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Journal

British Journal of Pharmacology, 174(24)

ISSN

0007-1188

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Publication Date

2017-12-01

DOI

10.1111/bph.13963

Peer reviewed

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# Inhibition of Nuclear Wnt Signaling: Challenges of an Elusive Target for Cancer Therapy

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**Abstract**

The highly conserved Wnt signaling pathway plays an important role in embryonic development and disease pathogenesis, most notably cancer. The “canonical,” or β-catenin-dependent Wnt signal initiates at the cell plasma membrane with the binding of Wnt proteins to Frizzled:LRP5/LRP6 receptor complexes, and is mediated by the translocation of the transcription co-activator protein, β-catenin, into the nucleus. β-catenin then forms a complex with TCF/LEF transcription factors to regulate multiple gene programs. These programs play roles in cell proliferation, migration, vasculogenesis, survival, and metabolism. Mutations in Wnt signaling pathway components that lead to constitutively active Wnt signaling drive aberrant expression of these programs and development of cancer. It has been a longstanding and challenging goal to develop therapies that can interfere with the TCF/LEF-β-catenin transcriptional complex. This review will focus on the i) structural considerations for targeting the TCF/LEF-β-catenin and co-regulatory complexes in the nucleus, ii) current molecules that directly target TCF/LEF-β-catenin activity, and iii) ideas for targeting newly-discovered components of the TCF/LEF-β-catenin complex and/or downstream gene programs regulated by these complexes.

**Abbreviations**

APC, adenomatous polyposis coli; CBP, cAMP response element binding protein (CREB)-binding protein; DVL, disheveled; LEF, Lymphoid Enhancer Binding Factor; LRP, low-density lipoprotein receptor-related protein; MCT, monocarboxylate transporter; TCF, T-cell factor; VEGF, vascular endothelial growth factor; WRE, Wnt Response Element

**Introduction**

Wnt signaling comprises a set of signal transduction cascades that are highly conserved across many different species including both non-vertebrates (such as nematodes and fruit flies) and vertebrates (frogs, mice, and humans). These signals play important roles not only in cell fate decisions during embryonic development and stem cell homeostasis in somatic niches of normal and injured tissues, but also in diseases such as cancer (Nusse and Varmus, 1982; Bodmer et al., 1987; McMahon and Moon, 1989; Rocheleau et al., 1997). A great deal of work has been performed to uncover the intricacies of the signal transduction steps at the cell surface and the cytoplasm to better understand the normal and dysfunctional activity of Wnt signaling. Recent reviews published elsewhere and in this issue describe our current understanding of these steps in detail (Niehrs and Acebron, 2012; DeBruine et al., 2017; Driehuis and Clevers, 2017; Nusse and Clevers, 2017; van Kappel and Maurice, 2017; Zimmerli et al., 2017).

In brief, the "canonical," or β-catenin-dependent Wnt signaling pathway is the primary source of dysregulated transcription in the disease setting. In the normal "on-state", this signal initiates at the cell surface when Wnts bind to Frizzled:LRP5/LRP6 receptor complexes, and it culminates in the nucleus where it triggers the formation of a powerful transcription-activating complex **(Figure 1)**. The main mediator of this cell surface-to-nucleus signal is β-catenin, a membrane/cytoplasmic armadillo repeat protein with no ability to promote transcription by binding DNA on its own (Niehrs and Acebron, 2012; Masuda and Ishitani, 2017; Nusse and Clevers, 2017). Instead, through a multitude of protein interactions, β-catenin moves to the nucleus where DNA-binding TCF/LEF transcription factors recruit it to promoters and enhancers of Wnt target genes (Cadigan and Waterman, 2012; Masuda and Ishitani, 2017). Once tethered to DNA by TCF/LEFs, β-catenin recruits co-activators and other regulatory components to activate transcription of the downstream genes known collectively as Wnt target genes. It is this increased transcription of coordinated sets of genes (i.e. gene programs) that directs cells to proliferate, self-renew, differentiate, and survive in different tissues and contexts. In normal cells and tissues, feedback inhibition results in this activity occurring only transiently, preventing over-activation of Wnt target gene transcription. Signal transduction is "turned off" in cells with low or absent Wnt because β-catenin becomes unstable: it is efficiently tagged in the cytoplasm for ubiquitination by the β-catenin destruction complex and degraded by proteasomes. However, in diseases (ie. colon cancer) with genetic mutations of one or more destruction complex

components (e.g. *APC, AXIN2* and *FAM123B/WTX*) or inactivating mutation of negative regulators of Wnt/receptor interactions (e.g. *RNF43/ZNRF3*), or mutations that enhance Wnt signaling (*RSPO2, and RSPO3*), Wnt signaling is improperly activated. These mutations negate the cytoplasmic feedback controls and create cells with constitutive, high levels of β-catenin and aberrantly high levels of Wnt target gene transcription that can initiate the transformation of colon epithelia into malignant cells (Cancer Genome Atlas Network et al., 2012; Polakis, 2012; Mazzoni and Fearon, 2014; Nusse and Clevers, 2017).

