Discovery of Potent, Orally Bioavailable, Small-Molecule Inhibitors of WNT Signaling from a Cell-Based Pathway Screen

Aurélie Mallinger, † Simon Crumpler, † Mark Pichowicz, † Dennis Waalboer, † Mark Stubbs, † Olajumoke Adeniji-Popoola, † Bozena Wood, † Elizabeth Smith, † Ching Thai, † Alan T. Henley, † Katrin Georgi, § William Court, † Steve Hobbs, † Gary Box, † Maria-Jesus Ortiz-Ruiz, † Melanie Valenti, † Alexis De Haven Brandon, † Robert TePoele, † Birgitta Leuthner, § Paul Workman, † Wynne Aherne, † Oliver Poeschke, † Trevor Dale, † Dirk Wienke, † Christina Esdar, § Felix Rohdich, § Florence Raynaud, † Paul A. Clarke, † Suzanne A. Eccles, † Frank Stieber, § Kai Schiemann, § and Julian Blagg* †

Supporting Information

ABSTRACT: WNT signaling is frequently deregulated in malignancy, particularly in colon cancer, and plays a key role in the generation and maintenance of cancer stem cells. We report the discovery and optimization of a 3,4,5-trisubstituted pyridine 9 using a high-throughput cell-based reporter assay of WNT pathway activity. We demonstrate a twisted conformation about the pyridine–piperidine bond of 9 by small-molecule X-ray crystallography. Medicinal chemistry optimization to maintain this twisted conformation, cognisant of physicochemical properties likely to maintain good cell permeability, led to 74 (CCT251545), a potent small-molecule inhibitor of WNT signaling with good oral pharmacokinetics. We demonstrate inhibition of WNT pathway activity in a solid human tumor xenograft model with evidence for tumor growth inhibition following oral dosing. This work provides a successful example of hypothesis-driven medicinal chemistry optimization from a singleton hit against a cell-based pathway assay without knowledge of the biochemical target.

INTRODUCTION

The WNT signaling network is a major regulator of mammalian development through control of cellular functions such as proliferation and differentiation. WNT signaling is frequently deregulated in malignancy, particularly in colon cancer. Binding of WNT ligands, a family of secreted glycosylated proteins, to the Frizzled and LRP families of cell surface receptors initiates a series of signaling events via the cytoplasmic protein Dishevelled (DSh). Upon activation, DSh recruits Axin and destabilizes the destruction complex that normally acts to degrade the transcriptional cofactor β-catenin. In the absence of degradation, β-catenin accumulates and translocates to the nucleus, where it recruits coactivators and associates with the LEF/TCF family of DNA-binding proteins, thereby altering expression of a variety of genes, including cyclin D1 and c-MYC. In cells not subject to stimulation by WNT ligands, β-catenin degradation by the destruction complex limits β-catenin-mediated gene transcription. Key components of the β-catenin destruction complex include Axin, APC (the protein product of the adenomatous polyposis coli tumor suppressor gene), and glycogen synthase kinase-3-β (GSK3-β), which phosphorylates β-catenin and thereby renders β-catenin a substrate for ubiquitination and degradation by the 26S proteasome.

Overexpression of WNT-regulated genes can cause transformation of mammalian epithelial cells. Clinically, 80% of colon cancers have defects in the APC gene, leading to high levels of β-catenin. A subset of colon cancers and melanomas harbor β-catenin mutations that prevent its phosphorylation

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and subsequent degradation. Many other cancers also show evidence of inappropriate WNT pathway activation, including raised levels of $\beta$-catenin. Validation of a pivotal role for WNT signaling plays a key role in the generation and maintenance of cancer stem cells. Defective WNT signaling plays a key role in the generation and maintenance of cancer stem cells, and compounds that dampen WNT pathway activity could provide useful molecularly targeted therapeutics for the treatment of cancer; indeed, the fully humanized monoclonal antibody OMP-18RS that targets Frizzled receptors is efficacious in patient-derived mouse tumor models and is currently in a phase 1 clinical trial. Despite the importance of the WNT pathway, it is only recently that small-molecule inhibitors have been identified and progressed toward clinical trial. Compound 1 (ICG001) blocks the interaction between $\beta$-catenin and the transcriptional coactivating protein CREB binding protein (CBP), leading to a reduction of colon adenoma formation in mouse models. The $\beta$-catenin–CBP interaction was shown to promote stem/progenitor marker expression, while the related $\beta$-catenin–p300 interaction (not inhibited by 1) promoted expression of genes involved in proliferative responses such as c-Myc. The $\beta$-catenin–CBP interaction has now been progressed to clinical trial. Compounds 2 (XAV939) and 3 (IWR-1) have been disclosed as inhibitors of WNT signaling via inhibition of the tankyrase activity required for degradation of Axin; 2 was discovered by target deconvolution from a cell-based pathway screen. 4 (IWP-2), a small-molecule inhibitor of porcupine, the acyltransferase essential for secretion of functional WNT ligands, was also discovered by cell-based pathway screening (Figure 1). Subsequent to the identification of tankyrase and porcupine as validated molecular targets, many small-molecule inhibitors have been reported. Examplar tankyrase inhibitors include 5 (NVP-TNKS656), 6 (G007-LK), and compound 7 from Amgen; the porcupine inhibitor 8 (LGK974) is currently in a phase I clinical trial.

Many WNT pathway mutations occur at, or upstream of, $\beta$-catenin. Therefore, if the pathway is blocked at or below this point, an inhibitor should be active against multiple tumors driven by a WNT-activating mutation. With this in mind, we set out to discover small-molecule inhibitors of WNT signaling and employed a cell-based pathway screening strategy to identify compounds that block WNT signaling at, or downstream of, $\beta$-catenin. While our work was in progress a number of reports appeared describing successful cell-based pathway screens against the WNT pathway and, for example, BMP signaling revealing hitherto undiscovered regulatory mechanisms, increasing our confidence in such an approach. We have previously reported our screening strategy and describe here the medicinal chemistry optimization of a 3,4,5-trisubstituted pyridine hit to give potent and orally bioavailable small-molecule inhibitors of WNT signaling. We demonstrate a 350-fold potency enhancement in a cell-based assay of WNT pathway inhibitors.

Figure 1. Small-molecule inhibitors of the WNT pathway: 1, 2, 3, 4, 5, 6, 7, and 8.
pathway activity through hypothesis-driven medicinal chemistry design respectful of physicochemical properties likely to elicit good cell permeability.

**CHEMISTRY**

Our synthetic strategy to most desired 3,4,5-trisubstituted pyridines involved SNAr displacement of the 4-chloro substituent in either 3,4,5-trichloropyridine (10) or 3-bromo-4,5-dichloropyridine (11) using the appropriate N-boc-protected pyrrolidine 12, piperidine 13, or NH-piperidine 14 to give the 4-substituted analogues 9, 15-22, 39, 41, 43, 45, 47, 49, 51, 53, 57, and 84 according to our previously published methodology. Subsequent palladium-mediated cross-coupling of appropriate aryl boronic acids or esters gave the corresponding 3,4,5-trisubstituted pyridines 26, 28-38, 40, 42, 44, 46, 48, 50, 52, 54, and 65-81 (Scheme 1). Compounds 59, 60, and 62 described in Table 5 were prepared by sequential cross-coupling at the 3- and 5-positions of 8-(3-bromo-5-chloropyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (57). Compounds 63 and 64 were prepared directly from 8-(3-chloro-5-(4-morpholin-4-ylphenyl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (44), while compound 56 was prepared by SnAr displacement of the 4-chloro substituent in 3-bromo-4-chloro-5-methylpyridine, followed by palladium-mediated cross-coupling at the 3-position. Preparations of non-commercial aryl boronic acids or esters are described in the Experimental Section and the Supporting Information.

**RESULTS AND DISCUSSION**

To discover inhibitors of WNT signaling, we used an inducible luciferase reporter assay in human embryonic kidney cells (HEK293) that contained both estrogen receptor-DSH and TCF-luciferase-IRES-GFP constructs. GFP fluorescence was used to sort cells by flow cytometry that were highly induced by estradiol. Clone 7dF3 was selected for use in HTS format and gave a 40-fold induction of luciferase activity when DSH protein expression was induced using 10 μM estradiol. We screened 63,040 compounds at an assay concentration of 20 μM and selected compounds that reproducibly gave >50% inhibition in the 7dF3 luciferase reporter assay (n = 2) and did not inhibit luciferase enzymatic activity nor lead to >50% cell death at 20 μM. A control cell line incorporating a thymidine kinase promoter-driven renilla luciferase reporter construct was also utilized to deselect compounds that demonstrated potent and efficacious inhibition of non-WNT-mediated transcription.

