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Benzimidazole inhibitors from the Niclosamide chemotype inhibit Wnt/ β -catenin signaling with selectivity over effects on ATP homeostasis

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Abstract

The Wnt signaling pathway plays a key role in organ and tissue homeostasis, and when dysregulated, can become a major underlying mechanism of disease, particularly cancer. We reported previously that the anthelmintic drug Niclosamide inhibits Wnt/ β -catenin signaling and suppresses colon cancer cell growth in vitro and in vivo. To define Niclosamide's mechanism of Wnt/ β -catenin inhibition, and to improve its selectivity and pharmacokinetic properties as an anticancer treatment, we designed a novel class of benzimidazole inhibitors of Wnt/ β -catenin signaling based on SAR studies of the Niclosamide salicylanilide chemotype. Niclosamide has multiple biological activities. To address selectivity in our design, we interrogated a protonophore SAR model and used the principle of conformational restriction to identify novel Wnt/ β -catenin inhibitors with less effect on ATP cellular homeostasis. These studies led to the identification of 4-chloro-2-(5-(trifluoromethyl)-1H-benzo[d]imidazol-2-yl) phenol (**4**) and related derivatives with greater selectivity for Wnt/ β -catenin signaling inhibition vs. differential effects on cellular ATP homeostasis. This is the first report that the Wnt signaling inhibitory activity of Niclosamide can be translated into a new chemical class and to show that its effects on ATP homeostasis can be separated from its inhibitory effects on Wnt signaling. These compounds could be useful tools to elucidate the mechanism of Niclosamide's inhibition of Wnt signaling, and aid the discovery of inhibitors with improved pharmacologic properties to treat cancer and diseases in which Niclosamide has important biological activity.

GRAPHICAL ABSTRACT

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Keywords

Niclosamide; Wnt signaling inhibitor; β -catenin; Small molecule; Cancer; Drug design; ATP; Protonophore; Oxidative phosphorylation; Mechanism

1. Introduction

The Wnt signaling pathway is critical to embryonic development and the maintenance of tissues.¹ Intense efforts to discover modulators of this pathway have taken place over the past two decades in an effort to discover treatments for multiple diseases.² In cancer, the pathway is often activated by mutation, particularly in colorectal cancer (CRC), where roughly 80% of colorectal cancers have mutations in key Wnt pathway proteins such as adenomatous polyposis coli (APC) or β -catenin.³ Mechanisms by which extracellular Wnt proteins transduce signals into the cell have been delineated.² In the canonical pathway, secreted Wnt proteins bind the transmembrane Frizzled receptor and a co-receptor LRP5/6 resulting in the internalization of the Frizzled receptor and activation of cytosolic proteins called Dishevelled (Dvl).^{4, 5} This leads to downstream signaling events that result in the stabilization of Axin and β -catenin proteins, and the translocation of cytosolic β -catenin into the nucleus. In the nucleus, β -catenin binds the transcription factor LEF/TCF to drive the transcription of Wnt/ β -catenin target genes. Overall, the signals in this pathway are transduced through a number of protein-protein interactions, a feature that has made it difficult to target with small drug-like molecules.^{6–8} As a result, drugs that specifically inhibit the pathway have yet to be approved even though the importance of Wnt signaling activity in cancer, in particular, has been known for decades.⁹

To identify Wnt signaling pathway inhibitors we reported the use of a high-throughput drug screen that assessed Fzd1-GFP internalization in U2OS cells by confocal microscopy. Upon screening a library of FDA approved drugs, we discovered that the anthelmintic drug Niclosamide (Figure 1) promoted Frizzled internalization.¹⁰ Subsequent studies found Niclosamide downregulates Dishevelled and β -catenin, and inhibits colon cancer cell growth in vitro and in vivo.^{10,11} SAR studies have further delineated the structural requirements of Wnt/ β -catenin inhibition by Niclosamide.^{12–14}

Niclosamide was approved by the FDA in 1982 to treat tapeworm infections in humans¹⁵ and is on the World Health Organization's list of essential medicines.¹⁶ Along with other members of its salicylanilide class of anthelmintic agents, Niclosamide has been used widely as a treatment in livestock.^{17, 18} The mechanism of Niclosamide's anthelmintic activity is reported to result from its impact on ATP energy balance related to its ability to uncouple oxidative phosphorylation and activate ATPases.^{17–19} Niclosamide is a potent uncoupler of oxidative phosphorylation,^{20, 21} and as a whole, the ability of salicylanilide anthelmintic agents to uncouple oxidative phosphorylation and modulate ATP levels is well-

documented.¹⁸ However, for a drug that has been used clinically for more than 30 years, it is surprising that its mechanism of anthelmintic activity is not better delineated. Niclosamide is poorly absorbed upon oral administration and is cleared rapidly once absorbed such that the majority of an oral dose stays in the GI tract.¹⁷ These pharmacokinetic properties are appropriate to remove worms in the gut, but present a challenge in repurposing Niclosamide to treat diseases in which systemic exposure is needed.

Since the discovery of Niclosamide's anthelmintic activity, the discovery of additional biological activities clearly indicate it is a multi-functional drug. These additional biological activities have led to efforts to repurpose it to treat cancer^{11, 22–30} and other human diseases.^{31, 32} Toward treatments of cancer, Niclosamide inhibits the proliferation of breast, colon, lung, prostate, ovary, blood and pancreatic tumor cells at concentrations that overlap with concentrations that inhibit Wnt/ β -catenin signaling.^{10, 11, 22, 23, 27, 33} Moreover, Niclosamide inhibits other important oncogenic signaling pathways²³ such as Notch,³⁴ mTOR,³⁵ NF- κ B,²⁴ and STAT-3³⁶ and is active against drug resistant cancers,^{11, 29, 37} As a result of these findings, Niclosamide is currently being evaluated as a cancer treatment in four clinical studies registered in ClinicalTrials.gov, two of which are in colorectal cancer.³⁸ Niclosamide's other clinically relevant biological activities include antibacterial³⁹, antituberculous⁴⁰ and drug resistant *Staphylococcus aureus* activity,³² antiviral activity,^{41, 42} and anti-trypanosomal activity.⁴³ Niclosamide is a protonophore and can impact metabolic processes such as oxidative phosphorylation and AMPK activation.^{31, 35, 41} In a mouse model of Type 2 diabetes and Non-Alcoholic Fatty Liver Disease (NAFLD), Niclosamide improved glycemic control, delayed disease progression, and reduced fatty deposits in the liver.³¹

Our initial SAR studies indicated that: 1) inhibition of Wnt signaling by Niclosamide appeared unique among structurally-related salicylanilide anthelmintic agents used commercially; 2) Wnt/ β -catenin functional response was dependent on small changes in the chemical structure of Niclosamide; and 3) ester derivatives provided improved absorption and significant plasma levels of Niclosamide from a single oral dose.^{12, 13} As part of our SAR studies, we also sought agents with better selectivity. We hypothesized that many of Niclosamide's biological activities may be related to its ability to modulate cellular ATP levels and uncouple oxidative phosphorylation given that no specific biological target has been shown to bind Niclosamide to account for many of the biological activities reported. To test this hypothesis we required compounds with less effect on cellular ATP concentrations. Niclosamide's protonophore activity may be the mechanistic driver underlying its effects on ATP homeostasis and on oxidative phosphorylation.^{44, 45} Thus, to improve the selectivity of Niclosamide-derived inhibitors, we interrogated a protonophore SAR model based on cLogP and pKa,⁴⁴ and used the principle of conformational restriction⁴⁶ to identify novel benzimidazole Wnt/ β -catenin inhibitors with less effect on ATP cellular homeostasis than Niclosamide and greater selectivity for inhibition of Wnt/ β -catenin.

