules used to target Wnt signaling. These compounds will be classified based upon their ability to stimulate or inhibit Wnt, their derivation (natural or synthetic), and their specific mechanism of action.

2. INTRODUCTION

In 1982, Roel Nusse and Harold Varmus published their seminal paper describing the int1 locus; a new oncogene discovered from what had been an old hypothesis (1). Later renamed as Wnt1, these groundbreaking studies led to the identification of 19 Wnt genes and the entire Wnt signaling pathway, a new means of proto-oncogene discovery via proviral tagging, and research that helped launch the field of

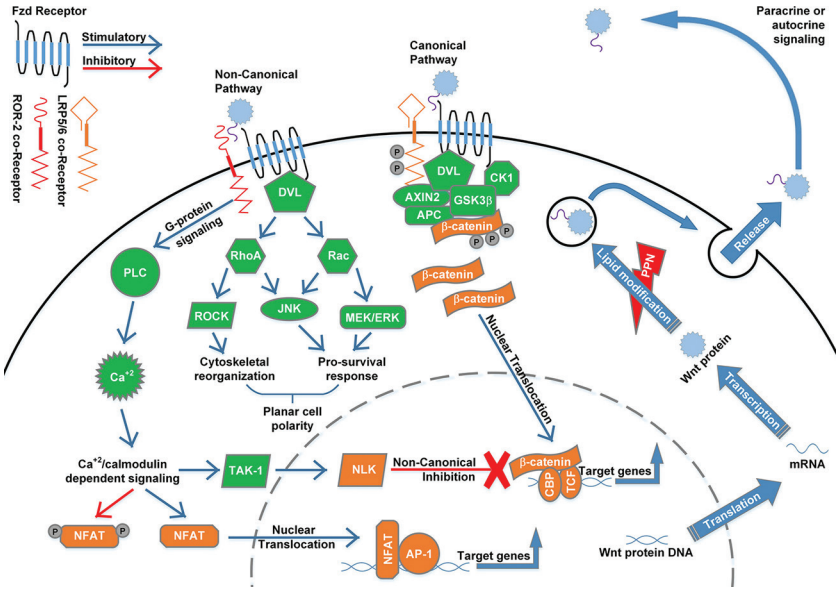

Figure 1. The Wnt signaling pathways depicted in their activated states. Separate signaling mechanisms are associated with ligand binding to the Lrp5/6 or ROR-1/2 co-receptors, distinguishing the canonical and non-canonical pathways, respectively.

Notch signaling. Involvement of Wnt signaling in human cancers was suspected from the very beginning (due to its roots in MMTV-driven tumors), but early searches for mutations or rearrangements that resulted in malignant lesions were not successful. A significant leap forward was made with the development of the Min mouse and the discovery of inactivating mutations in the adenomatous polyposis coli (APC) protein (2, 3). This realization led to further searches for targets that were downstream of the secreted Wnt proteins. Tumor-driver mutations in β-catenin, glycogen synthase kinase-3 beta (GSK-3β) and Axin yielded further insight and added significance to the role of Wnt in promoting development of many types of cancers. Wnt signaling ties to prominent tumor drivers have since been thoroughly established through the distinct Wnt signaling etiologies known as the canonical or non-canonical pathways. Canonical signaling (Figures 1 and 2), predominantly acting through the transcription factor β-catenin, facilitates the transcription of oncogenes such as c-myc and the ATP-binding cassette (ABC) transporters. Non-canonical Wnt signaling acts through two major pathways: the planar cell polarity (PCP) pathway, and G-protein mediated calcium signaling (Figures 3 and 4, respectively), both of which function to effect changes in cell fate and differentiation, cytoskeletal rearrangements, and metastatic behaviors.

3. THE Wnt CASCADE

Wnt signaling begins with translation and transcription of the 19 human Wnt proteins (Figure 1). These relatively small proteins (~350-400 amino acids and approximately 40kDa) are lipid modified in the endoplasmic reticulum (ER) by a membrane bound O-acyl transferase known as porcupine (PPN, or porcn). PPN transfers a palmitoleic acid to a conserved serine on each Wnt protein. The proper function of PPN appears to be highly important, as this lipid addition is necessary for Wnt protein exit from the cell. Further, based upon the reported crystal structure of *Xenopus Wnt8* in complex with the cysteine rich domain (CRD) of a Frizzled (Fzd) receptor, it appears that the lipid interacts directly with the Fzd8 receptor (4). This hypothesis is further supported by the work done by Takada, *et al*, which had previously shown that unmodified Wnt proteins were trapped inside the ER membrane (5). Following processing by PPN, Wnts are shuttled to the Golgi apparatus where they bind to Wntless, an interaction that is reported to require palmitoleic acid modification. Wntless facilitates Wnt release via retromer trafficking, and is recycled through the Golgi via retrograde transport. Wnts are secreted into the extracellular space and can act in a paracrine or autocrine manner to stimulate signaling via the various Wnt pathways. Initially, Wnt proteins bind to a Fzd receptor – members of the family of G-protein coupled receptors (GPCRs).Subsequently, this binding recruits an associated co-receptor – either the Lipoprotein receptor-related protein 5/6 (Lrp5/6) or the receptor tyrosine kinase-like orphan receptor-1/2 (ROR-1/2) for the canonical or non-canonical pathways, respectively. The co-receptor Ryk may also be utilized in the non-canonical pathway, but we will focus on the function of ROR-1/2 within this review for the sake of simplicity (6). Wnt proteins have often been categorized based upon their preference for stimulating canonical or non-canonical signaling. Contrary to this straightforward view of the pathway, recent studies have highlighted the ability of prototypical non-canonical Wnts to stimulate the