Hit compound 9 (IC_{50} = 1.76 μM) was the only exemplar of a 3,4,5-trisubstituted pyridine in our screening library at that time; however, despite being a singleton, it was among the most attractive chemicals, with a low molecular weight and acceptable physicochemical properties (Table 1 and Figure 2). Compound 9 demonstrated potency and efficacy in all these cell-based assays, similar to the potency observed in the 7dF3 primary assay and...
consistent with intracellular inhibition of WNT signaling at or below the TCF locale (IC50’s = 0.9–7.6 μM, Table 2).

In addition to promising potency in the 7dF3 luciferase cell-based reporter assay (IC50 = 1.76 μM), compound 9 demonstrated moderate in vitro metabolic stability in human and mouse liver microsomes (Figure 2). However, we were concerned that 9 may be a nonspecific pharmacophore for ion channels by virtue of the 4-aminopyridine motif. A measured pKₐ of the pyridine nitrogen (pKₐ = 3.5) indicated that 9 is essentially nonbasic, in contrast to 4-aminopyridine (pKₐ = 9.2) and 4-(piperidin-1-yl)pyridine (measured pKₐ = 9.42). The inductive electron-withdrawing effect of two m-chloro substituents is likely to significantly reduce the pKₐ (pyridine pKₐ = 5.24; 3-chloropyridine pKₐ = 2.84). We hypothesized that a twisted conformation about the pyridine–piperidine bond, driven by the steric bulk of the flanking chlorine atoms, may also reduce the basicity of compound 9 by abrogating electron donation from the 4-amino substituent. Indeed, a twisted conformation was evident in the small molecule X-ray crystal structure of 9 with a torsion angle of 54.4° observed between the plane of the piperidine and pyridine rings [Figure 3 and Supplementary Figure S4, Supporting Information (SI)]. By contrast, 4-(piperidin-1-yl)pyridine adopts a planar conformation about the piperidine–pyridine bond in two reported small-molecule X-ray crystal structures. Thus, the 3,5-dichloro substitution pattern in compound 9 reduces the pKₐ of the pyridine nitrogen (inductive effect) and induces a twisted conformation about the piperidine–pyridine bond (steric effect), which may further reduce the pKₐ. The measured pKₐ of compound 9 is consistent with an observed lack of in vitro ion channel pharmacology (hERG IC50 > 10 μM) and absence of CYP450 enzyme inhibition apart from weak inhibition of CYP3A4 (IC50 = 7 μM, Supplementary Table S1, SI); these observations are in marked contrast to classical 4-aminopyridine-based ion channel modulators.

In the absence of information about the precise biochemical target of compound 9, we followed a classical ligand-based design approach. We hypothesized, first, that the H-bond donor (HBD) and acceptor (HBA) properties of the primary amide moiety would be essential for activity; second, that the pyridine nitrogen would be an essential component of activity; and, third, that it would be important to maintain the observed twisted conformation about the pyridine–piperidine bond. We initially set out to test these three hypotheses, cognisant in our

Table 1. Modification of the Piperidine-4-carboxamide

| Entry | No | R     | X     | 7dF3 IC50 ±SD (μM) | MLM° | HLM° |
|-------|----|-------|-------|-------------------|------|------|
| 1     | 9  | O-NH₂ | N     | 1.76±0.796        | 41%  | 22%  |
| 2     | 15 | O-N   | N     | 12.5±1.59         | 57%  | 13%  |
| 3     | 16 | O-N   | N     | 26.0±5.59         | 99%  | 22%  |
| 4     | 17 | N     | >30   | ND                | ND   | ND   |
| 5     | 18 | O-N   | N     | >30   | ND   | ND   |
| 6     | 19 | O-NH₂ | N     | 44.6±2.26         | ND   | ND   |
| 7     | 20 | H     | N     | >30   | ND   | ND   |
| 8     | 21 | NH₂   | N     | 10.0±4.256        | ND   | ND   |
| 9     | 22 | O-NH₂ | CH    | 27.9±3.43         | ND   | ND   |

° MLM and HLM values indicate percent turnover after 30 min of incubation.
activation factor that is constitutively active in the absence of β-κατενin). See the Supplementary Information for assay protocols.

that binds to and inhibits GSK-3β (Axin-GSK3βDisheveled homologue and activator of WNT signaling), Axin-GID (constitutively activating component of the WNT receptor), Dvl-2 (a cell permeability.

Methylation of the primary amide in compound 9 to give the secondary amide 15 or tertiary amide 16 led to 7- and 15-fold loss in activity, respectively, suggesting that at least one of the H-bond donors is required for activity. The greater loss of activity with more sterically demanding amides 17–19 suggested a lack of space adjacent to the amide moiety. Removal of the carbamoyl group completely (compound 20) or removal of the carbonyl moiety (compound 21) led to a reduction in potency compared with compound 9, consistent with the hypothesis that both H-bond donor and acceptor functions of the carbamoyl are important components of the pharmacophore (Table 1).

Table 2. Assessment of Compounds 9 and 33 in Cell-Based Assays in Which WNT Signaling Is Activated at Distinct Levels by Inducible Expression of cDNAs Encoding Activating Components of the Canonical WNT Pathway

| WNT pathway activation level | reporter | IC50 (μM) 9 | IC50 (μM) 33 |
|-----------------------------|----------|-------------|--------------|
| Dvl-ER (7dF3 primary assay) | TCF-luc  | 1.76        | 0.037        |
| Tet-O-ΔN-LRP6               | TCF-luc  | 0.91        | 0.020        |
| Tet-O-Dvl2                  | TCF-luc  | 1.20        | 0.020        |
| Tet-O-Axin-GID              | TCF-luc  | 1.50        | 0.024        |
| Tet-O-ΔN-β-catenin          | TCF-luc  | 2.73        | 0.070        |
| Tet-O-VP16-TCF4             | TCF-luc  | 7.61        | 0.23         |

Activating components of the canonical WNT pathway: ΔN-LRP6 (a constitutively activating component of the WNT receptor), Dvl-2 (a Disheveled homologue and activator of WNT signaling), Axin-GID (Axin-GSK3β Interaction Domain, a dominant negative form of Axin that binds to and inhibits GSK-3β), ΔN-β-catenin (a stabilized form of β-catenin resistant to degradation), or VP16-TCF (a TCF transcription factor that is constitutively active in the absence of β-catenin). See the Supplementary Information for assay protocols.

compound design of the cell-based primary assay whereby true SAR versus an intracellular biochemical target may be masked by poor cell penetration. Hit compound 9 (HBD count = 2; HBA count = 4) has excellent passive cell permeability (Caco-2: Papp = 36 × 10^-6 cm s^-1, no efflux) and compounds with equivalent or lower HBD/HBA count were prioritized to avoid masking of SAR trends against an intracellular target by poor cell permeability.

Figure 3. Small-molecule X-ray crystal structure of 9 (panel A) depicting a twisted conformation about the pyridine–piperidine bond with the plane of piperidine ring tilted 54.4° against the plane of the aromatic ring (panel B and Supplementary Figure S4, SI). Data has been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 1020858).

To explore the extent to which the pyridine nitrogen is essential for activity, we prepared compound 22, where the pyridine nitrogen was replaced with a CH (Table 1, entry 9). A significant potency drop was observed, suggesting that the hydrogen bond acceptor capability of this nonbasic nitrogen is indeed crucial for activity.

We next turned our attention to the requirement for a twisted conformation about the pyridine–piperidine bond while the piperidine 4-carbonyl was kept constant. Replacement of chlorine atoms with protons at both the 3- and 5-positions on the pyridine ring led to loss of activity, while replacement of one chloride led to a 13-fold loss in potency (Table 3, entries 1 and 2). Pleasingly, replacement of one chlorine atom with a phenyl ring in compound 26 (Table 3, entry 4) resulted in a 20-fold increase in potency over compound 9; however, the combination of a 3-phenyl, 5-H (Table 3, entry 5) abrogated activity by 37-fold in comparison with 26 (Table 3, entry 4), although some activity loss exhibited by compound 28 (Table 3, entry 6) may result from the presence of an additional H-bond donor and consequent impaired cell penetration with respect to its matched pair (Table 3, entry 7). The thiophene analogue 30, while among the most potent replacements, exhibited unsurprising metabolic instability, and pyridine replacements had decreased potency (Table 3, entries 9 and 10). Substitution at the para-position of the phenyl ring proved more fruitful, with both electron-donating and -withdrawing substituents tolerated (entries 11–13); however, the strongly electron withdrawing sulfone was not tolerated (entry 14).
Table 3. Modification of the Pyridine 3- and 5-Substituents