2. Material and Methods

Inhibitors

Benzimidazole derivatives were prepared as described in the supplemental information by the coupling of aryl diamines with aldehydes using a published method.⁴⁷ Antimycin A (A8674), FCCP (C2920), Niclosamide (N3510), Oligomycin (O4876) were all obtained from Sigma Aldrich using the catalog numbers provided in parentheses.

Chemical Property Calculations

pKa values in water were calculated using Epik and cLogP values were calculated using Qikprop within Maestro (Version 10.3.015, Schrodinger, LLC).

Frizzled internalization assay

The Fzd1-GFP assay was performed following a procedure similar to that previously published.¹⁰ Briefly, U2OS cells stably expressing Frizzled1-GFP were plated in confocal dishes and incubated at 37°C with 5% CO₂. After 24 hours the cells were treated with 12.5µM of test compound in DMSO or DMSO as a control for 6 hours at 37°C and then fixed with 4% paraformaldehyde. The cells were then examined by microscopy using a LSM 510-Meta confocal microscope (Carl Zeiss, Thornwood, NY, USA) equipped with 40× and 100× apo chromat objectives. YFP was excited using a 488-nm argon laser line. Images were processed using the LSM software Image Browser (CarlZeiss, Thornwood, NY, USA). Plates were read twice in blinded fashion and scored using a 0–5 point scale: Punctate similar to control = 0, trace amount of punctate greater than control = 1, moderate = 3, strong = 5.

TOPFlash reporter assay

The procedure used was followed that adapted from Chen.¹⁰ Wnt-3A conditioned medium was prepared using L WNT-3A cells (ATCC® CRL-2647™) purchased from ATCC, and was obtained using published protocols (<http://www.atcc.org/Products/All/CRL-2647.aspx#culturemethod>).¹⁰ HEK293 cells were stably transfected with p8xTOPFlash, Renilla luciferase plasmid pRL-TK (Promega), and pLKO.1 as previously published.¹⁰ Briefly, stably transfected cells were seeded in 100 µl of cell growth medium (MEM, Sigma, catalog number M4655 supplemented with 10% FBS (Atlanta Biologicals, catalog number S11050), and 1µg/mL puromycin (Sigma, catalog number P8833), 100 U per ml penicillin and streptomycin (Invitrogen, catalog number 15140122)/well in 96-well plates at 100% confluency. Fifty microliters of Wnt-3A conditioned medium containing the chemical compounds to be tested or DMSO was added to each well. After 6 h treatment, the cells were washed once with PBS and lysed with 55 µl of Passive Lysis Buffer supplied in the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI). Twenty-five microliters of cell lysate was used for measuring luciferase activity in a 96-well plate reader (FluoStar Optima, BMG Labtech, Chicago, IL).

Western blot analysis of cytosolic β -catenin and Wnt/ β -catenin target gene products Axin2, c-Myc, Cyclin D1 and Survivin in HCT116 and SW480 cells

Cytosolic β -catenin and Axin2—6-well plates were incubated with 1ml poly-D-lysine solution (Sigma, P6407, 10 μ g/mL) for 30 minutes at room temperature after which time the poly-lysine solution was removed and the wells washed twice with 2 mL of distilled water. To the wells were added HCT-116 cells, (ATCC, CCL-247), maintained in McCoy's 5A medium (Invitrogen, 16600-082) supplemented with 10% FBS (Atlanta Biologicals, S11050), and Penicillin-Streptomycin (100 U/mL) (Invitrogen, 15140122), or SW480 cells (ATCC, CCL-228), maintained in MEM growth medium (Sigma, M4655) supplemented with 10% FBS and 100 U per ml penicillin and streptomycin. The cells were allowed to grow for 48 hours at 37°C, 5% CO₂ in an incubator. The cell confluency was approximately 60–80%. The media was then replaced with fresh media containing the indicated compounds in DMSO or DMSO. The final DMSO concentration was 0.1%. The cells were incubated for 18 hours. The media was removed and the cells washed 2 times with PBS, and the cytosolic fractions isolated using hypotonic buffer as described previously (Chen M, et. al. Biochemistry (2009) 48(43): 10267–10274). Cytosolic fractions were analyzed by Western blot using antibodies to β -catenin (Santa Cruz Biotechnology, SC-7963) or Axin2 (Santa Cruz Biotechnology, SC-20784). As a loading control, β -actin was analyzed with antibodies to β -actin (C-4, Santa Cruz Biotechnology, SC-47778).

c-Myc, Cyclin D1 and Survivin—HCT-116 and SW480 cells were maintained in culture as described above. The cells were plated in 6-well plates and incubated at the same time alongside cells used for analysis of β -catenin. The media was then replaced with fresh media containing the indicated compounds in DMSO or DMSO. The final DMSO concentration was 0.1%. The cells were incubated for 18 hours. The media was removed, the cells washed with PBS, and then lysed with 240 μ L 1X Laemmli sample buffer. Immunoblots using antibodies to c-Myc (Santa Cruz Biotechnology, SC-789), Cyclin D1, and Survivin (Cell Signaling Technology, cat#2978 and 2808) were used to detect c-Myc, Cyclin D1 and Survivin levels in the total cell lysates. Antibodies to β -actin (C-4, Santa Cruz Biotechnology, SC-47778) was used to measure levels of β -actin, which served as a loading control in all immunoblots.

Adenosine triphosphate (ATP) bioluminescent Assay

20,000 HCT-116 cells were added per well to poly-D-lysine solution coated 96-well plates and incubated at 37°C with 5% CO₂ in McCoy 5A medium (Invitrogen, catalog number 16600-082) supplemented with 10% FBS (Atlanta Biologicals, catalog number S11050) and 50 U per ml penicillin and streptomycin, and allowed to attach overnight. For experiments in media without glucose, the media was removed and test compounds dissolved in DMSO and diluted in the DMEM (Sigma, catalog number D5030) supplemented with 2mM sodium pyruvate (Invitrogen, catalog number 11360070) and 2mM L-glutamine (Invitrogen, catalog number 25030081) were added to the cells. In experiments with glucose, test compounds were added to cells dissolved in DMSO and DMEM media supplemented with 2mM sodium pyruvate, 2mM L-glutamine, and 5mM glucose (Sigma, catalog number G8769). The final DMSO concentration was 0.1%. The cells were incubated at 37°C with 5% CO₂, and after 3 hours treatment, the cells were washed with one time with 200 μ L PBS and lysed at room

temperature for 20 minutes with 100 μ L Somatic Cell ATP Releasing Reagent supplied in the Adenosine 5-triphosphate (ATP) bioluminescent somatic cell assay kit (Sigma, catalog identifier FLASC). 50 μ L cell lysates were used for measuring luciferase activity in a 96-well plate reader (FluoStar Optima, BMG Labtech, Chicago IL).

