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Inhibition of Wnt/β-Catenin Signaling by p38 MAP Kinase Inhibitors Is Explained by Cross-Reactivity with Casein Kinase Iδ/ε

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SUMMARY

Wnt/β-catenin signaling plays essential roles in embryonic development, adult stem cell maintenance, and disease. Screening of a small molecule compound library with a β-galactosidase fragment complementation assay measuring β-catenin nuclear entry revealed TAK-715 and AMG-548 as inhibitors of Wnt-3a-stimulated β-catenin signaling. TAK-715 and AMG-548 are inhibitors of p38 mitogen-activated protein kinase, which has been suggested to regulate activation of Wnt/β-catenin signaling. However, two highly selective and equally potent p38 inhibitors, VX-745 and Scio-469, did not inhibit Wnt-3a-stimulated β-catenin signaling. Profiling of TAK-715 and AMG-548 against a panel of over 200 kinases revealed cross-reactivity with casein kinase Iδ and ε, which are known activators of Wnt/β-catenin signaling. Our data demonstrate that this cross-reactivity accounts for the inhibition of β-catenin signaling by TAK-715 and AMG-548 and argue against a role of p38 in Wnt/β-catenin signaling.

INTRODUCTION

Wnt/β-catenin signaling is an evolutionarily conserved intracellular signal transduction pathway with important functions during embryogenesis and adulthood (Logan and Nusse, 2004). It is initiated by members of the Wnt protein family, a group of glycosylated extracellular ligands that comprises 19 members in mammals. Wnt proteins (Wnts) bind to a member of the Frizzled family of seven-transmembrane receptors and a representative of the low-density lipoprotein receptor-related protein family (LRP5/6) (Bhanot et al., 1996; Pinson et al., 2000; Wehrli et al., 2000). These surface proteins initiate an intracellular signaling cascade that ultimately leads to the cytoplasmic stabilization of the transcriptional modulator β-catenin. β-Catenin translocates to the nucleus where it interacts with proteins of the T cell factor/Lymphoid enhancer factor (TCF/LEF) family to influence the transcription of β-catenin-dependent genes (Molenaar et al., 1996). In quiescent cells, the nuclear accumulation of β-catenin is prevented by the action of a large protein assembly termed the "destruction complex." The destruction complex consists of several scaffolding proteins (e.g., Axin1, Axin2/Conductin and the protein product of the adenomatous polyposis coli gene [APC]) and the Ser/Thr kinases glycogen synthase kinase 3 (GSK3α/β) and casein kinase Iα (CKIα). De novo synthesized β-catenin is recruited to the destruction complex and then sequentially phosphorylated by CKIα and GSK3 at several N-terminal residues. These phosphate moieties serve as a cue for proteasomal degradation of β-catenin. As a result, cytoplasmic β-catenin levels in nonstimulated cells are typically low.

Aberrations in Wnt/β-catenin signaling activity are associated with several malignancies, most notably cancer (Barker and Clevers, 2006; Clevers, 2006). Invariably, these cancers display elevated levels of nuclear β-catenin and increased expression of β-catenin-dependent genes. Thus, inhibitors of Wnt/β-catenin signaling may have therapeutic utility for treatment of cancer. We recently described a cell-based screening assay to measure the cytoplasm-to-nuclear translocation of β-catenin using enzyme fragment complementation (EFC) (Verkaar et al., 2010). The β-catenin EFC assay measures the complementation of a nuclear-resident β-galactosidase deletion mutant (termed Δα-Nuc) with nuclear-translocated β-catenin C-terminally tagged with a corresponding β-galactosidase fragment (so-called α-peptide). β-Galactosidase activity generated in this assay quantitatively reflects nuclear translocation of β-catenin (Verkaar et al., 2010).

Using the β-catenin EFC assay, we have screened a library of 2300 low molecular weight (LMW) synthetic chemicals, including a panel of kinase inhibitor reference compounds. Our aim was to identify lead molecules capable of activating or inhibiting Wnt/β-catenin signaling. A second objective was to couple the effect of reference compounds in the EFC assay to possible kinase drug targets within the β-catenin pathway. Here, we describe assay optimization for automated screening. Furthermore, we identified inhibition of Wnt/β-catenin signaling as a novel biological activity of the anti-inflammatory drugs TAK-715 and AMG-548, which are inhibitors of p38 mitogen-activated protein kinase (MAPK) and have been in clinical trials for rheumatoid arthritis. We correlated the inhibition of Wnt/β-catenin signaling by TAK-715 and AMG-548 to cross-reactivity to casein kinase Iδ (CKIδ) and CKIε, two closely related CKI isoforms that have been shown

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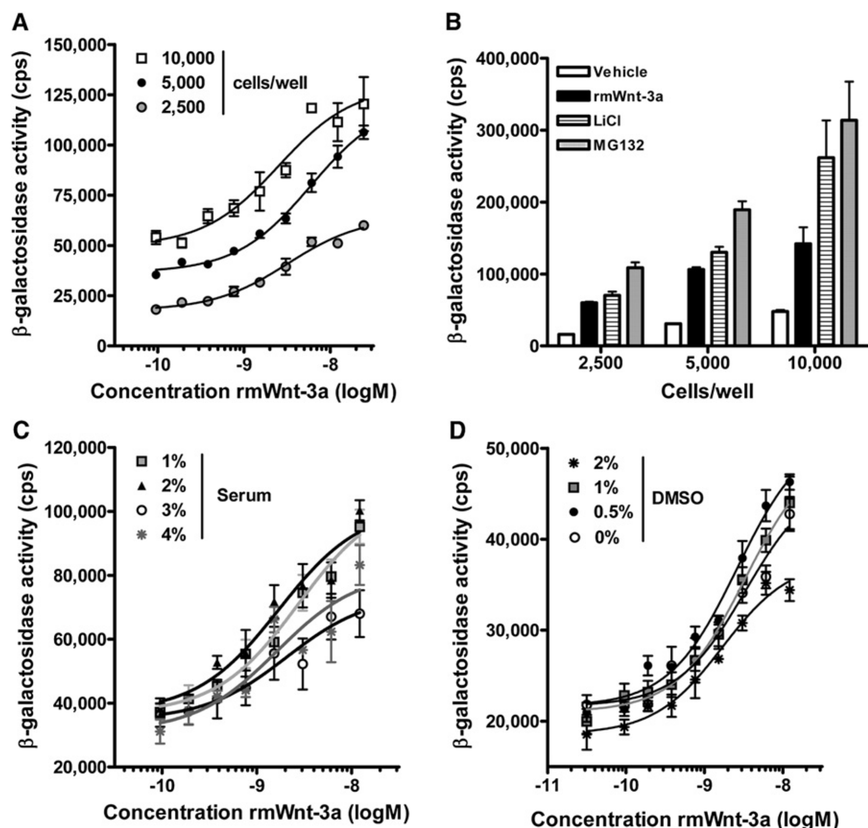

Figure 1. Optimization of Assay Conditions for the β-Catenin EFC Assay

(A–D) U2OS cells stably overexpressing a nuclear-resident β-galactosidase enzyme fragment and a β-catenin fusion protein C-terminally extended with the complementary β-galactosidase protein fragment (U2OS-EFC cells) were tested for β-galactosidase activity after 3 hr of stimulation with activators of the β-catenin pathway. Cells were plated at several cell densities and incubated with (A) increasing doses of recombinant mouse Wnt-3a (rmWnt-3a), (B) 30 mM lithium chloride (LiCl), or 20 μM MG132. Dose-responsiveness of the cells to rmWnt-3a was determined in the presence of varying serum (C) or DMSO (D) concentrations. Data are presented as mean ± SEM. See Figure S1 for results of antagonists, and Table S1 for assay performance.

to be necessary for proper activation of Wnt/β-catenin signaling (Peters et al., 1999; Sakanaka et al., 1999; Swiatek et al., 2004). This side activity may be explored to reposition TAK-715 and AMG-548 for other indications. Furthermore, our data call for a reevaluation of studies in which non-selective kinase inhibitors have been used to implicate p38 in Wnt/β-catenin signaling.