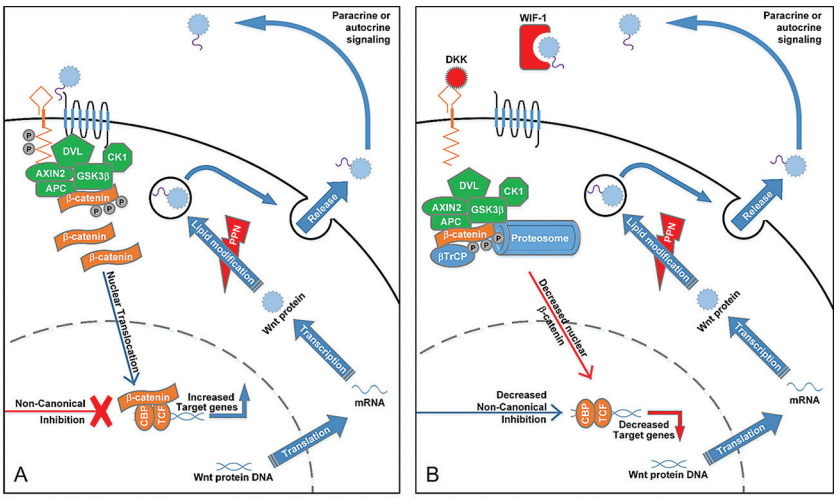

Figure 2. A: Active canonical Wnt signaling. B: Inactive canonical Wnt signaling.

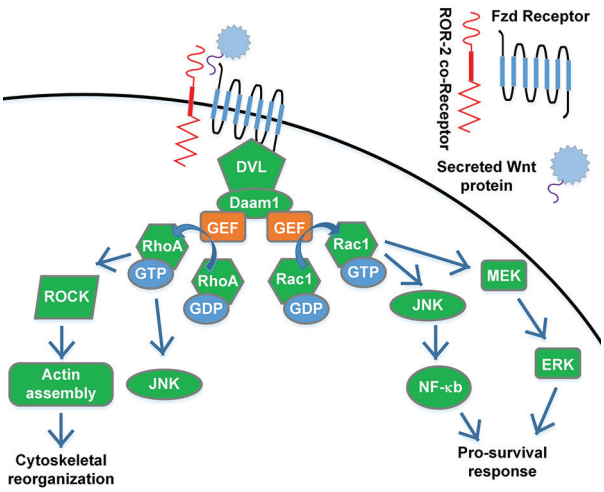

Figure 3. Non-canonical Wnt signaling: the planar cell polarity pathway.

canonical signaling pathway, and vice versa, depending on the population of receptors or co-receptors which are available (7). At the point of ligand-receptor binding, the Wnt pathway diverges into separate signaling networks – the canonical and non-canonical Wnt pathways.

Canonical Wnt is centered around β-catenin accumulation and nuclear translocation, or proteasomal destruction. When a Wnt ligand binds to Fzd, the Lrp5/6 co-receptor is recruited and Dishevelled (DVL) is able to bind to the Fzd/Lrp5/6 heterodimer complex (although it is reported that ligand binding is not necessary for DVL binding to Fzd) (8). Lrp5/6 and DVL are both phosphorylated by multiple isoforms of membrane bound casein kinase 1 (CK1), while Lrp5/6 is again phosphorylated by GSK3β (8-10). The phosphorylation events on Lrp5/6 enable its binding to Axin2 (11). It is currently thought that the entire β-catenin destruction complex, consisting of GSK3β, DVL, Axin2, APC, and captured β-catenin bind in concert to the Fzd/Lrp5/6 heterodimeric receptor complex (10). During active Wnt signaling (Figure 2A), the destruction complex is unable to facilitate proteasomal destruction of β-catenin due to its interaction with Fzd/Lrp5/6. It is believed that the activity of the destruction complex is the signal limiting factor in the canonical Wnt pathway (as compared to the production of β-catenin, or its ability to enter the nucleus). Thus, immobilization of the destruction complex via Wnt ligand binding stimulates signal propagation by increasing free β-catenin. Under these conditions, cytosolic β-catenin is able to translocate to the nucleus via a mechanism that is yet unclear, and bind with the T-cell factor (TCF)/Lymphoid enhancer factor (LEF) transcription factors to increase translation of select target genes (12). Notable among this group of β-catenin targets are genes such as c-myc, ATP-binding cassette transporter B1 (ABCB1) and Axin2 (13-15). Interestingly, β-catenin stimulates transcription of a member of its own destruction complex. Without Wnt ligand binding, or during inactive Wnt signaling (Figure 2B), cytosolic β-catenin is bound to the destruction complex and polyphosphorylated by GSK3β. The protein ligase beta-transducin repeat containing E3 ubiquitin protein ligase (βTrcP) is recruited to the complex where it can ubiquitinate β-catenin and target it for degradation in the proteasome. After release and degradation of β-catenin in the proteasome, the destruction complex is recycled.