Phospho-AMPK Western blot

12-well plates were incubated with 250 μ L poly-D-lysine solution (Sigma, P6407, 10 μ g/mL) for 30 minutes at room temperature, the poly-lysine solution was removed, and the wells washed twice with 500 μ L distilled water. HCT-116 cells were maintained in McCoy's 5A medium (Invitrogen, 16600-082) supplied with 10% FBS (Atlanta Biologicals, catalog number S11050), and Penicillin-Streptomycin (100 U/mL).

0.27 million HCT-116 cells were plated in the poly-D-lysine coated 12-well plates and allowed to attach overnight at 37°C, 5% CO₂ in the incubator. The next morning, the media was replaced with the indicated compound in DMEM medium (Sigma, D5030). For experiments in media without glucose, the media was removed and test compounds were dissolved in DMSO and diluted in the DMEM (Sigma, catalog number D5030) supplemented with 2mM sodium pyruvate (Invitrogen, catalog number 11360070) and 2mM L-glutamine (Invitrogen, catalog number 25030081). The final DMSO concentration was 0.1%. In experiments with glucose, test compounds were added to cells dissolved in DMSO and DMEM media supplemented with 2mM sodium pyruvate, 2mM L-glutamine, and 5mM glucose (Sigma, catalog number G8769). After 30 minutes treatment, the media was removed and the cells were lysed with 120 μ L 1X Laemmli sample buffer. Immunoblots using antibodies to phospho-AMPK (Cell Signaling Technology, catalog number 2535) was used to detect phosphorylated AMPK (p-Threonine 172) levels in the total cell lysates. Antibodies to β -actin (C-4, Santa Cruz Biotechnology, catalog number SC-47778) were used for loading controls in all immunoblots.

Evaluation of Wnt signaling and ATP levels in HEK293 cells

HEK293 TOPFlash cells were split prior to assaying for inhibition of Wnt/ β -catenin and ATP reduction. Inhibition of Wnt/ β -catenin signaling was evaluated in the TOPFlash reporter assay as described above. ATP levels were evaluated using the ATP bioluminescent assay kit. 20,000 TOPFlash stable HEK293 cells were plated per well in poly-D-lysine coated plates in 100 μ L cell growth medium (MEM, Sigma, catalog number M4655) supplemented with 10% FBS (Atlanta Biologicals, catalog number S11050), and 1 μ g/mL puromycin (Sigma, catalog number P8833), 100 U per ml penicillin and streptomycin (Invitrogen, catalog number 15140122) and incubated overnight at 37°C in a 5% CO₂ incubator. The media was removed and replaced with the indicated compound in DMEM medium (Sigma, D5030) supplemented with 2mM glutamine, 2mM Sodium pyruvate and incubated at 37°C. After 3 hours, the media was removed and the cells were washed once with PBS, and lysed with 80 μ L Somatic Cell ATP Releasing Reagent (Sigma, Catalog Number FLASC). Forty microliters of cell lysate were used for measuring ATP amount in a 96-well plate reader (FluoStar Optima, BMG Labtech, Chicago, IL).

3. Results

Based on the hypothesis that the protonophore activity of Niclosamide is the underlying mechanism responsible for uncoupling of oxidative phosphorylation and modulation of cellular ATP homeostasis we focused our inhibitor design strategy (Figure 1) on the salicylanilide protonophore/oxidative phosphorylation uncoupling SAR model developed by Terada.⁴⁴ Based on the Terada model, we sought to decrease hydrophobicity (i.e. decrease LogP) and decrease acidity (i.e. increase pKa) to reduce the SAR elements that support protonophore activity. We also sought to reduce the number of available conformations, a well-known design strategy to improve potency and selectivity.⁴⁶ Since previous SAR studies of Niclosamide indicated the 2-hydroxyl group, the 5-chloro group, and the 2'- and 4'-substituent of the anilide ring of Niclosamide were important for Wnt signaling inhibitory activity,^{12, 13} we maintained these SAR elements. In the inhibitor design, we then morphed the Niclosamide structure into a benzimidazole motif by connecting the oxygen of the amide to the 2'-chloro position of the anilide and then removed the chloro atom and replaced the amide oxygen with a nitrogen atom to maintain the correct atomic valences (Figure 1). In the process of making these molecular changes, the conformational rotational degrees of freedom and the cLogP were reduced and the calculated pKa of the phenolic hydrogen atom was increased, each changes expected to improve selectivity based on the design elements. To interrogate the impact of these structural changes on Wnt/ β -catenin signaling, similar to our previous SAR studies of Niclosamide derivatives, we used the Fzd1-GFP confocal microscopy internalization assay and the Wnt-stimulated TOPFlash assay.^{12, 13} To assess the impact of the structural changes on ATP homeostasis, we used an ATP bioluminescent assay in conjunction with a functional readout of changes in cellular ATP levels, the induction of p-AMPK levels by Western blot.⁴⁸

Inhibition of Wnt signaling

The benzimidazole derivatives required to test our design were readily prepared in one step from commercially available starting materials (Figure 2).⁴⁷ We first prepared benzimidazole derivative **2** and upon testing this compound in the Fzd1-GFP internalization assay in U2OS cells, we were pleased to find the compound induced a punctate pattern of Fzd1-GFP, consistent with earlier work that led to the discovery that Niclosamide inhibits Wnt/ β -catenin signaling¹⁰ (Table 1, Figure S1 supplemental information). Upon testing this compound for inhibition of Wnt/ β -catenin signaling in the Wnt-stimulated β -catenin gene transcription assay (TOPFlash assay), we were pleased to find benzimidazole **2** inhibited Wnt-stimulated signaling similar to Niclosamide, albeit with slightly less potency. Encouraged by this result, we then conducted a brief survey of the 5-position of the benzimidazole and the 4-position of the phenol ring with substituents studied at corresponding positions in the Niclosamide chemotype. We found that substitution of the nitro group at the 5-position of the benzimidazole with a trifluoromethyl substituent yielded a compound (**4**) that was active in the Fzd1-GFP internalization assay and was of similar potency to Niclosamide in the Wnt-stimulated TOPFlash assay. Substitution at the 5-position with fluorine, hydrogen or methoxyl (compounds **3**, **5**, **6**) produced compounds that were less active in both assays. In the phenol ring, chloro-substitution at the 4-position of the phenol ring (compound **2**) was preferred versus substitution of fluorine, hydrogen, methyl or