Given that the unifying feature of Wnt-linked cancer is aberrant target gene regulation, it has been a longstanding imperative for at least 20 years to develop a therapy that can interfere with TCF/LEF-β-catenin-regulated transcription (Polakis, 2012; Nusse and Clevers, 2017). In particular, there has been strong interest in designing an inhibitor that can act at the nuclear level and effectively target all Wnt-linked cancers, be it caused by receptor mutation or mutations of further downstream signaling components (destruction complex with APC or β-catenin mutations). As straightforward as this goal is, there have been numerous hurdles: first, the challenge of navigating the large, multi-component regulatory complex that β-catenin assembles (some of which is still unknown); second, designing a molecule that disrupts key protein-protein interactions within that complex; and third, finding a pharmacokinetically-favorable small molecule that can cross both the plasma membrane and nuclear membrane to disrupt those key interactions. These challenges have been formidable to the degree that despite a tremendous amount of academic and industry resources dedicated to the problem, not a single drug that inhibits TCF/LEF-β-catenin complexes is currently in clinical use. In addition, since Wnt signaling has a crucial role in stem cell proliferation and homeostasis of multiple organ systems (ie. intestinal, bone, skin, and hair), there has been concern that inhibiting this pathway may result in intolerable toxicities in the intestine or integrity of bone (Kahn, 2014; Nusse and Clevers, 2017). Despite these concerns, several molecules targeting various components of Wnt signaling have been developed and administered in clinical trials with minimal and tolerable toxicities to the gastrointestinal tract, bone, skin, and other organs in human patients (El-Khoueiry et al., 2013; Le et al., 2015; Mita et al., 2016). These molecules have successfully completed Phase I clinical trials (safety and toxicity evaluation) and have recently been approved by the FDA to proceed to Phase II clinical testing (El-Khoueiry et al., 2013; Le et al., 2015; Mita et al., 2016). Although only one of these molecules undergoing clinical studies acts at the nuclear

level (PRI-724, which is discussed in detail in this review), the collective results of the early phase clinical trials of these Wnt inhibitors are encouraging and provide a proof of principle that it is possible to inhibit the Wnt signaling pathway in human patients without causing severe toxicity. Furthermore, recent advances in our knowledge of gene programs that Wnt signaling targets for cell transformation, coupled with a better understanding of the composition and actions of TCF/LEF-β-catenin complexes, has led to the pre-clinical development of several small molecules that disrupt this activity and downstream effects. These advances are encouraging and suggest that the pace of drug development for Wnt-linked cancers is accelerating (Kahn, 2014; Zhan et al., 2016; Nusse and Clevers, 2017). This review will focus on the i) structural considerations for targeting TCF/LEF-β-catenin and co-regulatory complexes in the nucleus, ii) current molecules that directly target TCF/LEF-β-catenin activity, and iii) ideas for targeting newly discovered components of the TCF/LEF-β-catenin complex and/or downstream gene programs regulated by these complexes.

**General Principles in Designing Small Molecule Inhibitors of TCF/LEF-β-catenin**

β-catenin is recruited to target genes through direct recruitment interactions with the N-termini of TCF/LEF transcription factors (Cadigan and Waterman, 2012; Masuda and Ishitani, 2017). There are four mammalian TCF/LEFs (protein names: TCF-1, TCF-3, TCF-4 and LEF-1; gene names *TCF7*, *TCF7L1*, *TCF7L2*, and *LEF1* respectively), and the β-catenin binding domain is one of the most highly conserved features of the transcription factor family (Cadigan and Waterman, 2012; Masuda and Ishitani, 2017). It stands to reason that disrupting this interaction would be a good strategy for interfering with the overabundant TCF/LEF-β-catenin complexes that drive oncogenic Wnt signaling. However, structural studies of β-catenin binding to its myriad inhibitor/activator partners quite rightly predicted the challenges in identifying small molecules that can specifically disrupt binding to TCF/LEFs. The N-terminal ~50 amino acids of TCF/LEFs are intrinsically unstructured until they engage in extensive hydrophobic and salt-bridge interactions with the armadillo repeat array of β-catenin (Graham et al., 2000; Sun et al., 2000; Daniels et al., 2001; Poy et al., 2001; Choi et al., 2006). The interactions span from arm repeat 3 through arm repeat 10 – and given that there are only 11 armadillo repeats in β-catenin, this interaction represents an extensive “zipping together” of the β-catenin arm repeat array with the N-terminus of TCF/LEFs. The extensive binding interactions with multiple points of direct