While canonical Wnt has been defined to act mainly via β-catenin, non-canonical signaling has several modalities, some of which have yet to be clearly elucidated. Non-canonical Wnt can be categorized into two major divisions: the planar cell polarity (PCP) pathway and G-protein mediated signaling. The PCP pathway utilizes the interaction of Fzd and DVL to induce a cascade of signals after binding of a Wnt ligand. DVL interacts with

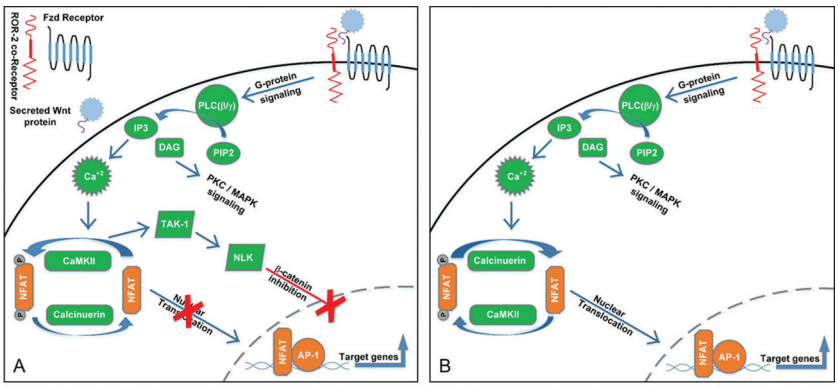

Figure 4. Models of non-canonical Wnt signaling via calcium. A: Wnt-calcium signaling through Calmodulin-dependent kinase II (CaMKII). B: Wnt-calcium signaling through Calcineurin.

the scaffolding protein Dishevelled-associated activator of morphogenesis (Daam1) to induce the activity of various guanine exchange factors (GEFs) which catalyze transfer of guanosine triphosphate (GTP) to the RhoA and Rac1 GTPases, producing their active, GTP-bound forms (Figure 3) (16). While several GEFs are involved, and it has been reported that Daam1 interaction may be non-essential, it is clear that both RhoA and Rac1 are activated by Wnt ligand/ROR-1/2/DVL signaling (17-19). RhoA can be initiated separately from Rac1, and vice versa; while in some cell types, it has been observed that non-canonical signaling can stimulate Rac1, but inhibit RhoA (20-22). RhoA activates the Rho-associated kinase (ROCK), which induces actin filament assembly and cytoskeletal changes (16, 19). Further, RhoA affects cell survival mechanisms through JNK signaling (18). Rac1 signals through MEK/ERK pathways and JNK/NF-κb pathways, resulting in a net proliferative/pro-survival stimulus (23-25).

Stimulation of the non-canonical Wnt receptor complex of Fzd/ROR-1/2 induces activation of various G-protein dependent signaling modalities via phospholipase C (PLC)-β and PLC-γ (Figures 1, 4A and 4B). PLCβ/γ activation causes hydrolysation of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG), binding of IP3 to the IP3-receptor and release of calcium from the ER. This intracellular calcium spike activates Calmodulin and various calcium/Calmodulin - dependent substrates. Both Fzd and ROR-1/2 are predicted to be able to separately stimulate G-protein signaling based upon their structural characteristics. While it would be expected that Fzd (a GPCR) and ROR-1/2 (a receptor tyrosine kinase-like receptor) would show preference for PLC-β and PLC-γ signaling, respectively, this hypothesis has never been directly studied, and it is hypothesized that their effects on Wnt signaling are equivalent (26).