methoxyl at this position (compound **7**, **8**, **9**, **10**). In each of the benzimidazole derivatives prepared, the calculated pKa of the phenol hydrogen was 1.4 log units less acidic than Niclosamide. To interrogate the impact the phenolic hydrogen pKa may play in inhibition of Wnt/ β -catenin signaling, we added a second chlorine atom in the phenol ring of compound **2**. This change produced benzimidazole derivative **11** in which the calculated pKa of the phenolic hydrogen is 7.1, now only less acidic than Niclosamide by 0.3 log units. Upon testing compound **11**, it was found to be less active than Niclosamide and compound **2** in the Fzd1-GFP and the TOPFlash assays. Whereas introduction of a chlorine atom at the ortho-position of the phenol could have a direct negative effect on potency, it appears that the pKa of the phenolic hydrogen may not, in and of itself, be a significant driver of inhibitor potency in the Wnt signaling assays.

Our previous SAR studies in the Niclosamide template indicated the phenol group was important for activity. Given that the phenol of Niclosamide is a major site of metabolism via glucuronidation,¹⁷ we explored sulfonamide derivatives as potential bioisosterics for the phenol group.⁴⁹ Toward this end, we prepared sulfonamide **12** (Table 2), and upon evaluation in the Fzd1-GFP and TOPFlash assays, were disappointed to find the compound was significantly less active. We then prepared a small array of sulfonamide derivatives that varied the sulfonamide substituent and the para-substituent in the context of 5-nitro or a 5-CF₃ substituent in the benzimidazole ring. We also prepared an analogous derivative of Niclosamide (**20**) in which the phenol was replaced with a toluenesulfonamide. In each case, replacement of the phenol by a sulfonamide led to a significant loss of Wnt/ β -catenin inhibitory activity.

To further validate the activity of this new class of Wnt/ β -catenin signaling inhibitors, a set of compounds selected from Table 1 were evaluated in HCT-116 and SW480 colorectal cancer cell lines, two cell lines with aberrant Wnt/ β -catenin signaling^{50, 51}. Consistent with the results of the Frizzled-Internalization and Wnt-stimulated TopFlash assays, treatment of both cell lines with either Niclosamide or benzimidazole **2**, **4**, or **10** and evaluation of the levels of cytosolic β -catenin, and downstream β -catenin target genes: Axin2, c-Myc, Cyclin D1, Survivin, led to a decrease in cytosolic β -catenin, and the levels of the Wnt/ β -catenin-target gene products relative to control in both cell lines (Figure 3). These data provide further evidence of the inhibitory activity of this new class of inhibitors of the Wnt/ β -catenin signaling pathway.

Reduction of cellular ATP levels

Having identified a number of novel benzimidazole inhibitors of Wnt/ β -catenin signaling, we next studied the effect of these structural changes on ATP levels in cells using a commercially available ATP-bioluminescence assay kit. To support the translation of our findings to a tumor model of colorectal cancer, we used HCT-116 cells.¹¹ As positive controls, we tested well-known inhibitors of ATP synthesis (Antimycin, Oligomycin)^{52, 53} and uncouplers of oxidative phosphorylation (FCCP, Niclosamide)⁴⁴ alongside a set of selected benzimidazole derivatives in standard DMEM media supplemented with glucose, pyruvate, and glutamine (Figure 4A). As expected, each of the ATP synthesis inhibitors and uncouplers of oxidative phosphorylation produced significant reduction in ATP levels in this

assay. Niclosamide in particular, produced a significant reduction in ATP levels vs control at all three concentrations tested. Gratifyingly, the benzimidazole derivatives produced effects ranging from little to no reduction of ATP levels (compound **4**, **8**, **10**), to a slight reduction (compound **2**), to a significant reduction of ATP (compound **11**). Of note, compound **11**, the benzimidazole derivative with the most potent reduction of ATP levels, had calculated properties (cLogP, pKa) closest to Niclosamide's values. To corroborate the results of the ATP bioluminescence assay, we evaluated phospho-AMPK (p-AMPK) levels^{48, 54} in HCT-116 cells treated with the same set of compounds (Figure 4B). Consistent with the results of the ATP bioluminescent assay, compounds including Niclosamide that produced a reduction of ATP demonstrated increased levels of p-AMPK, while compounds that had little to no reduction in ATP showed less increase in p-AMPK. Again, benzimidazole **11** was unique among the benzimidazole derivatives tested in that it produced a significant increase in p-AMPK levels. Before interrogating the SAR of ATP reduction in more detail, we then tested the compounds in DMEM media without glucose supplementation, based on the notion that magnitude of reduction of ATP by agents that modulate ATP synthesis could be limited if cells engage glycolysis to maintain cellular ATP levels in response to the test agents. Upon re-testing the same set of inhibitors in DMEM media without glucose supplementation, we found that the magnitude of ATP reduction was larger with the positive controls and test agents that had previously shown significant reduction of ATP in DMEM media supplemented with glucose (Figure 4C). Again, consistent with the reduction in ATP levels, levels of p-AMPK measured by Western blot were now also increased by the same agents while the agents that had little reduction in ATP levels showed little change in p-AMPK levels (Figure 4D). Based on these findings we used DMEM media without glucose supplementation in our further studies to avoid this compensatory change in cells that could confound interpretation of ATP levels and our SAR studies.

Upon reviewing the ATP reduction data in our initial studies, we noted that compounds with lower cLogP and higher calculated pKa produced less reduction of ATP levels, a trend consistent with the Terada SAR model,⁴⁴ while compound **11**, whose cLogP and calculated pKa values were closer to Niclosamide, produced a more significant reduction in ATP. To explore this observation further, we selected Niclosamide and 3 compounds (**10**, **11**, **20**) that varied by structural class and substitution to evaluate reduction of cellular ATP levels by dose response (Figure 5). Consistent with the prior ATP reduction results, Niclosamide, its related sulfonamide derivative **20** and benzimidazole **11** produced strong reduction of cellular ATP levels at each concentration tested (0.25 – 10 μ M). It is noteworthy that although these compounds differ structurally, they each share similar calculated pKa values and have cLogP values of ~3 or higher. Of the four compounds tested, one of the compounds, benzimidazole **10**, showed a weaker, dose dependent reduction of ATP that at the highest concentration tested (10 μ M) reached the level of ATP reduction seen with other three compounds at much lower concentration (0.25 μ M). When comparison of the calculated pKa of the most acidic hydrogens of benzimidazole **10** was made, the calculated pKa of the -OH and -NH groups were 9.3 and 9.2 respectively, approximately 2.5 log units less acidic than the pKa of the most acidic hydrogen in compounds that potently reduced cellular ATP levels. Moreover, the cLogP of benzimidazole **10** is 2.1, a value 0.7 log units lower than all 3 of the compounds that potently reduced ATP levels.