RESULTS

Automated Screening with the β-Catenin EFC Assay

U2OS cells genetically engineered to couple activation of the Wnt/β-catenin pathway to increases in β-galactosidase complementation (U2OS-EFC cells) have been described previously (Verkaar et al., 2010). These cells responded to recombinant mouse Wnt-3a (rmWnt-3a) with a dose-dependent increase in β-galactosidase activity (Figure 1A). Several parameters were varied to optimize the β-catenin EFC assay for automated screening. First, the number of cells per well was varied, before stimulating them with increasing concentrations of rmWnt-3a. As indicated in Figure 1A, both basal and rmWnt-3a-induced β-galactosidase activity increased with cell density but there was no noticeable change in signal-to-noise (S/N) ratio. The same trend was observed when several amounts of cells were stimulated with 30 mM lithium chloride (LiCl), which inhibits GSK3, and 20 μM MG132, which is a proteasomal inhibitor (Figure 1B). These treatments prevent the degradation of β-catenin and thereby mimic activation of the Wnt/β-catenin signaling cascade. We also derived dose-response relationships for rmWnt-3a when increasing concentrations of serum and DMSO were added. We found that at 3%–4% fetal bovine serum (FBS), the assay signal was slightly reduced (Figure 1C), and we therefore conducted all further experiments in the presence of 2% FBS. We found that DMSO up to 1% had no effect on assay response, whereas 2% slightly decreased assay signal (Figure 1D).

We performed the EFC assay in both agonistic and antagonistic setup with 2311 LMW compounds derived from lead optimization projects for G protein-coupled receptor (GPCR) or protein kinase targets, including a set of published kinase inhibitor reference compounds. The compounds were tested at 10 μM final concentration in 1% DMSO. To control for Wnt/β-catenin pathway activation, cells were treated with 12 nM rmWnt-3a (500 ng/ml; EC_{100}). In the antagonistic setup, cells were pretreated with 10 μM compound for 60 min before addition of 10 nM rmWnt-3a (420 ng/ml), which corresponded to EC_{80} pathway activation. Assay quality was verified by calculating Z' values (Zhang et al., 1999).

For the agonistic assay, the average S/N ratio was 4.3 with an average Z'-value of 0.52. For the antagonistic assay, the average S/N ratio and Z'-value were 3.4 and 0.57, respectively (see Table S1 available online). An active was defined as a compound producing >50% activation/inhibition. Compounds meeting this criterion were retested to confirm their activity. Three compounds were found to be active in the initial agonistic screen and all three could be confirmed upon retesting.

In the antagonistic assay, of the 93 initial actives, 63 were available for a second experiment. Of these, nineteen compounds displayed >50% pathway inhibition during the second test. Representative results for three compounds are depicted in Figure S1A. The compounds were also able to inhibit rmWnt-3a-stimulated reporter gene activity in HEK293T (Figure S1B) and U2OS cells (Figure S1C) stably transfected with a β-catenin-driven luciferase reporter gene (SuperTOPflash) (Veeman et al., 2003).

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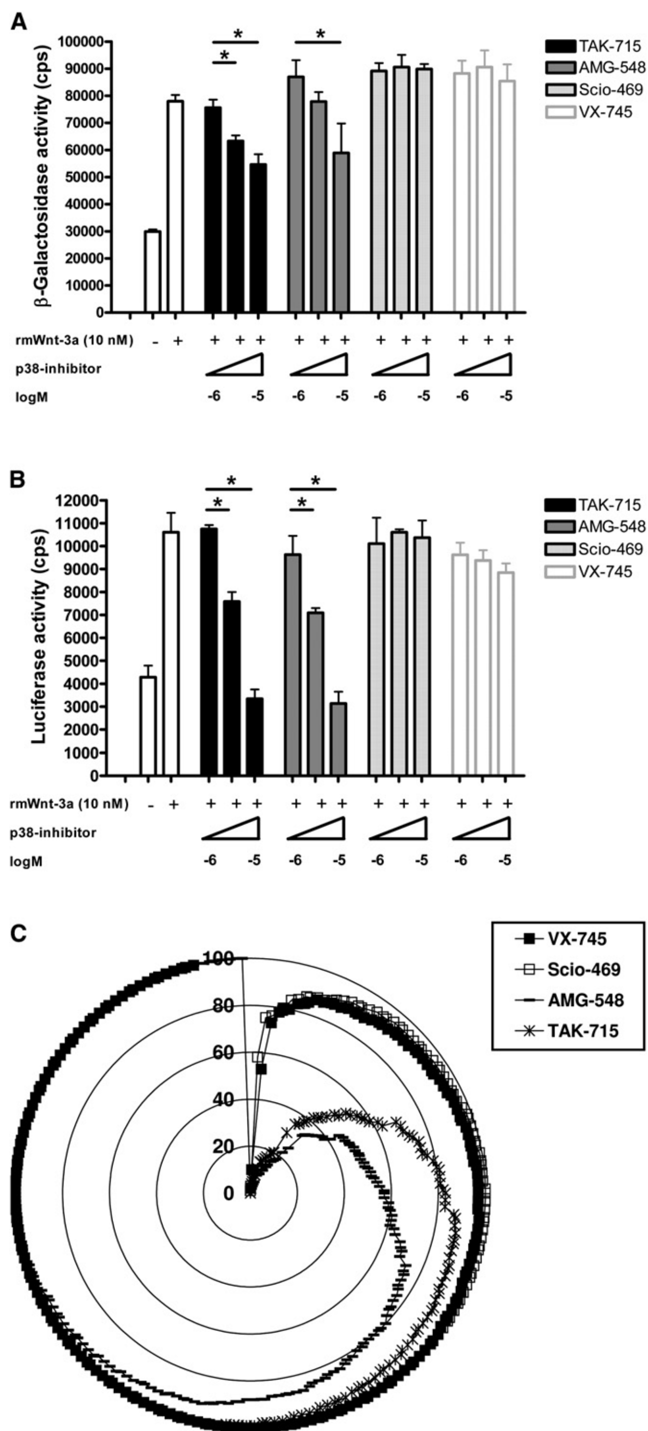

Figure 2. TAK-715 and AMG-548 Inhibit Wnt/β-Catenin Signaling

(A) U2OS-EFC cells were treated with p38 inhibitors at concentrations ranging from 1 to 10 μM in the presence of 10 nM rmWnt-3a for 3 hr, followed by measurement of β-galactosidase activity.

(B) U2OS cells stably transfected with SuperTOPflash reporter gene were treated with p38 inhibitors at the indicated concentrations in the presence of 10 nM rmWnt-3a for 5 hr, and were then tested for luciferase reporter gene activity.

(C) Selectivity wheel representing the percentage remaining kinase activity of over 200 purified human kinases tested in ^{32}P incorporation assays in the presence of p38 inhibitors at a concentration of 10 μM. Data are presented as mean ± SEM. Asterisks (*) represent statistically significant differences ($p < 0.05$).

See Figure S2 for structures of p38 inhibitors and Table S2 for their activities in p38 enzyme activity assay.