Interestingly, there are conflicting views on the outcomes of Wnt-stimulated calcium signaling, which center around the actions of two calcium-sensitive enzymes: Calmodulin-dependent kinase II (CaMKII) and Calcineurin. One proposed model (Figure 4A) asserts that calcium mediated signaling through Wnt results in an increase in CaMKII activity (27-29). This induction of CaMKII via Wnt results in phosphorylation and inhibition of the nuclear factor of activated T-cells (NFATs), as well as phosphorylation and stimulation of the transforming growth factor-β activated kinase-1 (TAK-1) (30, 31). The Nemo-like kinase (NLK) is phosphorylated by TAK-1 and, in turn, inhibits the β-catenin transcriptional hub by phosphorylation of TCF/LEF family proteins, and CBP (32-34). Thus, non-canonical Wnt activation of CaMKII is a negative regulator of both NFAT signaling and canonical Wnt/β-catenin signaling, an observation which has been widely reported (28, 35, 36). A different model (Figure 4B) claims that stimulation of the non-canonical Wnt pathway induces G-protein-mediated calcium release, which drives the activity of Calcineurin, a protein phosphatase which has high specificity to the NFATs (37-39). Through this mechanism, Wnt increases Calcineurin function, activating NFATs and promoting NFAT translocation to the nucleus where they are transcriptionally active (40). Subsequently, a number of studies have aided to establish this mechanism in various model systems (41-45). Thus, Wnt-stimulated calcium signaling activates Calcineurin and positively regulates NFAT signaling. Although seemingly contradictory, these models may very well be reconcilable as the differential responses may prove to be cellular context or organism dependent. These reports serve to further demonstrate the complexity of Wnt signaling, and its multitude of functions within any given cell type.

4. STRATEGIES TO INHIBIT WNT SAMPLING

4.1. Synthetic compounds

4.1.1. Porcupine inhibitors

For some time, the PPN enzyme has been viewed as an attractive therapeutic target within the Wnt pathway (46). The acyl-transferase enzymatic activity of

PPN appears to be specific to the Wnt proteins, and this ER membrane bound enzyme controls a rare bottleneck in the signaling pathway – all Wnts are forced through PPN (Figures 1 and 2). Without this specific palmitoleic acid addition to a conserved serine, Wnt proteins build up inside the cell and cannot induce signaling (5). In 2012, the first report of small molecule inhibitors of PPN (termed "Inhibitor of Wnt Production" or, IWPs) was released (47) Quickly following, several groups reported use of another PPN inhibitor, C59, and its more potent analog, LGK-974 (both developed by Novartis) in the context of mammary cancer, pancreatic ductal adenocarcinoma, and head and neck squamous cell carcinoma (48-50). Subsequently, these molecules have proven their utility not only as tools to treat Wnt-driven cancers, but also as useful means to elucidate the role of Wnt in various stem cell biology applications (51-55). Due to the initial efficacy of porcupine inhibitors used in mouse models of Wnt-dependent malignancies, a Phase 1 clinical trial was launched in 2012 by Novartis for the use of LGK-974 in pancreatic adenocarcinoma, BRAF mutant colorectal cancer, and other tumors reported to exhibit aberrant Wnt signaling.

4.1.2. Targeting endocytosis in Wnts

Wnt protein shuttling prior to secretion and subsequent endocytosis, the density of available Wnt receptors, and the density of Wnt co-receptors are all regulated by clathrin-dependent functions (56-59). Interestingly, clathrin dependent internalization of Wnt3a and Wg (drosophila) functions to promote Wnt signaling. Blockade of Wnt protein endocytosis by clathrin results in canonical Wnt signaling inhibition (60). Similarly, Lrp6 presence on the cell surface and its proper signaling is regulated by clathrin. Formation of Lrp6 signalosomes requires clathrin function, and specific tyrosines in the cytoplasmic portion of Lrp6 are required for clathrin mediated internalization of the receptors (59, 61)._ENREF_38 Molecules such as monodansylcadaverine function by inhibiting clathrin function, thereby blocking Wnt protein endocytosis and Lrp6 signalosome formation. In recent studies, the anti-helminthic drug niclosamide has been reported to inhibit Wnt signaling, and function as a potent anti-cancer agent. Niclosamide appears to inhibit Wnt signaling by inducing Fzd1 internalization, as well as decreasing expression of DVL (62, 63). In murine models of colorectal cancer and ovarian cancer, niclosamide was demonstrated to potently abrogate Wnt and decrease tumor burden (63, 64). Niclosamide is known to be limited by poor systemic bioavailability when given orally – a quality that is desirable for anti-helminthic treatment. In order to better leverage niclosamide activity, a recent effort has been made to synthesize niclosamide pro-drugs, which exhibit improved gut absorption and could be metabolized into the active drug. Mook, *et al* synthesized a number of derivatives based upon structure-activity relationships of niclosamide. Their study identified a compound which was metabolized into niclosamide, and exhibited more favorable pharmacokinetic properties in their mouse model (65). This first study in developing novel compounds targeted to inhibit Wnt by decreasing available receptors may lead the way for further work towards exploiting this aspect of Wnt signaling.