| Entry | No | R¹ | R² | 7dF3 IC₅₀ ±SD (μM) | MLM* | HLM* |
|-------|----|----|----|-------------------|------|------|
| 1     | 23 | H  | H  | >30               | ND   | ND   |
| 2     | 24 | Cl | H  | 23.0±2.90         | ND   | 8%   |
| 3     | 25 | Br | Br | 0.361±0.170       | 35%  | 13%  |
| 4     | 26 | Cl |     | 0.088±0.060       | 56%  | 6%   |
| 5     | 27 | H  |     | 3.26±1.31         | 23%  | 4%   |
| 6     | 28 | Cl | N  | 1.70±0.474        | 88%  | 6%   |
| 7     | 29 | Cl | N  | 0.778±0.283       | 27%  | 4%   |
| 8     | 30 | Cl |     | 0.040±0.028       | 97%  | 27%  |
| 9     | 31 | Cl |     | 1.11±0.551        | 37%  | 6%   |
| 10    | 32 | Cl |     | 0.728±0.261       | 40%  | 5%   |
| 11    | 33 | Cl | OMe| 0.037±0.013       | 84%  | 30%  |
| 12    | 34 | Cl |     | 0.123±0.102       | ND   | ND   |
| 13    | 35 | Cl |     | 0.087±0.026       | 52%  | ND   |
| 14    | 36 | Cl |     | 1.67±1.15         | 29%  | 0%   |
| 15    | 37 | Cl |     | 0.309±0.097       | 38%  | 39%  |
| 16    | 38 | Cl |     | 0.042±0.010       | 21%  | 19%  |

* MLM and HLM values indicate percent turnover after 30 min of incubation.
 Increased potency of the p-methoxy analogue 33 in the 7dF3 cell-based luciferase reporter assay (IC\textsubscript{50} = 0.037 μM) compared to 9 (IC\textsubscript{50} = 1.76 μM) was mirrored by increased potency and overall profile in cell-based assays incorporating different activating components of WNT signaling, suggesting that the locus of action on the WNT pathway had been maintained (Table 2).

Although compound 33 was among the most potent, its metabolic stability was poor; however, the p-morpholine derivative 38 (Table 3, entry 16) proved equipotent, with acceptable metabolic stability, while the corresponding meta-derivative 37 was 8-fold less potent (Table 3, entry 15). Despite acceptable metabolic stability, compound 38 demonstrated in vivo clearance greater than liver blood flow upon iv dosing in mouse pharmacokinetic studies; we suspected amidase-mediated cleavage of the primary amide, although this proved difficult to definitively confirm.

With compound 38 in hand, we were keen to further explore the piperidine carboxamide in order to understand the optimal geometry for the H-bond acceptor and donor motifs and to reduce the potential for putative amidase-mediated cleavage of the primary carboxamide. Replacement of the 4-carboxamide by nitrile, a highly directional H-bond acceptor, maintained activity in the reporter assay and reinforced the notion that an appropriately positioned H-bond acceptor motif was the dominant feature (Table 4, entry 1). Introduction of a geminal methyl group at the 4-position of the piperidine ring also maintained potency (Table 4, entry 2) and suggested that spirocyclic carboxamides might be tolerated. With this result in hand, a range of 6,5-spirocyclic ring scaffolds was explored and, pleasingly, resulted in compounds with good potency and metabolic stability. The 2,8-diazaspiro[4.5]decan-1-one 44 exhibited 25-fold improved potency over the isomeric spirocycle 46, consistent with a specific interaction from the amide motif (Table 4, entries 3 and 4). Methylolation of the amide (entry 5) reduced potency 24-fold, indicating that, in this spirocyclic scaffold, both H-bond donor and acceptor motifs are important for activity. An isomeric spirocyclic ring system at the 3-position of the piperidine ring lost significant potency (entry 6), although potency could be restored with the corresponding S,S-spirocyclic ring system incorporated as a racemate (entry 7). Improving the H-bond donor ability of the spirocyclic NH by incorporation of an additional carbonyl functionality to give the acidic imide 54 resulted in a very potent and metabolically stable cell-based inhibitor of WNT pathway activity; however, poor solubility (0.003 mg/mL in phosphate buffer at pH 7.4) was an unfavorable attribute and below our criteria for compound progression. Considering all the data together, compound 44 presented the most promising profile, and we were keen to explore the extent to which the chloro substituent could be varied (Table 5). Replacement with small alkyl, cycloalkyl, aryl, or heteroaryl groups (Table 5, entries 2−7), all of which were designed to maintain cell penetration, resulted in maintenance of potency for the prop-1-en-2-yl substituent in 59 (Table 5, entry 3) but a loss of potency for other exemplars, which we ascribed to a lack of toleration of steric bulk in this region of the putative binding site. In order to avoid potential metabolic liabilities with the presence of an alkene in compound 59, we henceforth adopted chloride as the preferred substituent.

To benchmark the chemical series, we conducted mouse in vivo pharmacokinetic studies on compound 44, which revealed improved in vivo clearance compared to compound 38; however, clearance remained unacceptably high with corresponding low oral bioavailability (Table 6). Despite an unfavorable pharmacokinetic profile, other properties of compound 44 were attractive: it was inactive when tested at 10 μM across a panel of 55 receptors, ion channels, and enzymes and demonstrated no significant activity at 1 μM.

### Table 4. Optimization of the Piperidine-4-carboxamide

| Entry | No | R | 7dF3 IC\textsubscript{50} ±SD (μM) | MLM\textsuperscript{a} | HLM\textsuperscript{a} |
|-------|----|---|-----------------|----------------|----------------|
| 1     | 40 | N | 0.070±0.026     | 78%            | ND             |
| 2     | 42 | O | 0.087±0.057     | 20%            | 21%            |
| 3     | 44 | N | 0.030±0.017     | 29%            | 20%            |
| 4     | 46 | N | 0.746±0.577     | ND             | ND             |
| 5     | 48 | N | 0.715±0.403     | ND             | ND             |
| 6     | 50 | N | 5.70±3.57       | ND             | ND             |
| 7     | 52 | Nh| 0.055±0.014     | 42%            | ND             |
| 8     | 54 | N | 0.006±0.004     | 25%            | 24%            |

\textsuperscript{a} MLM and HLM values indicate percentage parent remaining after 30 min of incubation.

\[ \text{MLM} = \frac{\text{Parent Remaining}}{\text{Initial Concentration}} \times 100 \]

\[ \text{HLM} = \frac{\text{Parent Remaining}}{\text{Initial Concentration}} \times 100 \]
morphinophenyl (Table 8, entries 11–13). Removal of either morpholine heteroatom reduced potency versus the parent compound 44 (Table 8, entries 1 and 2). Incorporation of an unsubstituted piperazine proved detrimental, whereas the N-methylpiperazine 68 restored potency with some improvement in metabolic stability; however, an unfavorable Caco-2 permeability profile (efflux ratio >500) precluded further progression of this compound.

Pleasingly, 5- or 6-membered heterocyclic replacements for the morpholine provided compounds with good potency and equivalent or improved metabolic stability. The electron-withdrawing oxadiazole 70 proved the least potent modification in the set, consistent with the poor activity of the electron-withdrawing sulfone 36 (Table 3, entry 14). The N-methylpyrazole 74 proved more potent than its desmethyl matched pair 73, possibly due to poorer cell penetration of the latter compound, while the regioisomeric pyrazole 71 also displayed lower potency. Replacement of the 4-morpholinophenyl moiety with fused 6,6- or 5,6-heterocycles (Table 8, entries 11–13) retained potency but at the expense of higher metabolic clearance compared to compound 74, which provided the optimal balance of potency and metabolic stability in this subseries.

We were keen to explore the combination of the preferred 1-methyl-4-phenyl-1H-pyrazole with selected spirocyclic carboxamides from Table 4. In comparison with compound 74, we noted that the corresponding 5,5-spirocyclic carboxamide 78 was significantly less metabolically stable as a racemate (Table 9). The most potent enantiomer 80 (absolute stereochemistry not assigned) displayed the highest metabolic stability of the pair; however, this was still inferior to the parent molecule 74 and halted further investigation of this compound. Combining a spirocyclic imide with our favored 1-methyl-4-phenyl-1H-pyrazole substituent gave the potent and stable inhibitor 81; however, in common with other imides prepared in this series, aqueous solubility proved to be very poor (<0.001 mg/mL in phosphate buffer at pH 7.4), which precluded further progression of this compound. 8-(3-Chloro-5-(4-(1-methyl-1H-pyrazol-4-yl)phenyl)-2,8-diazaspiro[4.5]decan-1-one (CCT251545, 74) was therefore selected for progression to in vivo studies.

