Cell Signaling

A Small-Molecule Agonist of the Wnt Signaling Pathway**

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The Wnt signaling pathway plays an important role in a variety of physiological and pathological processes by regulating genes involved in cellular adhesion, proliferation, and differentiation.[1,2] Many of the key components of the signaling pathway have been elucidated. The Wnt ligand activates downstream signal transduction through interaction with its receptor, Frizzled. This interaction leads to the inhibition of β-catenin phosphorylation by the axin–APC–GSK-3β complex and results in the accumulation of β-catenin in the cytoplasm.[3,4] β-Catenin then translocates to the nucleus and forms a complex with T-cell factor/lymphoid-enhancer factor (TCF/LEF), which drives target gene expression. Because Wnt signaling is essential for many aspects of early embryonic development, genetic alteration of this pathway often results in complicated embryonic defects in animal models, which complicates the interpretation of experimental results.[1] In contrast, small-molecule agonists or antagonists offer greater temporal control over Wnt signaling and provide useful tools for the studies of adult-tissue homeostasis and regeneration, and embryogenesis in animal models.[5] Herein we report the identification and characterization of a small-molecule agonist of Wnt that activates Wnt signaling in cellular assays as well as in a *Xenopus* model.

Combinatorial small-molecule libraries that consist of 100000 heterocyclic compounds (including substituted purines, pyrimidines, indoles, quinazolines, pyrazines, pyrrolopyrimidines, pyrazolopyrimidines, phthalazines, pyridazines, and quinoxalines) were screened for small-molecule modulators of Wnt signaling.[6–9] A reporter-based cellular assay was used to measure the transcriptional activity of β-

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catenin/TCF in 384-well cell culture plates. β-Catenin/TCF transcriptional activity is dependent on Wnt signaling and can be used to monitor the activity of the Wnt signaling pathway.[1] The reporter construct encodes the luciferase gene driven by β-catenin/TCF-responsive elements.[10] 293T cells were transiently transfected with the reporter construct in T75 cell culture flasks and replated onto 384-well cell culture plates after overnight culture; 24 h later, cells were treated with library compounds at a concentration of 10 μM. After incubation for another 24 h, luciferase activity was measured, and a number of compounds were identified as potent modulators of the Wnt/β-catenin signaling pathway. One such compound, 2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine (**1**; Figure 1a), induces β-catenin- and TCF-dependent transcriptional activity in a dose-dependent manner with an EC_{50} value of 0.7 μM (Figure 1b).

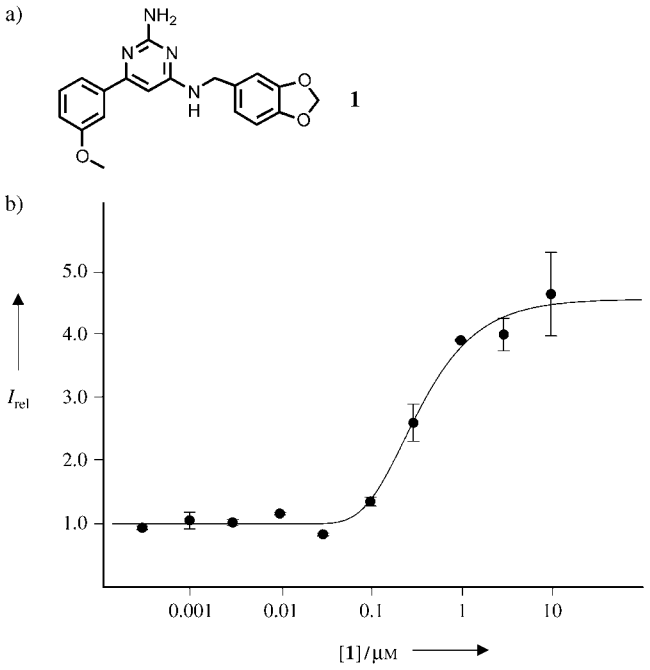

Figure 1. a) Structure of compound **1**; b) compound **1** induces the β-catenin/TCF-dependent reporter in a dose-dependent manner. I_{rel} = relative intensity of the luciferase signal, reflective of the fold activation in reporter gene expression.

zylamino group and the 2-(4-hydroxyphenyl)ethylamino group. Representative active and inactive analogues and their EC_{50} values are provided in the Supporting Information.