contact between β-catenin and TCF/LEFs make it challenging to design a small molecule inhibitor (Daniels et al., 2001; Xu and Kimelman, 2007; Kahn, 2014). An additional challenge comes from the discovery of a convergence of binding modes for inhibitory and activating partners of β-catenin; that is, the same armadillo repeats engage in extensive and similar interactions with E-cadherin and other inhibitory proteins such as (APC, AXIN, and ICAT (summarized in (Daniels et al., 2001)). Despite these challenges, there has been some success in identifying small molecule inhibitors, and more recently, success in moving a few of these promising hits through pre-clinical xenograft studies. Most successes have come chiefly through the development of high throughput screens for molecules that disrupt the transcription regulating activities of TCF/LEF-β-catenin complexes. These high- throughput screens most frequently utilize either a TCF/LEF-β-catenin-specific luciferase reporter construct (TOPFlash, SuperTOPFlash, etc.) or TCF/LEF-β-catenin binding ELISA assays to measure modulation in overall Wnt signaling output (Korinek et al., 1997; Lepourcelet et al., 2004; Kahn, 2014). Many screens have utilized cell lines with activated Wnt signaling (ie. SW480 and HCT116 colon cancer cell lines) in which to perform these assays. It should be noted that although these assays are useful as an initial rapid screening tool, the readouts use artificial promoter constructs containing multiple Wnt Response Elements (WREs), and therefore follow up studies are often required to assay for changes in endogenous expression of various Wnt signaling components and targets to further determine the site of action (extracellular, cytoplasmic or intranuclear) in the pathway (Kahn, 2014; Duchartre et al., 2016). Below, we summarize the screens that have used these types of strategies to identify lead compounds that directly target TCF/LEF-β-catenin activity (**Figure 2 and Table 1)**.

**Compounds Targeting TCF/LEF and β-catenin Interaction**

One early class of molecules that was discovered to specifically inhibit Wnt signaling, is a class that directly targets the transcription factor *LEF1* and interferes with its ability to interact with β-catenin. Ethacrynic acid (EA) was first discovered when 960 FDA approved drugs were screened for the ability to decrease the activity of a luciferase reporter (TOPFlash) in HEK293 cells overexpressing dishevelled (DVL; Wnt signaling activator) (Lu et al., 2009). The initial screen was complemented by a secondary screen using Wnt3A to activate signaling through Frizzled:LRP5/6 receptors on the plasma membrane, as well as by expression of a combination

of various other Wnt components such as Wnt1/LRP6, Wnt3/LRP6, DVL, or β-catenin (Lu et al., 2009). The results revealed that EA could inhibit Wnt signaling activity in a dose dependent manner in all activation models suggesting that its molecular action was either targeting β-catenin or disrupting events further downstream. EA was also found to have selective cytotoxicity towards Chronic Lymphocytic Leukemia (CLL) cells with an $IC_{50}$ of 8.56 ± 3 μM compared to 34.97 ± 15.97 μM in normal peripheral blood mononuclear cells (Lu et al., 2009). Furthermore, the authors found that EA could inhibit the expression of Wnt target genes *LEF1* and *CCND1*. Through co-immunoprecipitation experiments using an antibody to EA, and LEF-1, EA was found to directly bind to LEF-1 protein, leading to destabilization of LEF-1/β-catenin interactions (Lu et al., 2009). Unfortunately, the $IC_{50}$ for other cancer cell lines such as colon cancer (SW480, HCT116) were found to be higher at 68 and 58 μM respectively, suggesting that this molecule has less potent cytotoxicity in other cancers (Lu et al., 2009). The same group also showed that various amide derivatives of EA could be synthesized and these exhibited lower $IC_{50}$ when treating CLL cells (Jin et al., 2009), however so far, these higher affinity derivatives do not appear to have been developed further.