In vivo pharmacokinetic studies on compound 74 revealed moderate clearance in both mouse and rat with moderate to high oral bioavailability; the volume of distribution in both species was low, consistent with the pharmacokinetic profile of a neutral compound (Table 10). Compound 74 displayed high flux (Papp = 24 × 10^{-6} cm s^{-1}), acceptable efflux (ER = 2.5), and minimal hERG inhibition (IC_{50} > 10 μM); aqueous kinetic and thermodynamic solubilities were also sufficient for further progression (94 μM and 0.006 mg/mL, respectively, in pH 7.4 phosphate buffer). Compound 74 demonstrated minimal activity when tested across a panel of 55 receptors, ion channels, and enzymes at 1 μM (Table S4, SI); weak inhibition of CYPs 2C8, 2C9, and 3A4 was observed (see Supplementary Table S1, SI); however, this was not considered sufficient to preclude further investigation. Testing versus a panel of 291
	able{5. Variation of the 3-Chloro Substituent}{
| Entry | No | R | IC_{50} ±SD (μM) | MLM \(\%\) | HLM \(\%\) |
|-------|----|---|----------------|----------|----------|
| 1     | 44 | Cl | 0.030±0.017     | 29        | 20       |
| 2     | 56 | Me | 0.113±0.067     | 26        | 5        |
| 3     | 59 |   | 0.012±0.002     | 39        | ND       |
| 4     | 60 |   | 0.078±0.076     | 17        | 38       |
| 5     | 62 |   | 1.01±0.346      | ND        | ND       |
| 6     | 63 |   | >30             | ND        | ND       |
| 7     | 64 | N-pyrrolidine   | 1.20       | ND        | ND       |

\(\text{MLM and HLM values indicate percentage parent remaining after 30 min of incubation.}\)

across a broad panel of 209 nonmutated kinases (see Supplementary Tables S2 and S3, SI); in addition, 44 did not strongly inhibit the known intracellular WNT pathway target tankyrase (TNKS1 IC_{50} = 19.0 ± 0 μM, TNKS2 IC_{50} = 16.0 ± 1.41 μM). Permeability, as assessed by Caco-2 flux, was high (Papp = 37 × 10^{-6} cm s^{-1}) with no efflux, while kinetic and thermodynamic aqueous solubilities were acceptable (164 μM and 0.017 mg/mL in phosphate buffer at pH 7.4, respectively) and no plasma instability was observed. We concluded that the poor oral bioavailability was largely the result of high first pass clearance and adopted a more accurate measure of intrinsic metabolic clearance (Clint) across three species to better inform our selections for in vivo pharmacokinetic studies. In these in vitro metabolism studies, compound 44 showed high intrinsic clearance in the mouse consistent with our in vivo observations (Table 7).

Compound 44 also exhibited increased inhibition of CYP enzymes (CYP2C9, IC_{50} = 2.9 μM; CYP3A4, IC_{50} = 2.1 μM) in comparison with the original hit 9 and generated a glutathione adduct in an in vitro reactive metabolite assay (data not shown); we were therefore keen to replace the morpholine moiety that has been implicated in reactive metabolism formation. A diverse set of compounds was prepared bearing saturated rings or aromatic heterocycles as morpholine replacements.}

Table 6. Mouse Pharmacokinetics of Compound 44

| dose (mg/kg) | route | Cl (mL/min/kg) | Vss (L/kg) | Tmax (h) | AUC (h ng/mL) | bioavailability (%) | T_{1/2} (h) |
|-------------|-------|----------------|-----------|---------|---------------|---------------------|------------|
| 0.2         | iv    | 65             | 1.44      | 0.10    | 50.8          | NA                  | 0.31       |
| 0.5         | po    |                |           | 0.25    | 7.4           | NA                  | 6          |

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kinases at 1 μM revealed weak inhibition of GSK3α and -β as the only hits (IC50 = 0.462 and 0.690 μM, respectively; see Supplementary Table S5, SI), insufficiently potent to account for the observed effects on cell-based WNT pathway activity; moreover, inhibition of GSK3β may be expected to activate rather than inhibit the WNT signaling. Compound 74 demonstrated weak inhibition of tankyrase enzymes (TNKS1 IC50 >10 μM, TNKS2 IC50 = 15.0 ± 0 μM).

To confirm activity on the WNT pathway, a number of studies were undertaken with compound 74. RT-PCR of 7dF3 cells treated with 74 demonstrated a 12-fold inhibition of endogenous cMYC transcription (IC50 = 2.3 nM, Supplementary Figure S1, SI), a commonly studied WNT target gene.40 Compound 74 was also tested in human colorectal cancer cell lines LS174T (β-catenin mutant) and SW480 (APC mutant) that have constitutively activated WNT signaling or PA-1 human teratocarcinoma cells that are WNT ligand dependent; 74 potently inhibited reporter-based readouts of basal WNT pathway activity in LS174T and SW480 cells in the absence of WNT ligand or other stimulants (IC50 = 0.023 ± 0.011 μM and 0.190 ± 0.030 μM, respectively) and demonstrated potent inhibition of WNT3a ligand-dependent reporter readout in PA-1 cells (IC50 = 0.007 μM). No activity was observed in RKO-F1522 cells bearing a firefly luciferase reporter downstream of the EGF-α promoter, a commonly used, ubiquitously active, housekeeping promoter that has high activity in most mammalian cells, irrespective of their WNT dependence (IC50 > 10 μM). We developed an APC mutant human colorectal cancer cell line (COLO205-F1756 clone 4) engineered to express a modified luciferase-based WNT pathway reporter. Compound 74 demonstrated potent inhibition of WNT pathway activity in this clone (IC50 = 0.035 ± 0.003 μM, n = 8); in addition, we were able to demonstrate that compound 74 did not affect endogenous levels of TCF1 or TCF4 at concentrations that reduce luciferase expression in this cell line (Supplementary Figure S2, SI). Taken together, these data confirm that suppression of the WNT pathway activity by 74 is not unique to a specific cell line, is independent of exogenous WNT pathway stimulation, does not result from destabilization of TCF1 or TCF4, and is not due to interference with the luciferase-based reporter readout.

Extension of WNT pathway inhibition to the whole animal setting was achieved using the APC mutant human colorectal cancer cell line COLO205-F1756 clone 4. Mice bearing established COLO205-F1756 clone 4 tumor xenografts were treated with compound 74 (70 mg/kg po bid for 9 days), at which point WNT reporter activity was measured by whole body bioluminescent imaging and therapeutic response by excised tumor weights. Significant inhibition of WNT signaling was observed, and treated tumors were smaller than controls: flux normalized to tumor weight was reduced by 80% (P = 0.0003) and tumor weights by 37.5% (P = 0.0379, Mann–Whitney U test; Figure 4A,B). Plasma and tumor levels of compound 74 were determined 2 and 6 h after the last dose, and at these time points, total plasma and tumor levels significantly exceeded those required for inhibition of luciferase reporter activity in a cell-based assay. Taken together, these data demonstrate inhibition of WNT pathway activity in a human colorectal cancer xenograft model and some indication of tumor growth inhibition following oral dosing at exposures consistent with inhibition of WNT pathway activity.

**CONCLUSIONS**

We have described the optimization of a small-molecule inhibitor of the WNT pathway identified as a singleton from an HTS of a diverse 63 040 compound library versus a cell-based luciferase reporter assay. Application of classical hypothesis-driven, ligand-based SAR enabled the discovery of highly potent cell-based WNT pathway inhibitors, with compound 74 demonstrating good oral pharmacokinetics in mouse and rat. With knowledge from our cell-based assay cascade, we
hypothesized that this series of compounds acts at an intracellular target. Therefore, our medicinal chemistry optimization necessitated design criteria likely to maintain cell permeability to avoid masking true WNT-pathway SAR, and with this in mind, attention to H-bond donor and acceptor count was critical. The SAR is consistent with the requirement for a distinct twisted conformation about the pyridine—piperidine bond, as observed in the small-molecule X-ray