To further assess this trend, we then evaluated a larger set of compounds made up from compounds in Tables 1 and 2. In this analysis, we tested the compounds at three concentrations (0.5, 1 and 2 μM) in HCT-116 cells in DMEM media without added glucose using the ATP bioluminescent assay (Figure 6). We then plotted compounds by their cLogP and calculated pKa values (see limitations of calculated values in the Discussion section), and coded the compound by the magnitude of ATP reduction measured. Compounds with no or a small reduction of ATP (less than $< 25\%$ at all three concentrations) were plotted as green diamonds, compounds with an intermediate reduction of ATP ($> 25\%$ decrease in ATP at 2 μM and $< 25\%$ at 0.5 μM) were plotted as yellow squares, and compounds with a significant reduction of ATP ($> 50\%$ decrease at all concentrations) were plotted as red circles. As can be seen from the graph in Figure 6, compounds with the strongest reduction in ATP levels had calculated pKa values of 6.8 to 7.1, compounds with modest reduction generally had calculated pKa values of 7.1 to 8.4, and compounds with weaker reduction had calculated pKa values of 8.4 to 9.4. Overall, molecules that were less lipophilic and less acidic produced less reduction of ATP. From this data, it would appear that the calculated pKa has a stronger association with ATP reduction than cLogP. Although additional studies are needed to fully establish a correlation, we believe the use of calculated pKa and cLogP are useful in the design of new benzimidazole inhibitors of Wnt/ β -catenin to produce compounds with less effect on ATP homeostasis, at least within the chemical series represented here.

Comparison of inhibition of Wnt/ β -catenin signaling to ATP reduction

Our early pilot SAR experiments with Niclosamide and other commercially used salicylanilide anthelmintic agents suggested that inhibition of Wnt/ β -catenin signaling appeared to be separate from its anthelmintic activity, reportedly driven by uncoupling of oxidative phosphorylation and modulation of ATP levels.¹² The identification of novel benzimidazole inhibitors of Wnt/ β -catenin signaling with less reduction of cellular ATP levels described here provided an opportunity to test whether the SAR of the inhibition of Wnt/ β -catenin signaling and reduction of ATP levels are separate and whether these novel inhibitors of Wnt/ β -catenin signaling are more selective. To address these questions, we evaluated the inhibition of Wnt/ β -catenin signaling and the reduction of ATP levels using HEK293 TOPFlash cells in three separate side-by-side experiments. In these side-by-side experiments, TOPFlash cells for both experiments were split and plated from the same batch of cells and allowed to attach overnight. Different batches of cells were used in each of the three separate experiments (Figure 7). To evaluate inhibition of Wnt/ β -catenin signaling, the inhibitors were tested in the TOPFlash assay in Wnt-3A conditioned media, and to evaluate reduction of cellular ATP levels, the inhibitors were tested in the ATP bioluminescent assay in DMEM media without glucose. We evaluated Niclosamide and benzimidazoles **2**, **4** and **10** based on their potency in previous TOPFlash and ATP assays. Upon measuring inhibition of Wnt/ β -catenin signaling, again we found that Niclosamide and benzimidazoles **2**, **4** and **10** inhibited signaling in a dose-dependent fashion, producing IC_{50} s in these experiments of $0.12 \pm 0.02 \mu\text{M}$, $0.36 \pm 0.05 \mu\text{M}$, $0.23 \pm 0.02 \mu\text{M}$ and $0.62 \pm 0.06 \mu\text{M}$, respectively (Figure 7A–D). Upon measuring the reduction of ATP levels, each compound also reduced ATP levels in a dose-dependent fashion (Figure 7A–D), producing EC_{50} values of $0.056 \pm 0.018 \mu\text{M}$, $2.9 \pm 1.19 \mu\text{M}$, $5 \mu\text{M}$ and $>5 \mu\text{M}$ for Niclosamide, benzimidazole **2**, **4** and **10**,

respectively. Comparison of the dose response curve for inhibition of Wnt/ β -catenin signaling by Niclosamide to the dose response for reduction of ATP levels indicated the two dose-response curves were nearly coincident (Figure 7A). In contrast however, comparison of the dose response curve for inhibition of Wnt/ β -catenin signaling and ATP reduction by benzimidazole **2**, **4** and **10** indicated the dose response for reduction of ATP was shifted significantly to the right of the dose response for inhibition of Wnt/ β -catenin signaling for each compound (Figure 7B–D). The shift of the dose response curves for reduction of cellular ATP levels vs. inhibition of Wnt/ β -catenin signaling supports the assertion that the benzimidazole derivatives are more selective and have less effect on cellular processes involved in ATP homeostasis than Niclosamide. To further assess selectivity and to determine whether the SAR of the two effects were separate, we compared the ATP levels at specific concentrations of compounds that produced a similar reduction of Wnt/ β -catenin signaling. Niclosamide and benzimidazole **4** have similar potency (IC_{50}) in the TOPFlash assay (Figure 7A and 7B), and at 0.16 μ M concentration of each compound, they each produced 40–45% reduction in Wnt/ β -catenin signaling (Figure 7E). However, the reduction of ATP levels at 0.16 μ M produced by each compound is significantly different. In the case of Niclosamide, ATP levels are reduced by more than 80%, whereas the ATP levels of benzimidazole **4** are unchanged. The effect on ATP levels were not only significantly different but in an opposite direction relative to their effect on Wnt/ β -catenin signaling. Niclosamide reduced ATP levels more strongly than its inhibition of Wnt/ β -catenin signaling while benzimidazole **4** had significantly less effect on ATP levels at a similar level of inhibition of Wnt/ β -catenin signaling (Figure 7E). Moreover, the trend in which the benzimidazole derivatives produce stronger inhibition of Wnt/ β -catenin signaling than the reduction of ATP levels was similar with benzimidazoles **2** and **10** (Figure 7E). Benzimidazole **2** and **10** are less potent inhibitors of Wnt/ β -catenin signaling than Niclosamide, thus for this analysis, slightly higher concentrations of each compound are required to achieve the same level of inhibition of Wnt/ β -catenin as Niclosamide (Figure 7E). At 0.31 μ M, benzimidazole **2** inhibited Wnt/ β -catenin signaling by 40%, a level similar to Niclosamide at 0.16 μ M. However, benzimidazole **2** produced less effect (no reduction) on ATP levels, while Niclosamide had a stronger reduction of ATP levels than inhibition of Wnt/ β -catenin signaling. The same trend is observed when benzimidazole **10** is compared to Niclosamide. At 0.63 μ M, benzimidazole **10** inhibited Wnt/ β -catenin signaling to the same extent as Niclosamide at 0.16 μ M, and again the reduction of ATP levels produced by benzimidazole **10** was less (no reduction) than its reduction in Wnt/ β -catenin signaling compared to Niclosamide where the reduction of ATP levels was greater than its inhibition of Wnt/ β -catenin signaling. These results clearly show the benzimidazole derivatives produce different and opposite responses compared to Niclosamide. From these studies, we conclude that the benzimidazoles derivatives are more selective than Niclosamide at inhibiting Wnt/ β -catenin signaling vs. its effect on cellular ATP homeostasis and that the SAR of the two activities appear separate.

