

Pharmacologic regulation of Wnt/ β -Catenin Signaling in Stem and Progenitor Cells

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Abstract

Pharmacologic regulation of Wnt/ β -Catenin Signaling in Stem and Progenitor Cells

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The Wnt/ β -Catenin signaling pathway is a key regulator of proliferation, differentiation, and programmed cell death in stem and progenitor cells. Misregulated or aberrant signaling events in the pathway contribute to the pathology of diseases such as cancer and neurodegeneration. It stands to reason that if we can monitor and influence Wnt/ β -Catenin signaling in stem and progenitor cells with sufficient resolution, it will be possible to design rationally targeted therapies. The body of work presented in this thesis contributes to the realization of this goal through discovery of molecular agents for controlling Wnt/ β -catenin signaling, examination of the biological mechanisms linking this pathway to disease, and identification of cases where the pharmacological perturbation of Wnt/ β -Catenin signaling may have therapeutic utility.

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List of Abbreviations

hESC - human embryonic stem cell

pBAR - plasmid (DNA construct containing) β -Catenin Activated Reporter

FDA - (United States) Food and Drug Administration

NIH - National Institutes of Health

HTS - High Throughput Screen

PCR - Polymerase Chain Reaction

aNPC - Adult Neural Progenitor Cell(s)

ALS - Amyotrophic Lateral Sclerosis

CNS - Central Nervous System

TBI - Traumatic Brain Injury

Preface

This thesis is an encapsulation of the research that I conducted over the last six years under the mentorship of Dr. Randall Moon. The goal of my graduate work was to elucidate how Wnt/ β -catenin signaling directs behavior in stem and progenitor cells. Within this realm, I chose to investigate contexts in which findings may inform novel therapeutics. I selected three journal articles to include within this thesis, each directed towards this goal. My role in the first two papers was in the assay development, discovery, analysis, and hit validation steps. In the final paper, of which I am the primary author, I was responsible for making decisions in the direction of the project, and attempted to use guiding principals that I had established throughout my time in the Moon lab.

Acknowledgements

I would like to thank my advisor Randall Moon for his support and guidance and for providing me with the resources to pursue my interests in biological research. I'd also like to thank my committee members for their time, consideration, and patience in seeing that my work proceeded from proposal to execution. I am forever indebted to the members of the Moon lab, past and present, for teaching me how to properly run experiments and for providing useful perspective.

1 - Introduction

1.1 - Background

1.1.1 - Stem Cell Signaling and Disease

Stem cells are cells that divide both symmetrically and asymmetrically. This means that populations of stem cells hold the capabilities for self renewal, as well as production of differentiated cell types of various lineages (NIH, 2009). Human embryonic stem cells (hESCs) reside within developing human embryos and are pluripotent. These cells can differentiate into any of three germ layers, and ultimately are capable of yielding any of the cell types found in the body. In addition to embryonic stem cells, there are stem cells that reside in certain tissues during adulthood. These cells may be lineage restricted (multipotent), as opposed to pluripotent. Adult stem cells are also referred to as somatic stem cells or progenitor cells depending on their type (Young and Black, 2004). Adult stem cells are responsible for maintaining homeostasis in tissues. This is especially the case in areas with rapid cell turnover such as bone, hematopoietic tissue, hair follicles, and intestinal crypts (Potten, 1998) (Morris et al., 2004–4AD).

Stem cell populations in both developing and adult organisms are sometimes found within specialized subcompartments called niches (Scadden, 2006–6AD). Stem cell niches house stem cells as well as supporting cells. Supporting cells within niches are responsible for providing nutrients to stem cells, and also can provide signals that guide stem cell behaviors. Among these behaviors are proliferation, induction of differentiation, and lineage specification. At any given time, the mixture of signaling molecules present in the microenvironment of the niche guides stem cell behavior and affects the identity and rate of new cell formation (Morrison and Spradling, 2008).

Although we possess only a partial understanding of how these signals are integrated to regulate key behaviors in stem cells, it is clear that when signaling becomes misregulated there are often deleterious effects. Alterations in proliferation and differentiation that are

caused by genetic or environmental factors contribute to the pathology of a number of diseases. In cases where a cell type that is necessary for maintaining healthy tissue is no longer produced, tissue degeneration can occur. Alternatively, stem cell proliferation gone unchecked may spur tumorigenesis. A subfield of cancer biology has formed to investigate the hypothesis of cancer stem cells (Reya et al., 2001–11AD). This hypothesis holds that there are stem cells within a tumor that possess indefinite potential for self-renewal, and that the same pathways regulating self-renewal in normal stem cells have similar roles in cancer stem cells.

Given the importance of signaling molecules in guiding stem cells towards either healthy or disease phenotypes, a major goal of current research is to better understand the signals that influence stem cell decisions. This may aid in the development of novel therapies for these diseases. Moreover, if we possess pharmacological tools to direct stem cell behavior it may be possible to repair diseased tissue using cells derived from the patient or a donor.

1.1.2 - Wnt/ β -Catenin Signaling in Stem Cells

In the ensuing decades since the discovery of Wnt signaling, this pathway has been determined to play a central part in most aspects of embryonic development as well as in the homeostasis of adult tissues (Clevers, 2006). Wnts are a family of secreted glycoproteins that act as ligands for Fzd transmembrane receptors. Wnts were first discovered for their ability to regulate cell polarity in *Drosophila* and to induce body axes in *Xenopus* during development (reviewed in Chien et al., 2009a). Wnt/ β -catenin signaling, which is referred to as canonical Wnt signaling, involves the accumulation of β -catenin protein in the cytosol following membrane receptor activation by canonical Wnt ligands. β -catenin is then translocated to the nucleus where it binds a set of transcription factors, ultimately driving expression of various target genes. (Angers and Moon, 2009) In contrast, β -catenin independent, or non-canonical, Wnt signaling is thought not to direct gene expression.¹

¹The main focus of this thesis is on Wnt/ β -Catenin signaling, and unless otherwise noted 'Wnt/ β -Catenin' may heretofore be used interchangeably with 'Wnt' within this text.

1.1.3 - Wnt in Cancer Tumorigenesis

Misregulation of the Wnt pathway has been linked to the formation of different types of tumors. A well studied example of this is in colorectal cancer, where mutations in the gene *APC* are the most frequent cause (Korinek et al., 1997). *APC* is one of the proteins responsible for degrading β -Catenin when the pathway is not active. When *APC* cannot function there is an abnormally high level of Wnt signaling that causes overexpression of growth promoting genes (Najdi et al., 2011). Mutations in other core Wnt pathway genes *AXIN1*, *CTNNB1*, and *TCF7L2* have also been linked to tumor formation. Aberrations in Wnt signaling have been identified as contributing to pancreatic, mammary, ovarian, and skin cancers (Anastas and Moon, 2013).

In melanoma, as opposed to colorectal cancer, a high level of Wnt signaling was found to correlate with improved patient survival (Chien et al., 2009b). Melanoma tumors are commonly caused by mutations in the ERK signaling pathway, the most frequently observed being *BRAF V600E* mutation. It was discovered that Wnt has a high level of crosstalk with hyperactivated ERK signaling in melanoma cells (Biechele et al., 2012). It should also be noted that a high level of Wnt/ β catenin signaling has been found to induce tumorigenesis and metastasis in melanomas dependent on mutational status (Delmas et al., 2007) (Damsky et al., 2011).

A more in depth explanation of Wnt in melanoma is included in chapter 2 of this thesis.

1.1.4 - Wnt in cultured hESCs

Wnt governs the transition between self renewal and spontaneous differentiation in hESCs. It was initially reported that active endogenous Wnt signaling is necessary for embryonic stem cells to maintain an undifferentiated state (Sato et al., 2004–1AD). However, this finding was later determined to be specific to culture conditions used in the study. Endogenous Wnt signaling has since been shown to be dispensable for self-renewal, while activation of the pathway with exogenous Wnt leads cells to undergo spontaneous differentiation (Davidson et al., 2012). The conflicted nature of these findings speaks to the complexity of hESC culture, where multiple signaling pathways are highly dependant upon

one another. It has been suggested that the investigation of novel small molecule inhibitors of Wnt/ β -Catenin signaling may help to resolve this (Blauwkamp et al., 2012).

The development of protocols for directed hESC differentiation is an active area of research. Efforts to control when and how hESCs differentiate in culture are central to a number of novel therapeutic strategies. Directed differentiation protocols have been formulated that utilize manipulation of the Wnt pathway in order to produce myocardial cells (Paige, 2010), neuronal cells (Dhara and Stice, 2008), and retinal pigment epithelium cells (Li et al., 2009). Development of directed differentiation protocols require both the knowledge of how Wnt controls lineage specification, and tools that can be used to specifically regulate the pathway.

The effects of Wnt signaling in hESC differentiation are discussed in chapter 3 of this thesis.

1.1.5 - Wnt in Adult Neurogenesis

Until the 1990s, it was not widely accepted that cells continue to divide throughout adulthood in mammalian brains. This changed when number of studies using cell cycle markers identified dividing progenitor populations that reside in the certain brain regions. Adult neural progenitor cells (aNPCs) were found to generate new functioning neurons in various animal models (Gage, 2002). As with other adult stem cell populations, depletion of aNPCs was found to contribute to disease. For example, following traumatic brain injury, a drop in aNPC population is linked with outcomes like post traumatic stress disorder and memory loss. Genetic factors that negatively affect adult neurogenesis contribute to the etiology of various CNS disorders, including Alzheimer's and Schizophrenia (Reif et al., 2006) (Brinton and Wang, 2006).

The Wnt/ β -catenin pathway is a key regulator of adult hippocampal neurogenesis (Lie et al., 2005). Enhanced Wnt/ β -catenin signaling is sufficient to increase production of neurons from aNPCs. Moreover, Hippocampal neurogenesis is blocked in adult rats by a dominant-negative Wnt, which leads to deficits in spatial and object recognition memory. (Jessberger et al., 2009) In the normal aging process, secretion of negative regulators of

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Wnt signaling was found to increase throughout the lifespan of mice, and this correlated with a reduction in neurogenesis (Seib et al., 2013–2AD) (Jang et al., 2013–2AD).

A more detailed account of Wnt in adult neurogenesis is presented in chapter 4 of this thesis.

1.1.6 - Pharmacologic regulators of Wnt/ β -Catenin signaling

In spite of the fact that misregulation of the Wnt pathway has a well known role in cancer and degenerative diseases, there currently are no clinically approved therapies that specifically target Wnt signaling (Zimmerman et al., 2012). However, promisingly there are a handful of such compounds currently undergoing clinical trials (Hitt, 2013).

There are numerous difficulties that have stood in the way of progress towards developing Wnt targeted drugs. For one, progenitor cell populations such as in the intestine and in germinal regions of the brain require Wnt signaling for normal function, so inhibiting the pathway in order to block the growth of a tumor may not be clinically feasible (Watanabe and Dai, 2011). Another problem is the interconnectedness of Wnt pathway components with other signaling pathways. For example GSK3, which is a kinase and thus relatively easier to target with small molecules, is also integral to AKT signaling and performs an additional role in synthesizing glycogen (Takahashi-Yanaga, 2013).

Chapters 2 - 4 of this manuscript each discuss pharmacologic regulators of the Wnt pathway.

1.2 Approach

1.2.1 Discovering Small Molecule Regulators of Wnt/ β -Catenin Signaling

In the research presented in chapters 2-4, I looked to discover small molecule regulators of the Wnt/ β -catenin pathway. I was particularly interested in small molecules because of their applicability to both basic research and clinical drug development. As opposed to genetic approaches such as siRNAs and shRNAs to induce a gain or loss of function in cell signaling, small molecules can be included in an experimental paradigm with relatively

little overhead and higher relative predictability of effect (Weiss et al., 2007). This result carries over when administering small molecules to animals, which tends to be more efficacious than genetic materials. Although the delivery of RNAi molecules *in vivo* is possible, studies employing this method have relied upon sophisticated techniques and instrumentation (Beronja et al., 2010–7AD). Finally, small molecules have a long proven track record in treating human diseases.

Consistent with my overall goals, small molecules are highly amenable to discovery via automation. The use of arrayed libraries in microplates allows for high-throughput screens (HTS) to be performed by automated equipment, rather than being tested individually by hand (Sundberg, 2000). The contents of these libraries can be produced from curated lists, such as in the screen discussed in chapters 2 and 4 of this thesis. Libraries can also be produced combinatorially around a scaffold or particular pharmacophore (Thompson and Ellman, 1996), as is used in chapter 3.

Another reason that I prioritized the discovery of small molecule regulators of Wnt/ β -Catenin signaling is that the small molecules we report on serve as deliverables in and of themselves alongside journal articles. This means that future projects have a reliable physical starting place from which to conduct experiments. The molecules discussed in these works are commercially available, and in some cases are already approved for human use. Thus, the time it takes for another group to begin experiments based off of our findings will be minimized, and ongoing studies of these molecules such as in clinical trials will be able to draw inference from our projects.

1.2.2 Understanding Biological Mechanisms through Cell Signaling Events

The projects in which I participated attempt to further our understanding of the mechanisms of cell signaling. In each case, I used Wnt/ β -Catenin signaling as an entry point from which to address biological mechanisms that govern behavior. This entailed selectively monitoring and perturbing Wnt signaling. In order to monitor the pathway I made use of genetic reporter constructs whose expression are driven by nuclear β -Catenin. These were pBAR in cultured cells (Biechele and Moon, 2008) and BAT-GAL in mice

(Maretto et al., 2003). To determine the role of Wnt in homeostatic and disease processes, I perturbed Wnt signaling while monitoring behaviors such as cell death, proliferation, induction of differentiation, and lineage specification.

The lack of Wnt pathway directed drugs can be blamed, in part, on the need for more feasible drug targets. One way to find more targets is to seek to discover additional protein regulators of the pathway. To this end, I monitored Wnt signaling while perturbing candidate interactors, in order to find where multiple pathways may cross-talk with one another. In the work presented in chapters 2 and 4, we attempted to map the previously unknown connections between drug targets HMGCR and GRM1, and the Wnt signaling pathway. In chapter 3 of this thesis, biochemical assays revealed that the molecule under investigation was able to inhibit Wnt through its action on TNKS.

1.2.3 Exploiting Cell Signaling to Inform Novel Therapeutics

As a natural complement to learning about the role of Wnt/ β -Catenin signaling in disease and discovery of small molecules, an aim of my work was to gather pieces of pre-clinical evidence. This meant identifying opportunities where perturbation of Wnt signaling in stem and progenitor cells may help to inform the development of rational therapeutics.

An example of this in my work is in the context of metastatic melanoma. Until the FDA approval of the mutant BRAF inhibitor vemurafenib in 2011, this disease had no effective drug treatment. We investigated the possibility that small molecules that regulate Wnt signaling could be efficacious in this disease. Ultimately this helped to developed new inroads for the development of melanoma therapies. Our investigation of Wnt in melanoma was able to help explain how mutant *BRAF* contributes to disease pathology, and has provided clues as to how some patients' resistance to vemurafenib may be overcome (Biechele et al., 2012).

Another example of this approach is to attempt to repurpose drugs that had already passed through human safety studies. This has been widely discussed as a strategy that may successfully speed up development of therapies, and the NIH has promoted this approach in collaboration with the pharmaceutical industry (Huang et al., 2011). Chapters 2 and 4 of

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this thesis present investigation of previously undocumented effects FDA approved compounds.

2 - Chemical-genetic screen identifies riluzole as an enhancer of Wnt/ β -catenin signaling in melanoma

2.1 - Introduction

Upon joining the Moon lab, I began to analyze a dataset that came as a result of a high-throughput small molecule screen. This screen was performed by lab members Travis Biechele and Nathan Camp, in collaboration with Dr. Stephen Haggerty of the Broad Institute. The screen was conducted using a cell line that had been lentivirally transduced with the pBAR-Luciferase construct, a robust genetic reporter of β -Catenin signaling (Biechele and Moon, 2008). The motivation for this screen came, in part, from the finding by Dr. Andy Chien's group that enhanced Wnt/ β -catenin signaling correlates with improved outcome among melanoma patients (Chien et al., 2009b). The Moon and Chien labs were interested to identify small molecules that activate the Wnt pathway in melanoma cells, and to see whether this activation yielded beneficial results in models of the disease. We were most interested in molecules with clinical translatability.

The library of molecules used in the screen performed at the Broad Institute included ~2,000 compounds that had previously been approved for human use in the United States, Canada, or the European Union. The intention in including human-experienced compounds in the screen was twofold. First, in pursuit of compounds that are clinically feasible there is typically a high rate of attrition in the steps between HTS discovery and generation of lead compounds (Bleicher et al., 2003). Biechele and Camp looked to avoid this by focusing on compounds that already had proven safety and efficacy in humans. Second, these human-experienced compounds had been well researched prior to their use in the screen, meaning that hits were likely able to be matched with known mechanisms of action.

1. While the Broad Institute provided the facilities for Moon lab members to conduct the physical screen, we were left to our own devices in the analysis of the raw dataset. To proceed ahead with the project, it was imperative that we formulate a plan for hit prioritization, and secondary follow up validation. The majority of my work on this

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project was focused on these tasks. In particular, I was interested in combining statistical and bioinformatic approaches in order to make an informed decision about where to focus research energy.