Table 8. Optimization of the Pyridine 5-Substituent

| Entry | No | R       | 7df3 IC_{50} ±SD (μM) | Clint (μL/min/mg) |
|-------|----|---------|-----------------------|------------------|
|       |    |         |                       | Mouse | Rat | Human |
| 1     | 65 | [Image] | 0.141±0.092           | >1000 | 225 | 600   |
| 2     | 66 | [Image] | 0.154±0.045           | 532   | 111 | 226   |
| 3     | 67 | [Image] | 0.430±0.350           | 21    | ND  | 15    |
| 4     | 68 | [Image] | 0.016±0.009           | 101   | 10  | 64    |
| 5     | 69 | [Image] | 0.024±0.002           | 173   | 99  | 100   |
| 6     | 70 | [Image] | 0.225±0.166           | 366   | ND  | 41    |
| 7     | 71 | [Image] | 0.036±0.013           | 129   | 97  | 30    |
| 8     | 72 | [Image] | 0.050±0.011           | 813   | 136 | 244   |
| 9     | 73 | [Image] | 0.022±0.004           | 374   | ND  | 64    |
| 10    | 74 | [Image] | 0.005±0.002           | 141   | 54  | 84    |
| 11    | 75 | [Image] | 0.015±0.009           | 556   | ND  | 76    |
| 12    | 76 | [Image] | 0.028±0.007           | 422   | ND  | 102   |
| 13    | 77 | [Image] | 0.010±0.007           | 345   | 66  | 79    |
crystal structure of the original hit compound 9, and also is consistent with the lower pKₐ of the pyridine nitrogen and the lack of significant ion channel pharmacology across exemplars of the chemical series. Enhanced potency was achieved through maintenance of this twisted conformation and replacement of one of the chlorine atoms with a para-substituted aryl ring. Methylpyrazole was found to be the optimal para-substituent in terms of potency, metabolic stability, and membrane permeability. Combination of the favored methylpyrazole substituent with a 6,5-spirocyclic carboxamide, in place of the original piperidine carboxamide in hit compound 9, led to 74, a potent small-molecule inhibitor of the WNT pathway with good oral pharmacokinetics and in vivo activity against the WNT pathway. Compound 74 also demonstrated a lack of transporter-mediated efflux and a lack of activity across broad in vitro panels of enzyme, receptor, and ion channel targets. Further details of the in vivo pharmacological profile of this chemical tool, including studies to determine its molecular target, will be reported in due course.

The canonical WNT pathway is of significant interest in oncology, and the paucity of druggable targets on the pathway has hampered drug discovery efforts. Pathway-based screening approaches are becoming increasingly widespread as greater appreciation of the benefits of this strategy become apparent. In particular, hits from such screens, by necessity, have good cell permeability, and components of protein complexes are more likely to be in a relevant endogenous conformation, which may reveal new druggable binding sites or alternative targetable conformations of known sites; moreover, key signal-determining pathway nodes are more likely to be discovered as a result of cell-based readouts at the transcriptional level. Here we describe a successful example of hypothesis-driven medicinal chemistry optimization from a singleton hit against a cell-based assay readout for the WNT pathway in the absence of knowledge of the biochemical target.

### Table 9. Combination of Optimal Pyridine 4- and 5-Substituents

| Entry | R¹ | R² | 7df3 IC₅₀ ±SD (μM) | Clint (μL/min/mg) |
|-------|-----|-----|-------------------|------------------|
| 1     | 74  |    | 0.005±0.002       | 141              |
| 2     | 78  |    | 0.015±0.005       | 282              |
| 3     | 79  |    | 0.040±0.019       | 606              |
| 4     | 80  |    | 0.019±0.004       | 198              |
| 5     | 81  |    | 0.002±0.0003      | 32               |

¹Absolute stereochemistry undefined.

### Table 10. Pharmacokinetics of Compound 74

| species | dose (mg/kg) | route | Cl (mL/min/kg) | Vss (L/kg) | Tmax (h) | AUC (h ng/mL) | bioavailability (%) | t₁/₂ (h) |
|---------|--------------|-------|----------------|------------|----------|---------------|---------------------|----------|
| mouse   | 0.2          | iv    | 31             | 1.1        | 0.10     | 106.8         | NA                  | 0.55     |
| mouse   | 0.5          | po    | NA             | NA         | 0.25     | 143.0         | 54                  | NA       |
| rat     | 0.2          | iv    | 26             | 1.8        | 0.1      | 129.6         | NA                  | 0.97     |
| rat     | 0.5          | po    | NA             | NA         | 0.25     | 287.5         | 88                  | NA       |
EXPERIMENTAL SECTION

Chemistry. Commercially available starting materials, reagents, and dry solvents were used as supplied. Flash chromatography was performed using Merck silica gel 60 (0.025–0.04 mm). Column chromatography was also performed on a Biotage SP1 purification system using Thomson or Biotage Flash silica cartridges. Preparative TLC was performed on Merck plates. Ion exchange chromatography was performed using acidic Isolute Flash SCX-II columns or basic Isolute Flash NH2 columns. Preparative HPLC was conducted using a Phenomenex Luna column (5 μm, 250 × 21.2 mm, C18, Phenomenex, Torrance, CA) using a Gilson GX-281 Liquid Handler system combined with a Gilson 322 HPLC pump (Gilson, Middleton, WI), over a 15 min gradient elution from 10:90 to 100:0 MeOH:water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min or over a 15 min gradient elution from 40:60 to 100:0 MeOH:water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min.

UV−vis spectra were acquired at 254 nm on a Gilson 156 UV−vis detector (Gilson, Middleton, WI). Collection was triggered by UV signal and collected using a Gilson GX-281 Liquid Handler system (Gilson, Middleton, WI). Raw data was processed using Gilson Trilution Software. 1H NMR spectra were recorded on a Bruker Avance-500 or Bruker Avance-400. Samples were prepared as solutions in a deuterated solvent and referenced to the appropriate internal nondeuterated solvent peak. 13C NMR spectra were recorded at 126 MHz using an internal deuterium lock. Some internal references were used: CDCl3 (δC 77.0), CD3OD (δC 49.0), and DMSO-d6 (δC 39.5). Unobserved resonances for quaternary carbon atoms are denoted by “Cq not observed.” LC−MS and HRMS analyses were performed on an Agilent 1200 series HPLC and diode array detector coupled to a 6210 time-of-flight mass spectrometer with dual multimode atmospheric pressure CI/ESI source or coupled to an Agilent 6520 quadrupole-time-of-flight mass spectrometer with dual multimode atmospheric pressure CI/ESI source. LC−MS analysis was also performed on a Waters Alliance 2755 separations module and Waters 2487 dual wavelength absorbance detector coupled to a Waters/Micromass LCt time-of-flight mass spectrometer with ESI source. Analytical separation was carried out according to the following methods. For method A, analytical separation was carried out at 40 °C on a Merck Purospher STAR column (RP-18e, 30 × 4 mm) using a flow rate of 3 mL/min in a 2 min gradient elution with detection at 254 nm. The mobile phase was a mixture of methanol (solvent A) and water containing formic acid at 0.1% (solvent B). Gradient elution was as follows: 1:9 (A/B) to 9:1 (A/B) over 1.25 min, 9:1 (A/B) for 0.5 min, and then reversion back to 1:9 (A/B) over 0.15 min, and finally 1:9 (A/B) for 0.1 min. For method B, analytical separation was carried out at 30 °C on a Merck Chromolith SpeedROD column (RP-18e, 50 × 4.6 mm) using a flow rate of 2 mL/min in a 4 min gradient elution with detection at 254 nm. The mobile phase was a mixture of methanol (solvent A) and water containing formic acid at 0.1% (solvent B). Gradient elution was as follows: 1:9 (A/B) to 9:1 (A/B) over 2.5 min, 9:1 (A/B) for 1 min, and then reversion back to 1:9 (A/B) over 0.3 min, and finally 1:9 (A/B) for 0.2 min. For method C, analytical separation was carried out at 30 °C on a Merck Purospher STAR column (RP-18e, 30 × 4 mm) using a flow rate of 1.5 mL/min in a 4 min gradient elution with detection at 254 nm. The following reference masses were used for HRMS analysis: caffeine [M + H]+ 195.087652; hexakis(1H,1H,3H-tetrafluoropentoxy)phosphazene [M + H]+ 922.009798 and hexakis(2,2-difluoroethoxy)phosphazene [M + H]+ 622.02896 or reserpine [M + H]+ 609.280657. All compounds submitted for biological testing were determined to be >95% pure by method A, B, or C.