The Wnt signal is transduced through its receptor and results in the formation of a heterodimeric complex of β-catenin and TCF that drives downstream target gene expression.[1,3,4] To determine if the active compounds induce reporter gene expression in a TCF-dependent manner, a dominant-negative TCF4 was constructed and transfected into cells in the absence or presence of compound. The dominant negative TCF4 contains a DNA-binding domain but lacks the β-catenin-interaction motif, which leads to the sequestration of TCF-binding sites and subsequent blockage of β-catenin- and TCF-dependent transcriptional activity.[10] The gene encoding the dominant negative TCF4 was generated by PCR and cloned into the eukaryotic expression vector pcDNA3. As shown in Figure 2, β-catenin- and TCF-

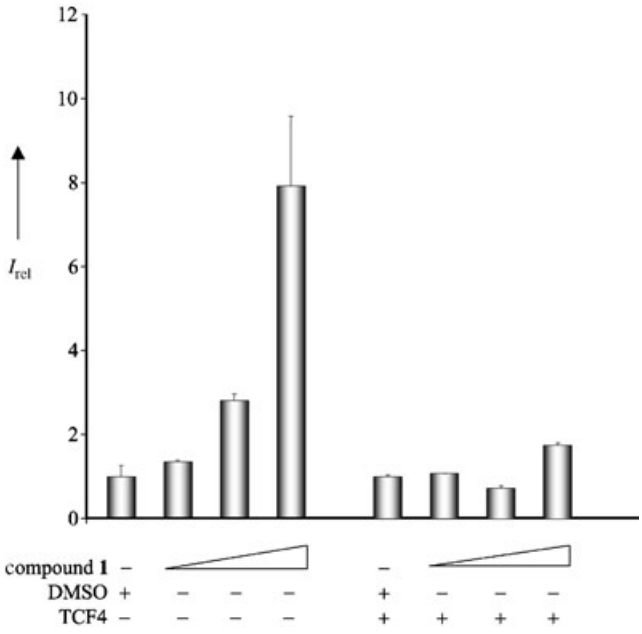

Figure 2. Activity of compound **1** blocked by the presence of a dominant-negative TCF4. 293T cells were treated with DMSO or **1** (0.2–5 μM) in the presence or absence of a dominant negative TCF4. I_{rel} = relative intensity of the luciferase signal, reflective of the fold activation in reporter gene expression.

To further explore the structure–activity relationship, 124 similar compounds bearing the core 4,6-disubstituted 2-aminopyrimidine scaffold were analyzed in the same reporter assay for activation of the Wnt/β-catenin signaling pathway. Only a fraction of these structurally related compounds function as Wnt agonists. The active compounds have small aryl substituents (such as phenyl or methoxyphenyl groups) at position 6 of the pyrimidine ring. Substitution with bulky aryl groups at this position dramatically decreases activity. The introduction of an additional O or NH linkage between the pyrimidine ring and the aryl groups at position 6 also leads to a loss in activity. Only two substituents at position 4 were tolerated in active compounds: the 3,4-(methylenedioxy)ben-

dependent reporter gene expression induced by compound **1** was blocked in the presence of the dominant negative TCF4, thus suggesting that the active compound functions in the canonical Wnt pathway through the TCFs.

A number of known agonists of the Wnt pathway act through inhibition of the activity of the kinase GSK-3β.[7,11,12] As GSK-3β is involved in multiple signaling pathways other than Wnt, a small molecule that blocks GSK-3β could have disparate effects in cellular and organism models. To determine if compound **1** is an inhibitor of GSK-3β, its activity in an in vitro GSK-3β kinase assay was measured. As described previously,[13] purified GSK-3β was incubated with its substrate, a peptide derived from glycogen synthetase, and γ-^{33}P-

labeled ATP with or without compound **1**. The kinase activity of GSK-3β can be blocked by the known inhibitor, staurosporine (Supporting Information).[11] The pyrimidine analogues have no such effect, which suggests that they act independently of GSK-3β. Although the molecular targets of the active compounds and their mechanism of action are still under investigation, current results indicate that the active compounds activate Wnt without inhibiting GSK-3β.

We next tested the ability of pyrimidine **1** to function in a whole-organism model. During early *Xenopus* development, Wnt antagonism plays an essential role in head specification. The expression of Wnt inhibitors results in enlarged heads and forebrains.[14–20] Conversely, inactivation of Wnt inhibitors, or overexpression of Wnt activators leads to microcephalic embryos.[21] This developmental process provides an excellent experimental means to test the ability of a compound to modulate Wnt signaling in vivo. Embryos were treated with compound **1** (10 μM) or the vehicle, DMSO, at stage 10.5 (gastrulation stage) for 24 h, and allowed to develop until stage 40 (tadpole stage). All the vehicle-treated embryos ($n = 29$) appeared normal (Figure 3). All

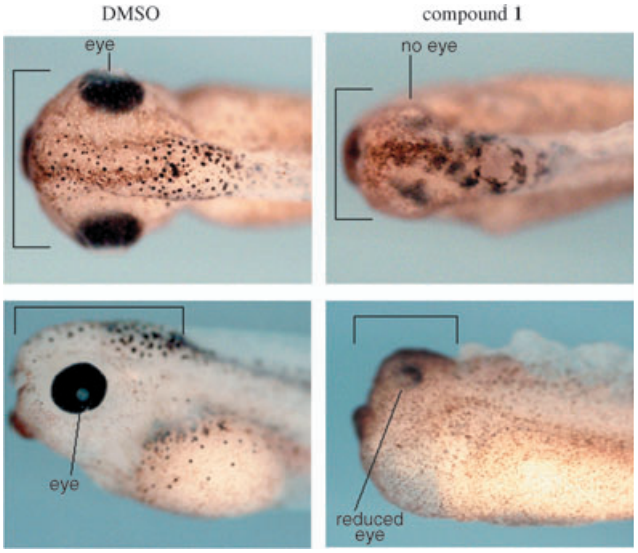

Figure 3. Compound **1** affected *Xenopus* embryonic head specification. Embryos were treated with compound **1** (10 μM) or DMSO from developmental stage 10.5 to stage 40. All DMSO-treated embryos appeared normal ($n = 29$). All drug-treated embryos ($n = 36$) had substantial head defects.