Wnt/β-catenin Signaling Is Not Affected by p38 MAPK Inhibition

Among the actives in the antagonistic EFC and SuperTOPflash reporter gene assays were two inhibitors of p38 MAPK: TAK-715 and AMG-548 (Figures 2A and 2B) (Miwatashi et al., 2005; Dominguez et al., 2005). Because earlier research has suggested that p38 facilitates Wnt/β-catenin signaling (Bikkavilli et al., 2008b), we examined the role of p38 in Wnt/β-catenin signal transduction by testing two other p38 inhibitors, VX-745 and Scio-469 (Dominguez et al., 2005). Figure S2 depicts structures of all four p38 inhibitors. VX-745 and Scio-469 inhibited p38 enzyme activity in immobilized metal assays for phosphochemicals (IMAP) with similar nanomolar potencies as TAK-715 and AMG-548 (Table S2). However, VX-745 and Scio-469 did not appreciably inhibit Wnt/β-catenin signaling in the EFC or the SuperTOPflash reporter gene assay (Figures 2A and 2B), suggesting that the activity of TAK-715 and AMG-548 was due to cross-reactivity with another kinase. To examine this, the four p38 inhibitors were screened against a panel of more than 200 kinases at 10 μM in biochemical ^{32}P incorporation assays (performed at Millipore, Dundee, UK). AMG-548 and TAK-715 inhibited, respectively, 17 and 22 kinases by more than 80%, whereas VX-745 and Scio-469 only inhibited the α- and β-isoforms of p38 by more than 80% (Figure 2C). Five kinases (CKIδ, JNK3, MINK, NLK, and YES) were inhibited by both AMG-548 and TAK-715, but not by VX-745 or Scio-469. Of these kinases, Nemo-like kinase (NLK) has been associated with inhibition of Wnt/β-catenin signaling (Ishitani et al., 1999; Ishitani et al., 2003), whereas CKIδ, and its closest homolog CKIε, have been demonstrated to be required for activation of Wnt/β-catenin signaling (Peters et al., 1999; Sakanaka et al., 1999; Swiatek et al., 2004). Although both CKI homologs potentially possess nonoverlapping roles in Wnt/β-catenin signal transduction, both proteins will on occasion be referred to here as CKIδ/ε for convenience.

We tested the activity of six different CKI isoforms (i.e., α1, δ, ε, γ1, γ2, and γ3) in the presence of ascending concentrations of the four p38 inhibitors in Z'-lyte biochemical assays (Rodems et al., 2002). Although all four inhibited p38α, only TAK-715 and AMG-548 inhibited CKIδ and CKIε (Figures 3A–3D). Other CKI isoforms were not, or only marginally, inhibited by these compounds (Figures 3A–3D and data not shown).

Wnt-activated CKIδ/ε induces the phosphorylation of the cytoplasmic scaffolding protein human Dishevelled-2 (hDvl-2), which can be visualized by a retarded mobility of this protein on western blots (Gonzalez-Sancho et al., 2004; Bryja et al., 2007). As a positive control for inhibition of the pathway, cells were cotreated with rmWnt-3a and 1 μg/ml human Wnt inhibitory factor-1 (WIF1), which has been shown to inhibit Wnt/β-catenin signaling by associating with Wnt proteins (Hsieh et al., 1999). Treatment with 1 μg/ml WIF1 completely abrogated rmWnt-3a-induced nuclear translocation of β-catenin in the EFC assay, as did treatment with 2 μg/ml of the soluble Wnt antagonist

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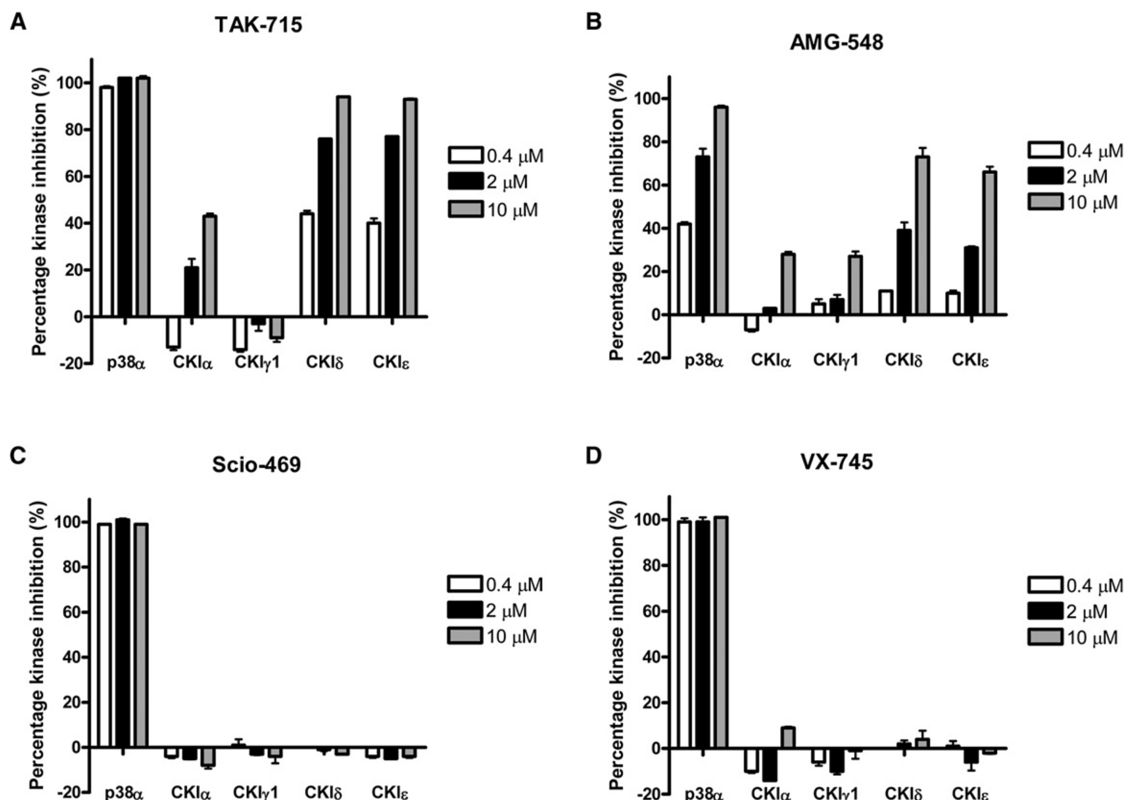

Figure 3. TAK-715 and AMG-548 Inhibit Casein Kinase I Isoforms

(A–D) The activity of purified p38, casein kinase Iα (CKIα), CKIγ1, CKIδ, and CKIε was measured in the presence of ascending concentrations of p38 inhibitor: TAK-715 (A), AMG-548 (B), Scio-469 (C), and VX-745 (D). Data are presented as mean ± SEM.

recombinant human Dickkopf-1 protein (rhDkk-1) (Figure S3). Stimulation of U2OS-EFC cells with 10 nM rmWnt-3a resulted in a distinct shift of the mobility of hDvl2 to a lower-mobility form, which was inhibited by cotreatment with WIF1 (Figure 4A). To demonstrate that the mobility shift of hDvl2 was CKIδ/ε dependent, we used two published CKI inhibitors, IC261 and D4476, which inhibit CKIδ/ε with micromolar potency (Mashhoon et al., 2000; Rena et al., 2004). Moreover, IC261 displays bias for inhibition of CKIδ and CKIε over other CKI isoforms (Mashhoon et al., 2000). Cotreatment of U2OS-EFC cells with 10 nM rmWnt-3a and ascending concentrations of IC261 or D4476 resulted in a dose-dependent inhibition of the rmWnt-3a-induced