To do this, I wrote MATLAB scripts to compute statistical scores that are appropriate when considering large datasets (Malo et al., 2006). I incorporated categorical variables into the sorting and filtering process such as drug approval status across government agencies. I also attached semantic data, mined from publicly available databases such as Drugbank (Wishart et al., 2006) and STITCH (Kuhn et al., 2008), about the known targets and mechanisms of hit compounds.

Once we had filtered and sorted the hit list we began to perform secondary validation steps. It would have been prohibitively expensive to perform *in vivo* tumor forming assays with mice for each of the dozens of compounds that passed the initial filtering process. As an orthogonal approach to pBAR, I relied on quantitative PCR analysis of Wnt target genes. This was necessary in order to eliminate false positives and identify the candidate hits showing the most robust activation of the Wnt pathway. Ultimately we decided to pursue riluzole, an inhibitor the glutamate receptor GRM1 indicated to treat ALS, as the primary target of our inquiry. We based this choice on a combination of riluzole's results in secondary validation, as well as our perceived feasibility of the compound for clinical use that came from bioinformatic analysis.

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2.2 - Journal Article: Chemical-genetic screen identifies riluzole as an enhancer of Wnt/ β -catenin signaling in melanoma

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2.2.1 - Summary

To identify new protein and pharmacological regulators of Wnt/ β -catenin signaling, we used a cell-based reporter assay to screen a collection of 1857 human-experienced compounds for their ability to enhance activation of the β -catenin reporter by a low concentration of WNT3A. This identified 44 unique compounds, including the FDA-approved drug riluzole, which is presently in clinical trials for treating melanoma. We found that treating melanoma cells with riluzole in vitro enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease cell proliferation. Furthermore riluzole, like WNT3A, decreases metastases in a mouse melanoma model. Interestingly, siRNAs targeting the metabotropic glutamate receptor, GRM1, a reported indirect target of riluzole, enhance β -catenin signaling. The unexpected regulation of β -catenin signaling by both riluzole and GRM1 has implications for the future uses of this drug.

2.2.2 - Highlights

A cell-based screen identified 44 compounds that enhance WNT/ β -catenin signaling
Riluzole, approved for treating ALS, was found to enhance WNT/ β -catenin signaling
Riluzole, in clinical trials for melanoma, decreases melanoma metastases in mice GRM1 negatively regulated WNT/ β -catenin signaling in melanoma cells

2.2.3 - Introduction

Wnt proteins act as ligands for members of the Frizzled family of serpentine receptors and for the LRP5/6 coreceptors. Activation of the pathway results in β -catenin (CTNNB1) stabilization and nuclear accumulation. Aberrant Wnt/ β -catenin signaling arising from

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either hyper- or hypoactivation has been linked to numerous clinical conditions, most notably cancers (Moon et al., 2004). Several types of cancer have been linked to mutations in core Wnt/ β -catenin pathway genes that result in constitutive ligand-independent activation of the pathway (Moon et al., 2004). However, as described below for melanoma, elevation of Wnt/ β -catenin signaling correlates with or may promote favorable biological or clinical outcomes.

Wnt/ β -catenin signaling is a major regulator of melanocyte differentiation (Thomas and Erickson, 2008), so it is not surprising that this pathway is also involved in the pathogenesis of malignant melanoma. In a mouse model of malignant melanoma, Wnt/ β -catenin signaling itself is insufficient for melanocyte transformation, but activation of this pathway can work synergistically with active Ras signaling to promote tumor formation (Delmas et al., 2007). By contrast, several studies using patient-derived tumor samples have reported that melanoma progression is associated with the loss of nuclear β -catenin, a clinical marker of Wnt/ β -catenin activation. Furthermore, improved survival is seen in patients with higher levels of cytosolic or nuclear β -catenin (Bachmann et al., 2005, Chien et al., 2009, Kageshita et al., 2001 and Maelandsmo et al., 2003), suggesting that active Wnt/ β -catenin signaling in patients predicts improved prognosis. Interestingly, activation of Wnt/ β -catenin signaling by Wnt3a in a mouse melanoma model results in decreased proliferation *in vitro* and *in vivo*, along with increased expression of melanocyte differentiation markers usually lost with melanoma progression, pointing to potential mechanisms that might explain the improved prognosis of patients with elevated β -catenin (Chien et al., 2009). Collectively, these observations underscore the potential importance of determining whether any existing approved drugs enhance Wnt/ β -catenin signaling and whether they may have therapeutic benefit in treating melanoma.

In the present study, we used a cell-based screen to identify several small molecule enhancers of Wnt/ β -catenin signaling. We focused on one enhancer compound, riluzole, an FDA approved therapeutic for amyotrophic lateral sclerosis that is under investigation in clinical trials as a melanoma therapy (Yip et al., 2009).

2.2.4 - Results and Discussion

Using the β -catenin-activated luciferase reporter (BAR) (Biechele and Moon, 2008) (see Figure S1A available online) in a cell-based assay, we screened for small molecules that enhance the activation of the reporter by exogenous WNT3A (Figure 1 inset). We optimized the WNT3A stimulus to obtain an EC20 (Figure S1B) in HT22 neuronal cells and screened a library of 1857 human experienced chemicals (1500 unique). We identified 47 (44 unique) chemicals that enhanced the WNT3A stimulus greater than 1.5-fold (Figure 1A; Supplemental Database), several of which displayed a dose-dependent enhancement of Wnt/ β -catenin signaling upon rescreening (Figures S1C and S1E).

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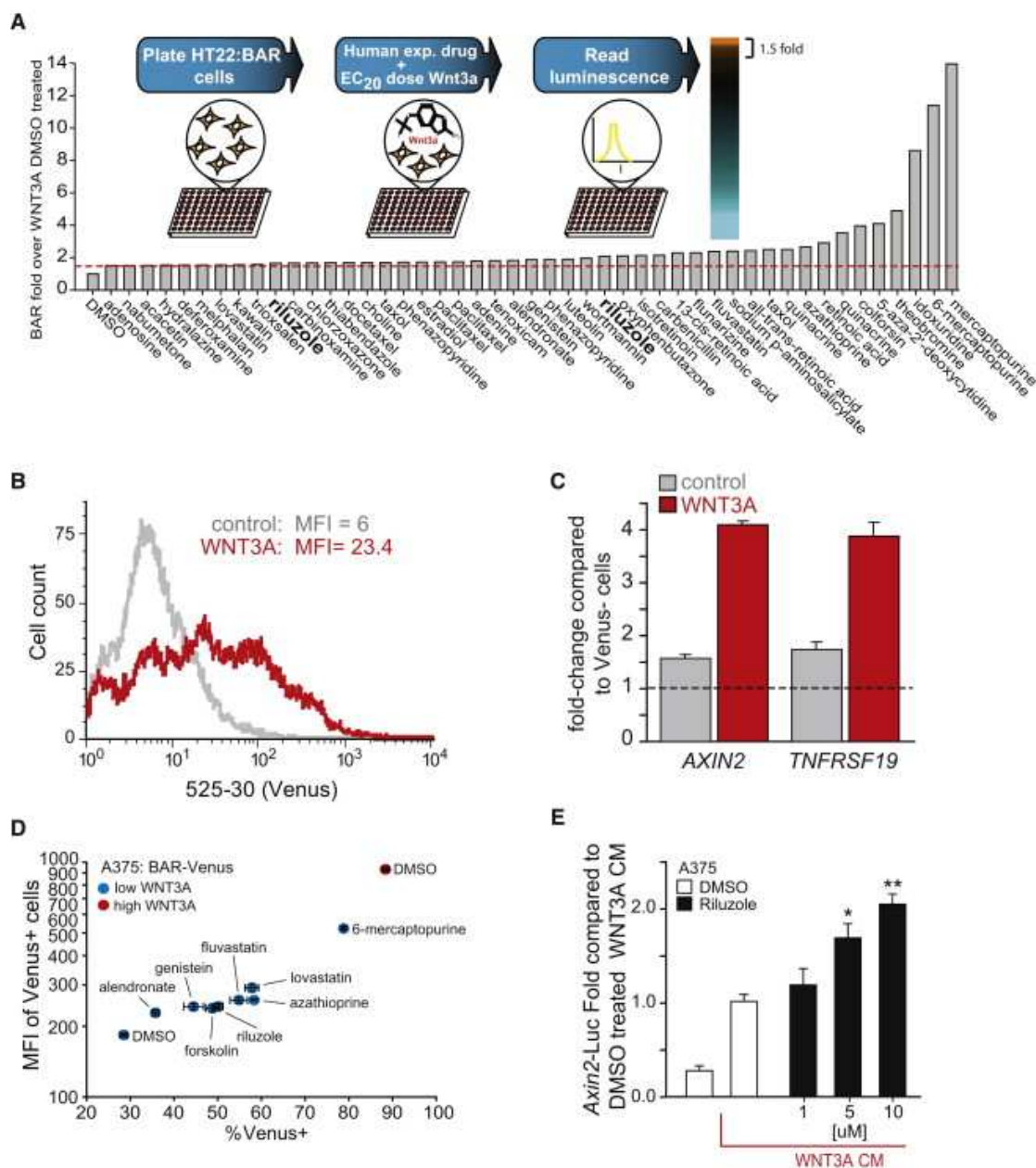


Figure 1. Chemical Screen Identifies Human-Experienced Small Molecule Enhancers of Wnt/ β -Catenin Signaling (A) A human-experienced collection of chemicals was screened in HT22 cells stably expressing BAR for enhancers of Wnt/ β -catenin signaling. (B) Histogram representation of flow cytometric analysis of A375 melanoma cells stably expressing the BAR-Venus reporter in the absence and presence of WNT3A. (C) A375:BAR-Venus cells were treated with control conditioned media (CM) or WNT3A CM and sorted for Venus-positive and -negative based on a gate set by A375 cells stably expressing a control reporter (*fuBAR-Venus*). *AXIN2* and *TNFRSF19* expression was determined by qRT-PCR. (D) Cells were treated with 10 μ M of each compound (100 μ M alendronate) for 18 hr, harvested, and analyzed by flow cytometry. (E) A375 melanoma cells transiently transfected with BAR were treated with WNT3A CM and DMSO or riluzole at the indicated doses. In (D) and (E) Data represent the mean value of triplicate experiments \pm SD. \square $p < 0.003$, \blacksquare $p < 0.0002$. See also Figure S1.

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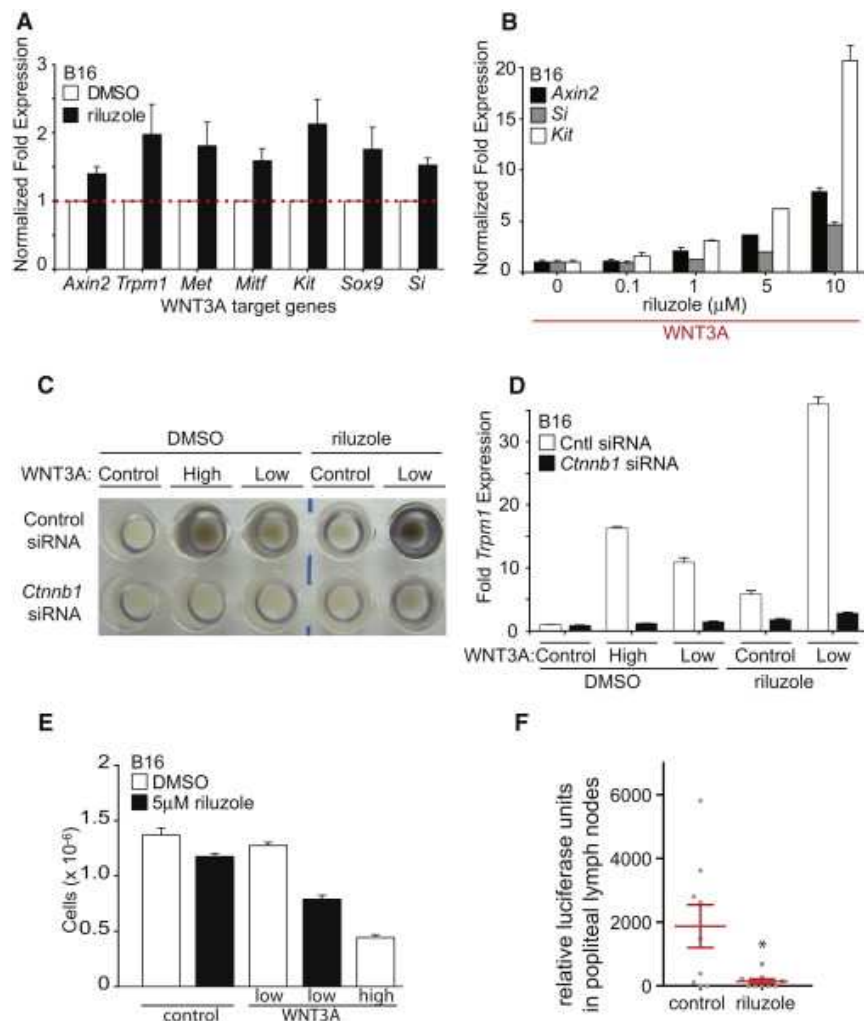
To rule out the formal possibility that some of the primary hits from the screen act directly on luciferase activity rather than on the Wnt/ β -catenin pathway, we used a second cell-based Wnt/ β -catenin reporter system in which Venus fluorescent protein is the reporter gene (Rekas et al., 2002). WNT3A stimulation of cells stably expressing this reporter promoted an increase in both the number of Venus positive cells and their mean fluorescent intensity (MFI) by FACS analysis (Figure 1B). To validate that Venus expression is tightly linked to β -catenin signaling, the expression of endogenous β -catenin target genes in Venus-positive and -negative cells was compared. Consistent with a positive correlation, the Venus-positive population of untreated cells, or the Venus-positive population of cells that had been treated with WNT3A-conditioned media prior to FACS sorting, showed elevated expression of the β -catenin target genes AXIN2 and TNFRSF19 when compared with Venus-negative cells (Figure 1C). In this assay, several primary screen hits enhanced the WNT3A-mediated increase in the percentage of Venus-positive cells and their MFI (Figure 1D).

Candidate hits from the primary screen were analyzed with respect to their being mentioned in the literature and in clinical trials databases, enabling us to generate a list of diseases where these bioactive molecules were being studied. We compared this list to the diseases being treated with lithium chloride, a classic inhibitor of GSK3 which is the only available patient-experienced drug that is known to activate Wnt/ β -catenin signaling. As a result of this analysis, we focused on the drug riluzole (brand name Rilutek) (Figure S1D), an FDA-approved drug used to slow the progression of amyotrophic lateral sclerosis (ALS) (Aggarwal and Cudkowicz, 2008). Riluzole is under intensive evaluation as a treatment for bipolar disorder, a condition where lithium chloride is a first-line treatment (Pittenger et al., 2008). Lithium is also being evaluated as a treatment for ALS (Fornai et al., 2008), which is also consistent with the possibility that these two unrelated compounds may regulate the same signaling pathway(s).

Riluzole enhanced Wnt/ β -catenin signaling in both the primary screen in HT22 neuronal cells (Figure S1E) and in adult hippocampal progenitor cells (Figure S1F). Furthermore, riluzole enhanced the stabilization and nuclear localization of β -catenin in U2OS cells (Figures S2A and S2B). As riluzole is in clinical trials for treatment of metastatic melanoma

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(Yip et al., 2009) and decreased Wnt/ β -catenin in clinical samples has been associated with melanoma progression (Chien et al., 2009), we focused on validating this hit and investigating its mechanism of action in the context of melanoma. We first demonstrated that riluzole enhances Wnt/ β -catenin signaling in a dose-dependent manner in A375 melanoma cells stably expressing BAR confirming the observations in neuronal cells (Figure S2C). As a control, we showed that riluzole does not activate a control reporter construct (fuBAR) in which the TCF/LEF sites required for responding to β -catenin have been mutated (Figure S2C). We next demonstrated that riluzole enhances the WNT3A-dependent activation of the promoter of a known direct target gene of β -catenin, Axin2 (Figure 1E). Moreover, riluzole, like WNT3A, upregulates several genes involved in melanocyte development (Trpm1, Met, Mitf, Sox9, Kit, and Si) alone (Figure 2A) and in synergy with exogenous WNT3A (Figure 2B).