4. Discussion

To translate the inhibition of Wnt/ β -catenin signaling by Niclosamide into the clinic, our program has sought to understand Niclosamide's mechanism of action and identify

compounds with improved potency, selectivity and pharmacokinetic properties as future drug candidates. Niclosamide was first discovered to be an inhibitor of Wnt/ β -catenin signaling via a high-throughput reverse chemical genetic screen, and as a result, the target responsible is unknown and the mechanistic details are not immediately understood. In our initial SAR studies we found that structurally-related anthelmintic agents did not inhibit Wnt signaling. This suggested to us that Niclosamide's mechanism of inhibition of Wnt/ β -catenin signaling may be different from its anthelmintic mechanism of action, reportedly due to effects on ATP homeostasis and uncoupling of oxidative phosphorylation. If true, then it should be possible to define SAR elements that separate effects on ATP homeostasis from Wnt/ β -catenin inhibitory activity to improve the Wnt/ β -catenin selectivity. Moreover, we postulated that modulation of Niclosamide's effects on ATP homeostasis may further improve its selectivity based on the hypothesis that many of Niclosamide's other non-Wnt biological activities may also result from its ability to modulate cellular ATP levels and uncouple oxidative phosphorylation. Thus to improve the selectivity of inhibition of Wnt/ β -catenin signaling and reduce the potential for dose-limiting toxicities in the clinic, we sought to decrease the effect of our Wnt/ β -catenin inhibitors on cellular ATP homeostasis and on uncoupling of oxidative phosphorylation.

To test these hypotheses, we prepared benzimidazole derivatives of Niclosamide based on SAR information gained in our previous studies. In previous SAR studies, the amide linker was found to be important for Wnt/ β -catenin inhibition such that a number of modifications of the amide led to a significant diminution of activity. Here the use of a benzimidazole bioisostere was found to be a suitable replacement of the amide and anilide to maintain Wnt/ β -catenin inhibitory activity while at the same time reducing the conformational flexibility of the anilide ring and modulating the cLogP and calculated pKa to enable testing of our hypothesis. Upon comparing the SAR of the benzimidazole derivatives to the previous Niclosamide SAR, we found the SAR trends were similar. Electron-withdrawing substituents in the benzimidazole ring that correspond to the 4'-position of the anilide in Niclosamide were similarly preferred. For example, trifluoromethyl and nitro substituents were more active, and fluorine, hydrogen, and methoxyl were less active. In the pendant phenol ring, similar SAR trends to the Niclosamide SAR were also observed. Specifically, a 4-chloro substituent enhanced potency, while fluoro, methyl, hydrogen and methoxyl at this position were less active. In previous SAR studies within the Niclosamide chemotype, we observed a good correlation between activity in the Fzd1-GFP assay and activity in the TOPFlash assay. In the studies herein, we observed for the first time molecules with low micromolar activity in the Wnt/ β -catenin TOPFlash assay that did not produce a Fzd1-GFP punctate pattern. The mechanistic underpinning for this observation is not understood at this time. In our SAR studies, we focused on compounds that both induced internalization of Fzd1-GFP and inhibited Wnt/ β -catenin signaling in the TOPFlash assay in order to identify compounds with mechanisms of Wnt/ β -catenin inhibition similar to Niclosamide. Overall, the Wnt/ β -catenin SAR trends observed in the Niclosamide chemotype appeared to translate into the benzimidazole chemotype.

In our previous SAR studies, the phenol group of Niclosamide was important for Wnt/ β -catenin inhibitory activity; however, it was not understood what feature of the OH group was important and how the pKa of the phenol factored into inhibition of Wnt signaling. Data in

the literature indicate the phenol of Niclosamide is a weak acid with a pKa of 5.6.¹⁶ The calculations used here also indicate the phenol is acidic, though the magnitude differs from the reported experimental value. Calculation of the pKa in water using Epik (Schrodinger, LLC) provided a pKa value of 6.8 ± 0.6 , whereas calculations from ACD labs software provide a calculated value of 7.45 ± 0.43 .⁵⁵ Although each calculated value is higher than the reported experimental value, the calculated and experimental values each indicate the OH proton is acidic and is significantly dissociated at neutral pH. For consistency, pKa values from Epik are used within. Based on the calculated values of the acidity of the phenol and the limited set of compounds tested, the pKa of the phenol may not be a sensitive determinant of Wnt/ β -catenin inhibitory activity (Table 1). Compounds that are calculated to be less acidic by more than 1.6 log units are similarly active to Niclosamide.

As part of the design of more selective inhibitors, cLogP was an important design parameter to understand. Here again it is noteworthy that the cLogP values used within also differ from the reported experimental values. The cLogP of Niclosamide using Qikprops (Schrodinger, LLC) is 3.1, while the calculated value using ACD Labs software is 3.66.⁵⁵ The reported experimental value of Log P is 4.48 (pH7).¹⁶ Thus, the calculated LogP values tend to underestimate the lipophilicity of these derivatives, perhaps because they underestimate the contribution of intramolecular hydrogen bonding in these derivatives. Again whereas the magnitudes vary, the calculated and experimental values both indicate that Niclosamide is lipophilic, such that the trends identified from calculated values can be useful when comparing compounds. It is not clear from our studies how cLogP values influence inhibition of Wnt/ β -catenin.

Previous studies by Terada et al. in the salicylanilide structural class provided an SAR model of uncoupling oxidative phosphorylation in mitochondria and protonophore activity.⁴⁴ In this model, the acid dissociation constant (pK_a), the partition coefficient between octanol and water (LogP) and the ability of the conjugate base to delocalize a negative ionic charge are important for activity. Both hydrophobic and electron-withdrawing properties were found to be essential for induction of potent uncoupling activity. We interrogated this model by evaluating changes in cellular ATP levels, a downstream index of protonophore activity and uncoupling of oxidative phosphorylation rather than directly evaluating protonophore activity or uncoupling of oxidative phosphorylation. Initially, we measured ATP levels in cells incubated in media containing glucose but later focused on measuring ATP levels in cell media without glucose when it was found that glucose in the cell media attenuated the reduction of cellular ATP levels and could confound interpretation of the results. The trends obtained in the ATP assay were the same regardless of the presence of glucose in the cell media. Overall, we found the benzimidazole inhibitors of Wnt/ β -catenin signaling caused less reduction in ATP levels than Niclosamide. Molecules that contained OH or NH groups calculated to be weak acids with pKa values approaching 7, regardless of structural class, that had similar or greater lipophilicity than Niclosamide, reduced ATP levels more than molecules calculated to be less acidic. Of particular note was the activity of benzimidazole **11** in which a simple insertion of a chlorine atom ortho to the phenol in a compound with weak effects on ATP levels (benzimidazole **2**), produced a compound (benzimidazole **11**) with a strong reduction of ATP levels. The calculated acidity of the phenol and the