Preparation of Compounds in Table 1 Exemplified by Compound 9. 1-(3,5-Dichloropyridin-4-yl)piperidine-4-carboxamide (9). To a stirred suspension of 3,4,5-trichloropyridine (10) (500 mg, 2.74 mmol) and piperidine-4-carboxamide (350 mg, 2.73 mmol) in NMP (15 mL) at ambient temperature was added triethylamine (770 μL, 5.48 mmol) in one portion. The reaction was heated to 220 °C for 60 min under microwave irradiation. Once cooled, the reaction mixture was partitioned between EtOAc and sat. NaHCO3. The separated aqueous layer was extracted with EtOAc (2 × 100 mL), and the combined organic extracts were washed with water (50 mL) and brine (50 mL), dried over magnesium sulfate, filtered, and concentrated in vacuo to give a crude oily solid. Purification was accomplished by flash chromatography on silica gel eluting with CH2Cl2/1 M methanolic ammonia (95:5 to 92:8) to yield impure title.
Preparation of Compounds in Table 4 Exemplified by Compounds 26 and 38. 1-(3-Chloro-5-phenylpyridin-4-yl)piperidine-4-carboxamide (26). To a stirred suspension of 1-(3,5-dichloropyridin-4-yl)piperidine-4-carboxamide (9) (100 mg, 0.365 mmol), phenylboronic acid (49 mg, 0.401 mmol), and triethylamine (450 μL, 0.326 mmol), the reaction was heated to 150 °C under microwave irradiation for 30 min. Once cooled, the reaction was concentrated in vacuo and azeotroped with toluene (2 × 50 mL) to give a yellow solid. Purification was accomplished by flash chromatography on silica gel eluting with CH2Cl2/MeOH (98:2) to obtain the title compound (19 mg, 19% yield) as a white solid: 1H NMR (500 MHz, CDCl3) δ 1.53–1.60 (m, 4H), 2.04–2.11 (m, 1H), 2.49–2.57 (m, 2H), 3.00–3.06 (m, 2H), 3.15–3.20 (m, 4H), 3.73–3.78 (m, 4H), 6.73 (br s, 1H), 7.03 (d, J = 8.7 Hz, 2H), 7.18 (d, J = 8.7 Hz, 3H), 8.13 (br s, 1H), 8.40 (br s, 1H). 13C NMR (126 MHz, CDCl3) δ 29.2, 42.4, 50.9, 127.8, 130.0, 134.1, 137.6, 149.6, 150.7, 174.8. LC–MS (method B, ESI, m/z) tR = 0.99 min, 316/318 (M + H)+; ESI-HRMS calc for C17H19ClN3O+ (M + H)+: C17H19ClN3O (M + H)+ 316.1211, found 316.1206.

Preparation of Compounds in Table 4 Exemplified by Compounds 44 and 54. 8-(3,5-Dichloropyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (54). Ten microwave vials were prepared as follows: To a stirred suspension of 8-(3,5-dichloropyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (53) (300 mg, 0.764 mmol), 4-morpholinophenylboronic acid (222 mg, 0.963 mmol), and triethylamine (450 μL, 0.326 mmol), 2,8-diazaspiro[4.5]decane-1,3-dione hydrochloride (206 mg, 62% yield) as a white solid: 1H NMR (500 MHz, CDCl3) δ 1.48–1.54 (m, 2H), 2.07–2.14 (m, 2H), 2.55 (s, 2H), 2.75 (t, J = 11.7 Hz, 2H), 3.88–3.92 (m, 4H), 6.97–7.05 (m, 2H), 7.17–7.24 (m, 2H), 8.10 (s, 1H), 8.25 (s, 1H). 13C NMR (126 MHz, CDCl3) δ 33.3, 40.6, 44.2, 47.2, 48.7, 66.8, 115.2, 127.8, 130.0, 148.1, 150.0, 150.9, 174.9, 181.4. LC–MS (method B, ESI, m/z) tR = 2.00 min, 441/443 (M + H)+; ESI-HRMS calc for C17H17ClN3O2+ (M + H)+: C17H17ClN3O2 (M + H)+ 441.1688, found 441.1693.

Preparation of Compounds in Table 5 Exemplified by Compounds 56, 59, and 60. 8-(3-Bromo-5-methylpyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (55). A solution of 3-bromo-5chloro-5-methylpyridine (200 mg, 1.45 mmol), triethylamine (610 μL, 4.36 mmol), and 2,8-diazaspiro[4.5]decane-1,3-dione hydrochloride (491 mg, 1.53 mmol) in NMP (4.4 mL) was heated to 220 °C under microwave irradiation for 5 h at 220 °C. Once cooled, the reaction was partitioned between EtOAc and sat. NaHCO3, then dried over MgSO4, filtered, and concentrated in vacuo to give a crude yellow solid. The solid was recrystallized in hot EtOAc, filtered, washed with ether, and dried in vacuo to yield 8-(3-bromo-5-methylpyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (55) (44 mg, 9% yield) as an off white solid.
1H NMR (500 MHz, CDCl3) δ 1.51−1.58 (m, 2H), 2.06−2.15 (m, 2H), 2.16 (t, J = 6.8 Hz, 2H), 2.31 (s, 3H), 3.18 (br s, 2H), 3.33 (br s, 2H), 3.39 (t, J = 6.8 Hz, 2H), 6.18 (br s, 1H), 8.21 (br s, 1H), 8.45 (br s, 1H); LC−MS (method C, ESI, m/z) tR = 1.42 min, 324/326 (M + H)⁺.

8-(3-Chloro-5-(4-morpholin-4-ylphenyl)-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (57). 8-(3-Chloro-5-(4-morpholin-4-ylphenyl)-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (57) (268 mg, 0.943 mmol), and Pd(dppf)Cl2·CH2Cl2 (3.7 mg, 5.1 μmol) were loaded in a microwave vial under microwave irradiation for 2.5 h at 220 °C. The solvent was evaporated and the crude material was dry-loaded and purified via Biotage column chromatography (CH2Cl2/EtOH 3:1) to give the title compound 57 (35 mg, 0.01 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)piperazine (34 mg, 0.11 mmol), and Pd(dppf)Cl2·CH2Cl2 (3.7 mg, 5.1 μmol) were loaded in a microwave vial under microwave irradiation, and then degassed MeCN (1.8 mL) and 0.5 M aqueous sodium carbonate solution (284 μL, 0.142 mmol) were added. The reaction mixture was heated at 150 °C for 30 min under microwave irradiation. The solvent was evaporated and the crude material was purified via Biotage column chromatography (DCM/EtOH 98:2 to 90:10) to give the title compound 59 as a colorless oil (24.5 mg, 44% yield). 1H NMR (500 MHz, CDCl3) δ 1.21−1.25 (m, 2H), 1.80−1.88 (m, 2H), 1.96 (t, J = 6.9 Hz, 2H), 2.13 (s, 3H), 2.72−2.79 (m, 2H), 3.11−3.16 (m, 2H), 3.21−3.24 (m, 4H), 3.27 (t, J = 6.9 Hz, 2H), 3.87−3.91 (m, 4H), 5.00−5.02 (m, 1H), 5.24−5.26 (m, 1H), 5.52 (s, 1H), 7.00 (d, J = 8.8 Hz, 2H), 7.30 (d, J = 8.8 Hz, 2H), 8.17 (s, 1H), 8.18 (s, 1H); LC−MS (method C, ESI, m/z) tR = 1.85 min, 435 (M + H)+; ESI-HRMS calcd for C26H35N4O2 (M + H)+ 440.2212, found 440.2215.

8-(3-Isopropyl-5-(3-(4-morpholin-4-ylphenyl)-4-yl)-4-yl)-2,8-diazaspiro[4.5]decan-1-one (60). 8-(3-(4-Morpholinophenyl)-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (45) (126 mg, 0.580 mmol) was solubilized in ethanol (0.5 mL) and a few drops of DCM. Then 1 M HCl (35 μL, 0.035 mmol) and palladium 10% on carbon (3 mg, 0.01 mmol) were added. The reaction was evaporated under a balloon of hydrogen at rt for 2 days and filtered over Celite. The crude material was filtered on a flash NH4 column and concentrated to give the title compound 60 as a colorless oil (4 mg, 80% yield). 1H NMR (500 MHz, CDCl3) δ 1.28−1.35 (m, 5H), 1.58−1.62 (m, 4H), 2.06−2.09 (m, 4H), 2.39 (s, 3H), 2.60−2.65 (m, 2H), 3.11 (t, J = 8.3 Hz, 2H), 3.25 (m, 2H), 3.33 (m, 2H), 3.45 (m, 1H), 3.73−3.79 (m, 1H), 3.88−3.94 (m, 4H), 4.96−4.98 (m, 1H), 5.56−5.69 (m, 2H), 6.99−6.99 (m, 2H), 7.20 (d, J = 8.3 Hz, 2H), 8.15 (s, 1H), 8.42 (s, 1H); 13C NMR (126 MHz, CDCl3) δ 24.0, 26.2, 30.5, 32.4, 38.6, 42.0, 48.5, 49.2, 66.9, 115.1, 130.4, 130.6, 134.3, 140.9, 147.9, 150.3, 150.5, 154.5, 181.6; LC−MS (method C, ESI, m/z) tR = 1.85 min, 435 (M + H)+; ESI-HRMS calcd for C26H35N4O2 (M + H)+ 440.2212, found 440.2215.