4.1.3. Tankyrase inhibitors

The tankyrase enzymes (TANK1 and TANK2) are members of the superfamily of poly(ADP-ribose) polymerase (PARP) proteins. Other prominent members of this family include PARP1 and PARP2, among other PARP enzymes. Relative to Wnt signaling, the TANK enzymes function to regulate the destruction of Axin via PARsylation of Axin, binding of TANK1/2 with Axin to the ubiquitin ligase RNF143 and subsequent destruction in the 26S proteasome (66-68). Axin destruction mediated by the TANK enzymes limits function of the β-catenin destruction complex, and thereby promotes canonical Wnt signaling (Figure 2). Inhibition of the TANKs blocks Wnt signaling by increasing available Axin and inhibiting β-catenin accumulation and nuclear translocation. In late 2009, and following in 2010, synthetic inhibitors of the TANK enzymes were developed (XAV939, IWR-1 and IWR-2) as means to antagonize Wnt signaling through β-catenin (69, 70). These molecules have since been shown to be highly potent inhibitors of TANKs, as well as promising therapeutics in initial studies (71). Further, many applications of TANK inhibitors in developmental biology and stem cell fields highlight the significance of these molecules for studying Wnt signaling. (72, 73)

4.1.4. Targeting casein kinase 1-alpha

In a screen for molecular inhibitors of Wnt signaling, Thorne, *et al* discovered that pyrvinium, an FDA-approved drug for treating pinworm, was an activator of casein kinase 1-alpha (CK1α) and a potent Wnt antagonist (74). While pyrvinium does not appear to activate other isoforms of CK1 (e.g. CK1δ and CKε, see section 5.1.2.), it has been shown to bind directly to CK1α and increase kinase activity, thus inhibiting canonical Wnt signaling. Subsequently, a number of studies have explored the use of pyrvinium in preclinical models of cancer, with some success (75, 76). Although initial work with pyrvinium has been promising, and its path to the clinic may be more straightforward as it has been FDA approved for other purposes, pyrvinium also acts as an androgen receptor antagonist (77, 78). This property of pyrvinium action may foreshadow severe side effects of extended exposure during rounds of chemotherapy in patients, while, simultaneously, androgen receptor antagonism has sparked interest in applications for treating prostate cancer (79). It is essential to note the reported functional difference between CK1 isoforms, which has been well reviewed by Cruciat, and is further discussed in Section 5.1.2. of this review (80). Due to the importance of CK1 enzymatic activity for proper canonical Wnt signaling, it seems that research into both stimulators and inhibitors

of CK1 isoforms will prove to be an area of great interest in the near future.

4.1.5. β-catenin transcriptional inhibition

In a search for molecules which can effectively inhibit the Wnt pathway in colon cancer, *in vitro*, the small molecule ICG-001 was discovered (81). ICG-001 binds to the Creb-binding protein (CBP), inhibiting its interaction with β-catenin (Figure 2, A and B). CBP, along with p300, TCF, and LEF, are necessary to facilitate β-catenin induced transcription of canonical Wnt target genes (82). Thus, ICG-001 inhibition of CBP significantly reduces canonical Wnt signaling through β-catenin. Initial studies utilizing ICG-001 *in vitro* and *in vivo* as a potential therapeutic agent for treatment of Wnt-regulated cancers have achieved promising results (83, 84). Other small molecules which function similarly have been developed more recently (85-87). The CBP is not Wnt-specific, as it interacts with a number of other proteins to facilitate transcription of genes. Thus, even molecules which are highly monogamous in their interaction with CBP will broadly block other signaling networks, outside of Wnt. Still, targeting of CBP or p300 functions has remained highly appealing (88). While useful for certain experiments in studying Wnt, continued work is being done to assess the potential biological applications of these molecules.

4.2. Natural compounds

4.2.1. G-protein and calcium signaling blockade

Pertussis toxin protein (PTX) is an exotoxin derived from the bacterium responsible for causing whooping cough, *Bordatella pertussis*. PTX has been well characterized as an inhibitor of G-protein signaling, as it functions to block cAMP via stimulation of ADP-ribosylation of the α-subunit of G-inhibitor (G_i) proteins (89, 90). Currently, PTX is contained in vaccines against *B. pertussis* (90, 91). PTX has been used as a tool to study non-canonical Wnt signaling, as it interferes with G-protein mediated PLC and Ca^{+2}/Calmodulin signaling, which depend on GPCR-mediated stimulation via G_i proteins (7, 92, 93). Additionally, Cyclosporin A (CsA), an inhibitor of Calcineurin, has been used to study further downstream effects of Wnt mediated Ca^{2+} release on NFAT signaling (Figure 4) (94). While both PTX and CsA are frequently utilized, neither molecule is highly specific to Wnt signaling. To our knowledge, very few small molecule inhibitors of non-canonical signaling exist, and none have been widely reported.

4.2.2. 2-Methoxyestradiol

2-Methoxyestradiol (2ME) is an estrogen metabolite that appears to have very little to no affinity for the estrogen receptor. 2ME has been widely researched due to its ability to inhibit hypoxia inducible factor 1-alpha (HIF1α), interferon, stimulation of JNK signaling, and inhibition of β-catenin (95-100). Although molecular targets of 2ME appear to be numerous and relatively non-specific, its action on β-catenin signaling and indirect Wnt targeting via interferon warrant classification as a Wnt inhibitor. The tumor inhibitory function of 2ME has generated intrigue in the scientific community, as it is has been broadly effective *in vitro* and *in vivo* against a number of malignancies (97, 101, 102). Further study of this molecule will aid in the elucidation of its various mechanistic effects, and characterization of its potential as a chemotherapeutic agent.