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Figure 2. Riluzole Enhances Wnt/ β -Catenin Signaling to Promote Markers of Differentiation and Decrease the Proliferation of Melanoma Cells (A) B16 melanoma cells were treated with 10 μ M riluzole or DMSO for 24 hr and qRT-PCR was performed. All data were normalized to Gapdh expression and represent the mean value of triplicate experiments \pm SD. (B) B16 melanoma cells were treated with a low dose of WNT3A conditioned media and DMSO or the indicated doses of riluzole for 6 hr and profiled for the expression of Axin2, Si/gp100, and Kit by qRT-PCR. All data were normalized to gapdh expression, expressed as fold over the corresponding DMSO control, and represent the mean value of triplicate experiments \pm SD. (C and D) B16 melanoma cells transfected with control or Ctnnb1 siRNA were treated for 3 days with the indicated conditions and analyzed for pigmentation (C) and Trpm1 expression (D). The pigmentation data are representative of three independent experiments and Trpm1 expression represents the mean value of triplicate experiments \pm SD. (E) Riluzole synergizes with WNT3A to decrease the proliferation of melanoma cells. B16 cells were treated for 4 days in the indicated conditions and then harvested and counted. Data represent the mean value of six experiments \pm SD. (F) B16 cells were injected into footpads of C57BL/6 mice, and treatment with riluzole was initiated 1 week postinjection. Sentinel lymph nodes in the popliteal fossa adjacent to the injected foot were assayed for the presence of metastases as measured by Firefly luciferase. Bars represent the mean and standard deviation of ten mice for each group and indicate that tumors from mice treated with riluzole exhibited significantly decreased metastasis compared to control mice with no treatment (unpaired two-tailed t test) $\square p < 0.02$. See also Figure S2.

Consistent with the reported ability of WNT3A to elevate levels of transcripts involved in melanocyte differentiation (Chien et al., 2009), treating B16 melanoma cells with WNT3A leads to a dose- and β -catenin-dependent increase in cellular pigmentation (Figure 2C; Figure S2D). Treatment of these cells with riluzole also increases pigmentation, and does so in a β -catenin-dependent manner that is synergistically enhanced by cotreatment with WNT3A (Figure 2C; Figure S2D). Analysis of riluzole-treated B16 cells in which siRNAs have been used to deplete endogenous β -catenin showed that Wnt-3a and riluzole synergize in a β -catenin-dependent manner to elevate the expression of Trpm1 (Figure 2D).

We have previously demonstrated that elevated Wnt/ β -catenin signaling in melanoma cell lines cultured in vitro, and in melanoma patients, correlates with a reduction in cell proliferation (Chien et al., 2009), raising the question as to whether riluzole would also affect proliferation. Synergy between riluzole and WNT3A was observed in the reduction of proliferation in B16 cells (Figure 2E). We also observed a decrease in the proliferation of several human melanoma cell lines in the presence of riluzole (Figure S2E).

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We then tested whether riluzole inhibits melanoma progression *in vivo* using B16 melanoma cells implanted into the footpads of mice, followed by treating the mice with riluzole. There was a striking reduction in metastases compared with vehicle-treated mice, as measured by the detection of cells in the sentinel popliteal lymph node (Figure 2F). Collectively, the data show that riluzole both mimics and enhances the ability of WNT3A to promote differentiation of melanoma cells toward a more melanocyte-like state, that riluzole decreases the proliferation of melanoma cells *in vitro*, and that riluzole reduces the metastasis of melanoma cells *in vivo*.

To better understand the mechanism by which riluzole enhances Wnt/ β -catenin signaling in melanoma cells, we explored known signaling targets and pathways previously linked to riluzole. In a phase 0 trial of riluzole for treatment of melanoma, it was shown that tumors from patients treated with riluzole exhibited decreased phosphorylation of ERK (Yip et al., 2009). However, treatment of A375 human melanoma cells with riluzole resulted in no change in ERK phosphorylation (Figure S3A). Another possible mechanism for riluzole to enhance Wnt/ β -catenin signaling involves the activation of PKA through a riluzole-mediated increase in cAMP levels (Duprat et al., 2000 and Taurin et al., 2006). To test this mechanism, A375 cells were treated with riluzole or with forskolin, a small molecule known to activate adenylyl cyclase and thus PKA (Seamon et al., 1981). Cell lysates from forskolin, but not riluzole-treated cells, showed a significant enhancement in PKA-phosphorylated CTNNB1 as well as other protein substrates relative to controls (Figure S3B).

Previously, a mouse with a high incidence of spontaneous melanoma-like lesions was found to carry a mutation that led to elevated expression of metabotropic glutamate receptor 1 (GRM1) (Pollock et al., 2003). Riluzole has been reported to inhibit glutamate release and reuptake and thereby inhibit activation of metabotropic glutamate receptors (Doble, 1996). If riluzole enhances Wnt/ β -catenin signaling through a mechanism involving GRM1 then reducing GRM1 expression might enhance Wnt/ β -catenin signaling. To test this hypothesis, we transfected A375:BAR melanoma cells with two independent siRNAs targeting GRM1, or with siRNAs targeting CTNNB and AXIN1/2 as controls. Both GRM1 siRNAs depleted GRM1 transcript levels to approximately 10% of control siRNA

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were treated with WNT3A CM and several doses of riluzole (1, 5, 10, 20 μ M), A841720 (0.1, 1, 10, 20 μ M), LY456236 (0.1, 1, 10, 20 μ M), or Bay 36-7620 (0.5, 1, 5, 10 μ M). (C and D) B16 melanoma cells were treated for 3 days with the indicated conditions. Riluzole and Bay 36-7620 were used at 10 μ M final concentration. Cells were trypsinized and pelleted for imaging (C) followed by resuspension and 405 nm absorbance measurement (D). In (A), (B), and (D), data represent the mean value of triplicate experiments \pm SD. In (C), data are representative of three independent experiments. See also Figure S3.

2.2.5 - Significance

This article describes the identification and characterization of the FDA-approved drug riluzole as an enhancer of Wnt/ β -catenin signaling in melanoma cells. In melanoma cells, riluzole enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease proliferation. Furthermore, riluzole, like WNT3A, decreased metastases in vivo in a mouse melanoma model. Investigating the mechanisms of action of riluzole revealed that endogenous metabotropic glutamate receptor GRM1 but not GMR5 represses Wnt-mediated activation of β -catenin signaling in melanoma cells. Given that riluzole is in clinical trials for treating melanoma, our data that riluzole modulates β -catenin signaling, combined with prior data that β -catenin signaling has complex effects in different cancers, should stimulate further investigation into the use of riluzole in cancer therapies.

2.2.6 - Experimental Procedures

Full details of materials and experimental procedures used in this study can be found in the Supplemental Experimental Procedures that accompany this Brief Communication (available online).

2.2.7 - Acknowledgments

A.J.C. was supported by the National Cancer Institute (K08CA128565) and R.T.M. by the HHMI. This project was supported by the National Cancer Institute's Initiative for Chemical Genetics under Contract No. N01-CO-12400 and by the Department of Defense (W81XWH-07-1-0367). We thank Stephanie Norton for technical assistance.

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2.3 - Conclusion

Our publication identifying riluzole as a Wnt enhancer in melanoma resulted from a successful implementation of a high throughput screening campaign followed by thorough validation and analysis of mechanism. We were able to reuse this pattern in later projects, as seen in the proceeding chapters.

Typical for high throughput workflows, there was a high level of attrition between each filtering step. This meant that a great deal of the energy that I spent validating compounds did not end up contributing to the publication. The ramifications of this may be better accepted in commercial settings, where HTS had been used far more extensively than in academic biology labs. Having undergone the experience, I was able to form a perspective about the ideal ratio of effort to spend at each step of the workflow, which I carried on to later projects.

We attempted to use siRNAs as well as additional small molecules directed at effectors proximal to GRM1 in order to pinpoint the link between this receptor and Wnt signaling. However, the results of these experiments were not entirely consistent. Unfortunately, we did not come away from our investigation of riluzole with a clear understanding of how GRM1 inhibition feeds into the Wnt pathway. This came as a surprise, as we had chosen to focus on hit compounds with known targets particularly to aid in elucidation mechanism.

An upside to our use of HTS was that this primary screen was able to seed multiple investigations of interesting compounds. In addition to riluzole, in this screen we identified statins as enhancers of the Wnt pathway. This is covered in chapter 4 of this thesis.

3 - WIKI4, a novel inhibitor of tankyrase and Wnt/ β -catenin signaling

3.1 - Introduction

Following my participation in the riluzole investigation, I worked in collaboration with others in the Moon lab to discover additional Wnt regulators. The opening of the Quellos High Throughput Screening Facility at the University of Washington enabled our group to prototype and perform our own series of screens.

One such screen utilized a library of ~7,000 small molecules that were selected based on the presence of pharmacophores predicted to interact with the ATP binding pocket of protein kinases. So, although this library was made up of compounds that had not been previously tested for biological activity, it was thought that hit compounds would likely function through inhibition of kinases. As in other screens, we used the pBAR construct as our primary assay in order to identify compounds with either an activating or inhibiting effect on the Wnt pathway.

The Moon lab's interest in identifying kinase inhibitors that regulate Wnt signaling related to complementary studies that were concurrently underway in the lab. Within the time-period of the study presented in this chapter, we had run screens of pBAR cell lines using multiple siRNA libraries targeting all individual kinases. We also subjected these same cell lines to mass-spectrometry based quantitative phosphoproteomic analysis under a variety of conditions. Thus, we had gathered a sizeable amount of data describing kinase regulation of the pathway.

Given the importance of regulating Wnt in directed differentiation protocols of hESCs, we sought to identify small molecules that could inhibit Wnt in these cells. We also sought to address the lack of available molecules with the potential to inhibit Wnt in animal models. This instructed our decision to follow the molecule WIKI4 through the course of this investigation.

3.2 - Journal Article: WIKI4, a novel inhibitor of tankyrase and Wnt/ β -catenin signaling

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3.2.1 - Abstract

The Wnt/ β -catenin signaling pathway controls important cellular events during development and often contributes to disease when dysregulated. Using high throughput screening we have identified a new small molecule inhibitor of Wnt/ β -catenin signaling, WIKI4. WIKI4 inhibits expression of β -catenin target genes and cellular responses to Wnt/ β -catenin signaling in cancer cell lines as well as in human embryonic stem cells. Furthermore, we demonstrate that WIKI4 mediates its effects on Wnt/ β -catenin signaling by inhibiting the enzymatic activity of TNKS2, a regulator of AXIN ubiquitylation and degradation. While TNKS has previously been shown to be the target of small molecule inhibitors of Wnt/ β -catenin signaling, WIKI4 is structurally distinct from previously identified TNKS inhibitors.

3.2.2 - Introduction

Wnt family genes encode highly conserved secreted glycoproteins, which activate downstream signal transduction pathways important in development and tissue homeostasis. Wnts can signal through one of several pathways, including the conserved Wnt/ β -catenin pathway. The Wnt/ β -catenin pathway is activated by Wnt ligands binding to Frizzled serpentine receptors and to LRP5/6 co-receptors, leading to the post-translational regulation of the stability of β -catenin (encoded by CTNNB1) (reviewed in [1]). In the absence of a Wnt signal, cytosolic CTNNB1 is bound by the scaffolding proteins Adenomatous Polyposis Coli (APC) and AXIN1, and the kinases Casein Kinase 1 (CSNK1A1) and Glycogen Synthase Kinase (GSK). Sequential phosphorylation of CTNNB1 by CSNK1A1 and GSK3 leads to its recognition by a ubiquitin ligase protein complex and its subsequent degradation by the proteasome. Upon activation of Wnt/ β -catenin signaling, this

“destruction complex” is inhibited, resulting in accumulation of newly translated CTNNB1, which then translocates to the nucleus where it acts as a co-activator during transcription of target genes that ultimately lead to context-dependent changes in cell proliferation, specification, or differentiation.

Wnt/ β -catenin-dependent transcription plays critical roles in both embryonic development and in adults [2], [3]. Examination of mice and zebrafish that are transgenic for β -catenin-dependent reporters has revealed that β -catenin signaling is spatially and temporally regulated [4]–[9]. Not surprisingly, Wnt/ β -catenin signaling plays many roles in development, including patterning of all three germ layers [10]–[15]. In addition, we and others have shown that ectopic activation of the Wnt/ β -catenin pathway can drive differentiation of human embryonic stem cells (hESCs) towards mesodermal and endodermal lineages [16], [17]. Lastly, Wnt/ β -catenin signaling is activated by acute injury and functions in regenerative responses [18], as well as in diverse chronic diseases including cancers (colorectal cancer [19], liver cancer [20], [21], Wilms tumor [22], [23], lymphoma [24], [25], myeloma [26], [27], [28], leukemias [29], [30]) and neuropsychiatric diseases [31].

There have been a growing number of small molecule inhibitors of Wnt/ β -catenin signaling (reviewed in [32]), which at a minimum should provide tools for modulating the pathway in vitro. For example, Huang and colleagues have described a small molecule inhibitor of Wnt/ β -catenin signaling that works by inhibiting the adenosine di-phosphate (ADP) ribosylase protein, Tankyrase (TNKS) [33], [34], [35]. Inhibiting the activity of TNKS leads to elevation of levels of AXIN, thereby promoting the degradation of CTNNB1 and inhibiting Wnt/ β -catenin signaling [33], [34], [35].

In an effort to identify additional small molecule inhibitors of Wnt/ β -catenin signaling, we screened A375 melanoma cells stably transduced with a β -catenin-activated reporter (BAR). To ensure Wnt pathway-specificity, we cross-screened A375 cells containing luciferase reporters activated by different signaling pathways and eliminated those compounds that inhibited multiple pathways. Using this approach we identified a novel Wnt inhibitor, Wnt Inhibitor Kinase Inhibitor 4 (WIKI4), which effectively blocks Wnt/ β -

catenin reporter activity in diverse cell types, including cancer cells that display elevated β -catenin signaling due to activating APC mutations. WIKI4 inhibits the expression of Wnt target genes as well as the functional effects of Wnt/ β -catenin signaling in colorectal carcinoma cells and hESCs. We subsequently established that WIKI4 antagonizes Wnt/ β -catenin signaling via inhibition of TNKS activity.

3.2.3 - Materials and Methods

Reagents

The reporters described in this manuscript are lentiviral plasmids containing 12 binding sites for transcription factors downstream of the Wnt/ β -catenin (5'-AGATCAAAGG-3') (previously described in [36]), Nuclear Factor Kappa B (NF- κ B, 5'-GGGAATTTCC-3'), Transforming Growth Factor Beta (TFG β , 5'-AGCCAGACA-3'), and Retinoic Acid (RA, 5'-GGTTCACCGAAAGTTCA-3') signaling pathways which are each separated by distinct 5-base pair linkers. The transcriptional binding cassettes are located upstream of a minimal thymidine kinase promoter and the firefly open reading frame. Each reporter also contains a separate phosphoglycerate kinase promoter that constitutively drives the expression of a puromycin resistance gene. To engineer stable cell lines that express the reporters, cells were infected with un-concentrated virus, and selected with puromycin (2 μ g/mL).

H1 (WiCell) and H1-BAR hESC lines were maintained on irradiated MEF feeders in 20% Knockout Serum Replacement medium +8 ng/ml FGF2 (KSR medium) and passaged weekly using dispase as previously described [17]. NALM6 human pre-B cells (DSMZ) were grown in RPMI 1640 with 10% fetal bovine serum (FBS) and 55 μ M β -mercaptoethanol, A375 malignant melanoma cells (ATCC) were grown in RPMI 1640 with 5% FBS. DLD1 colorectal carcinoma (ATCC), SW480 colorectal carcinoma (ATCC), U2OS osteosarcoma (ATCC) cells were grown in DMEM/F12 with 10% FBS. Wnt3A conditioned medium (CM) and control L CM were generated as previously described [17] from L cells and L-Wnt3A cells (ATCC).

The following antibodies were used: AXIN1 (2087; Cell Signaling Technology), AXIN2 (2151; Cell Signaling Technology), CTNNB1 (9562; Cell Signaling Technology),

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S33/37T41P-CTNNB1 (9561; Cell Signaling Technology), S45P-CTNNB1 (9564; Cell Signaling Technology), GCTM2 (kind gift from Martin Pera, University of Melbourne, Australia; GCTM-2 antibody previously described in [37], [38], [39]), CD9 (mAB4427; Millipore), TUBB1 (T7816; Sigma-Aldrich), UBIQUITIN (SC-8017, Santa Cruz Biotechnology). The following compounds were used in this study: MG132 (474790, EMD Millipore), XAV-939 (S1180, Selleck Chemical), U0126 (U-6770, LC Labs), and WIKI4 (7990417, Chembridge). All sequences used for real-time PCR or siRNA transfection are listed in Table S1 or previously published [17]. All molecules used in the structure activity relationship analysis are detailed in Table S2.

High Throughput Small Molecule Screen

Screening was performed using the facilities of the Quellos High Throughput Screening Facility at the Institute for Stem Cell and Regenerative Medicine in Seattle, WA. Compounds dissolved in DMSO were obtained from Chembridge (a custom selection of 6,492 entities from Chembridge's KINASet library). For the primary screen, performed in duplicate, A375 malignant melanoma cells stably expressing BAR were cultured in growth medium (DMEM/5%FBS/1%antibiotic). 4000 cells per well were transferred to clear bottom 384-well plates (BD Falcon; Fisher Scientific 08-772-004) in 30 μ L of growth media using a Matrix WellMate (ThermoScientific). The following day 50 nL of each compound (final concentrations of 370 nM and 10 μ M) and 10 μ L of Wnt3A-conditioned media (EC20 dose) was transferred to the cells. On the third day, 10 μ L of resazurin (final concentration 0.1 mg/mL) was added to the cells, and after a three hour incubation viability was assessed by quantifying the fluorescent reduction product of resazurin using an Envision Multilabel plate reader (PerkinElmer). Finally, 5 μ L of Steady-Glo (Promega) was added to each well, and luciferase was quantified using the Envision Multilabel plate reader. The fold-increase over the background of DMSO controls for viability and luciferase was calculated. Inhibitors were chosen for further analysis if they inhibited Wnt3A-dependent luciferase production at the 370 nM concentration (normalized Steady-Glo fold change <0.5) but did not decrease resazurin reduction at the 10 μ M concentration (viability fold change >0.9). For the Z-factor calculation (Z'), we used the following equation [40]:

In this equation μ equals the mean calculated from the positive (c+) and negative (c-) control replicates, and σ equals the standard deviation calculated from the positive (c+) and negative control replicates (c-). For the secondary screening, compounds that satisfied our hit criteria were re-screened for their ability to inhibit stimulation of reporters for the Wnt/ β -catenin, NF- κ B, TGF β , and RA pathways stably expressed in A375 melanoma cells as described above.