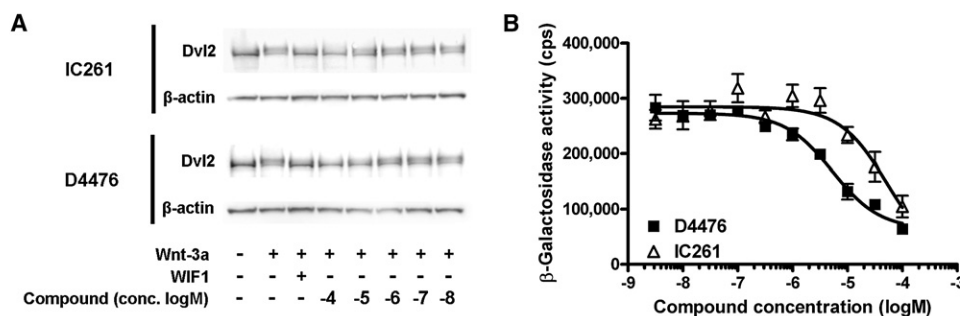

Figure 4. Inhibition of CKIδ/ε Inhibits rmWnt-3a-Induced Phosphorylation of Human Dishevelled 2 and Blocks Signaling Through the Wnt/β-Catenin Pathway

(A) U2OS-EFC cells were treated with increasing concentrations of the CKI inhibitors IC261 (upper panel) and D4476 (lower panel) in the presence of 10 nM rmWnt-3a for 1 hr. Cell lysates were immunoblotted for human Dishevelled-2 (hDvl2) and β-actin. As a control for Wnt/β-catenin pathway inhibition, cells were cotreated with 10 nM rmWnt-3a and 1 μg/ml human Wnt inhibitory factor-1 (WIF1). See also Figure S3.

(B) U2OS-EFC cells were treated with increasing concentrations of IC261 and D4476 in the presence of 10 nM rmWnt-3a for 3 hr, followed by measurement of β-galactosidase activity. Data are presented as mean ± SEM. See also Figures S4 and S5.

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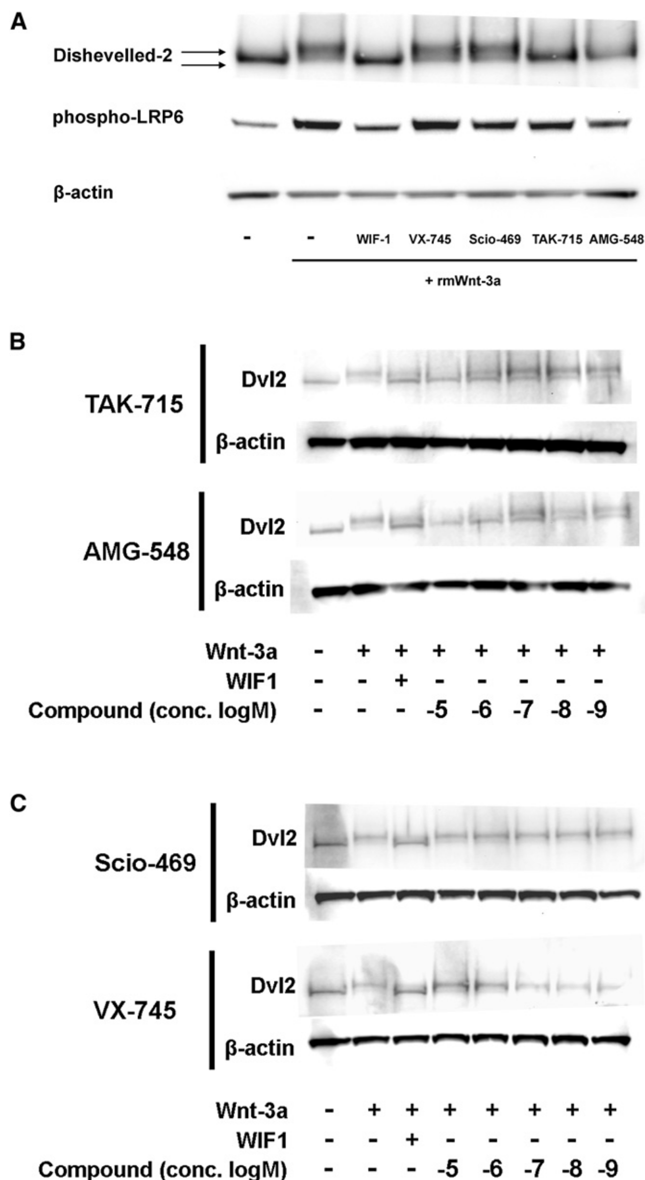

Figure 5. TAK-715 and AMG-548 Inhibit CKIδ/ε Activity in U2OS-EFC Cells

(A) U2OS-EFC cells were treated with vehicle or 10 nM rmWnt-3a in the presence of 1 μg/ml WIF1, or p38 inhibitors at a concentration of 10 μM. Subsequently, cellular extracts were analyzed for hDvl2, phospho-Ser1490 low-density lipoprotein receptor-related protein 6 (LRP6) and β-actin by western blot.

(B) U2OS-EFC cells were stimulated with 10 nM rmWnt-3a in the presence of increasing concentrations of TAK-715 (upper panel) and AMG-548 (lower panel) for 1 hr. Cell lysates were probed for hDvl2 and β-actin.

(C) U2OS-EFC cells were cotreated with 10 nM rmWnt-3a and increasing concentrations of Scio-469 (upper panel) and VX-745 (lower panel) for 1 hr, followed by western blotting for hDvl2 and β-actin.

See Figure S6 for quantification of peak intensities of low versus high mobility hDvl2 of blots in Figure 5B and C.

hDvl2 mobility shift (Figure 4A). Figure S4 represents quantifications of the peak intensity of phosphorylated versus nonphosphorylated hDvl2 on these blots. When tested in the EFC (Figure 4B) and TOPflash assay (data not shown), IC261 and D4476 inhibited activation of Wnt/β-catenin signaling with a potency similar to that observed for the inhibition of hDvl2 phosphorylation. Additionally, we transfected U2OS-EFC cells with siRNAs targeting p38 or CKIδ/ε and verified knock-down of these proteins by western blotting (Figure S5A). We observed that knock-down of CKIδ/ε, but not p38, inhibited the rmWnt-3a-dependent mobility shift of hDvl2 (Figure S5A; see Figure S5B for a quantification of the Dvl2 peak intensity on this blot). These results confirmed that CKIδ and CKIε are responsible for hDvl2 phosphorylation (Bryja et al., 2007). In addition, we demonstrated that inhibition of CKIδ and CKIε can account for the inhibition of Wnt/β-catenin signaling observed in U2OS-EFC cells treated with TAK-715 or AMG-548.

We subsequently tested the ability of the four p38 inhibitors to inhibit the Wnt-3a-induced hDvl2 mobility shift in U2OS-EFC cells. TAK-715 and AMG-548 inhibited the hDvl2 shift at 10 μM, whereas Scio-469 and VX-745 did not (Figure 5A). Upon Wnt treatment, Ser1490 of LRP6 is phosphorylated by the sequential activity of GSK3 and CKIγ (Tamai et al., 2004; Davidson et al., 2005). We did not observe any change in LRP6 phosphorylation by TAK-715 or AMG-548 (Figure 5A), indicating that inhibition of CKIγ did not contribute to the inhibitory effect of these compounds on Wnt/β-catenin signaling. This is consistent with the inability of both compounds to inhibit CKIγ in biochemical assays (Figures 3A and 3B). We subsequently studied the dose-dependency of the TAK-715- and AMG-548-mediated inhibition of Wnt-3a-induced hDvl2 phosphorylation. TAK-715 and AMG-548 inhibited the hDvl2 shift at micromolar concentrations (Figure 5B). In contrast, Scio-469 and VX-745 did not inhibit the Wnt-3a-induced hDvl2 mobility shift at concentrations up to 10 μM (Figure 5C). The peak intensity ratio of nonphosphorylated versus phosphorylated hDvl2 of these blots is depicted in Figure S6. Thus, the concentrations of TAK-715 and AMG-548 necessary to inhibit CKIδ/ε in cells closely approximate those required to inhibit Wnt/β-catenin signaling in the TOPflash and EFC assays. This finding, along with the biochemical profile of TAK-715 and AMG-548, strongly suggests that the effect of these compounds on Wnt/β-catenin signaling is mediated through CKIδ/ε cross-reactivity.