The Shivdasani group performed a high throughput screen of ~7,000 natural compounds using a custom-designed TCF-4 and β-catenin ELISA assay to detect small molecules which could disrupt their association (Lepourcelet et al., 2004). Eight compounds were identified with reproducible $IC_{50}$ < 10 uM and of these, 6 compounds (PKF115-584, CGP049090, PKF222-815, PKF118-744, PKF118 310, and ZTM000990) were found to inhibit TOPFlash reporter activity in the HCT116, human colon cancer cell line (Lepourcelet et al., 2004). Immunoblot experiments showed that these compounds could inhibit both CCND1 and C-MYC protein expression (Wnt target genes) in a dose-dependent manner while cellular β-catenin protein levels remained intact. Furthermore, the authors also showed that these compounds could inhibit the *in vitro* proliferation of colon cancer cell lines (HCT116 and HT29) and prostate cancer cell lines (PC-3 and DU-145). To date however, there are no clinical trials using these molecules or next-generation derivatives.

Using information from co-crystallization studies of β-catenin and the β-catenin-binding fragment of the TCF-4 N-terminus, the An group performed a virtual screen to identify 200 potential compounds from a library of 1,990 compounds that could bind to three potential regions within the TCF-4 and β-catenin interaction domains that had been previously proposed

(Fasolini et al., 2003; Tian et al., 2012). These 200 compounds were then screened in a TOPFlash luciferase reporter assay in the HCT116colon cancer cell line. Of these compounds, BC21 was found to be most effective compound in inhibiting TOPFlash luciferase reporter activity. This compound reduced the endogenous expression of Wnt target genes *CCND1* and *C-MYC* at the mRNA and protein level with an $IC_{50}$ of 15 □M. At the time of this publication however, there is no evidence showing that BC21 has advanced to clinical trials.

The DasGupta group screened 14,977 compounds and selected molecules that could disrupt β-catenin-dependent transcription activation of a TOPFlash-like luciferase reporter (dTF12) in Drosophila Cl8 cells (Gonsalves et al., 2011). Several compounds were identified (iCRT3, iCRT5, and iCRT14) and their mode of action was shown to interfere directly with the binding of TCF-4 to □-catenin. The anti-tumor effects of these compounds were validated using primary human colon cancer specimens in a microspheroid-based 3D assay for cell survival. The average $IC_{50}$ across six patient samples was determined to be comparable to other commonly used chemotherapy drugs in colon cancer such as 5-FU (Gonsalves et al., 2011). Unfortunately, there is no evidence that these compounds have advanced beyond the preclinical stage.

A few years later, the Dale group screened 63,00 compounds in HEK293 cultures with activated Wnt signaling (Dishevelled-Estrogen Receptor fusion protein) to identify small molecules that could inhibit a luciferase reporter assay containing three endogenous Wnt response elements in the xNR3 promoter (Ewan et al., 2010). Secondary screens with three additional assays in which Wnt signaling was activated via manipulation at various cellular levels (extracellular: LRP6, cytoplasmic: AXIN2, and intracellular: TCF-4) confirmed a final set of 20 compounds that demonstrated Wnt inhibition (Ewan et al., 2010). Of these compounds, CCT031374 exhibited the strongest suppression of cell proliferation in human cancer cell lines that are dependent on Wnt signaling (HCT116, SW480, HT29, and SNU475). The related 3-indolylmethaneamine compound, CCT036477, was effective in blocking Wnt-dependent phenotypes in zebrafish and *Xenopus* embryos. The authors did not further characterize which components of the TCF/LEF complex were directly targeted by these compounds and there is no current evidence that these leads have advanced beyond the preclinical stage. However, a highly related set of 3-indolylmethaneamine compounds has recently been shown to be effective in blocking the growth of HL-60 promyelocytic and SKOV-3 ovarian cancer xenograft tumors in mice (Guthrie et al., 2015).

In 2016, the Birchmeier group completed an ELISA screen of 16,000 synthetic compounds to identify molecules that could inhibit the binding of β-catenin to a TCF4-derived peptide (Fang et al., 2016). Amongst the screened compounds, LF3 was found to have the strongest selective, disruptive activity. Further studies showed that it could inhibit TOPFlash reporter activity as well as endogenous protein interactions between β-catenin and TCF-4 or LEF-1 (Fang et al., 2016). This compound reduced the protein and mRNA expression of Wnt target genes *AXIN2*, *C-MYC*, and *CCND1* in a dose dependent manner and it also inhibited cell proliferation of HCT116 and HT29 colon cancer cell lines with an approximate $IC_{50}$ of 30 μM (Fang et al., 2016). Importantly, an *in vivo* xenograft mouse model using SW480 colon cancer cells showed that LF3 strongly inhibited tumor growth by approximately 40% at 40 days (study end-point) suggesting that at least in mice, this compound has anti-tumor effects. Interestingly, LF3-treated tumors also appeared to be more differentiated compared to untreated control tumors. It was encouraging that the authors did not find any signs of obvious system toxicity, such as weight loss, and it leaves potential for future study using this drug in clinical development.