Functional Cell Assays

For the DLD1 colony forming assays, single DLD1 cells were plated at 1000 cells per well (6-well) and cultured overnight in DMEM containing 0.5% FBS. The next day, compounds were added to the media, and the cells were subsequently cultured for ten days with refreshment of the media and compound occurring every two days. At the end of the culture period, the colonies in each well were counted.

For the flow cytometric analysis of cell surface markers in hESCs, H1-BAR-VENUS hESCs were seeded as small clusters on MEFs in KSR media at \sim 35,000 cells per square cm. The following day, the medium was replaced with 50% (vol/vol) conditioned medium (control L or Wnt3A CM) in KSR media with or without inhibitors, XAV-939 or WIKI4. DMSO served as a vehicle control for the compounds. The medium was replenished daily. After 6 days of treatment, hESCs were isolated as single cells with TrypLE Express (Invitrogen) and counted. 500,000 cells were immunolabeled with 100 μ l primary antibodies: GCTM2 (hybridoma supernatant, 1:2, a kind gift from Martin Pera, University of Melbourne, Australia; GCTM-2 antibody previously described in [37], [38], [39]) and CD9 (TG30 clone, 1:100, Millipore). Cells were then incubated with isotype-specific secondary antibodies (Invitrogen): goat anti-mouse IgM-Alexa 647 (1:100) and goat anti-mouse IgG2a-biotin (1:5000), followed by PE-Cy7-streptavidin (1:250). hESCs were resuspended in 140 ng/ml DAPI in KSR media, then passed through a cell strainer prior to analysis on a BD FACSCanto II flow cytometer. Results were quantified using FlowJo software. The percentage of GCTM2 and CD9 double-positive hESCs was determined from the DAPI-negative (viable), DsRED-positive gated population. DsRED is constitutively expressed in H1-BAR-VENUS cells, thus this gating strategy serves to exclude any MEFs.

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For the gene expression analysis in hESCs, total RNA was isolated using TRIZOL according to the manufacturer's protocol (Invitrogen). 2.5 μ g RNA was used for cDNA synthesis with RervertAid First Strand cDNA Synthesis kit (Fermentas). cDNA was diluted 100-fold, then used as template for quantitative PCR (2 μ l cDNA per 10 μ l reaction) using Applied Biosystems SYBR Green-based detection according to the manufacturer's protocol on a Roche Lightcycler 480 instrument. Duplicate reactions were performed for each sample. Transcript copy numbers were normalized to GAPDH for each sample, and fold expression over the untreated control was calculated for each gene of interest. Primer sequences are previously published [17] or listed in Table S1.

For all low-throughput siRNA experiments, siRNAs were reverse-transfected at a final concentration of 10 nM using RNAiMAX (#13778-075, Invitrogen) according to the manufacturer's instructions.

Biochemistry

For the compound wash-off experiments, ten million SW480 or DLD1 colorectal carcinoma cells were treated overnight with DMSO (D9170; Sigma-Aldrich), WIKI4, or XAV-939 at the concentrations indicated. Cells were then washed off and treated for one hour with the indicated treatments, and then lysed in RIPA buffer (50 mM Tris-cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 5 mM ADP-HPD and 5 mM N-ethyl maleimide). Lysates were immunoprecipitated overnight with the indicated antibodies and analyzed by western blot. The in vitro TNKS2 assay was acquired from commercial sources (80565; BPS Bioscience).

3.2.4 - Results and Discussion

Identification of WIKI as a Small Molecule Inhibitor of Wnt/ β -catenin Signaling

To make an assay for Wnt/ β -catenin signaling suitable for high throughput screening, we generated A375 melanoma cells stably infected with a β -catenin-activated luciferase reporter (BAR) [23], [36] and selected populations in which luciferase activity is increased at least 4,000-fold by WNT3A. We tested the robustness of our assay by calculating the Z-

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factor (Z') values [40] using probes that are known to enhance (U0126 [41], Riluzole [42], and GSK3B inhibitor IX [43]) or inhibit (XAV-939 [33]) Wnt/ β -catenin signaling (Figure S1A). For all control probes, we found the Z' values to be greater than .45 (Figure S1A), a value considered robust in high throughput screening assays [40]. Following validation of our assay, we then screened A375 melanoma cells at two concentrations of a small molecule library in the presence of a twenty percent effective concentration (EC20) dose of WNT3A. We focused on small molecules that reduced expression of the luciferase reporter at a low dose (330 nM) and that did not kill cells at a high dose (10 μ M) relative to controls treated with dimethyl sulfoxide (DMSO), with the expectation that these criteria would filter out compounds that inhibited BAR due to cellular toxicity. Five compounds met our criteria for further study by significantly decreasing Wnt/ β -catenin signaling without causing toxicity at either dose (Fig. 1A).

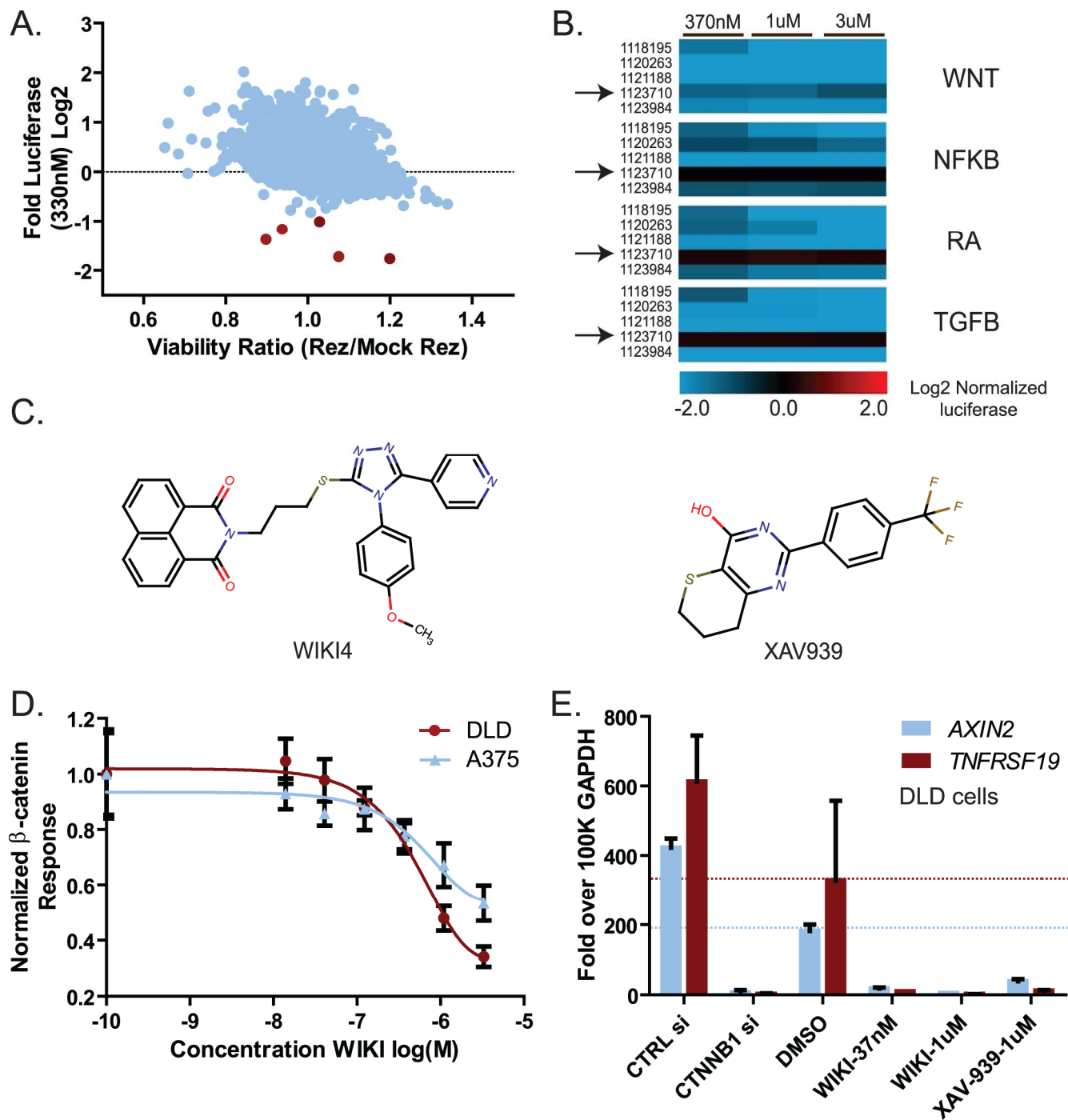


Figure 1. WIKI4 is identified as a novel small molecule inhibitor of the Wnt/ β -catenin pathway. (A) Scatter plot of a small molecule screen in human A375 melanoma cells stably expressing the β -catenin Activated Reporter (BAR) driving firefly luciferase with each dot representing a single compound. The red dots represent compounds that exhibited decreased luciferase signal (two standard deviations below the sample mean), and unchanged cell viability as measured by resazurin. (B) A heat map showing the effects of five Wnt/ β -catenin inhibitors on reporters for the Wnt/ β -catenin, Nuclear Factor Kappa B (NF- κ B), Retinoic Acid

(RA), and Transforming Growth Factor β (TGFB) pathways. WIKI4 (arrow) is the only compound that specifically inhibits Wnt/ β -catenin signaling. (C) Chemical structure of WIKI4 (left) and XAV-939 (right). (D) Dose response curves showing that WIKI4 inhibits β -catenin reporter activity in DLD1 colorectal carcinoma cells and Wnt-stimulated A375 melanoma cells. (E) Inhibition of the expression of the β -catenin target genes AXIN2 and TNFRSF19 by WIKI4 as assessed by quantitative PCR. DLD cells were transfected with CTNNB1 siRNA as a control 72 hours prior to harvesting for RNA; cells were treated with compounds or DMSO for 16 hours prior to harvesting. The experiments in (D) and (E) are representative of three independent experiments and the error bars represent standard deviation from four technical replicates

We next asked whether any of the five compounds preferentially modulated Wnt/ β -catenin signaling by comparing the repression of BAR in A375 cells relative to luciferase reporters for the Nuclear Factor Kappa B (NF- κ B), Transforming Growth Factor Beta (TGF β), and Retinoic Acid (RA) signaling pathways (Fig. 1B). Of the five candidate Wnt/ β -catenin inhibitors that we tested, WIKI4 (left panel, Fig. 1C) was the only inhibitor of BAR that did not also inhibit the reporters for NF- κ B, TGF β , and RA (Fig. 1B). Furthermore, WIKI4 has demonstrated activity in one of nine published assays (<http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2984337>), supporting our contention that WIKI4 is not a general inhibitor of activity in high throughput screening assays. We then demonstrated that WIKI4 inhibits Wnt/ β -catenin signaling in several other cell lines, including DLD1 colorectal cancer cells (Fig. 1D), NALM6 B cells (Figure S1B), U2OS osteosarcoma cells (Figure S1B) and hESCs (Figure S1C). In all cell types tested, we observed that WIKI4 potently inhibited Wnt/ β -catenin signaling and that its half-maximal response dose was \sim 75 nM.

We next investigated whether WIKI4 is sufficient to inhibit expression of Wnt/ β -catenin target genes in DLD1 colorectal carcinoma cells, which express a truncated form of the Wnt/ β -catenin inhibitor APC [44]. We found that incubation of DLD1 cells overnight with either WIKI4 or the structurally distinct TNKS inhibitor, XAV-939 (right panel, Fig. 1C) [33], resulted in decreased steady-state abundance of AXIN2, and TNFRSF19 (Fig. 1E), which is consistent with WIKI4 acting as an inhibitor of Wnt/ β -catenin signaling. Furthermore, we observed that WIKI4 is sufficient to inhibit WNT3A-dependent increases in the expression of AXIN2 and TNFRSF19 in hESCs (Figure S1D, S1E). Thus we have identified WIKI4 as a new inhibitor of Wnt/ β -catenin signaling that regulates the pathway in several cell types.

To determine which chemical groups in WIKI4 are required for its ability to inhibit Wnt/ β -catenin signaling, we next performed a structure activity relationship analysis (Figure S2). WIKI4 has a molecular weight of 522 and a calculated partition coefficient of 4.8, putting it near the limits of “druglikeness” by Lipinski’s Rule of Five [45]. WIKI4’s mass and complexity is greater than XAV-939 (Fig. 1C), and identification of small active WIKI4 analogs could provide more opportunities for modification while maintaining its druglike properties. To identify less complex WIKI4 analogs and to determine which portions of WIKI4 are required for activity, we searched for commercially available analogs. We queried the ZINC [46] and eMolecule (www.emolecules.com) databases and identified 62 WIKI4 analogs for further testing (Table S3). We assayed the Wnt/ β -catenin inhibitory activity of a subset of these compounds (Figure S2). Our results indicate that the traizole’s 4-pyridyl and 4-methoxyphenyl groups tolerate some modification, but the latter group could not be removed (Figure S2A). Additionally, substitution of the 1,8-naphthalimide group with a phthalimide group eliminated activity as did replacement of the 1,8-naphthalimide group with a methyl or phenyl group (Figure S2B).

WIKI4 Inhibits the Cellular Responses to Wnt/ β -catenin Signaling

We next asked whether cells treated with an effective dose of WIKI4 would show a reduction in Wnt/ β -catenin-mediated responses at the cellular level. As DLD1 colorectal cancer cells require β -catenin signaling for growth in limiting culture experiments [47], these cells provide an excellent functional model of the pathway in which to test small molecules. We found that WIKI4 inhibits growth of DLD1 cells relative to DMSO controls in media containing low serum (Fig. 2A). This result demonstrates that WIKI4 inhibits a known cellular response to Wnt/ β -catenin signaling.