4.2.3. Quercetin

Quercetin is a member of the flavonoid family, a group of low-molecular weight natural products of plant origin that are found in many fruits and vegetables (103). Other flavonoids include geinstein and kaempferol, which are also considered dietary constituents and have a variety of biological effects. While the effects of various flavonoids on Wnt signaling have been described, quercetin is one of the more well-studied flavonoids with Wnt-inhibitory properties (104, 105). It suppresses the Wnt pathway via blockade of β-catenin/TCF signaling, leading to down-regulation of the expression of target genes such as Cyclin D1 and survivin (Figure 2) (105-108). Recent results also indicate that in addition to directly inhibiting Wnt signaling as shown by reduced levels of β-catenin, APC, Axin1 and LEF mRNA levels, quercetin additionally upregulates expression of Dkks which also leads to inhibition of Wnt/β-catenin signaling (109, 110).

5. STRATEGIES TO STIMULATE Wnt SIGNALING

5.1. Synthetic compounds

5.1.1. *Glycogen synthase kinase 3-beta* (GSK3β) inhibitors

For many years, the protein kinase glycogen synthase kinase 3-beta (GSK3β) has been of significant interest to a number of fields of study, including diabetes, neurodegenerative disorders, cardiovascular diseases, and cancer (111-114). GSK3β functions in two major capacities: during active Wnt signaling GSK3β is required for phosphorylation of Lrp5/6, inducing formation of signalosomes and enabling signal propagation. Secondarily, GSK3β is necessary for polyphosphorylation of bound β-catenin, which is a signal inhibitory function. These opposing functions of GSK3β confound its utility as a means to block Wnt, as our current model of Wnt signaling predicts that GSK3β acts as both a stimulator and inhibitor. Despite predictions of conflicting or negating activities, a number of small molecule inhibitors of GSK3β have been synthesized and tested to date (115-120). Most reports indicate that GSK3β inhibition induces a net Wnt-stimulating response – inducing TCF/LEF (TopFlash), Axin2 expression, and increases in total β-catenin (121-123). Clinically, GSK3β inhibitors have found initial promise in Alzheimer's disease, as a dose escalation pilot study, and a phase II clinical trial have been reported for the GSK3β inhibitor Tideglusib (124, 125). Further, phase I and II clinical trials

of Tideglusib in the treatment of progressive supranuclear palsy have been completed, although results show no significant efficacy in treatment groups (126). As with many of the molecules discussed here, the clinical utility of GSK3β inhibitors remains to be widely tested. With a wide array of potential applications, it is certain that many exciting advances in this field are waiting to be made.

5.1.2. Targeting casein kinase 1-delta/epsilon

While all CK1 isoforms act in inhibitory roles in Wnt, CK1δ and CK1ε functions are distinct from the activity of CK1α (80). CK1α phosphorylates β-catenin, initiating the process of its destruction (127). In contrast, primary substrates of CK1δ/ε include DVL, APC, and Axin (128). Additionally, Lrp6 is phosphorylated by CK1δ/ε, resulting in signal potentiating or signal inhibiting changes, depending on the phosphorylation site (129). In similar fashion, other researchers have found CK1 to have dual roles in Wnt signal propagation (130). It is clear that the effects of CK1 inhibition are determined by the isoform being blocked, the tissue type, and possibly other factors such as high/low expression of CK1 substrates. More detailed understanding of the structure of CK1 isoforms and their function have led to the development and testing of an array of small molecule inhibitors (131, 132). While a number of these new compounds have been synthesized and roughly shown to have anti-proliferative or differentiation inducing effects, their ability to stimulate Wnt signaling remains largely untested. CK1δ/ε functions in many contexts, and the consequences of its targeting require further analysis.

5.2. Natural compounds

5.2.1. Curcumin

Curcumin is a naturally occurring phenol found in turmeric spice. A highly lipophilic molecule, curcumin exists in 1,3-diketo and two enol tautomers, and displays relatively low bioavailability due to degradation of the enol tautomer and rapid excretion from the body (133, 134). Many strategies have been attempted to increase curcumin bioavailability, including nanoparticulate formulations, synthesis of curcumin analogues, co-treatment with piperine (a UDP-glucuronosyl transferase inhibitor), and packaging in micelles (133, 135-138). Although curcumin exhibits a number of potential mechanisms of action, a significant body of work has established its effect on Wnt signaling (135, 139-144). Curcumin has been shown to inhibit expression of GSK3β, as well as increase TCF/LEF transcriptional activity. Further, *in silico* work indicates that curcumin may interact with the endogenous Wnt inhibitors Dkk and Wif-1, thus inhibiting their function (135). While the specific biological effects of curcumin remain diverse, curcumin is well tolerated both *in vitro* and *in vivo*, and has been widely reported to be efficacious in a number of disease states including models of neurodegeneration, diabetes, and cancer (135, 139-141, 143-145). Gupta, *et al* have written an extensive review of the various clinical trials of curcumin over the years (146). Further work and the results of ongoing trials will determine the future of curcumin as a therapeutic molecule or an interesting side piece among an array of biologically active natural products.