Also recently, the S.W. Lee group performed a screen of 22,000 compounds for their ability to inhibit TOPFlash reporter activity in HCT116 cells (Hwang et al., 2016). MSAB was identified as the best candidate molecule and further validation showed that it inhibits proliferation of multiple Wnt-dependent cell lines but not Wnt-independent cell lines such as breast epithelial cells and human dermal fibroblasts. *In vivo* studies showed that MSAB inhibits tumor growth of xenografts from HCT116, HT115, and H23 cell lines, and in addition it inhibits the endogenous expression of β-catenin at the protein level (Hwang et al., 2016). Furthermore, the authors also showed how this compound could inhibit expression of Wnt target genes AXIN2 and CMYC at both the protein and mRNA level (Hwang et al., 2016). Mechanistic studies revealed that MSAB binds directly to β-catenin and promotes its degradation by preventing its binding to the TCF/LEF transcription factors (Hwang et al., 2016). The *in vivo* xenograft mouse models showed no obvious signs of systemic toxicity upon administration of the drug, which is encouraging and shows potential for future clinical studies.

**Compounds Targeting Wnt Co-factors**

The identification of a complete list of co-regulatory factors that assemble with TCF/LEFs at Wnt response elements in promoters and enhancers associated with -catenin dependent Wnt signaling has been an active area of research for many years. Early genetic studies in *Drosophila* and subsequent confirmation in other model systems identified a few of these players including the direct association of Groucho/TLE transcription co-repressors with TCF/LEFs in the absence of Wnt signaling (Levanon et al., 1998; Brantjes et al., 2001), and CBP/p300 transcription co-activators in the presence of active Wnt signaling (Waltzer and Bienz, 1998; Hecht et al., 2000; Sun et al., 2000; Takemaru and Moon, 2000). In addition, the *Drosophila* co-regulators Pygopus and Legless were discovered to have mammalian homologs (*PYGO1, PYGO2,* and *BCL9*, *B9L* respectively) (Willis et al., 1998; Kramps et al., 2002; Thompson et al., 2002; Townsley et al., 2004). Other studies have identified additional regulatory complexes, such as the DOTCOM complex, that contribute to β-catenin regulation of transcription (Mohan et al., 2010), but complexes such as these are more broadly acting and not necessarily dedicated to β-catenin-TCF/LEF complexes (Cadigan and Waterman, 2012). More recently, there have been exciting advances in identifying the specific complex of proteins established by TCF/LEFs on chromatin and understanding how that complex is modified by Wnt signaling and β-catenin (Chodaparambil et al., 2014; Fiedler et al., 2015; van Tienen et al., 2017) (**Figure 1**). The old model of β-catenin displacing Groucho/TLE repressors from TCF/LEFs to activate gene expression clearly needs refinement as current studies suggest that co-repressors and co-activators might exist simultaneously. The entry of β-catenin into the complex modifies pre-existing, structurally-based interactions and activities to exert transcription activating functions. The insertion of small molecules that selectively disrupt the activation process is a challenge and rather complex to identify and define mechanistically.

Some of the newly identified TCF/LEF-β-catenin complex associating factors contain enzymatic or scaffolding activity and have been exploited as potential targets for small molecule inhibitors. Of these co-factors, the scaffolding, protein-protein interaction (PPI) between BCL9 and β-catenin has been found to be an effective target for small molecule inhibition by various groups. The Bienz group used a customized BCL9 peptide fragment (homology domain 2) and -catenin binding ELISA assay to screen through a library of 46,250 compounds and found that carnosic acid (CA), which is a natural occurring component in rosemary, had the highest and most specific inhibitory activity with a Ki value of 3.3 1.8 M (de la Roche et al., 2012). This

compound could also inhibit the transcription of the Wnt target gene *AXIN2* in cells with high levels of Wnt signaling such as LiCl-treated HeLa cells, SW480 cells, and HCT116 cells. In addition, CA was found to decrease TOPFlash luciferase reporter activity by ~90% in SW480 cells compared to the DMSO treated control. Although this study did not investigate if CA could inhibit tumor growth in mice, there has been ongoing studies by other groups to determine if this molecule and other natural components of rosemary may have anti-neoplastic benefits.