Chapter 3 - WIKI4, a novel inhibitor of tankyrase and Wnt/ β -catenin signaling

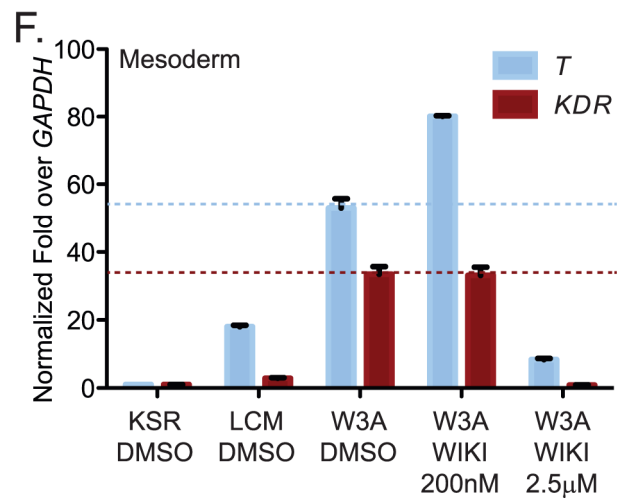
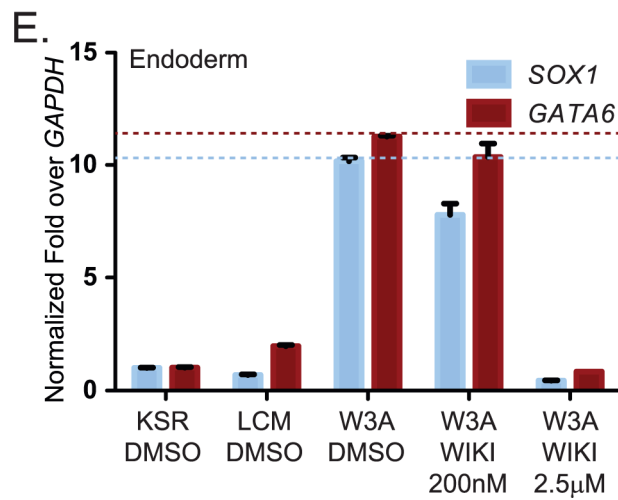
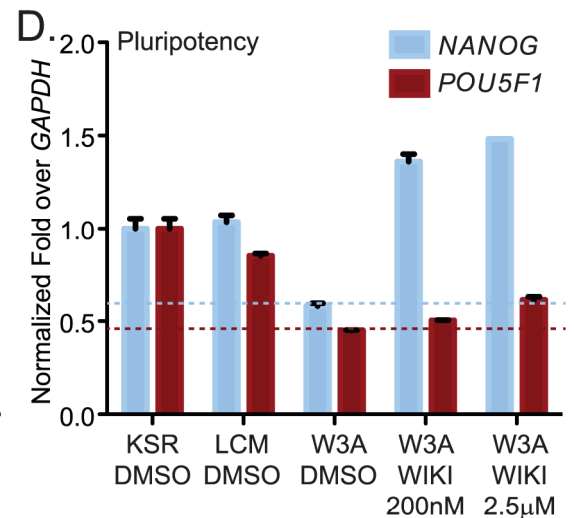
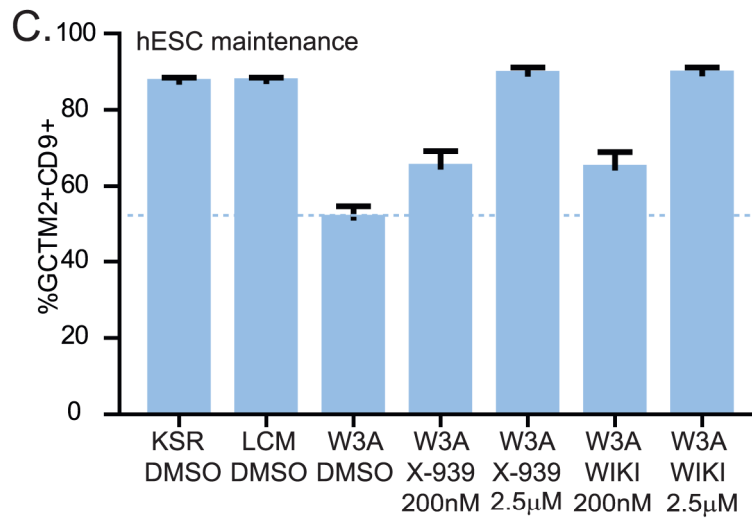
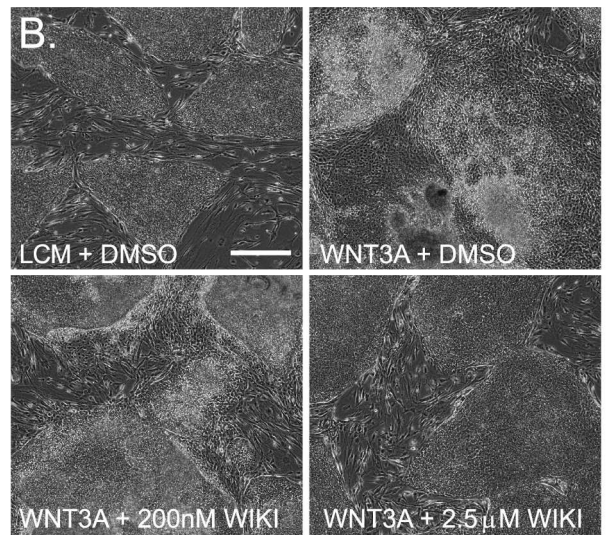
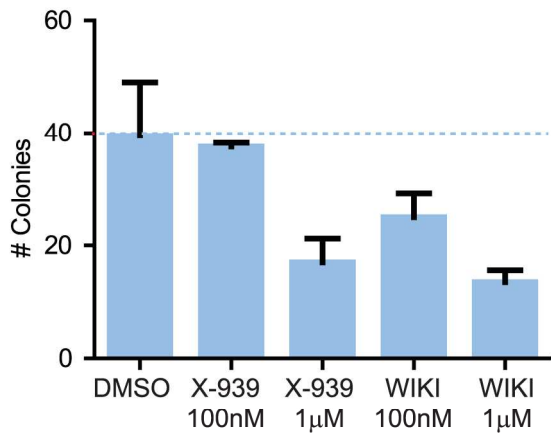


Figure 2. WIKI4 inhibits the functional outcomes of Wnt/ β -catenin signaling. (A) WIKI4 inhibits colony formation of DLD1 colorectal cancer cells. DLD1 cells were plated individually in 0.5% serum containing medium, and treated with the indicated concentrations of WIKI4 and XAV-939. This experiment is representative of three independent experiments and the error bars represent standard deviation of three technical replicates. (B-F) WIKI4 prevents Wnt3A-dependent differentiation of H1 human embryonic stem cells (hESCs). (B) Culturing hESCs for six days with Wnt3A causes marked morphological changes that are rescued by treatment with WIKI4. Scalebar = 500 μ m. (C) Treatment with WIKI4 prevents the decrease in co-expression of markers of undifferentiated hESCs following Wnt3A stimulus. hESCs were stimulated with the indicated treatments and expression of GCTM2 and CD9 was assessed by flow cytometry following six days of treatment. (D-F) The effect of WIKI4 treatment on the expression of genes that are altered during Wnt3A-dependent differentiation of hESCs was assessed by qPCR. hESCs were treated for the indicated conditions for six days, and then analyzed by qPCR for markers of undifferentiated stem cells (NANOG, POU5F1) (D), endoderm (SOX17, GATA6) (E), and mesoderm (T, KDR) (F). The data was normalized to 100,000 copies of GAPDH and plotted as a ratio to the untreated hESCs (cultured in KSR media). The data in the experiments presented in B-F are representative of three independent experiments and the error represents standard deviation of technical replicates. In B-F, LCM = control L cell CM, WNT3A = Wnt3a CM; both 50% (vol/vol) in KSR medium.

Given that cellular responses to Wnt/ β -catenin signaling are diverse and context-dependent, we next examined the effects of WIKI4 on hESCs. Activation of Wnt/ β -catenin signaling in hESCs alters their cell fate and causes them to differentiate into early mesoderm and endoderm lineage cells [16], [17]. Upon stimulation with Wnt3A for 6 days, hESC colonies exhibit overt phenotypic changes that include loss of compact colony structure (top panels, Fig. 2B), decreased co-expression of cell surface markers of undifferentiated hESCs (GCTM2 and CD9, Fig. 2C) and decreased steady-state RNA abundance of pluripotency genes (NANOG and POU5F1, Fig. 2D). Additionally, treatment of hESCs with WNT3A leads to increased expression of genes associated with endoderm (SOX17 and GATA6, Fig. 2E) and mesoderm (T and KDR, Fig. 2F) differentiation. We found that in hESCs treated with both Wnt3A and WIKI4, the WNT3A-dependent effects that we typically observe on colony morphology (bottom panels, Fig. 2B), expression of cell surface markers (Fig. 2C) and expression of markers of pluripotency and differentiation (Fig. 2D, 2E, 2F) were eliminated. We conclude that WIKI4 inhibits Wnt/ β -catenin-mediated processes in hESCs, as well as in DLD1 cells, suggesting that WIKI4 acts on a conserved component of the Wnt/ β -catenin signaling pathway.

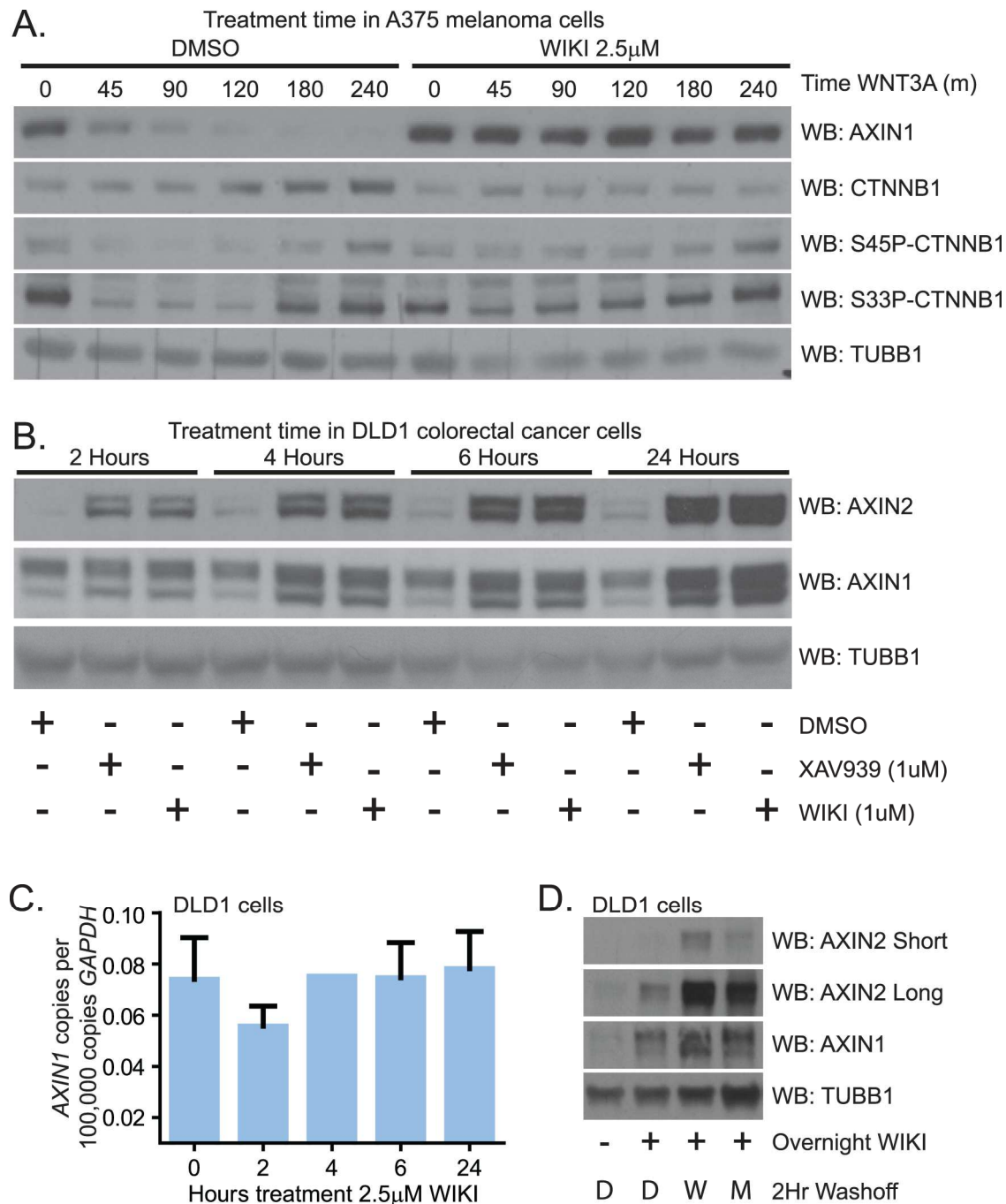


Figure 3. WIKI4 increases the steady-state abundance of the Wnt/ β -catenin inhibitory protein, AXIN1. (A) WIKI4 prevents degradation of AXIN1 following stimulation with Wnt3A. A375 melanoma cells were stimulated with 10% (vol/vol) Wnt3A CM for the indicated time periods with or without WIKI4 treatment, lysed and analyzed by western blot using the indicated antibodies. (B) WIKI4 increases the steady-state abundance of AXIN1 and AXIN2 protein. DLD1 colorectal carcinoma cells were incubated with DMSO, WIKI4 or XAV-939 for the indicated times, lysed and analyzed by western blot. (C) WIKI4 does not significantly affect the steady-state RNA abundance of AXIN1. DLD1 colorectal carcinoma cells were incubated

with WIK4 for the indicated times, and processed for qPCR to assess changes in the steady-state abundance of AXIN1 transcript. This data is representative of two independent experiments and the error bars represent standard deviation. (D) WIKI4-dependent increases in AXIN1 protein abundance can be maintained by treatment with a proteasome inhibitor. DLD1 colorectal carcinoma cells were treated overnight with WIKI4, and after washing were then incubated for two hours with DMSO (D), WIKI4 (W), or the proteasome inhibitor MG132 (M). The cells were lysed and analyzed by western blotting for the indicated antibodies.

WIKI4 Increases Steady-state Abundance of AXIN1

After stimulation of A375 melanoma cells with Wnt3A, we observed that the steady-state abundance of the scaffold protein AXIN1 is reduced (left time course, Fig. 3A) and conversely, abundance of cytosolic CTNNB1 increases (left time course, Fig. 3A).

Additionally, we observed that the abundance of CTNNB1 that is phosphorylated at sites that are regulated by the destruction complex components CSNK1A1 (S45, left time course Fig. 3A) and GSK3B (S33, left time course, Fig. 3A) is decreased following Wnt3A stimulation. We next investigated whether WIKI4 regulates the biochemical changes associated with Wnt/ β -catenin signaling. We found that WIKI4 inhibits WNT3A-dependent increases in the steady-state abundance of cytosolic CTNNB1, inhibits Wnt3A-dependent decreases in steady-state abundance of AXIN1, and inhibits Wnt3A-dependent decreases in abundance of phosphorylated β -catenin (S33 and S45) (right time course, Fig. 3A).

Taken together, our findings indicate that WIKI4 modulates Wnt-dependent changes in the abundance and phosphorylation of known core components of the Wnt/ β -catenin signaling pathway.

We next examined whether WIKI4 alters steady-state abundance of AXIN1 and the related AXIN2 in another cell type. Increases in the steady-state abundance of the AXIN scaffolding proteins have been shown to correlate with decreases in the steady-state abundance of cytosolic CTNNB1, even in APC-mutant colon cancer cells [33], [34]. To test the effects of WIKI4 on AXIN levels in APC-mutant cells, DLD1 colorectal cancer cells were treated with WIKI4 for two, four, six or 24 hours and processed for western blotting. We observed that WIKI4 significantly increased the steady-state abundance of AXIN1 and AXIN2 (Fig. 3B) to levels similar to those seen with treatment with the TNKS inhibitor XAV-939.

To further investigate how WIKI4 regulates AXIN protein abundance, we queried whether WIKI4 treatment promotes expression of AXIN mRNA or whether it prevents the degradation of AXIN by the proteasome. Using quantitative PCR (qPCR) analyses of DLD1 colorectal carcinoma cells, we found that steady state levels of AXIN1 (Fig. 3C) and AXIN2 (Fig. 1D) transcripts were not increased upon treatment with WIKI4. To test whether WIKI4 inhibits AXIN protein turnover, we treated DLD1 cells overnight with WIKI4, and then released them from treatment the next day for two hours (wash-off). We found that cells continuously treated with WIKI4 during the wash-off period exhibited increased abundance of AXIN1 and AXIN2 relative to cells treated with DMSO (Fig. 3D, compare lanes two and three), suggesting that WIKI4 prevents turnover of the AXIN proteins. When DLD1 cells were treated with the proteasome inhibitor MG132 during the wash-off period, AXIN1 and AXIN2 protein abundance remained elevated (Fig. 3D, compare lanes 3 and 4). Taken together, the qPCR and wash-off experiments suggest that WIKI4 increases the steady-state abundance of AXIN proteins by preventing their degradation by the proteasome.

WIKI4 Blocks the Activity of TNKS2 and Prevents AXIN Ubiquitylation

AXIN1 is modified sequentially by two enzymes in order for it to be recognized by the proteasome for degradation. First, AXIN1 is ADP-ribosylated by the TNKS1 and TNKS2 enzymes [33]. Subsequently, ADP-ribosylated AXIN1 is bound by the E3 ubiquitin ligase RNF146, which specifically catalyzes its ubiquitylation (Fig. 4A, [48], [49]). To test whether WIKI4 prevents ubiquitylation of AXIN protein, we treated SW480 (Fig. 4B) and DLD1 (Figure S3A, S3B) colorectal carcinoma cells overnight with WIKI4, and subsequently incubated for two hours with either MG132 alone or MG132 and WIKI4 (wash-off). We found that inhibition of the proteasome during the wash-off period with MG132 led to an increase in the abundance proteins bound to ubiquitin (Fig. 4B, Fig. S3A, S3B left panels). We further observed that WIKI4 treatment during the wash-off period reduced the detection of ubiquitin in immunoprecipitated AXIN2 (Fig. 4B, Figure S3A) and AXIN1 (Figure S3B), suggesting that WIKI4 indeed inhibits AXIN ubiquitylation.