embryos treated with compound **1** ($n = 36$) had substantial head defects that ranged from significantly diminished heads and complete loss of eyes, to reduced eyes and heads; the posterior structures of tadpoles remained largely intact. This phenotype is similar to that which results from Wnt overexpression in head specification during early embryonic development.[21] These results suggest that the small-molecule Wnt agonist indeed mimics the effects of Wnt at the whole-organism level.

In conclusion, the small-molecule Wnt agonist identified in this study functions both in cell culture and a *Xenopus* model. Determination of the molecular mechanism of **1** may reveal novel components of Wnt signaling, including the effects of oscillating pathway activities.[22,23] Furthermore, small-molecule Wnt modulators should be useful tools for studies of biological processes that involve Wnt signaling, such as hematopoietic stem cell renewal, mesenchymal progenitor cell differentiation, and embryonic limb development. They may also reveal new physiological effects of Wnt signaling in model organisms, and might ultimately lead to novel therapeutic targets.[24]

Experimental Section

1: All chemicals were purchased from Aldrich. 2-Amino-4,6-dichloropyrimidine (100 mg, 0.61 mmol) was dissolved in 1-butanol (5 mL). Diisopropylethylamine (117 μL, 0.67 mmol) and piperonylamine (92.2 mg, 0.61 mmol) were then added to the mixture. The reaction mixture was heated at 70 °C for 12 h, and the desired product (2-amino-4-chloro-6-(1,3-benzodioxol-5-ylmethylamino)pyrimidine) was purified by column chromatography (85 % yield). 2-Amino-4-chloro-6-(1,3-benzodioxol-5-ylmethylamino)pyrimidine (10 mg, 0.036 mmol), $[Pd(PPh_3)_4]$ (2.1 mg, 0.0018 mmol), Na_2CO_3 (15.2 mg, 0.144 mmol), acetonitrile/water (1:1, 2 mL), and 3-methoxyphenylboronic acid (11 mg, 0.072 mmol) were mixed together under Ar. The reaction mixture was then heated at 150 °C for 20 min in a microwave reactor. The product was then purified by using reversed-phase HPLC with trifluoroacetic acid (0.1 % in H_2O) and MeCN as solvents. A linear gradient of MeCN (5 %→95 %) over 5 min was used, in which **1** has a retention time of 3.5 min. The corresponding fractions were collected and lyophilized to yield pure **1** as a white powder (80 % yield). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 3.84$ (s, 3H), 4.54 (d, 2H, $J = 5.8$ Hz), 6.0 (s, 2H), 6.39 (s, 1H), 6.85 (m, 2H), 6.96 (s, 1H), 7.19 (m, 1H), 7.28 (m, 2H), 7.52 (m, 1H), 9.13 ppm (s, 1H); HRMS (ESI-TOF): $C_{19}H_{19}N_4O_3$ $[M+H]^+$ calcd: 351.1457, found: 351.1455.

Cell culture, transfection, and high-throughput screening: Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with fetal bovine serum (10 %) and the antibiotics penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). Cells were cultured in a humidified incubator at 37 °C in CO_2 (5 %). Cells were transiently transfected with FuGene 6 (Roche) at a FuGene/DNA ratio of 3:1 according to the manufacturer's instructions. Luciferase activity was measured with a BrightGlo or DualGlo luciferase kit (Promega). For high-throughput screening, 293T cells were transiently transfected with the reporter construct (Upstate) in T75 cell culture flasks. After overnight culture, cells were harvested and replated onto 384-well plates with 1.5×10^3 cells in each well; 24 h later, compound solution (1 mM, 500 nL) was added to each well. After incubation for another 24 h, luciferase activity was measured with BrightGlo (Promega).

Plasmid construction: A dominant negative TCF4 was generated by PCR with primer F1: GCGCGC GGATCC GCCGCCACC ATG ACGA ATCAAAACAG CTCCTC and primer R1: GGGTTAGG-GATAGGCTTACC TTCTAAAGACTTGGTGACGA. The product was used in a second round of PCR with primer F1 and primer R2 GCCGCC TCTAGA TTA CGTAGAATCGAGACCGAGGA-GAGGGTTAGGGATAGGCTTACC to add a V5 epitope tag at the C terminus. It then was digested with BamH1 and XbaI, inserted into the pcDNA3 vector (Invitrogen), and sequenced.

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