The Carrosco group used a rational target design approach to design a synthetic peptide which could interfere with the binding of the □-helical HD2 (homology domain 2) of BCL9 (residues 351 to 374) and surface groove formed by □ helices 2 and 3 of the armadillo repeat 1 of β-catenin (Sampietro et al., 2006; Takada et al., 2012). The investigators mutated the residues at the BCL9 binding interface (H358A or R359A) and designed a series of cell permeable peptides (SAH-BCL9, stabilized □ helix of BCL9 peptides), which could inhibit the ability of BCL9 to bind to β-catenin (Takada et al., 2012). Of these peptides, SAH-$BCL9_B$ was found to be the most effective at targeting β-catenin and could selectively disrupt BCL9/ β-catenin complex in a dose-dependent manner as measured by ELISA and immunoprecipitation in two different human colon cancer cell lines (Colo320 and DLD-1) (Takada et al., 2012). This molecule inhibited Wnt signaling in a dose dependent manner in Colo320 colon cancer cells as measured by decreased TOPFlash luciferase reporter activity and mRNA levels of various Wnt target genes (*AXIN2, LGR5, LEF1, VEGF-A,* and *C-MYC*) (Takada et al., 2012). Furthermore, SAH-$BCL9_B$ inhibited tumor growth, angiogenesis, and metastasis in mouse xenograft models of colon cancer (Colo320) and multiple myeloma (INA-6) (Takada et al., 2012). The administration of SAH-$BCL9_B$ did not appear to cause any obvious systemic toxicity such as cytopenia or severe weight loss (Takada et al., 2012). These findings are encouraging and demonstrate potential for future clinical studies.

Using a similar approach the Ji group was able to identify a small molecule, 4′-fluoro-N-phenyl-[1,1′-biphenyl]-3-carboxamide that could inhibit the BCL9/β-catenin PPI (Hoggard et al., 2015). They then used this molecule as a generic scaffold to design 30 small molecule inhibitors that were more specific for the BCL9/β-catenin PPI (Hoggard et al., 2015). These molecules were then tested against carnosic acid (see above), and compound 22 was found to be most effective at inhibiting BCL9/β-catenin PPI at 2.1 □ 0.41 □M (Hoggard et al., 2015). Compound 22 was found to inhibit endogenous transcription of several Wnt target genes (*AXIN2, LGR5,*

*LEF1,* and *CCND1*) by qRT-PCR in a triple negative breast cancer cell line (MDA-MB-231) in dose dependent manner (Hoggard et al., 2015). The authors also showed that compound 22 inhibited cell proliferation in colorectal (SW480, HCT116, and HT29) and triple negative breast cancer cell lines (MDA-MB-231 and MDA-MB-436) with an $IC_{50}$ of ~ 2-3 μM (Hoggard et al., 2015). However, the authors did not further study this molecule in an *in vivo* model and there is no current evidence that these compounds have advanced beyond the preclinical stage in development.

The Li group used a TOPFlash reporter assay in HEK293 cells treated with Wnt3a to screen a library of 4000 compounds. They identified the small molecule NC043 as having the highest inhibitory activity of TOPflash reporter activity and endogenous mRNA levels of Wnt target genes (*AXIN2* and *SURVIVIN*) in Wnt dependent colon cancer cell lines (SW480 and Caco-2) (Wang et al., 2011). This molecule also inhibited tumor growth in a SW480 colon cancer xenograft mouse model in a dose dependent manner by up to ~70%. A detailed follow up study with the goal of determining the inhibitory mechanism of NC043 found that this molecule covalently binds to CARF (collaborator of ARF), a protein with context-dependent effects on proliferation (He et al., 2017). Through a series of biochemical and proteomic studies, CARF was found to interact directly with DVL in the nucleus and to potentiate the formation of the TCF/LEF-β-catenin complex and transcription of Wnt target genes (He et al., 2017). The authors concluded that NC043 could covalently bind to CARF and disrupt its interaction with DVL, which then led to inhibition of β-catenin-dependent Wnt signaling in the nucleus. These findings show potential for future clinical studies.