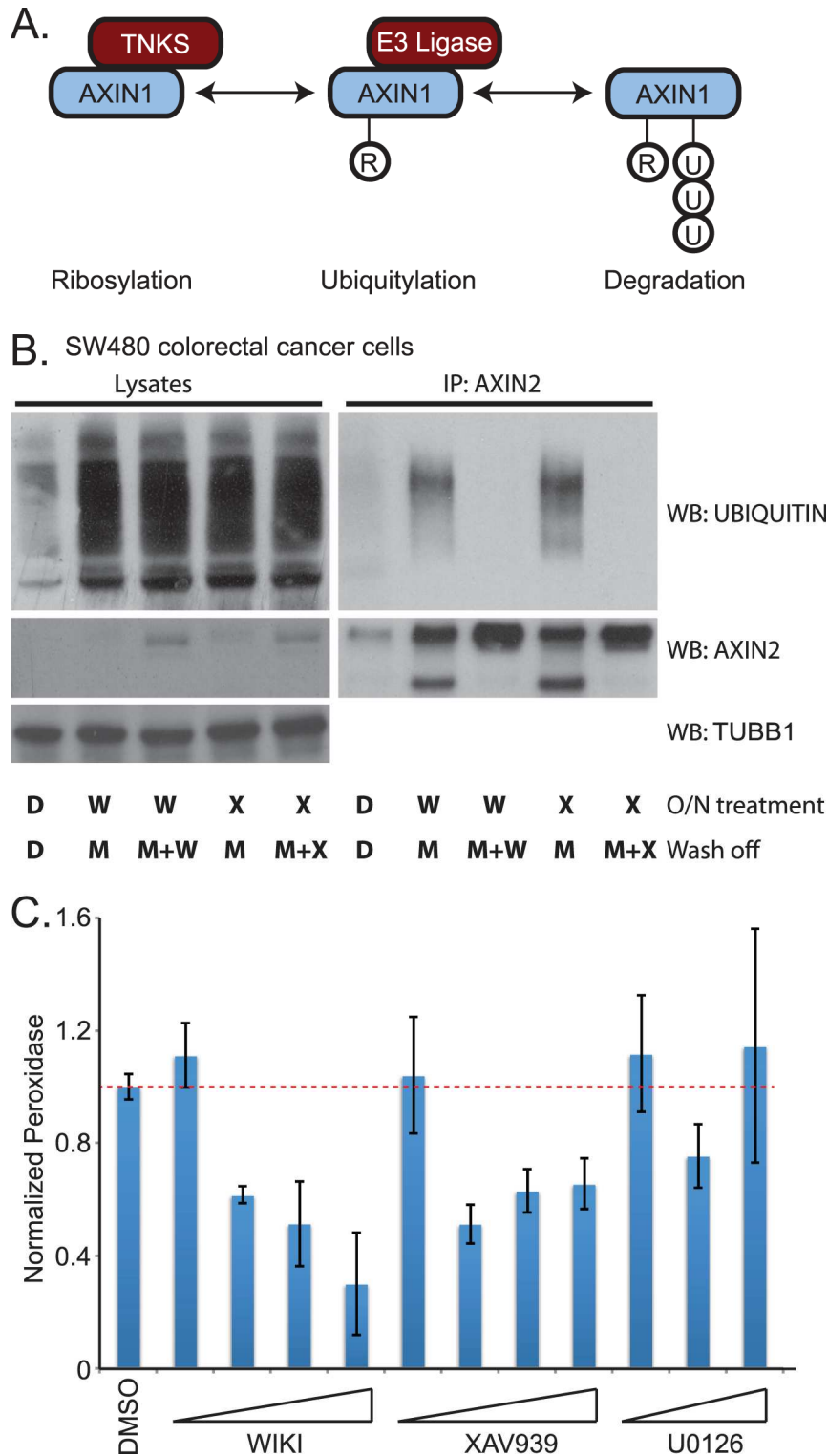


Figure 4. WIKI4 prevents ubiquitylation of AXIN and inhibits the enzymatic activity of TNKS2. (A) Schematic showing a model of how AXIN proteins are sequentially ADP-Ribosylated and then poly-ubiquitylated prior to their degradation by the proteasome. (B) WIKI4 inhibits ubiquitylation of AXIN2 in SW480 colorectal carcinoma cells. SW480 cells were treated

overnight with DMSO (D), 2.5 μ M WIKI4 (W) or 2.5 μ M XAV-939 (X). Following a brief wash, the cells were then incubated for two hours with DMSO (D), 10 μ M MG132 (M) or MG132 and one of the Wnt/ β -catenin pathway inhibitors. Lysates and AXIN2 immunoprecipitates from this experiment were processed for western blotting with the indicated antibodies. (C) WIKI4 inhibits the enzymatic activity of TNKS2. Recombinant GST-TNKS2 was bound to 96-well plates coated with glutathione. Auto-ADP-ribosylation assays were carried out using biotinylated substrate in the context of the indicated treatments. The amount of TNKS2 auto-ribosylation was quantified by performing chemiluminescent detection of the reaction between streptavidin conjugated to horseradish peroxidase and biotinylated substrate. U0126 was used as a negative control.

One possible explanation for WIKI4-dependent inhibition of AXIN ubiquitylation is that WIKI4 directly inhibits TNKS-mediated ADP-ribosylation of AXIN. The ADP-ribosylation activity of TNKS proteins can be assayed in vitro by quantifying their ability to catalyze auto ADP-ribosylation. To investigate the hypothesis that WIKI4 inhibits the catalytic activity of TNKS proteins, we performed in vitro auto-ADP-ribosylation assays using recombinant TNKS2. Similar to what is observed for the known TNKS inhibitor XAV-939, we found that WIKI4 prevents auto-ADP-ribosylation of TNKS2 at an IC₅₀ of \sim 15 nM (Fig. 4C). In contrast to the effects of XAV-939 and WIKI4, a second ATP analog, U0126, failed to inhibit auto-ADP-ribosylation of TNKS2, demonstrating that our assay is specific (Figure 4C). Taken together, our data suggest that WIKI4 inhibits Wnt/ β -catenin signaling by inhibiting tankyrase activity, and thus preventing the ubiquitylation and degradation of AXIN proteins.

3.2.5 - Conclusions

In summary, we have identified and characterized WIKI4, a novel small molecule inhibitor of Tankyrase that leads to inhibition of Wnt/ β -catenin signaling in multiple cell lines and in hESCs. As the structure of WIKI4 is distinct from the other published Tankyrase inhibitors [33], [34], [35], it is unlikely to share off-target effects with those molecules. Therefore, WIKI4 will be useful as a complementary biological probe for researchers who wish to inhibit the Wnt/ β -catenin pathway by inhibiting Tankyrase.

3.2.6 - References

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3.3 - Conclusion

As alluded to in the introduction of this chapter, the screen in which WIKI4 was discovered was one amongst many screens that we had performed using a similar set of conditions. Here we utilized A375 melanoma cells for the primary screen, and we then looked to see whether hit compounds had activity in the H1 hESC line.

I also worked extensively to screen kinome targeted siRNA libraries under multiple drug conditions. I performed entire siRNA screens in duplicate, where in once case all cells were treated beforehand with a drug and in the other case all cells were treated with vehicle control. The idea, termed 'modifier-screening' was to identify synergistic enhancement of pBAR when combining a drug such as riluzole or simvastatin and an siRNA targeting a kinase. I used this strategy in hopes of highlighting kinases involved in the intermediary connection between the targets of these drugs and known Wnt pathway components. The use of these siRNA libraries did help us to identify novel biology, which we went on to characterize in a later paper (Biechele et al., 2012). However, the modifier-screening approach in the end was not fruitful.

As discussed in the introductory chapter of this thesis, reports have conflicted as to the precise role of canonical Wnt signaling in hESC self renewal and differentiation. We hoped to discover novel biology by testing compounds discovered through HTS in hESCs. Through biochemical investigation of the mechanism of this WIKI4, we found that it imparts an inhibitory effect on Wnt/ β -catenin signaling by inhibiting enzymatic activity of the protein TNKS. A compound called XAV939 had recently been reported on (Huang et al., 2009), which also inhibited Wnt via this mechanism, and as such our findings did not significantly inform any novel Wnt biology. However, WIKI4 is structurally distinct from XAV939, and so it may yet afford unique utility upon further investigation.

4 - Simvastatin Promotes Adult Hippocampal Neurogenesis by Enhancing Wnt/ β -Catenin Signaling

4.1 - Introduction

In addition to riluzole, the results of the screen performed by Biechele and Camp (discussed in chapter 2) indicated that multiple compounds from the drug class statins are Wnt enhancers. Statins are known to inhibit the metabolic enzyme HMGCR, which is involved in cholesterol synthesis. Statins are typically prescribed to patients for prevention and treatment of hyperlipidemia and other cholesterol disorders (Reiner, 2013–8AD).

During my work performing secondary validation on many of the hits from the screen, I saw that statins indeed exhibited a robust enhancement of the Wnt/ β -Catenin pathway. I was intrigued by this finding based on the amount of pre-existing knowledge of these drugs' mechanism of action, and because statins are among the most commonly prescribed medications in the United States (2011).

I tested to see whether the effect was consistent across multiple statins, and affirmed Wnt/ β -Catenin pathway enhancement with three different drugs in this class: lovastatin, mevastatin, and simvastatin. I performed experiments with statins in a number of biological contexts in which the Moon lab was interested in finding pharmacologic enhancers of Wnt. I saw that the effect of statin on Wnt held for melanoma cells, hESCs, and neurally derived cells using the pBAR reporter.

Ultimately, I chose to focus on the neural context in order to proceed ahead with my investigation. Simvastatin had previously been investigated for possible effects in the CNS that were independent of inhibition of cholesterol synthesis. A body of evidence existed suggesting that statins both enhance neurogenesis, and have therapeutic potential for neurological disorders (see chapter 1 and also intro section of journal article). I aimed my research towards answering the question of whether enhancement of Wnt/ β -Catenin signaling by simvastatin could explain these phenomena.

4.2 - Journal Article: Simvastatin Promotes Adult Hippocampal Neurogenesis by Enhancing Wnt/ β -Catenin Signaling

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4.2.1 - Summary

Statins improve recovery from traumatic brain injury and show promise in preventing Alzheimer disease. However, the mechanisms by which statins may be therapeutic for neurological conditions are not fully understood. In this study, we present the initial evidence that oral administration of simvastatin in mice enhances Wnt signaling *in vivo*. Concomitantly, simvastatin enhances neurogenesis in cultured adult neural progenitor cells as well as in the dentate gyrus of adult mice. Finally, we find that statins enhance Wnt signaling through regulation of isoprenoid synthesis and not through cholesterol. These findings provide direct evidence that Wnt signaling is enhanced *in vivo* by simvastatin and that this elevation of Wnt signaling is required for the neurogenic effects of simvastatin. Collectively, these data add to the growing body of evidence that statins may have therapeutic value for treating certain neurological disorders.

4.2.2 - Introduction

A large body of evidence indicates that statins, a class of drugs typically used to treat hyperlipidemia, are therapeutically beneficial for neurological disorders. Statins have been shown to improve outcome following traumatic brain injury and stroke (Chen et al., 2003, Karki et al., 2009, Lu et al., 2007, Mahmood et al., 2009 and Wu et al., 2008). Simvastatin rescues cerebrovascular and memory-related deficits in mouse models of Alzheimer disease (AD) (Li et al., 2006, Tong et al., 2009 and Tong et al., 2012), and recent meta-analysis of clinical studies concluded that statins provide a slight benefit in the prevention of AD and all-type dementia (Wong et al., 2013). While these effects have been attributed to reduction of inflammation, reduced oxidative stress, upregulated PI3K/AKT signaling, and

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enhanced neurogenesis, the mechanisms by which statins are beneficial in neurological disorders are not fully understood.

Previously, we reported a chemical genetic screen that revealed that several statins activate a β -catenin-responsive luciferase reporter (BAR) in a cell-based assay (Biechele et al., 2010). This result supports prior *in vitro* studies that have shown that statins modulate Wnt/ β -catenin signaling (henceforth referred to as Wnt signaling) in human neuronal cells (Salins et al., 2007), in rat mesangial cells (Lin et al., 2008), and in mouse embryonic stem cells (Qiao et al., 2011). Given that Wnt signaling is a key regulator of adult hippocampal neurogenesis (Jang et al., 2013, Kuwabara et al., 2009, Lie et al., 2005, Luo et al., 2010, Mao et al., 2009 and Seib et al., 2013), we sought to determine whether statin-mediated enhancement of the Wnt pathway can occur in this region of the brain and to characterize any downstream effects on neurogenesis. We chose to focus on simvastatin (simva), as it is a lipophilic statin capable of crossing the blood-brain barrier (Tamai and Tsuji, 2000) and is commonly studied in neural contexts.

We find that simva enhances Wnt signaling in the adult hippocampus and that Wnt signaling is required for statins to enhance neuronal specification in differentiating adult neural progenitor cells (aNPCs). Through examination of various stage-specific markers *in vivo*, we determine that simva treatment increases the number of newborn neurons in the dentate gyrus (DG) by enhancing proliferation of intermediate precursor cells (IPCs) in the subgranular zone (SGZ). Finally, we determine that the effect of simva on the Wnt pathway is independent of cholesterol and is mediated by inhibition of isoprenoid biosynthesis.

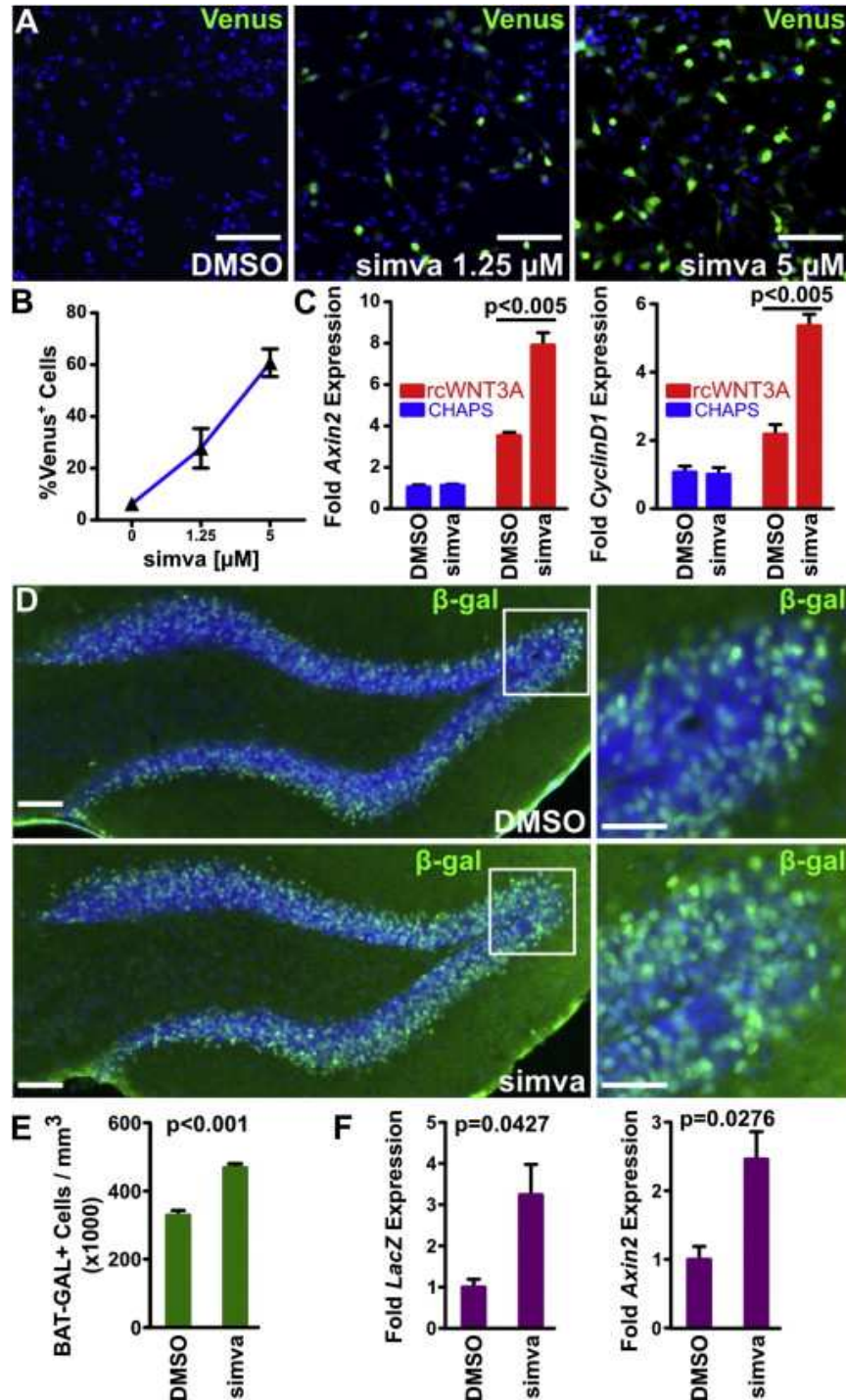
4.2.3 - Results

Simva Enhances Wnt Signaling *In Vitro* and *In Vivo*

We recently reported that lovastatin and fluvastatin enhance Wnt signaling (Biechele et al., 2010). In the present study, we extended our analysis to simva, a statin of clinical relevance to neurological disease. To monitor Wnt activity, we transduced cultured aNPCs with BAR driving expression of Venus fluorophore. Consistent with prior studies (Wexler et al., 2009), we saw no reporter activity under basal culture conditions, and addition of simva by

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itself did not induce reporter expression (not shown). However, in combination with a low dose (20 ng/ml) of recombinant WNT3A ligand (rcWNT3A), simva promoted a dose-dependent increase in the percentage of Venus+ cells following 4 days of treatment (Figures 1A and 1B).



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Figure 1. Simva Enhances Wnt Signaling In Vitro and In Vivo (A) Venus reporter expression in aNPCs treated with various concentrations of simva in combination with rcWNT3A (20 ng/ml), counterstained with Hoechst (scale bars, 100 μ m). (B) Quantification of Venus+ cells (n = 5 independent wells). (C) Expression of Wnt target genes in aNPCs treated with 5 μ M simva or DMSO in combination with rcWNT3A or CHAPS (n = 3 independent wells). (D) β -gal staining of DG from BAT-GAL mice treated with simva or DMSO, counterstained with DAPI (left scale bars, 100 μ m; right-inset scale bars, 50 μ m). (E) Quantification of β -gal+ cell density (n = 7–8 mice per group). (F) Real-time PCR analysis of Wnt target genes in hippocampi of BAT-GAL mice (n = 3 mice per group). Results are presented as mean \pm SEM (B, C, E, and F). Statistical analysis was performed with the Student's unpaired t test.