Earlier reports have implicated p38 in Wnt/β-catenin signaling on the basis of experiments studying the effect of the small molecule p38 inhibitors SB203580 and SB239063 on F9 mouse teratocarcinoma cells and HEK293 cells (Bikkavilli et al., 2008a; Bikkavilli et al., 2008b; Cervenka et al., 2011). In line with these reports, F9 cells transiently transfected with SuperTOPflash reporter gene construct and subsequently cotreated with rmWnt-3a and increasing concentrations of SB203580 or SB239063 displayed a dose-dependent decrease in reporter gene activity (Figure 6A). Similarly, SB203580 and SB239063 dose-dependently inhibited β-galactosidase activity in U2OS-EFC cells induced by rmWnt-3a (Figure 6B). We investigated whether the inhibition of Wnt/β-catenin signaling in these cells was due to inhibition of CKIδ/ε. First, we tested SB203580 and SB239063 in Z'-lyte biochemical assays and observed that both compounds inhibited CKIδ/ε with micromolar potency (Figures S7A and S7B). In addition, treatment of F9 cells with 10 μM SB203580 or SB239063 inhibited the rmWnt-3a-induced mobility shift of Dvl2 (Figures 6C and 6D). Similarly, HEK293T

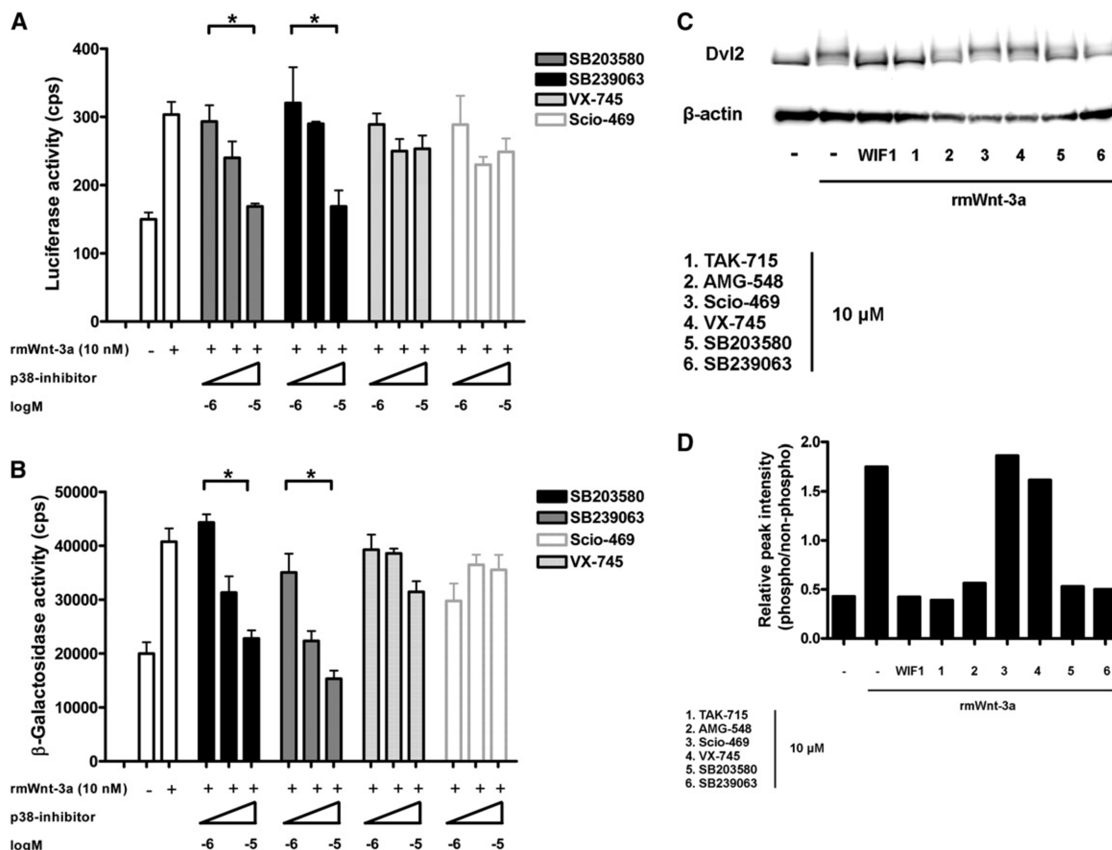

Figure 6. The p38 Inhibitors SB203580 and SB239063 Inhibit Wnt/β-Catenin Signaling in F9 Mouse Teratocarcinoma Cells and U2OS-EFC Cells Through Inhibition of CKIδ/ε

(A) F9 cells were transiently transfected with SuperTOPflash reporter gene construct. Forty-eight hours after transfection, cells were stimulated with 10 nM rmWnt-3a and increasing concentrations of SB203580, SB239063, Scio-469, or VX-745 for 5 hr before measurement of luciferase activity.

(B) U2OS-EFC cells were treated with 10 nM rmWnt-3a in the presence of increasing concentrations of SB203580, SB239063, Scio-469, or VX-745 for 3 hr prior to measurement of β-galactosidase activity.

(C) F9 cells were stimulated with 10 nM rmWnt-3a in the presence or absence of 1 μg/ml WIF1 or 10 μM p38 inhibitor as indicated in the figure for 1 hr. Lysates were probed for Dvl2 and β-actin.

(D) A quantification of the ratio between phosphorylated and nonphosphorylated Dvl2 derived from the blot in (C). Data are presented as mean ± SEM. Asterisks (*) represent statistically significant differences ($p < 0.05$).

See also Figures S7 and S8.

cells (Figures S8A and S8B) and U2OS-EFC cells (Figures S8C and S8D) treated with SB203580 or SB239063 also displayed impaired mobility of hDvl2 in response to rmWnt-3a. Thus, CKIδ/ε cross-reactivity is a common trait for p38 inhibitors that may have confounded the interpretation of previously reported results.

p38 Is Not Activated upon Treatment with Wnt-3a

Our data argue against a role for p38 in activation of Wnt/β-catenin signaling, but they do not exclude the possibility that p38 is activated upon Wnt treatment. To directly test this, U2OS-EFC cells were treated with Wnt-3a for 15 and 60 min before the cells were lysed, and lysates were analyzed for phosphorylated p38. As a positive control for p38 activation, cells were incubated with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA). We also assessed the phosphorylation status of extracellular stimulus-regulated kinase 1/2 (ERK1/2) and Akt/protein kinase B. ERK1/2 is activated by PMA (Graves et al., 1995), whereas activation of Akt can be accomplished by treatment with insulin (Cross et al., 1995).