Preparation of Compounds in Table 8 Exemplified by Compounds 68 and 74. 8-(3-Chloro-5-(4-(4-methylpiperazin-1-yl)phenyl)-4-yl)-2,8-diazaspiro[4.5]decan-1-one (68). 8-(3-Chloro-5-(4-morpholin-4-ylphenyl)-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (45) (55 mg, 0.10 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)piperazine (34 mg, 0.11 mmol), and Pd(dppf)Cl2·CH2Cl2 (3.7 mg, 5.1 μmol) were loaded in a microwave vial under microwave irradiation for 2.5 h at 220 °C. The solvent was evaporated and the crude material was purified via Biotage column chromatography (CH2Cl2/EtOH, 99.8:0.2 to 97:3) to give 8-(3-Chloro-5-(4-morpholin-4-yl)phenyl)-4-yl)-2,8-diazaspiro[4.5]decan-1-one (58). 8-(3-Chloro-5-(4-morpholin-4-yl)phenyl)-4-yl)-2,8-diazaspiro[4.5]decan-1-one (58) (200 mg, 0.580 mmol), isopropenylboronic acid (120 μL, 0.638 mmol), and Pd(dppf)Cl2·CH2Cl2 (24 mg, 0.029 mmol) were loaded in a microwave vial under argon, and then acetonitrile (10.4 mL) and 0.5 M aqueous sodium carbonate solution (160 mL, 0.812 mmol) were added. The reaction mixture was heated at 120 °C for 60 min under microwave irradiation, concentrated, and purified via Biotage column chromatography (DCM/EtOH 99:1 to 95:5) to give 8-(3-Chloro-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (58) as a colorless oil (122 mg, 69% yield). 1H NMR (500 MHz, CDCl3) δ 1.50−1.56 (m, 2H), 2.07−2.14 (m, 2H), 2.16 (t, J = 6.9 Hz, 2H), 3.15−3.20 (m, 2H), 3.36−3.42 (m, 4H), 4.97−4.98 (m, 1H), 5.26−5.28 (m, 1H), 5.81 (s, 1H), 8.15 (s, 1H), 8.39 (s, 1H); LC−MS (method C, ESI, m/z) tR = 1.78 min, 306/308 (M + H)+.

8-(3-(4-Morpholin-4-ylphenyl)-5-(prop-1-en-2-yl)pyridin-4-yl)-2,8-diazaspiro[4.5]decan-1-one (74). 8-(3-Chloro-5-(4-morpholin-4-yl)phenyl)-4-yl)-2,8-diazaspiro[4.5]decan-1-one (57) (250 mg, 0.725 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)N-2H-morpholine (82) (268 mg, 0.943 mmol), and Pd(dppf)Cl2·CH2Cl2 (30
mg, 0.036 mmol) were introduced into a microwave vial, and then MeCN (13 mL) and aqueous sodium carbonate solution (0.5 M, 2.00 mL, 1.02 mmol) were added. The reaction mixture was heated under microwave irradiation at 120 °C for 1 h and concentrated. The crude material was purified via Biotech column chromatography (CH2Cl2/ EtOH, 96:4 to 91:9), and trituration with hot EtOAc gave the title compound (81% yield). 76% yield as a white solid: 8-(3-bromo-5-chloropyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (83) (754 mg, 97%) as a white solid: H NMR (500 MHz, DMSO-d6) δ 1.41 (dd, J = 12.5, 1.4 Hz, 2H), 1.65 (td, J = 12.5, 3.9 Hz, 2H), 2.44–2.53 (m, 2H), 2.56 (2H, 2.86, δ (J = 12.5 Hz, 3.9 Hz, 2H)) LC–MS (method B, ESI, m/z) tR = 0.17 min, 169 (M+H)+. 8-(3-Bromo-5-chloropyridin-4-yl)-2,8-diazaspiro[4.5]decane-1,3-dione (84). 8-Bromo-4,5-dichloropyridine (11) (1.00 g, 4.41 mmol) and 2,8-diazaspiro[4.5]decane-1,3-dione (83) (1.10 g, 6.61 mmol) were introduced in a microwave vial, and then NMP (11 mL) and triethylamine (1.90 mL, 13.2 mmol) were added. The reaction mixture was heated under microwave irradiation for 1 h at 220 °C. The reaction mixture was poured into water (110 mL) and the precipitate was filtered by the omission of NADPH and UDPGA from the incubation reaction. The percentage compound turned over was determined after analysis by LC–MS. Mouse Liver Microsomal Stability. Compounds (10 μM) were incubated with male CD1 mouse liver microsomes (1 mg/mL) protein in the presence of 1 mM NADPH, 2.5 mM UDP-glucuronic acid (UDPGA), and 3 mM MgCl₂ in 10 mM PBS at 37 °C. Incubations were conducted for 0 and 30 min. Control incubations were generated by the omission of NADPH and UDPGA from the incubation reaction. The percentage compound turned over was determined after analysis by LC–MS. Human Liver Microsomal Stability. Compounds (10 μM) were incubated with mixed-gender, pooled human liver microsomes (1 mg/mL) protein in the presence of 1 mM NADPH, 2.5 mM UDPGA, and 3 mM MgCl₂ in 10 mM PBS at 37 °C. Incubations were conducted for 0 and 30 min. Control incubations were generated by the omission of NADPH and UDPGA from the incubation reaction. The percentage compound turned over was determined after analysis by LC–MS. Mouse, Rat, and Human Clint Microdetermination. Microsomes (final concentration 0.5 mg/mL), 50 mM phosphate buffer pH 7.4, and compound (final concentration 1 μM) were added to the assay plate and allowed to preincubate for 5 min at 37 °C. The reaction was initiated by the addition of NADPH (final concentration 1.5 mM) and the plate was shaken at 800 rpm at 37 °C. After 0, 5, 10, 20, and 30 min, aliquots were taken, and the reaction was stopped using cold acetonitrile. The samples were centrifuged at 4000 rpm for 30 min at 4 °C and analyzed by LC–MS/MS. Four test compounds were pooled for analysis. The in vitro intestinal clearance was calculated from the rate of compound disappearance. Caco-2 Papp Determination. Caco-2 cells (TC7 clone) were maintained in DMEM in an atmosphere of 8.5% CO₂. For transport experiments, 0.125 × 10⁵ cells/well were seeded on polycarbonate filter inserts and allowed to grow and differentiate for 10–14 days before the cell monolayers were used for experiments. Drug transport experiments were carried out using a cocktail approach in a 4-dimensional setting. Apparent permeability coefficients were determined for A > B and B > A directions with and without the presence of cyclosorbine A as a transporter inhibitor. Up to five test items and reference compounds were dissolved in Hank’s balanced salt solution at pH 7.4 to yield a final concentration of 1 μM. The assays were performed in HBSS containing 25 mM HEPES (pH 7.4) in an atmosphere of 5% CO₂ at 37 °C. Prior to the study, the monolayers were washed in prewarmed HBSS. At the start of the experiments, prewarmed HBSS containing the test items was added to the donor side of the monolayer and HBSS without test items was added to the receiver side. The plates were shaken at 150 rpm at 37 °C during the experiment. After 2 h, the transwell insert containing the monolayer was carefully removed and placed in a new plate, and aliquots of both the receiver and donor sides were taken and diluted with an equal volume of ACN containing the internal standard. The mixture was centrifuged and the supernatant analyzed by LC–MS/MS. The apparent permeability coefficients (Papp) were calculated using the
formula Papp = \((V_{ec}/A \times C_{donor}) \times \Delta C_{ec}/dt \times 10^6\) with \(\text{dC}_{ec} / \text{dt}\) being the change in concentration in the receiver compartment with time, \(V_{ec}\) the volume of the sample in the receiver compartment, \(C_{donor}\) the concentration in the donor compartment at time 0, and \(A\) the area of the compartment with the cells.

**Protein Binding Assays.** Protein binding of test compounds was determined by ultrafiltration using serum from mouse, rat, and human. The test items (final concentration 5 μM) were incubated in triplicate with three different serum dilutions (1:2, 1:5, and 1:10) for 30 min at 37 °C using slight agitation. After the incubation, the 96-well filter plates were centrifuged for 45 min at 3500 rpm and 37 °C; 25 μL portions of the filtrate samples were treated with 50 μL of ethanol and 50 μL of internal standard solution and analyzed by LC–MS/MS. The fraction unbound was calculated from the drug concentrations in the filtrate samples.

**In Vivo Mouse PK in-Life Phase.** Female NMRI mice (n = 5) were each given a single intravenous (bolus) injection or a single oral administration (by gavage) of the compound in a cocktail preparation. Doses of 0.2 mg/kg (per compound, intravenous) and 0.5 mg/kg (per compound, orally) were given as solutions in DMSO/PEG 200/200 water. Consecutive blood samples were taken retroorbitally under isoflurane inhalation from n = 3 animals per route of administration after 0.1 (iv only), 0.25 (po only), 0.5, 1, 2, 4, and 6 h and were further processed to obtain plasma. Plasma samples were spiked with internal standard, and the analyte was detected directly via the increase in HTRF signal. The full value used was the inhibitor-free fraction unbound was calculated from the drug concentrations in the filtrate samples.