5.2.2. Lithium chloride

Since 1996, lithium chloride has been recognized as an inhibitor of GSK3β (147). Previous to the work by Klein, *et al* the effects of LiCl on embryonic development were evident, but mechanistically poorly characterized (148, 149). Elucidation of the effect of LiCl on Wnt allowed a more clear explanation of these phenotypic changes (150). LiCl has been consistently used with significant success in the treatment of bipolar disorder. Despite side effects including renal and thyroid toxicities, as well as teratogenicity, the utility of LiCl in many cases of bipolar disorder remains unquestionable. Subsequently, LiCl has been used widely as a tool to study Wnt signaling, particularly for stimulation of Wnt *in vitro*. Although relatively high concentrations of LiCl (>1mM) are used in most contexts to increase Wnt signaling *in vitro*, its use has historically been of great importance. While LiCl effectively inhibits GSK3β, and is used clinically for other applications, such as in treatment of bipolar disorder, it seems unlikely that LiCl will find utility as a high-fidelity Wnt-targeted therapy in a clinical setting (151). The high concentrations needed for efficacious inhibition and off-target effects are severe limitations which complicate *in vitro* experiments with LiCl, and highlight the need for new molecules which replicate the on target effects of LiCl. Due to the development of highly specific GSK3β inhibitors, it is possible that the use of LiCl to study Wnt signaling will be considered old hat. Newer molecules which enable more precise experimentation may marginalize LiCl in the rising frontiers of Wnt signaling.

6. EXTERNAL SIGNALING INFLUENCES

The Wnt signaling pathway is not a closed system. Inputs from a number of distinct signaling cascades influence Wnt through several mechanisms. The bone morphogenetic protein (BMP), transforming growth factor-beta (TGFβ), and hedgehog (Hh) pathways have been demonstrated to act as external regulators of Wnt (152). A significant volume of research has established not only the necessity of Wnt signals for effective BMP signaling, and vice versa, but also the effect of BMPs on Wnt itself. Tang, *et al* found that osteogenic differentiation of MSCs, induced by BMP-9, was enhanced by Wnt3a, and diminished by loss of β-catenin (153). Other reports reached similar conclusions, finding that BMP-2 interacted with Wnt5a, while expression of sclerostin, an endogenous Wnt antagonist, was regulated by the presence of BMP-receptor 1A (154, 155). Invariably tied to BMP signaling, the TGFβ signaling cascade also exerts its cellular effects via Wnt signaling, and in coordination with Wnt. Smad7, an antagonistic mediator of TGFβ signaling stimulates β-catenin destruction,

while Axin interacts with Smad7 and facilitates its destruction (156, 157). Axin also phosphorylates the TGFβ signal transducing protein, Smad3 (158). For a more comprehensive review of BMP and TGFβ pathways, please note the recent and thorough articles by Rahman, *et al,* and Piersma, *et al* (159, 160). Additionally, the Hh signaling pathway has been established to depend on active Wnt, while also influencing Wnt signaling itself. Hh signaling directly regulates expression of Tcf3/4, which is required for canonical Wnt signaling through β-catenin (161). Canonical Wnt signaling in mesenchymal cells is also required for Hh mediated stimulation of alkaline phosphatase expression, a marker of osteoblast differentiation (162). The interplay of Hh and Wnt in driving epithelial-to-mesenchymal transitions, as well as their roles in various cancers, have also been well characterized (163, 164).

7. DISCUSSION

In recent years, our ability to selectively target components of the Wnt signaling pathway has expanded tremendously. The development of novel synthetic compounds, as well as characterization of natural molecules has allowed better understanding of various pathologies, compounding upon deeper mechanistic knowledge of Wnt functions. It is interesting to note that a striking disparity exists in the number of molecules developed to inhibit Wnt signaling, compared to the number of those developed to stimulate Wnt signaling. One could surmise that this is due to the many cancers known to exhibit aberrantly activated Wnt signaling, and the widespread search for potential chemotherapeutic agents for those malignancies. To the contrary, many other fields of research would benefit greatly from the discovery of various, potent Wnt stimulators; thus, a pressing need exists for all types of Wnt targeting agents. Molecules capable of activating Wnt would be a huge boon to researchers investigating cardiac regeneration, diabetes, or neurodegenerative disorders, among other disciplines. Alternatively, this difference in volume of available molecules may be caused by an inherent property of Wnt signaling: it is possible that it is simply more difficult to exogenously stimulate Wnt signaling compared to inhibiting Wnt. Although this conjecture potentially explains the variance in successful development of Wnt targeting molecules, it is a hypothesis which remains difficult to test or prove. For now, it seems reasonable to acknowledge the surprising variance in quantity of tools available to stimulate or inhibit Wnt, and identify a significant need for further research.