If, as suggested above, simva were able to synergize with Wnt signaling, then one would predict that such treatment should increase the expression of endogenous Wnt target genes. To test this, we treated aNPCs with simva or DMSO, in combination with either rcWNT3A or vehicle control (CHAPS), and used quantitative real-time PCR to monitor the levels of two genes that are known to be directly regulated by Wnt signaling in adult neural cells, Axin2 and CyclinD1 (Mao et al., 2009). As expected, rcWNT3A promoted increases in Axin2 and CyclinD1 expression compared to CHAPS, while simva alone had negligible effects. However, the expression of these genes was greatly enhanced by the combination of simva and rcWNT3A, similar to the synergy seen with the BAR reporter. We observed a 7.7-fold increase in Axin2 and a 5.2-fold increase in CyclinD1 expression (Figure 1C). Thus, both BAR reporter and expression levels of β -catenin target genes show that simva enhances Wnt signaling in cells where the pathway is activated at a low level.

We then directly investigated whether dosing mice with simva via oral gavage enhances Wnt signaling in the brain. Based on our in vitro data, we focused on the DG of the hippocampus, one of the germinal brain regions where aNPCs reside and where WNT3A is secreted throughout adulthood (Garbe and Ring, 2012 and Lie et al., 2005). We treated adult C57BL/6J mice harboring a reporter transgene (BAT-GAL) that drives expression of nuclear-targeted β -galactosidase (β -gal) in response to Wnt signaling (Maretto et al., 2003), and which has been used to measure Wnt signaling in the DG (Garbe and Ring, 2012 and Mazumdar et al., 2010). Based on previous studies, we treated BAT-GAL mice with either 10 mg/kg simva or DMSO by oral gavage daily for 7 days (Chen et al., 2003 and Karki et al., 2009). Mice were sacrificed 4 hr after the final drug treatment. We observed a 1.4-fold increase in the density of nuclei containing β -gal in the DG of mice treated with simva

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(Figures 1D and 1E), while the volume of the DG was unchanged between treatment groups (not shown). To test whether these results reflect changes in expression levels of Wnt targets, we collected total RNA from the hippocampus and performed real-time PCR analysis. We measured a significant increase in the average expression of both reporter gene LacZ and endogenous target gene Axin2 in mice treated with simva compared with vehicle-treated mice (Figure 1F). Together with the BAT-GAL immunostaining, these results demonstrate that systemic administration of simva enhances Wnt signaling in the DG of adult mice.

Simva Enhances Neuronal Specification via Wnt Signaling

We then investigated the effect of simva on aNPCs during differentiation. We induced differentiation in aNPCs as described previously (Luo et al., 2010, Palmer et al., 1999 and Smrt et al., 2007), in the presence of drug treatment for 4 days and stained for lineage-specific markers (Figure 2A). While simva slightly reduced the total number of cells detected following differentiation (Figure 2B), simva treatment lead to a 3.6-fold increase in the number of TUJ1+ neurons and a significant decrease in the number of GFAP+ astroglial cells (Figures 2C and 2D). To further assess the effect of simva on aNPC differentiation, we measured expression of lineage-specific genes using real-time PCR. mRNA levels of the neuron-specific genes Tuj1 and NeuroD1 were significantly increased following differentiation in aNPCs treated with simva (Figure 2E). Levels of the astroglial genes Gfap and Aqp4 were lower in simva-treated aNPCs, but the difference was not statistically significant (Figure 2F). These results, consistent with previous reports of Wnt enhancement during aNPC differentiation (Luo et al., 2010), indicate that simva influences lineage specification in aNPCs toward increased production of neurons and decreased production of astroglia.

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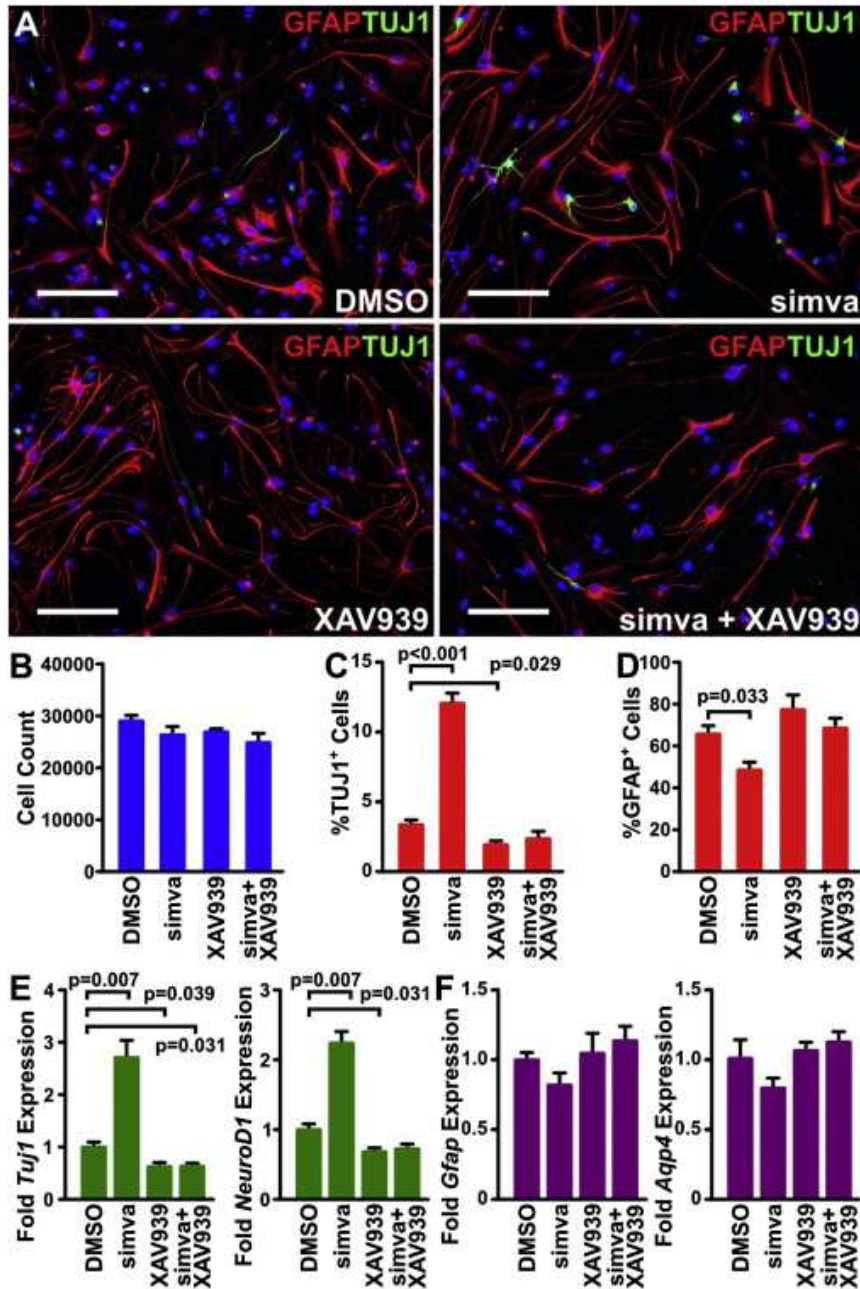


Figure 2. Simva Enhances Neuronal Specification via Wnt Signaling (A) Staining for astroglial marker GFAP and neuronal marker TUJ1 in differentiated aNPCs treated with DMSO, 5 μ M simva, 2.5 μ M XAV939, or a combination of simva and XAV939, counterstained with Hoechst (scale bars, 100 μ m). (B) Cell count for each condition following differentiation. (C) Quantification of TUJ1⁺ cells. (D) Quantification of GFAP⁺ cells. (E) Real-time PCR analysis of neuronal-specific genes. (F) Real-time PCR analysis of astroglial-specific genes. Results are presented as mean \pm SEM, n = 3 independent wells (B–F). Statistical analysis as performed with the Student's unpaired t test. See also Figure S1.

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In order to test whether the effect of simva on aNPC differentiation is due to enhanced Wnt signaling, we also tested simva in combination with a Wnt pathway antagonist. For this, we employed the small molecule XAV939. Importantly, XAV939 blocks simva-mediated enhancement of Wnt signaling in aNPCs (Figure S1 available online). We reasoned that if simva enhances neuronal differentiation via enhanced Wnt signaling, then a combination of XAV939 and simva should not elicit this effect.

As expected, XAV939 treatment significantly decreased the number of neurons formed following aNPC differentiation. We found that in aNPCs treated concurrently with XAV939 and simva, the percentage of TUJ1+ cells was equivalent to DMSO-treated cells and significantly reduced as compared to cells treated with simva alone (Figures 2A and 2C). Additionally, levels of the neuronal genes Tuj1 and NeuroD1 were decreased with combined simva and XAV939 treatment compared with DMSO (Figure 2D), while levels of the astrocytic genes Gfap and Aqp4 were not significantly changed (Figure 2E). These data demonstrate that blocking Wnt signaling abolishes the ability of simva to enhance neuronal differentiation and suggest that this effect of simva is Wnt signaling-dependent.

Simva Enhances Adult Hippocampal Neurogenesis

Having observed that simva enhances Wnt signaling in the DG of adult mice and increases neuronal specification in cultured aNPCs, we next assessed the effects of the simva treatment described above on in vivo hippocampal neurogenesis. To assess overall cell proliferation in the SGZ, we first examined the DNA replication marker MCM2 (Figure 3A). Quantification of cell numbers using confocal z stack images revealed that mice treated with simva had \approx 1.7-fold more MCM2+ cells per DG compared to control (Figure 3B). To test whether the simva-mediated increase in DG cell proliferation affects the formation of new neurons, we examined the immature neuron marker DCX. We did not find a significant difference in the number of DCX+ cells per DG for simva versus control (not shown). However, when we injected mice with a single dose of EdU (50 mg/kg) 24 hr prior to perfusion to specifically label cells in S phase toward the end of treatment, we saw that the number of DCX+/EdU+ cells per DG was increased 1.4-fold with simva treatment (Figures 3C and 3D). To test whether simva treatment affects cell survival, we counted the apoptotic

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nuclei within the same area of the DG. Cleaved caspase-3 (cCASP3) staining revealed no significant difference in the number of cCASP3+ cells per DG in simva-treated mice (Figure S2).

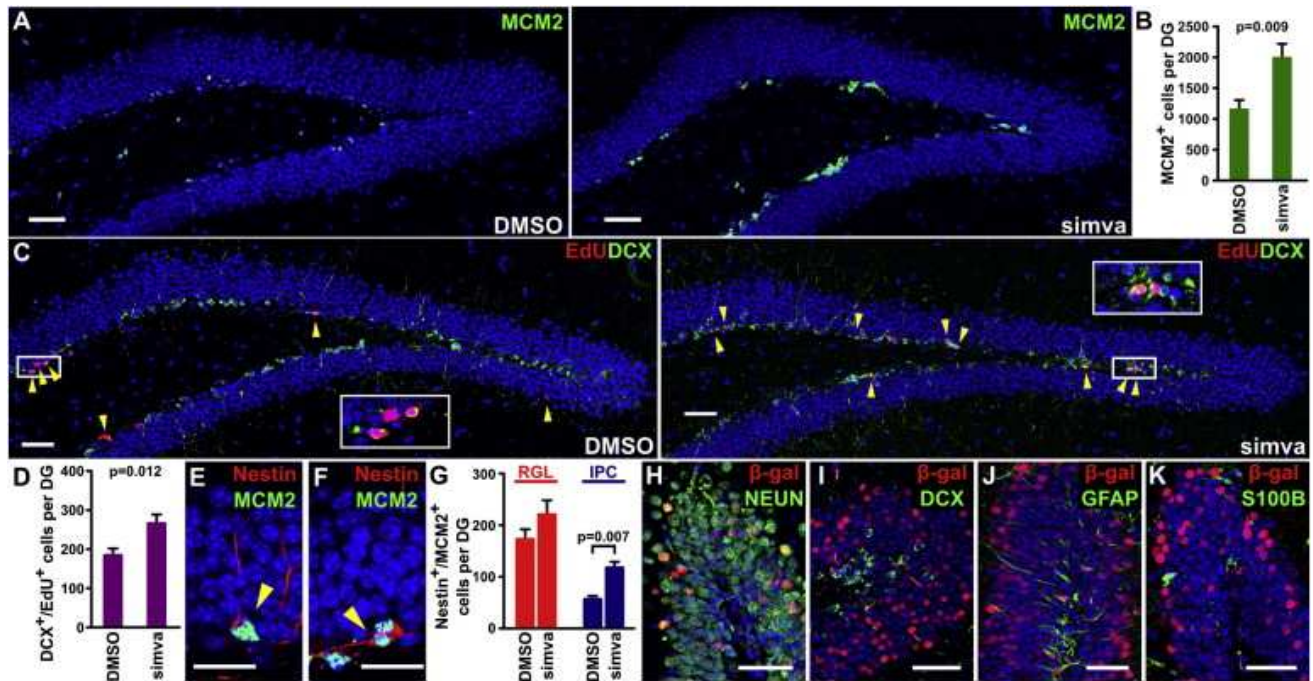


Figure 3. Simva Enhances Adult Hippocampal Neurogenesis (A) Staining for proliferation marker MCM2 in the DG of mice treated with simva or DMSO, counterstained with DAPI (scale bars, 50 μ m). (B) Quantification of MCM2 cell number. (C) Costaining for immature neuron marker DCX in cells labeled with a single dose of EdU 24 hr prior to sacrifice. (Insets show colocalization, yellow arrows indicate DCX+/EdU+ cells; scale bars, 50 μ m.) (D) Quantification of DCX+/EdU+ cell number. (E) Yellow arrow: activated early precursor cell (RGL), Nestin+/MCM2+ with radial morphology (scale bar, 25 μ m). (F) Yellow arrow: proliferating intermediate precursor cell (IPC), Nestin+/MCM2+ with horizontal morphology (scale bar, 25 μ m). (G) Quantification of activated RGL and proliferating IPC cell number. Results are presented as mean \pm SEM, $n = 8$ mice per group, statistical analysis performed with Student's unpaired t test (B, D, and G). (H–K) β -gal costaining with (H) mature neuron marker NEUN, (I) DCX, (J) astroglial and immature precursor marker GFAP, and (K) astrocyte marker S100B. Scale bars, 50 μ m. Counterstaining with DAPI (A, C, E, F, and H–K). See also Figure S2.

The increase in the number of proliferating cells within the SGZ that we observed as a result of simva treatment could be due to increased IPC proliferation or increased production of IPCs from radial-glia like early precursors (RGLs) (Bonaguidi et al., 2011). We looked to see what effect simva has on these populations by labeling for progenitor marker Nestin alongside MCM2. Activated RGLs, which asymmetrically divide to form new

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IPCs, were identified as Nestin⁺/MCM2⁺ with radial morphology (Figure 3E). Proliferating IPCs were identified as Nestin⁺/MCM2⁺ cells with horizontal morphology (Figure 3F). We observed a modest, but not statistically significant, increase the number of activated RGLs per DG in simva-treated mice. Meanwhile, the number of proliferating IPCs per DG was increased 2-fold in simva-treated mice (Figure 3G).

BAT-GAL staining revealed that Wnt signaling is active in some, but not all, of the cells in the DG of both simva- and control-treated mice (Figure 1D). To identify the cell types that exhibit active Wnt signaling following simva treatment, we costained tissue for β -gal and a panel of cell-type-specific markers. We observed that a majority of nuclear β -gal expression occurred in cells labeled with the mature neuron-specific protein NEUN in the granule cell layer (GCL) (Figure 3H), with very little β -gal in immature neurons (DCX⁺) (Figure 3I). We saw significant β -gal expression in GFAP⁺ cells in both the GCL and SGZ (Figure 3J). While GFAP can label both astrocytes and immature precursors, cells with nuclear β -gal expression were rarely labeled with the astrocyte marker S100B (Figure 3K).

We found that simva increases overall cell proliferation in the SGZ, leading to an increase in the number of newly formed neurons. When we looked to delineate between different progenitor pools, we found that IPC proliferation was significantly increased. Additionally, we observed Wnt reporter expression in both GFAP⁺ and NEUN⁺ cells following simva treatment. Taken together, these data suggest that oral simva treatment enhances adult neurogenesis in the mammalian hippocampus.

Simva Enhances Wnt Signaling via Depletion of Isoprenoids

Finally, we investigated the mechanism by which simva can enhance Wnt signaling. Statins antagonize HMG-CoA-reductase (HMGCR), the rate-limiting enzyme in the sterol biosynthetic pathway. This pathway is responsible for de novo synthesis of cholesterol as well as isoprenoids (Endo, 1992). To test whether inhibition of sterol biosynthesis enhances Wnt signaling, we used small interfering RNAs (siRNAs) to knock down HMGCR in human neuroblastoma (SH-SY5Y) cells harboring BAR driving luciferase. Similar to our aNPC data, we found that simva enhances Wnt signaling in these cells with a low dose of

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WNT3A conditioned media (CM), but not with control CM (Figure S3). We tested three unique siRNAs to knock down HMGCR (Figure S4) and a control nontargeting siRNA. Cells transfected with HMGCR siRNAs and treated with WNT3A CM showed significantly increased reporter induction (Figure 4A).