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**Biochemical Activity Testing of TNKS1 and -2 (Autoparyyla- lassay).** The autoparylation assay was run in two steps: first, the enzymatic reaction in whichGST-tagged TNKS1 or TNKS2 transferred biotinylated ADP-ribose to itself from biotinylated NAD as the cosubstrate and, second, the detection reaction where a time-resolved FRET between cryptate-labeled anti-GST bound to the GST tag of the enzyme and Xlent-labeled streptavidin bound the biotin-parylation residue was analyzed. The autoparylation activity was detectable directly via the increase in HTRF signal. The autoparylation assay was performed in 384-well HTRF (Cisbio, Codolet, France) assay format in Greiner low-volume 384-well microtiter plates. 250 nM GST-tagged TNKS1 (1023–1327 aa) or 250 nM GST-tagged TNKS2 (873–1166 aa) and 5 μM bio-NAD (Biolog, Life science Inst., Bremen, Germany) as cosubstrate were incubated in a total volume of 5 μL (50 μM HEPES, 4 mM MgCl₂, 0.05% Pluronic F-68, 1.4 mM DTT, 0.5% DMSO, pH 7.7) in the absence or presence of the test compound (10 dilution concentrations) for 90 min at 30 °C. The reaction was stopped by the addition of 1 μL of 50 mM EDTA solution, and 2 μL of the detection solution [1.6 μM SA-Xlent (Cisbio, Codolet, France), 7.4 mM Anti-GST-K (Eu-labeled anti-GST, Cisbio, Codolet, France) in 50 mM HEPES, 800 mM KF, 0.1% BSA, 20 mM EDTA, 0.1% CHAPS, pH 7.0] was added. After 1 h of incubation at rt the HTRF was measured with an Envision multimode reader (PerkinElmer LAS Germany GmbH) at an excitation wavelength of 340 nm (laser mode) and emission wavelengths of 615 and 665 nm. The ratio of the emission signals was determined. The full value used was the inhibitor-free reaction. The pharmacological zero value used was XAY-939 (Tocris) in a final concentration of 5 μM. The inhibitory values (IC₅₀) were determined using either the program Symyx Assay Explorer or Condosso from GeneData.

**Tagman Assays.** 7D3 cells were seeded at 20 000 cells/well into 96-well culture plates. After overnight incubation the cells were treated with 74 at concentrations ranging from 9.1 μM to 0.068 mM. After 2 or 6 h of incubation, the cells were washed with PBS and stored at −80 °C. Cells were lysed in Cells-to-cDNA II Cell lysis buffer (Life Technologies Ltd., Paisley, UK) and transferred to PCR plates. Lysis was completed by heating at 75 °C for 15 min. Lysates were DNase treated (1 μL/well Life Technologies) at 37 °C for 15 min and heat inactivated at 75 °C for 5 min. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). TaqMan reactions were performed in 384-well plates using 1.5 μL cDNA and 1X FAM-labeled primer probe for the measurement of target gene and 1X VIC-labeled primer probe for LRPO and were multiplexed in each well as the internal reference control with TaqMan Universal Master Mix (Life Technologies). Plates were run for 40 cycles using an ABI Via7 Sequence Detection System and software. Ct values for target genes were normalized relative to the LRPO control before being expressed relative to the vehicle treated control sample.

**In Vivo Cell-Based Reporter Assays.** 7D3 Luciferase Reporter Assay. 7D3 cells were seeded at 5000 per well in 50 μL of Dulbecco’s modified Eagle’s medium (DMEM) with 4 mM supplemental L-glutamine in 384-well white tissue culture plates (Corning 3570). After incubating overnight at 37 °C/5% CO₂, compounds ranging in final concentration from 90 μM to 0.3 nM were added in duplicate to the wells. After 2 h of further incubation as above, β-estradiol was added to a final concentration of 10 μM. The cells were incubated for 24 h at 37 °C/5% CO₂ and then 25 μL of luciferase reagent (SteadyGlo, Promega) was added, and the cells were mixed. After leaving the plate for 60 min at room temperature, luminescence was read on a plate luminescence reader (TopCount, PerkinElmer). The percentage inhibition for each compound concentration was calculated using total counts (no compound) and low counts (no β-estradiol) as highs and lows, respectively. IC₅₀ was subsequently determined using a curve-fitting software package in Excel (ExcelSoft, IDBS).

**Tet-O-WNT Reporter Lines.** T-Rex HEK293 Tet-O cells (Life Technologies) were transfected with the TCF-firefly luciferase-IRESGFP (TLIG) reporter, and a highly responsive reporter clone was derived using an analogous process to that previously used to derive the HEK293-based 7D3 cell line. Expression constructs coding for WNT inducers that act at different levels of the pathway were introduced downstream of the Tet-O promoter by FLP-FRT recombination. This generated a set of cell lines that contain an identical TCF-luciferase reporter in combination with Tet-inducible cDNAs encoding ΔN-LRP6, Dvl2, Axin-GID, ΔN-β-catenin, and VP16-TCF4.

**Assay Protocol for Tet-O-WNT Reporter Cell Lines.** Cells were seeded from frozen at 40 000/well into 96-well plates in 80 μL of medium. On day 3, eight compound concentrations ranging from 30 μM to 14 nm were prepared at 33xX final concentration in DMSO and then diluted to 5X final concentration in OptiMEM (Life Technologies) supplemented with 0.05 μg/mL doxycycline hyclate (Sigma-Aldrich). These solutions were then pipetted at 20 μL/well into each well of the plate. On day 4, medium was removed, and cells were lysed in 30 μL/well GLOysis buffer (Promega) for 30 min. BrightGLO firefly luciferase substrate (Promega) was pipetted at 30
human colorectal cancer cells were cotransfected with Topflash and CMV-lacZ reporter plasmids as described previously. The RKO (ATCC CRL-2577) cells were transfected with plasmid F1522 encoding PEStestabilized firefly luciferase (luc2P, designated Fluc2P; Promega) and IRES-driven PEST-stabilized renilla (hrLuc; Promega) downstream of the EF-1α promoter. The LS174T (ATCC CL-188) and COLO205 (ATCC, CCL-222) human colorectal cancer cells were transfected with a TCF/LEF lentiviral firefly luciferase reporter construct. This construct was generated by synthesizing and cloning 16 repeats of the TCF/LEF responsive element (−AGATCAAGG−3′) upstream of the minimal pTA promoter driving PEST-stabilized firefly luciferase Fluc2P (6 μg/mL puromycin and stable colonies left to grow for 2 weeks. Colorectal cancer cells were trypsinized and seeded as described previously. The luciferase reporter pathway activation by addition of Wnt-3A supernatant was measured in vitro in 384-well plates using Steady-Glo luciferase reagent (Promega) for 24 h before reporter assays were conducted. Cell viability was assayed in parallel using Alamar blue (Promega). PA-1 human teratocarcinoma cells were stably transfected with a TOPflash TCF reporter plasmid (Merck Millipore) that was modified by introduction of a neomycin resistance marker. Single clones were selected and tested for WNT-driven reporter pathway activation by addition of Wnt-3A supernatant produced by L. Wnt-3A cells (ATCC, CRL-2647). Reporter activity was measured in 96-well plates using Steady-Glo luciferase reagent (Promega) for firefly luciferase. Cells were exposed to compound for 24 h before reporter assays were conducted. Cell viability was assayed in parallel using Alamar blue (Promega). PA-1 human teratocarcinoma cells were stably transfected with a TOPflash TCF reporter plasmid (Merck Millipore) that was modified by introduction of a neomycin resistance marker. Single clones were selected and tested for WNT-driven reporter pathway activation by addition of Wnt-3A supernatant produced by L. Wnt-3A cells (ATCC, CRL-2647). Reporter activity was measured in 96-well plates using Steady-Glo luciferase reagent (Promega). Compound concentration was measured in vivo in 384-well plates using Steady-Glo luciferase reagent (Promega) for firefly luciferase. Cells were exposed to compound for 24 h before reporter assays were conducted. Cell viability was assayed in parallel using Alamar blue (Promega). PA-1 human teratocarcinoma cells were stably transfected with a TOPflash TCF reporter plasmid (Merck Millipore) that was modified by introduction of a neomycin resistance marker. Single clones were selected and tested for WNT-driven reporter pathway activation by addition of Wnt-3A supernatant produced by L. Wnt-3A cells (ATCC, CRL-2647). Reporter activity was measured in 96-well plates using Steady-Glo luciferase reagent (Promega). Compound concentration was measured in vivo in 384-well plates using Steady-Glo luciferase reagent (Promega).
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