Treatment with 10 μM PMA caused a marked increase in phosphorylated p38 (Thr180/Tyr182) and ERK1/2 (Thr202/Tyr204) (Figure 7). However, 10 nM rmWnt-3a had no effect on phosphorylation of these kinases (Figure 7). Phosphorylation of Akt/PKB (Ser473) was induced by 100 ng/ml insulin but not by treatment with rmWnt-3a (Figure 7). In contrast, rmWnt-3a activated phosphorylation of LRP6, whereas other treatments did not (Figure 7). These data demonstrate that p38 is not activated during Wnt/β-catenin signaling in U2OS-EFC cells. We also did not detect phosphorylation of GSK3β at Ser9 after Wnt-3a treatment (Figure 7), which has been suggested to mediate the effect of p38 on Wnt/β-catenin signaling (Bikkavilli et al., 2008b). Phosphorylation of GSK3β was readily observed following treatment with insulin and PMA (Figure 7), as has been reported previously (Cross et al., 1995). We extended our analysis by examining the phosphorylation of p38 and ERK1/2 in F9 cells and HEK293T

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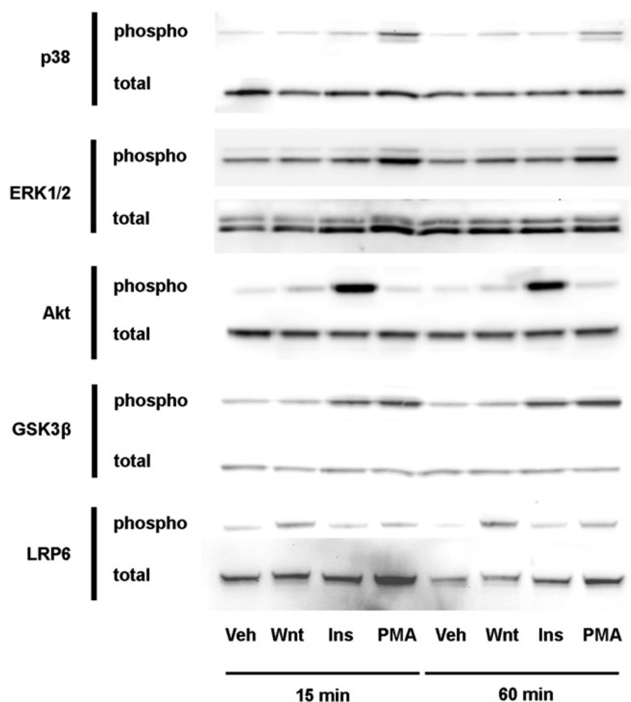

Figure 7. p38 Is Not Activated by rmWnt-3a Treatment in U2OS-EFC Cells

U2OS-EFC cells were treated with vehicle (Veh), 10 nM rmWnt-3a (Wnt), 100 ng/ml insulin (Ins), or 10 μM phorbol 12-myristate 13-acetate (PMA) for 15 or 60 min. Lysates were analyzed for phospho-Thr180/Tyr182 p38, phospho-Thr202/Tyr204 extracellular–regulated kinase 1/2 (ERK1/2), phospho-Ser473 Akt/protein kinase B, phospho-Ser9 glycogen synthase kinase 3β (GSK3β), and phospho-Ser1490 LRP6 by western blotting. See also Figure S9.

cells following treatment with rmWnt-3a. Similar to U2OS-EFC cells, F9 cells (Figure S9A) and HEK293T cells (Figure S9B) did not activate p38 and ERK1/2 following rmWnt-3a-treatment, whereas phosphorylation at Ser1490 of LRP6 and the Wnt-3a-induced mobility shift of Dvl2 were readily observed.

We conclude that inhibition of Wnt/β-catenin signaling by TAK-715 and AMG-548 is not due to their primary activity on p38, but most likely due to cross-reactivity with CKIδ/ε. Furthermore, we have found no evidence of p38 activation following Wnt/β-catenin signaling in F9, HEK293T, and U2OS cells.

DISCUSSION

High-throughput screening revealed inhibition of Wnt/β-catenin signaling as a side activity of the p38 inhibitors TAK-715 and AMG-548. Two other equally potent, but more selective p38 inhibitors, VX-745 and Scio-469, did not inhibit Wnt/β-catenin signaling. We could attribute the activity of TAK-715 and AMG-548 to cross-reactivity with CKIδ/ε. The ATP-binding pockets of p38 and CKIδ appear to be structurally similar, because CKIδ/ε cross-activity is commonly observed for p38 inhibitors (Godl et al., 2003; Hasegawa and Cahill, 2004; Bain et al., 2007). Reciprocally, several CKI inhibitors cross-react with p38 (Bain et al., 2007). The implications of this are two-fold.

First, the inhibitory effect on Wnt/β-catenin signaling and their cross-reactivity with CKIδ may be explored in drug discovery with the final aim to reposition TAK-715 and AMG-548 for other indications, such as cancer (Barker and Clevers, 2006; Clevers, 2006). For instance, mutations in CKIε have recently been linked to breast cancer, and the proliferation of several breast cancer cell lines has been shown to be CKIε dependent (Kim et al., 2010; Foldynova-Trantirkova et al., 2010). In addition, CKIδ and CKIε are key regulators of the mammalian circadian clock, and inhibitors of these kinases may potentially alleviate misalignment of the internal clock, as experienced during jet lag, depression, and seasonal affective disorder (Barnard and Nolan, 2008), provided these compounds pass the blood-brain barrier. SB239063 has been shown to display efficacy in reducing ischemic cerebral damage (Barone et al., 2001a; Barone et al., 2001b), which implies that it enters the brain, but whether TAK-715 and AMG-548 cross the blood-brain barrier has not been reported.

Second, our data argue against a role of p38 in Wnt/β-catenin signaling, in apparent contrast to suggestions by others (Caverzasio and Manen, 2007; Thornton et al., 2008; Bikkavilli et al., 2008b). These discrepancies may be explained by the fact that in earlier studies, activation of p38 in F9 cells was assessed by determining the phosphorylation of the p38 substrate activating transcription factor 2 (ATF2) (Bikkavilli et al., 2008b), whereas we determined p38 activity directly. ATF2 phosphorylation may occur upon activation of other signaling pathways, such as c-*jun* N-terminal kinase (JNK) (Gupta et al., 1995). Bikkavilli and colleagues reported that wild-type F9 cells did not activate JNK signaling (Bikkavilli et al., 2008a). However, they employed F9 cells stably expressing rat Frizzled-1 for their analysis of p38 signaling (Bikkavilli et al., 2008b), and these cells were previously reported to activate JNK signaling upon Wnt-3a-treatment (Bikkavilli et al., 2008a). Thus, activation of JNK, and not p38, may account for the ATF2 phosphorylation observed in these experiments.

p38 was suggested to activate Wnt/β-catenin signaling by inactivation of GSK3β through phosphorylation at Ser9 in F9 (Bikkavilli et al., 2008b) or Thr390 in HEK293 cells and mouse thymocytes (Thornton et al., 2008). We did not observe phosphorylation of GSK3β at Ser9 or Thr390 in U2OS-EFC cells stimulated with Wnt-3a for 15 or 60 min (Figure 7 and data not shown), in line with earlier reports (Ding et al., 2000; McManus et al., 2005). We also did not observe activation of ERK1/2 in U2OS-EFC cells treated with Wnt-3a, which has been reported to occur in several cell lines after stimulation with Wnt-3a-conditioned medium or transfection of Wnt-3a-coding vector (Civenni et al., 2003; Almeida et al., 2005; Yun et al., 2005; Caverzasio and Manen, 2007; Kim and Choi, 2007). It should be noted that conditioned media can be heterogeneous in composition and the ERK1/2 phosphorylation observed by others may be due to the presence of factors other than Wnt-3a present within the conditioned medium.

SIGNIFICANCE

Overly active Wnt/β-catenin signaling plays a critical role in many diseases (Barker and Clevers, 2006; Clevers, 2006). We found that the unanticipated cross-reactivity of the p38-inhibiting drugs TAK-715 and AMG-548 accounted for their ability to inhibit the Wnt/β-catenin pathway. TAK-715

and AMG-548 may be developed into therapeutics for β-catenin- and CKIδ/ε-dependent indications. TAK-715 and AMG-548 have been evaluated in clinical trials for rheumatoid arthritis and have been shown to possess good pharmacokinetic properties (Miwatashi et al., 2005; Dominguez et al., 2005). This makes TAK-715 and AMG-548 attractive starting points for chemical optimization.