A challenge in developing this review, and a possible limitation of its utility, are the many conflicting reports on specific sub-topics within Wnt. Many drugs studied and reviewed here, as well as Wnt pathway proteins, have been reported to act in contradictory roles in certain settings (35, 40). In contrast to the mechanisms of the canonical Wnt signaling pathway, upon which researchers seem to have attained general agreement, non-canonical signaling is much less well characterized (as the name might imply). Numerous conflicting reports exist concerning the Wnt-related and Wnt-unrelated roles of ROR-1/2, DVL, and their effector proteins, as well as the specificity of Fzd-Wnt protein interactions within canonical or non-canonical settings. Although mechanistic diagrams such as those within this review often appear to demonstrate certainty, these must be viewed with a critical eye, as specific mechanisms are only relevant within certain biological contexts. The multiple etiologies of non-canonical Wnt signaling are not necessarily distinct, nor exclusive to Wnt stimuli. As noted in Section 6, the interactions of other signaling pathways further challenge the development of a concise picture of broad Wnt signaling (159, 161, 165). Thus, we have provided an up-to-date perspective on the existing consensus within these fields, while simultaneously highlighting well-contested conflicting viewpoints.

The array of small molecules which have been developed to target Wnt signaling still leaves something to be desired. Wnt signaling is spearheaded by a group of GPCRs, the Frizzled receptors, which share significant structural homology and have been mechanistically well studied. To date, no molecule has been developed which directly targets any Frizzled receptor, despite the overall success of inhibiting GPCRs in other contexts. In addition, the Wnt co-receptors, Lrp5/6, ROR-1/2, and Ryk remain unavailable for direct targeting with our current regimen of molecules. The recent reports surrounding innovative molecules targeting porcupine and GSK3β have been very exciting, and hopefully will stimulate more research into further potential targets within Wnt, and their various applications (126, 166).

8. CONCLUSION

In sum, many novel developments have driven this field forward – creating new knowledge of cellular functions as well as disease states, and opening opportunities for clinical application of important findings. The discovery, development, and use of small molecules to both stimulate and inhibit Wnt remains vital for mechanistic findings within Wnt to be applied to clinical questions. Continued characterization of our currently available molecules will enable us to better test experimental models, and allow accurate prediction of off-target effects and potential patient outcomes in the future. We look forward to further progress within the whole of Wnt signaling.

9. ACKNOWLEDGEMENTS

Special thanks to the Mayo Clinic College of Medicine, the Mayo Graduate School, and the Mayo Clinic Department of Molecular Pharmacology and

Experimental Therapeutics. Funding for this work was provided by the John and Posy Krehbiel Endowed Professorship of Orthopedic Surgery and Biomedical Engineering (Michael J. Yaszemski), and by the Mayo Clinic Pharmacology T32 Training Grant (NIH T32 GM072474) (Carl T. Gustafson). The funding organizations had no role in manuscript topic, structure, preparation, or decision to publish.

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Abbreviations: Dkk, Dickkof; sFRPs, secreted Frizzled-Related Proteins; WIF-1, Wnt Inhibitory Factor-1; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3 beta; ABC, ATP-binding cassette; PCP, planar cell polarity; ER, endoplasmic reticulum; PPN, porcupine; CRD, cysteine rich domain; GPCRs, G-protein coupled receptors; Fzd, Frizzled; Lrp5/6, Lipoprotein receptor-related protein 5/6; ROR-1/2, receptor tyrosine kinase-like orphan receptor-1/2; DVL, Dishevelled; CK1, casein kinase 1; TCF, T-cell factor; LEF, Lymphoid enhancer factor; ABCB1, ATP-binding cassette transporter B1; βTrcP, beta-transducin repeat containing E3 ubiquitin protein ligase; Daam1, Dishevelled-associated activator of morphogenesis 1; GEFs, guanine exchange factors; GTP, guanosine triphosphate; ROCK, Rho-associated kinase; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; CaMKII, Calmodulin-dependent kinase II; NFATs, nuclear factor of activated T-cells; TAK-1, transforming growth factor-β activated kinase-1; NLK, Nemo-like kinase; TANK, tankyrase; PARP, poly(ADP-ribose) polymerase; CBP, Creb-binding protein; PTX, Pertussis toxin protein; CsA, Cyclosporin A; 2ME, 2-Methoxyestradiol; BMP, bone morphogenetic protein; TGFβ, transforming growth factor-beta; Hh, hedgehog

Key Words: Wnt Pathway, Small Molecule, Canonical Signaling, Non-Canonical Signaling, Beta-catenin, Review

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