lipophilicity of benzimidazole **11** compared with benzimidazole **2** are now closer to the calculated acidity and lipophilicity of Niclosamide, and now the effects on ATP and p-AMPK are also more similar (Table 1 and Figure 4). In contrast to the Wnt/ β -catenin SAR, the calculated pKa distinguished compounds with effects on ATP levels. Compounds that were calculated to be less acidic by more than 1.6 log units produced much less reduction of ATP levels compared to Niclosamide. Within the range of cLogP values of the compounds studied it did not appear that cLogP was a sensitive index to predict reduction of ATP levels. Overall the ability to predict a compounds effect on ATP levels using calculated LogP and pKa values were consistent with the Terada model; molecules that were less lipophilic and less acidic produce less reduction of ATP.

To address the question whether the Niclosamide SAR of Wnt/ β -catenin inhibition can be separated from the effects on ATP levels, side-by side analysis of Wnt/ β -catenin inhibition and reduction of ATP in the same batch of HEK293 TOPFlash cells showed a consistent trend that supports the conclusion the SAR is separate. The benzimidazole analogs of Niclosamide were more selective, producing less reduction of ATP levels at a similar level of inhibition of Wnt/ β -catenin signaling compared to Niclosamide. Based on the profile of benzimidazole **4**, a compound of similar Wnt/ β -catenin potency to Niclosamide, and benzimidazole **2** and **10** that are less potent inhibitors of Wnt/ β -catenin signaling, we conclude that the benzimidazoles derivatives are more selective inhibitors of Wnt signaling than Niclosamide with regard to effects on cellular ATP homeostasis. These studies support the conclusion that the SAR of Wnt inhibition by Niclosamide can be separated from the effects on cellular ATP homeostasis; conclusions consistent with our initial SAR studies of Niclosamide that suggested the Wnt/ β -catenin inhibitory effect of the Niclosamide appeared to be separate from the mechanism attributed to its anthelmintic effect.

Niclosamide is a multi-functional drug that possesses many valuable biological activities that could be useful in the treatment of a number of important diseases. Molecules identified here possessing greater selectivity provide tools for further studies to understand what processes drive Niclosamide's biological activities and to test the hypothesis that modulation of cellular ATP levels may be an underlying mechanism driving many of Niclosamide's biological activities. The studies here support the notion that inhibition of Wnt/ β -catenin signaling is not driven by Niclosamide's effect on ATP metabolism. Further studies are required to understand the mechanism underlying this selectivity. At present it is unknown how the structural changes in the benzimidazole class impact protonophore activity, uncoupling of oxidative phosphorylation activity, or activation of ATPases. Moreover it is not known how these structural changes effect the pharmacokinetic properties and systemic exposure. Based on fundamental medicinal chemistry principles, benzimidazole derivatives such as compound **4**, a compound with less acidity and propensity to ionize and with the major site of Niclosamide's metabolism replaced (-NO₂ replaced with -CF₃), have the potential for improved pharmacokinetic properties and improved systemic exposure.

5. Conclusion

The studies here establish for the first time that an SAR understanding of Wnt/ β -catenin inhibitory activity of Niclosamide can be translated into a different chemotype. Moreover,

the studies within establish that SAR models of protonophore activity based on cLogP and pKa are useful in translating the SAR to the design of new Wnt/ β -catenin inhibitors with less impact on cellular ATP homeostatic mechanisms and greater selectivity toward Wnt/ β -catenin inhibition than Niclosamide. Overall, the ability to translate the Wnt/ β -catenin inhibitory activity of Niclosamide into a new chemotype provides an opportunity to bring forward new inhibitors of Wnt/ β -catenin signaling with improved potency, selectivity, and pharmacokinetic properties to treat cancer and other systemic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	Adenomatous Polyposis Coli
CRC	colorectal cancer
Dvl	Dishevelled
FCCP	trifluorocarbonylcyanide phenylhydrazone
LEF/TCF	Lymphoid enhancer factor/T cell factor
Fzd1	Frizzled1
GFP	green fluorescent protein
SAR	structure-activity relationships

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Figure 1. Inhibitor design

cLogP and pKa values were calculated using Maestro (Schrodinger, LLC). cLogP values are QPLogP (octanol/water) calculated in Qikprop. The pKa values are the phenol substituent calculated in water using Epik.

Figure 2.
Synthesis of benzimidazole derivatives.

Figure 3. Reduction of β -catenin and β -catenin target gene levels in HCT-116 and SW480 cells by Niclosamide or benzimidazole inhibitors

Cells were treated with compound in DMSO or DMSO for 18 hours. Cell lysates were analyzed by Western blot. β -actin was used as a loading control.

Figure 4. Reduction of ATP levels and induction of phospho-AMPK by known inhibitors of ATP synthesis, uncouplers of oxidative phosphorylation, and benzimidazole Wnt/ β -catenin inhibitors in HCT-116 cells in DMEM media with and without glucose

(A) ATP levels in DMEM media with glucose at 3 hr measured by Adenosine triphosphate (ATP) bioluminescent assay normalized to DMSO control (B) Phospho-AMPK levels in DMEM media with glucose at 30 min with each agent at 2 μ M. β -actin used as a loading control. (C) ATP levels in DMEM media without glucose. (D) Phospho-AMPK levels in DMEM media without glucose with each agent at 2 μ M.

Figure 5. Reduction of ATP levels by inhibitors in different chemotypes

ATP levels measured at 3 hr by Adenosine 5-triphosphate (ATP) bioluminescent assay in HCT-116 cells in DMEM media without glucose.

Figure 6. Comparison of ATP lowering effects of compounds by calculated pKa and cLogP
ATP levels in HCT 116 cells in DMEM media without glucose were measured by ATP bioluminescent assay 3 hr after 2 μ M, 1 μ M and 0.5 μ M treatment with compounds in Table 1 and 2. Each compound is plotted by its calculated pKa and cLogP, and the reduction of ATP indicated by the symbol. Compounds with no or a small reduction of ATP (less than < 25%) at all three concentrations are depicted as green diamonds, compounds with an intermediate reduction of ATP (> 25% decrease in ATP at 2 μ M and < 25 % at 0.5 μ M) are depicted as yellow squares, and compounds with significant reduction of ATP (> 50 % decrease) at all concentrations are depicted as red circles. Chemical properties were calculated in Maestro (Version 10.3.015, Schrodinger, LLC) using Qikprops for cLogP (QPLogP(o/w)) and Epik for pKa in water.