Wnt/β-catenin signaling has proved difficult to target with small molecule compounds because of the unavailability of suitable drugable targets (Garber, 2009). Combining high-throughput screening of small molecule kinase inhibitors with biochemical profiling of kinase targets, as described here, could serve to quickly select a target set that can then be assessed using siRNA or gene knock-out approaches for target validation. To our knowledge, this chemo-genomics approach has not been previously explored for drug discovery on Wnt/β-catenin signaling.

Finally, our results should create awareness of the limitations of using small molecule ATP-competitive kinase inhibitors as tools for pathway analysis. The data presented here form an example of how cross-reactivities can potentially lead to misinterpretation of results unless verified by using multiple kinase inhibitors from structurally distinct classes and by correlating the cellular potency of the inhibitor to the observed effect.

EXPERIMENTAL PROCEDURES

Cell Lines

HEK293T, U2OS, and F9 cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM F12 containing 10% fetal bovine serum (FBS; Cambrex, Verviers, Belgium), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands). The generation of U2OS-EFC cells has been described elsewhere (Verkaar et al., 2010). This cell line was cultured in DMEM F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml hygromycin, and 500 μg/ml geneticin (Invitrogen).

LMW Compounds and Recombinant Proteins

Synthetic organic LMW compounds were selected from the Merck Research Laboratories compound collection (Oss, The Netherlands) according to previously determined activity on various G protein–coupled receptor (GPCR) or protein kinase targets. IC261 was purchased from Calbiochem (Gibbstown, NJ). D4476 and SB239063 were obtained from Tocris Cookson Inc. (St. Louis, MO). Recombinant mouse Wnt-3a (rmWnt-3a), recombinant human Wnt inhibitory factor-1 (WIF1), and recombinant human Dickkopf-1 (rhDkk-1) were purchased from R&D Systems (Abingdon, UK).

β-Galactosidase Fragment Complementation Assays

The agonistic β-catenin EFC assay has been described previously (Verkaar et al., 2010). For antagonistic testing, cells were seeded in 384-well CulturPlates (PerkinElmer, Boston, Massachusetts) with 0.5 × 10^4 U2OS-EFC cells in 22.5 μl DMEM F12 containing 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (assay medium). After overnight incubation at 37°C in a humidified incubator (5% CO_2, 95% humidity), 2.5 μl compound or DMSO control in assay medium was added to the cells and the plate was returned to the incubator for 1 hr. Cells were then stimulated with 2.5 μl of rmWnt-3a in assay medium (final concentration of 420 ng/ml [10 nM]) and returned to the incubator for 3 hr. Cells were disrupted using 12.5 μl of substrate-containing lysis buffer from the PathHunter Detection Kit in the formulation specified by the supplier (DiscoveRx). Plates were incubated in the dark for 1 hr at room temperature before measurement of β-galactosidase activity on an Envision multilabel plate reader (PerkinElmer).

SuperTOPflash Reporter Gene Assays

Transient transfection of DNA was described previously (Verkaar et al., 2009). Transfection of siRNA was described elsewhere (Verkaar et al., 2010). siRNAs targeting p38, CKIδ, and CKIε and nontargeting control siRNAs were purchased from Thermo Fischer Scientific (Waltham, Massachusetts). For stable transfections, HEK293T and U2OS cells were transfected with pTA-superTOPflash and pIRESpuro2 (Clontech; Mountain View, CA) at a 10:1 molar ratio using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, growth medium was replaced by medium containing 2 μg/ml puromycin (Calbiochem, San Diego, California) and cells were dispensed into 96-well plates for single cell clone selection by means of limited dilution. Single-cell clones were seeded in 384-well CulturPlates (PerkinElmer) at 10^4 (HEK293T) or 0.5 × 10^4 (U2OS) cells/well in 10 μl DMEM F12 supplemented with 1 μg/ml insulin (Sigma-Aldrich, Steinheim, Germany), 5 μg/ml apo-transferrin (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (IT-medium). After overnight incubation, cells were stimulated with 5 μl of compound or DMSO in IT-medium for 1 hr, followed by addition of 5 μl of rmWnt-3a or vehicle control in IT-medium. Plates were incubated at 37°C for 5 hr and then allowed to cool to room temperature before addition of 15 μl of SteadyLite reagent (PerkinElmer). After a 30 min incubation at room temperature in the dark, chemiluminescent activity was measured on an Envision multilabel plate reader.

Western Blotting

Isolation of cell lysates, followed by western blotting, was described previously (Verkaar et al., 2010). The following antibodies were used at the indicated dilutions: mouse anti-β-actin, 1:5000; mouse anti-casein kinase Iδ, 1:1000; and mouse anti-casein kinase Iε, 1:1000 (Abcam, Cambridge, UK); rabbit anti-dishevelled-2, 1:2000; rabbit-anti-Akt, 1:1000; rabbit anti-GSK3β, 1:1000; rabbit anti-phospho-Ser473 Akt, 1:1000; rabbit anti-phospho-Ser9 GSK3β, 1:1000; rabbit anti-phospho(Thr202/Tyr204)-ERK1/2, 1:1000; rabbit anti-LRP6, 1:1000; and rabbit anti-phospho-Ser1490 LRP6, 1:1000 (Cell Signaling Technology, Danvers, Massachusetts); mouse-anti-p38, 1:1000; rabbit anti-phospho(Thr180/Tyr182)-p38 and rabbit anti-ERK1/2 (Invitrogen), anti-mouse-HRP conjugate, 1:2000; and anti-rabbit-HRP conjugate, 1:2000 (Promega, Leiden, The Netherlands).

Kinase Assays

Immobilized metal assays for phosphochemicals (IMAP) for p38 kinase activity were performed as described previously (Loomans et al., 2003). Assays for casein kinase isoforms (Rodems et al., 2002) were performed at Invitrogen (Paisley, UK). Whole kinome profiling was performed at Millipore (Dundee, UK).

Data Analysis

Concentrations of rmWnt-3a were calculated from the expected molecular mass of recombinant Wnt-3a (41 kDa). Data were analyzed using Graphpad Prism 4.0 software. All data are represented as averages ± standard error of the mean (SEM). Statistical significance of observed differences was determined using Student's t test and indicated in the figures with asterisks (*); $p < 0.05$ was regarded as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes nine figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2011.01.015.

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REFERENCES

Almeida, M., Han, L., Bellido, T., Manolagas, S.C., and Kousteni, S. (2005). Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J. Biol. Chem. *280*, 41342–41351.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem. J. *408*, 297–315.

Barker, N., and Clevers, H. (2006). Mining the Wnt pathway for cancer therapeutics. Nat. Rev. Drug Discov. *5*, 997–1014.

Barnard, A.R., and Nolan, P.M. (2008). When clocks go bad: neurobehavioural consequences of disrupted circadian timing. PLoS Genet. *4*, e1000040.

Barone, F.C., Irving, E.A., Ray, A.M., Lee, J.C., Kassis, S., Kumar, S., Badger, A.M., Legos, J.J., Erhardt, J.A., Ohlstein, E.H., et al. (2001a). Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia. Med. Res. Rev. *21*, 129–145.

Barone, F.C., Irving, E.A., Ray, A.M., Lee, J.C., Kassis, S., Kumar, S., Badger, A.M., White, R.F., McVey, M.J., Legos, J.J., et al. (2001b). SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. J. Pharmacol. Exp. Ther. *296*, 312–321.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225–230.

Bikkavilli, R.K., Feigin, M.E., and Malbon, C.C. (2008a). G alpha o mediates WNT-JNK signaling through dishevelled 1 and 3, RhoA family members, and MEKK 1 and 4 in mammalian cells. J. Cell Sci. *121*, 234–245.

Bikkavilli, R.K., Feigin, M.E., and Malbon, C.C. (2008b). p38 mitogen-activated protein kinase regulates canonical Wnt-beta-catenin signaling by inactivation of GSK3beta. J. Cell Sci. *121*, 3598–3607.