Finally, the molecule that has advanced the most along the bench-to-bedside path is a compound that disrupts the interaction of β-catenin and a ubiquitous transcription co-activator protein named CBP (for CREB Binding Protein). To discover this compound, the Kahn group screened a small molecule library of 5,000 molecules for the ability to inhibit TOPFlash in SW480 colon cancer cells (Emami et al., 2004). From this library, three molecules showed promising Wnt inhibition, and of these, ICG-001 was the most potent with an $IC_{50}$ of 3 μM (Emami et al., 2004). An affinity assay with a biotinylated version of ICG-001 found that it interacted only with CBP and not its highly related paralog p300. Further studies using ICG-001 synthesized with the radioactive isotope $C^{14}$ found that it specifically inhibited CBP (Emami et al., 2004). The authors determined that ICG-001 acted as a competitive inhibitor of the β-

catenin:CBP interaction, and when applied in cell culture, could inhibit the expression of Wnt target genes *CCND1* and *SURVIVIN* at the mRNA and protein levels (Emami et al., 2004). Furthermore, the authors showed that ICG-001 could selectively inhibit growth of human colon cancer cells (SW480 and HCT116) both *in vitro* and *in vivo (Emami et al., 2004)*. Prism Pharmaceuticals® recently developed a second generation β-catenin:CBP inhibitor, PRI-724, and readied this molecule for clinical studies in human patients. In a Phase Ia clinical study, PRI-724 exhibitedan acceptable toxicity profile and it could decrease *SURVIVIN* and *BRC5* expression in circulating tumor cells (El-Khoueiry et al., 2013). Of the 18 patients who were enrolled and treated in the Phase Ia clinical trial, adverse effects were limited to hyperbilirubinemia (2 patients, 11%), diarrhea (2 pts; 11%), hypophosphatemia (2 patients, 11%), nausea (1 patient, 6%), fatigue (1 patient, 6%), anorexia (1 patient, 6%), thrombocytopenia (1 patient, 6%), and elevated alkaline phosphatase (1 patient, 6%) (El-Khoueiry et al., 2013). Most recently, PRI-724 has successfully completed Phase Ib and is proceeding to Phase II clinical trials (NCT02413853) to test the efficacy of this agent in combination with standard cytotoxic chemotherapy and bevacizumab in metastatic colon cancer patients (McWilliams et al., 2015; Duchartre et al., 2016).

As discussed at the beginning of this section, the TCF/LEF-β-catenin transcriptional complex has been found to be tightly regulated in a context-dependent manner by different co-regulatory factors that assemble at Wnt response elements in promoters and enhancers of target genes **(Figure 1) (Levanon et al., 1998; Waltzer and Bienz, 1998; Hecht et al., 2000; Sun et al., 2000; Takemaru and Moon, 2000; Brantjes et al., 2001; Chodaparambil et al., 2014; Fiedler et al., 2015; van Tienen et al., 2017)**. These findings have led to a 'Wnt enhanceosome model'(Fiedler et al., 2015). In this model, multiple proteins assemble at Wnt Response Elements throughout the genome and use BCL9/B9L as scaffolding proteins to establish an enhanceosome complex. It is this enhanceosome complex that captures newly stabilized, nuclear-localized β-catenin. The binding of β-catenin then triggers structural rearrangements to BCL9, negates TLE repressive functions, and activates the TCF/LEF-β-catenin transcriptional complex and transcription of Wnt target genes (Chodaparambil et al., 2014; Fiedler et al., 2015; van Tienen et al., 2017). Based on this model, one potential strategy for future drug development would be to design small molecule inhibitors that disrupt key protein-protein-interactions that drive the activity of the Wnt enhanceosome complex.

**Targeting downstream gene programs regulated by TCF/LEF-β-catenin**

In addition to directly inhibiting the Wnt signaling pathway itself, downstream Wnt target gene programs regulated by TCF/LEF-β-catenin complexes also show potential as therapeutic targets. Interfering with specific Wnt-controlled gene programs allows for targeting key activities that cancer cells exploit and , a strategy that might minimize adverse effects in non-malignant cells. Wnt signaling has been shown to play key roles in cell proliferation, survival, and migration (metastasis), all of which are cellular phenotypes driven by aberrantly active Wnt gene programs that could prove to be effective drug targets in tumors (Reya and Clevers, 2005; Barker et al., 2009; Nguyen et al., 2009). However, Wnt target genes that drive proliferation such as *C-MYC* and *CCND1* are often viewed as un-druggable since they lack enzymatic activity that can be inhibited and because they are required in non-malignant cells (Musgrove et al., 2011; Rennoll and Yochum, 2015; Koh et al., 2016). Instead, recent efforts have started to investigate whether *C-MYC* binding factors and/or downstream *C-MYC* targets could be inhibited (Rennoll and Yochum, 2015; Koh et al., 2016). Drug development focused on inhibitors of cyclin dependent kinases (CDK) *CDK4* and *CDK6*, rather than directly targeting *CCND1*, has been successful and led to two FDA approved drugs (palbociclib and ribociclib) for patients with advanced breast cancer (Finn et al., 2015, 2016; Turner et al., 2015; Hortobagyi et al., 2016). Whether these drugs are also effective in Wnt-linked cancer such as colon cancer, is a potentially promising possibility that should be explored.