Figure 7. Side-by side comparison of inhibition of Wnt/β-catenin signaling and reduction in cellular ATP levels by Niclosamide, benzimidazole 2, 4 and 10
 HEK293 cells stably expressing a TOPFlash and a Renilla luciferase reporter were split and evaluated side-by-side by TOPFlash and Adenosine triphosphate bioluminescent assays. TOPFlash assay: Cells were treated with compounds for 6 hours in Wnt3A-conditioned media. Adenosine triphosphate bioluminescent assay: Cells were treated with compounds for 3 hours in DMEM media without glucose. In each graph inhibition of Wnt signaling is depicted as black squares and reduction of ATP as red triangles. Panel (A) Niclosamide (B) Benzimidazole 4 (C) Benzimidazole 2 (D) Benzimidazole 10. (E) Inhibition of Wnt/β-catenin signaling and reduction of ATP levels at a specific concentration of the inhibitor near the IC₅₀ concentration that produced similar reduction of Wnt/β-catenin signaling.

Table 1

Inhibition of Wnt/ β -catenin signaling by benzimidazole derivatives.

Compound number	Structure	cLogP ^a	Calculated pK _a $\pm$ error ^d	-NH	Fzd1-GFP Internalization at 12.5 μ M ^b	Inhibition Wnt/ β -catenin transcription TopFlash IC ₅₀ (μ M) $\pm$ SE ^c
1		3.1	6.8 $\pm$ 0.6	10.8 $\pm$ 0.8	5	0.28 $\pm$ 0.06
2		2.3	8.4 $\pm$ 0.9	9.2 $\pm$ 0.8	3	0.48 $\pm$ 0.04
3		3.0	8.4 $\pm$ 0.9	12.3 $\pm$ 0.7	0	2.67 $\pm$ 1.08
4		4.0	8.4 $\pm$ 0.9	11.3 $\pm$ 0.7	3	0.27 $\pm$ 0.03
5		3.1	8.4 $\pm$ 0.9	12.4 $\pm$ 0.7	0	1.27 $\pm$ 0.39
6		3.2	8.4 $\pm$ 0.9	11.8 $\pm$ 0.7	0	7.65 $\pm$ 0.25
7		1.9	9.4 $\pm$ 0.7	9.5 $\pm$ 0.8	0	4.44 $\pm$ 0.91
8		2.2	9.7 $\pm$ 0.9	9.7 $\pm$ 0.8	0	1.69 $\pm$ 0.43

Compound number	Structure	cLog ^a	Calculated pKa ± error ^d		Fzd1-GFP Internalization at 12.5 μM ^b	Inhibition Wnt/β-catenin transcription TopFlash IC ₅₀ (μM) ± SE ^c	
			-OH	-NH			
9		2.0	9.6 ± 0.9	9.4 ± 0.8	0	4.19 ± 1.39	
10		2.1	9.3 ± 0.9	9.2 ± 0.8	2	1.15 ± 0.2	
11		2.9	7.1 ± 1.47	9.3 ± 0.8	2	1.54 ± 0.35	

^a pKa and cLogP values were calculated using Epik and Qikprop within Maestro (Version 10.3.015, Schrodinger, LLC). cLogP is QPlog(o/w). Calculated pKa values in water are reported. The pKa of the NH is calculated without ionization of the -OH group.

^b Fzd1-GFP assay: Internalization of Frizzled1-GFP stably expressed in U2OS cells was determined by confocal microscopy. Cells were treated with compounds for 6 hours, fixed, and scored visually by the amount of punctate observed versus DMSO control. Punctate similar to control = 0, trace amount of punctate greater than control = 1, moderate = 3, strong = 5. Images in Supplemental Information.

^c Inhibition of Wnt3A-stimulated Wnt/β-catenin transcription was determined by TOPFlash assay using HEK293 cells stably expressing a TOPFlash luciferase reporter and a Renilla luciferase reporter control. Cells were stimulated with Wnt3A-conditioned medium in the presence of DMSO or compounds from 0.04 to 10 μM. The TOPFlash reporter activity with DMSO treatment in Wnt3A-conditioned media was set as 100%. Data were fit using GraphPad Prism (mean ± SEM, n = 3).

Table 2

Inhibition of Wnt/ β -catenin signaling by sulfonamide derivatives.

Compound number	Structure	cLogp ^a	Calculated pK _a ± error ^d -N ¹ H -N ² H	Fzd1-GFP Internalization at 12.5 μM ^b	Inhibition Wnt/β-catenin transcription TopFlash IC50 (μM) ± SE ^c	
12		3.2	9.3 ± 0.8	7.9 ± 0.9	0	48 % inhibition at 10 μM ±2.4
13		4.8	7.5 ± 0.7	7.9 ± 0.9	0	3.2 ±1.9
14		2.7	11.6 ± 0.8	7.7 ± 0.9	0	15 % inhibition at 10 μM ±3.3
15		4.3	7.3 ± 0.7	7.7 ± 0.9	0	8 % inhibition at 10 μM ±2.0

Compound number	Structure	cLogP ^a	Calculated pKa ± error ^a -NH -N ² H	Fzd1-GFP Internalization at 12.5 μM ^b	Inhibition Wnt/β-catenin transcription TopFlash IC50 (μM) ± SE ^c	
16		2.7	9.1 ± 0.8	8.4 ± 1.5	0	53 % inhibition at 10 μM ±8.3
17		4.3	11.6 ± 0.8	8.4 ± 1.5	0	57 % inhibition at 10 μM ±5.1
18		2.9	11.6 ± 0.8	8.2 ± 2.2	0	74 % inhibition at 10 μM ±3.5
19		1.8	9.1 ± 0.8	8.2 ± 2.2	0	50 % inhibition at 10 μM ±7.6
20		3.9	6.7 ± 0.8	7.1 ± 0.9	3	1.5 ±0.4

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^a pKa and cLogP values were calculated using Epik and Qikprop within Maestro (Version 10.3.015, Schrodinger, LLC). Calculated pKa values in water are reported. The pKa of the second NH group is calculated without ionization of the first -NH group.

^b Fzd1-GFP assay: Internalization of Frizzled1-GFP stably expressed in U2OS cells was determined by confocal microscopy. Cells were treated with compounds for 6 hours, fixed, and scored visually by the amount of punctate observed versus DMSO control. Punctate similar to control = 0, trace amount of punctate greater than control = 1, moderate = 3, strong = 5. Images in Supplemental Information

^c Inhibition of Wnt3A-stimulated Wnt/β-catenin transcription was determined by TOPFlash assay using HEK293 cells stably expressing a TOPFlash luciferase reporter and a Renilla luciferase reporter control. Cells were stimulated with Wnt3A-conditioned medium in the presence of DMSO or compounds from 0.04 to 10 μM. The TOPFlash reporter activity with DMSO treatment in Wnt3A-conditioned media was set as 100%. The percent inhibition of Wnt signaling at the highest concentration tested is given when inhibition was weak and did not permit calculation of an IC₅₀. Data were fit using GraphPad Prism (mean ± SEM, *n* = 3).