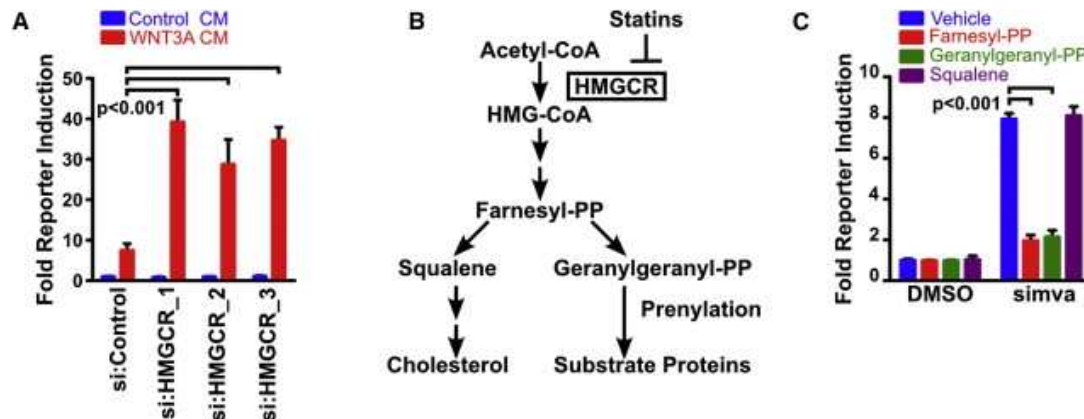


Figure 4. Simva Enhances Wnt Signaling via Depletion of Isoprenoids (A) BAR luciferase assay in SH-SY5Y cells transfected with control (nontargeting) siRNA or one of three independent siRNAs targeting HMGCR and treated with WNT3A CM or control CM. (B) The sterol biosynthetic pathway is blocked by statins, leading to depletion of isoprenoids and cholesterol. (C) BAR luciferase assay in SH-SY5Y cells treated with simva in combination with products of the sterol biosynthetic pathway: 10 μ M GGPP and 10 μ M FPP, and 10 μ M squalene. Results are presented as mean \pm SEM; $n = 3$ independent wells (A and C). Statistical analysis was performed with the Student's unpaired t test. See also Figures S3 and S4.

Downstream of HMGCR, sterol biosynthesis bifurcates to produce either the cholesterol-precursor squalene or the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fears, 1981) (Figure 4B). Statins, via inhibition of the sterol biosynthetic pathway, deplete cellular pools of cholesterol, FPP, and GGPP (Hughes, 1996). To test whether any of these products are involved in simva enhancement of Wnt signaling, we treated SH-SY5Y BAR cells with 5 μ M simva, WNT3A CM, and exogenous FPP, GGPP, or squalene. We reasoned that if statin-mediated depletion of any of these metabolites caused the enhancement of the Wnt pathway, then adding the responsible metabolite back to the cells would inhibit the ability of simva to enhance BAR activity. Notably, addition of 10 μ M GGPP or 10 μ M FPP to simva-treated cells significantly reduced simva-mediated Wnt enhancement, while 10 μ M squalene did not have an effect (Figure 4C). Since FPP is used to synthesize GGPP, the effect may be due to depletion of GGPP alone or a combination of FPP

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and GGPP depletion. Therefore, we conclude that simva enhances Wnt signaling via depletion of isoprenoids and not cholesterol.

4.2.4 - Discussion

Simva is under investigation for its potential therapeutic effects outside of hyperlipidemia treatment. While statins have been reported to enhance Wnt signaling *in vitro*, it was heretofore not known whether statins can enhance this pathway *in vivo* and in the context of neurogenesis. Here we provide evidence that oral simva treatment enhances Wnt signaling in the mammalian adult hippocampus. This is significant in that aside from lithium, no other clinically approved compound has been demonstrated to enhance Wnt signaling in the brain (Zimmerman et al., 2012).

The observations in this study are consistent with reports of increased hippocampal neurogenesis due to both simva treatment (Chen et al., 2003, Lu et al., 2007 and Wu et al., 2008) and increased Wnt signaling (as cited in introduction). Importantly, we demonstrate a link between these phenomena by probing Wnt's role in simva enhancement of neurogenesis *in vitro*, and subsequently investigating the effect of enhanced Wnt signaling during multiple stages of *in vivo* adult hippocampal neurogenesis.

While we showed a requirement for Wnt signaling in increased neuronal differentiation among simva-treated aNPCs, it remains possible that additional signaling pathways play a role in simva's effect on overall neurogenesis. Further, while others have demonstrated beneficial neurological effects of simva in disease models (as cited in introduction), the present study was performed using healthy animals and did not monitor later stages of neuronal development or behavioral outcomes.

To help map the biological connection between enhanced Wnt signaling and enhanced neurogenesis, we examined costaining of BAT-GAL with various cell-type-specific markers following simva treatment. However, a comparison showing differences in temporal expression patterns between different *in vivo* Wnt reporters presents a potential caveat to this approach (Garbe and Ring, 2012).

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The mechanism underlying statin enhancement of Wnt signaling had not been previously reported. Providing initial insight, we show that HMGCR loss of function is sufficient to enhance the Wnt pathway. Furthermore, we demonstrate that simva acts on Wnt signaling by depleting isoprenoids, rather than through a cholesterol-dependent mechanism. Prenylation guides membrane localization of small GTPases such as RAS and RHO-associated kinases and other signaling proteins (Zhang and Casey, 1996), and serves as a regulatory mechanism for these enzymes that can be targeted therapeutically (Gelb et al., 2006). To this point, recent studies have measured an age-dependent increase of isoprenoid levels in brains of mice (Hooff et al., 2012) and have identified an overabundance of isoprenoids in the brains of AD patients (Eckert et al., 2009). The identity of the specific prenylated protein or proteins responsible for the effect of simva on the Wnt pathway remains elusive. However, there are a number of prenylated proteins known to regulate Wnt signaling (e.g., RAC1 and RHOA) that may serve as candidates for future studies (Schlessinger et al., 2009).

4.2.5 - Experimental Procedures

BAR Venus Experiment with aNPCs

aNPCs were plated on polyornithine- (Sigma-Aldrich) and laminin-coated (Life Technologies) optical imaging plates (Corning) in proliferation media containing FGF (Life Technologies) and epidermal growth factor (EGF; PeproTech) (see Supplemental Experimental Procedures). Drugs were added as indicated 24 hr after plating. Following 4-day drug treatment, Hoechst 33342 dye (Sigma-Aldrich) was added at 1 μ g/ml.

Mouse Experiments

Eight- to 10-week-old C57BL/6J WT and BAT-GAL mice were used in this study. All animal-related procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington and were conducted in accordance with the guidelines of the National Institutes of Health. For oral gavage, simva was dissolved in DMSO and diluted in 1% carboxymethylcellulose (Sigma-Aldrich) in water. For EdU experiments, mice were

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injected intraperitoneally with 50 mg/kg EdU dissolved in DMSO. For details on tissue collection, see the Supplemental Experimental Procedures.

aNPC Differentiation

To induce differentiation, aNPCs were plated on polyornithine- and laminin-coated optical imaging plates with differentiation media containing 5 μ M forskolin (Sigma-Aldrich) and 1 μ M retinoic acid (Sigma-Aldrich) and lacking EGF and fibroblast growth factor (see Supplemental Experimental Procedures).

Antibody Staining, Imaging, and Quantification BAR Venus aNPCs were imaged with a fluorescence microscope (Nikon), and antibody-stained aNPCs were imaged with a Nikon A1 confocal microscope. TUJ1+ and GFAP+ aNPCs were manually counted using ImageJ, and percentage was determined by dividing by total number of nuclei. BAT-GAL+ cell density and DG volume was determined using stereology software and semiautomated counting with a fluorescence microscope. To quantify MCM2+, DCX+, EdU+/DCX+, and Nestin+/MCM2+ cells we collected z stacks and counted manually using ImageJ (see Supplemental Experimental Procedures).

Acknowledgments

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4.3 - Conclusion

This study, investigating whether simvastatin can enhance Wnt/ β -catenin signaling in the adult brain, is another example implementation of the research pattern that I have outlined throughout this thesis. I surfaced statins as an interesting hit from a primary screen, took the compounds through secondary validation, and investigated the biological effect in an *in vivo* model. Finally, I was able to make inroads to learning the mechanism of how statin inhibition of HMGCR connects to Wnt signaling.

Prior to conducting the *in vivo* experiments presented in this paper, I was most interested to see whether simvastatin could rescue deleterious phenotypes in a model of neurological disease. I sought advice on this subject from a former Moon lab member, Bryan White, and a collaborator of the lab, Phillip Horner, who had undertaken an investigation of Wnt signaling in traumatic brain injury (TBI) (White et al., 2010). I was informed of the within-group noise in the experimental results that White acquired. This was attributed to the inconsistent response between animals in this TBI model. A power analysis using White's variability numbers revealed that if I were to conduct a 4 group study testing each combination of simvastatin, injury, and controls, I would need a prohibitively large number of mice in order to reach statistical significance. Therefore, in order to stay within the scope of time, cost, and my own capabilities as a graduate student, I conducted the study with healthy adult mice, as opposed to a disease model.

In the submission process for this article, I fielded complaints from reviewers that my findings were incremental. This was based in the fact that I was trying to tie together two different ideas, each which were somewhat novel but not entirely new. It had previously been demonstrated in cell culture models that simvastatin enhances Wnt signaling. Also, a number of papers had shown neurological effects of statins. However in spite of what is already known, it remains that we do not possess a clear understanding of the mechanisms explaining reported neurological benefits of statins. My work showed for the first time that simvastatin enhances Wnt signaling *in vivo* and demonstrated a biological effect of this enhancement on neurogenesis, and thus contributed important new knowledge to the field.

5 - Conclusion

5.1 - Summary of Results

The journal articles covered in this thesis are connected in their approach and goals. Projects began with a high-throughput screen to discover molecules that regulate Wnt/ β -catenin signaling in decidedly interesting contexts. The molecules with the greatest activity in the screen were chosen as hits, and were fed into a pipeline of filtering and validation steps. Finally, the activity of the most promising molecules was characterized in a series of cell culture, animal, and disease models. From project to project, this pattern was iteratively improved upon. I looked to maximize the amount of usable information gathered, and to optimize relevance to disease using experimental models.

My contribution of original knowledge in the field of translational stem cell biology is the discovery of various molecules that can be used to manipulate Wnt/ β -Catenin signaling. This includes repurposed clinically-approved compounds that were not previously known to affect Wnt, and novel compounds whose biological mechanisms were later determined. In addition, I developed methods that can be used for conducting large scale screens and for performing automated experiments with stem and progenitor cells.

5.2 - Successes of my Approach

The central theme of the Moon Lab is Wnt signaling. This influenced the direction of my research, such as in deciding which primary screen hits I deemed most interesting to follow, and which types of libraries to screen. I found that, throughout the course of my research, there was a significant benefit to my progress by focusing specifically on Wnt signaling. There was a synergistic effect of having a well curated set of reagents and a wealth of human expertise on the subject of Wnt signaling in house. Notably, I was able to use the BAR construct as a first line of discovery in a diverse set of contexts.

The focus on discovery of small molecules means that alongside each publication presented in this thesis there is a tangible molecular entity. The small molecules that we reported on

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reliably elicit an effect, are widely available and are relatively inexpensive. In the case of WIKI4, where we identified a Wnt inhibitor from among a large set of novel compounds, this molecule is now commercially available alongside other Wnt inhibitors. As such, researchers looking to reproduce or build upon our findings will not have to rely upon proprietary formulations or methodologies. Further, clinicians investigating the therapeutic effects of riluzole or simvastatin may be able to contextualize their own findings with the biological mechanisms that we reported. These points, in sum, bolster the impact of the projects that I worked on.

5.3 - Shortcomings of my Approach

Aside from the benefits discussed above, choosing to investigate biological mechanisms and disease through the lens of a single signaling pathway imposes some difficulties in research. It is common that mechanisms contributing to the pathology of a disease span indiscriminately across signaling pathways. We found this to be the case in our investigation of melanoma, presented in chapter 2 of this thesis and in follow up studies, where ERK signaling and Wnt signaling both play crucial roles in cell proliferation and survival (Biechele et al., 2012). A major facet of cancer is its ability to adapt to unfavorable environments, and thus it is not surprising that tumors frequently develop insensitivity to drugs targeting specific pathways (Hutchinson, 2014–2AD).

In contrast to the positive aspects of prioritizing discovery of small molecules, I found that this restricted the number possible solutions that could be utilized to solve the problems that I faced in my research. In trying to determine the mechanisms connecting the targets of riluzole and simvastatin with the Wnt pathway, I came up short. With Riluzole, we found convincing evidence that its effect on Wnt was due to inhibition of metabotropic glutamate receptors, but we could not fully resolve this using other known small molecule inhibitors of the pathway. In our investigation of WIKI4 presented in chapter 3 of this thesis, we identified the mechanism in large part because a molecule with a similar mechanism of Tankyrase inhibition had recently been reported (Huang et al., 2009). In the case of the simvastatin, I was able to confirm that the effect on Wnt was due to inhibition of its known

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target HMGCR, but when I attempted to trace the steps back to Wnt signaling I could not connect all the dots using small molecules alone.

As is discussed throughout this thesis, I relied heavily on HTS as an initial discovery step in my inquiries. I found throughout the course of my studies that there is a finite limit to the utility of this method, and that there are certain downsides that apply to the use of this method in an academic settings. The nature of secondary followup of high throughput screens involves repetitive work that is absent of forward movement or intellectual consideration of results. This is not a problem in a commercial setting, but in an academic lab this often runs counter to the aspirations of the person conducting experiments. Graduate students and post-docs seek to learn the skills of how to best ask questions based on careful interpretation of result, and how to formulate methods to inquire and answer those questions. Manually grinding through lists of results in order to move to the next step in a research project does not work these muscles.

Finally, in cases where a single experiment represents the expenditure of a significant portion of lab resources, the challenges of resource management are amplified. The chapters in this thesis demonstrate that just as a single screen can spawn multiple projects leading to publications, it can also happen that multiple screens lead to projects yielding zero publications.

5.4 - Suggestions for Future Work

5.4.1 - Automate Wherever Possible

Although I've outlined above some of the potential pitfalls in relying on large screening campaigns, I believe that automation is heavily under-utilized in academic labs. While our understanding of complex biological mechanisms has grown massively in scale over the past decade, the tools that we use to make discoveries on a day to day basis have in large part remained constant. Hand pipetting, non-miniaturized assays, and manual data entry remain commonplace. These represent missed opportunities for cost and time savings.

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This recommendation does not imply that graduate students and post-docs should be expected exert any less effort, or that we should rely on machines to do the work of scientific discovery in place of human intellect. Rather, it is an acknowledgement that the time biologists spend on work is most effective when focused on the tasks of interpreting data, followed thorough consideration and planning of future experiments. The equipment to perform automated experiments will continue to become less expensive to own and operate, either by individual labs or institutional core facilities. Thus, I recommend that we should expedite its adoption.

5.4.2 - Expand Beyond the Pathway Paradigm

The work that I undertook was largely through the lens of Wnt/ β -catenin and other signaling pathways. The consideration of cell signaling pathways has aided in developing targeted therapies, and has drastically widened our understanding of cell function. However, in this chapter I have pointed out cases where the emphasis on studying cells at the level of the pathway can become a self-imposed restriction. Notably, cancer research has revealed that the folly in the expectation that chemical inhibition of a single pathway can eradicate a tumor. Moreso, the complex mixture of molecules that govern hESCs and aNPCs highlights the nonlinearity of signaling events in cells.

The solution, then, is to view cell signaling as an interconnected network rather than a series of pathways. The term ‘cross talk’ is used to refer to links between multiple cell signaling pathways. Based upon the above observations, this interconnectedness should be considered the norm rather than the exception. The most important implication of widening the scope of cell signaling research to de-emphasizing the individual pathway is that we may avoid biases that have stood in the way of discovering effective therapies.

5.4.3 - A new framework for collaboration

The adoption of these new paradigms in biology research require us to rethink the way that collaborations are formed, carried out, and reported on. At present, it is too difficult for researchers on the forefront of disease biology to connect with researchers possessing the technical skills involved in automation. The current journal publication and authorship

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model does not offer the correct set of incentives for these wide collaborations, particularly in its emphasis on a single lead author. Thus, we must find a way to properly distribute credit for these kinds of projects, which is both fair and accurately reflects the contribution of each individual.

Additionally, we must emphasize fast and open sharing of data from these large experiments. It often takes years in order for a research finding to make it to press, and during this time there can be troves of data left to sit in isolation. A potential solution would be to allow for rapid publication of large datasets upon acquisition. This can help to prevent us from repeating others' work, and to allow biologists to focus on their particular areas of expertise.

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