Bryja, V., Schulte, G., Rawal, N., Grahn, A., and Arenas, E. (2007). Wnt-5a induces Dishevelled phosphorylation and dopaminergic differentiation via a CK1-dependent mechanism. J. Cell Sci. *120*, 586–595.

Caverzasio, J., and Manen, D. (2007). Essential role of Wnt3a-mediated activation of mitogen-activated protein kinase p38 for the stimulation of alkaline phosphatase activity and matrix mineralization in C3H10T1/2 mesenchymal cells. Endocrinology *148*, 5323–5330.

Cervenka, I., Wolf, J., Mašek, J., Krejci, P., Wilcox, W.R., Kozubík, A., Schulte, G., Gutkind, J.S., and Bryja, V. (2011). Mitogen-activated protein kinases promote WNT/beta-catenin signaling via phosphorylation of LRP6. Mol. Cell Biol. *31*, 179–189.

Civenni, G., Holbro, T., and Hynes, N.E. (2003). Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. EMBO Rep. *4*, 166–171.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469–480.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785–789.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A., and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. Nature *438*, 867–872.

Ding, V.W., Chen, R.H., and McCormick, F. (2000). Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. J. Biol. Chem. *275*, 32475–32481.

Dominguez, C., Powers, D.A., and Tamayo, N. (2005). p38 MAP kinase inhibitors: many are made, but few are chosen. Curr. Opin. Drug Discov. Devel. *8*, 421–430.

Foldynova-Trantirkova, S., Sekyrova, P., Tmejova, K., Brumovska, E., Bernatik, O., Blankenfeldt, W., Krejci, P., Kozubik, A., Dolezal, T., Trantirek, L., and Bryja, V. (2010). Breast cancer-specific mutations in CK1epsilon inhibit Wnt/beta-catenin and activate the Wnt/Rac1/JNK and NFAT pathways to decrease cell adhesion and promote cell migration. Breast Cancer Res. *12*, R30.

Garber, K. (2009). Drugging the Wnt pathway: problems and progress. J. Natl. Cancer Inst. *101*, 548–550.

Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003). An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. Proc. Natl. Acad. Sci. USA *100*, 15434–15439.

Gonzalez-Sancho, J.M., Brennan, K.R., Castelo-Soccio, L.A., and Brown, A.M. (2004). Wnt proteins induce dishevelled phosphorylation via an LRP5/6-independent mechanism, irrespective of their ability to stabilize beta-catenin. Mol. Cell. Biol. *24*, 4757–4768.

Graves, J.D., Campbell, J.S., and Krebs, E.G. (1995). Protein serine/threonine kinases of the MAPK cascade. Ann. N Y Acad. Sci. *766*, 320–343.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science *267*, 389–393.

Hasegawa, M., and Cahill, G.M. (2004). Regulation of the circadian oscillator in *Xenopus* retinal photoreceptors by protein kinases sensitive to the stress-activated protein kinase inhibitor, SB 203580. J. Biol. Chem. *279*, 22738–22746.

Hsieh, J.C., Kodjabachian, L., Rebbert, M.L., Rattner, A., Smallwood, P.M., Samos, C.H., Nusse, R., Dawid, I.B., and Nathans, J. (1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature *398*, 431–436.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H., and Matsumoto, K. (1999). The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature *399*, 798–802.

Ishitani, T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003). Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. Mol. Cell. Biol. *23*, 1379–1389.

Kim, S.E., and Choi, K.Y. (2007). EGF receptor is involved in WNT3a-mediated proliferation and motility of NIH3T3 cells via ERK pathway activation. Cell. Signal. *19*, 1554–1564.

Kim, S.Y., Dunn, I.F., Firestein, R., Gupta, P., Wardwell, L., Repich, K., Schinzel, A.C., Wittner, B., Silver, S.J., Root, D.E., et al. (2010). CK1epsilon is required for breast cancers dependent on beta-catenin activity. PLoS ONE *5*, e8979.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Loomans, E.E., van Doornmalen, A.M., Wat, J.W., and Zaman, G.J. (2003). High-throughput screening with immobilized metal ion affinity-based fluorescence polarization detection, a homogeneous assay for protein kinases. Assay Drug Dev. Technol. *1*, 445–453.

Mashhoon, N., DeMaggio, A.J., Tereshko, V., Bergmeier, S.C., Egli, M., Hoekstra, M.F., and Kuret, J. (2000). Crystal structure of a conformation-selective casein kinase-1 inhibitor. J. Biol. Chem. *275*, 20052–20060.

McManus, E.J., Sakamoto, K., Armit, L.J., Ronaldson, L., Shpiro, N., Marquez, R., and Alessi, D.R. (2005). Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. EMBO J. *24*, 1571–1583.

Miwatashi, S., Arikawa, Y., Kotani, E., Miyamoto, M., Naruo, K., Kimura, H., Tanaka, T., Asahi, S., and Ohkawa, S. (2005). Novel inhibitor of p38 MAP kinase as an anti-TNF-alpha drug: discovery of N-[4-[2-ethyl-4-(3-methylphenyl)-1,3-thiazol-5-yl]-2-pyridyl]benzamide (TAK-715) as a potent and orally active anti-rheumatoid arthritis agent. J. Med. Chem. *48*, 5966–5979.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell *86*, 391–399.

Peters, J.M., McKay, R.M., McKay, J.P., and Graff, J.M. (1999). Casein kinase I transduces Wnt signals. Nature *401*, 345–350.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature *407*, 535–538.

Rena, G., Bain, J., Elliott, M., and Cohen, P. (2004). D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. EMBO Rep. *5*, 60–65.

Rodems, S.M., Hamman, B.D., Lin, C., Zhao, J., Shah, S., Heidary, D., Makings, L., Stack, J.H., and Pollok, B.A. (2002). A FRET-based assay platform for ultra-high density drug screening of protein kinases and phosphatases. Assay Drug Dev. Technol. *1*, 9–19.

Sakanaka, C., Leong, P., Xu, L., Harrison, S.D., and Williams, L.T. (1999). Casein kinase iepsilon in the wnt pathway: regulation of beta-catenin function. Proc. Natl. Acad. Sci. USA *96*, 12548–12552.

Swiatek, W., Tsai, I.C., Klimowski, L., Pepler, A., Barnette, J., Yost, H.J., and Virshup, D.M. (2004). Regulation of casein kinase I epsilon activity by Wnt signaling. J. Biol. Chem. *279*, 13011–13017.

Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z., and He, X. (2004). A mechanism for Wnt coreceptor activation. Mol. Cell *13*, 149–156.

Thornton, T.M., Pedraza-Alva, G., Deng, B., Wood, C.D., Aronshtam, A., Clements, J.L., Sabio, G., Davis, R.J., Matthews, D.E., Doble, B., and Rincon, M. (2008). Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science *320*, 667–670.

Veeman, M.T., Slusarski, D.C., Kaykas, A., Louie, S.H., and Moon, R.T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. *13*, 680–685.

Verkaar, F., van Rosmalen, J.W., Smits, J.F., Blankesteijn, W.M., and Zaman, G.J.R. (2009). Stably overexpressed human Frizzled-2 signals through the beta-catenin pathway and does not activate Ca(2+)-mobilization in Human Embryonic Kidney 293 cells. Cell. Signal. *21*, 22–33.

Verkaar, F., Blankesteijn, W.M., Smits, J.F., and Zaman, G.J. (2010). Beta-galactosidase enzyme fragment complementation for the measurement of Wnt/beta-catenin signaling. FASEB J. *24*, 1205–1217.

Wehrli, M., Dougan, S.T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature *407*, 527–530.

Yun, M.S., Kim, S.E., Jeon, S.H., Lee, J.S., and Choi, K.Y. (2005). Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. J. Cell Sci. *118*, 313–322.

Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. *4*, 67–73.

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