Angiogenesis, which plays an important role in tumor development and maintenance, is another gene program driven by Wnt signaling (Zhang et al., 2001; Gore et al., 2011). Wnt signaling triggers angiogenesis through activation of *VEGFA* and chemokine interleukin-8 as well as glycolysis (Zhang et al., 2001; Masckauchán et al., 2005; Schmidt et al., 2009; Gore et al., 2011; Pate et al., 2014). This process has been successfully targeted leading to the development of multiple anti-angiogenesis agents that are FDA approved and widely used in the clinic (Goel et al., 2012; Jain, 2014). One of the most commonly used anti-angiogenesis agents is bevacizumab (Avastin®), a VEGF monoclonal antibody that was approved by the FDA in 2004, and is now used in the treatment of multiple cancers known to have overactive Wnt signaling (glioblastoma, colon, and non-small cell lung cancer) (Hurwitz et al., 2004; Sandler et al., 2006; Vredenburgh et al., 2007; Aghajanian et al., 2012).

Recent work has shown that Wnt signaling can also play a direct role in regulating cancer cell metabolism (Lee et al., 2012, 2016; Pate et al., 2014; Sherwood et al., 2014; Sprowl-Tanio et al., 2016). For example, Wnt signaling controls cancer cell metabolism via regulation of pyruvate dehydrogenase kinase 1 (PDK1) and monocarboxylate transporter 1 (MCT1/*SLC16A1*) (Pate et al., 2014; Sprowl-Tanio et al., 2016). Regulation of both these genes results in cancer cells favoring aerobic glycolysis or Warburg metabolism, which potentiates a unique therapeutic target in Wnt-high cancers. Additionally, mathematical modeling and *in vitro* assays show that a combination therapy targeting Wnt signaling and glycolytic activity has a synergistic effect (Lee et al., 2016). Currently there are at least a dozen cancer metabolism drugs in clinical trials, some of which are promising - such as those targeting isocitrate dehydrogenase (IDH1) (Agios®), glutaminase inhibitor CB-839 from Calithera®, and fatty acid synthase inhibitor TVB-2640 from 3-V Biosciences® (Garber, 2016; Mullard, 2016). Also in early phase clinical trials is AZD3965 from AstraZeneca®, which inhibits the Wnt target and lactate transporter MCT-1/*SLC16A1* (Sprowl-Tanio et al., 2016). Currently, the biggest hurdle facing clinical trials for many of these metabolism inhibitors are toxicity issues and a lack of appropriate biomarkers to predict disease outcomes (Garber, 2016; Mullard, 2016). These considerations aside, efforts to targets cancer metabolism activities that are driven by Wnt signaling could be a promising new therapeutic goal.

**Conclusion**

In this review we have summarized recent developments in inhibiting Wnt signaling in the nucleus as a cancer therapy. We focused particularly on inhibitors targeting transcription regulation by interfering with β-catenin, TCF/LEFs, and CBP, and also briefly highlighted efforts to target downstream gene programs that are important for cancer development and progression. Targeting Wnt signaling in the nucleus, and in particular the transcriptional machinery that it directs, is therapeutically difficult and to date there has been only one agent (PRI-724) to proceed into Phase II clinical trials. Continued research of Wnt at the nuclear level and the downstream gene programs exploited by cancer cells (i.e. proliferation, migration, and metabolism) will provide better understanding of the potential future therapeutic targets which can hopefully benefit cancer patients.

**Acknowledgements**

This work was supported by NIH grants (CA096878 and CA108697), California CRCC award CRR-17-429379, P30-CA062203 NIH grant to the Center for Complex Biological Systems, and a P30CA062203 to the UC Irvine Chao Family Comprehensive Cancer Center. YL and ANH are supported by CA-T32 009054 from the National Cancer Institute. The work of GTC and MLW was supported by NIH Grant CA200298. ANH and MLW are supported by NIH grant CA177651. ANH is supported by NSF grant DGE-1321846. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Science Foundation, National Cancer Institute, or the National Institutes of Health.

**Author Contributions**

YL, ANH, and MLW wrote the manuscript text. GTC designed and illustrated the figures.

**Conflict of Interest**

The authors declare no conflicts of interest.

**Nomenclature of Targets and Ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

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