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The Anti-Helminthic Niclosamide Inhibits Wnt/Frizzled1 Signaling†

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Abstract

Wnt proteins bind to seven-transmembrane Frizzled receptors to mediate the important developmental, morphogenetic, and tissue-regenerative effects of Wnt signaling. Dysregulated Wnt signaling is associated with many cancers. Currently there exist no drug candidates, or even tool compounds that modulate Wnt-mediated receptor trafficking, and subsequent Wnt signaling. We examined libraries of FDA-approved drugs for their utility as Frizzled internalization modulators, employing a primary imaged-based GFP-fluorescence assay that uses Frizzled1 endocytosis as the readout. We now report that the anti-helminthic niclosamide, a drug used for the treatment of tapeworm, promotes Frizzled1 endocytosis, down regulates Dishevelled-2 protein, and inhibits Wnt3A-stimulated β -catenin stabilization and LEF/TCF reporter activity. Additionally, following niclosamide mediated internalization, the Frizzled1 receptor co-localizes in vesicles containing Transferrin and agonist-activated β_2 -adrenergic receptor. Therefore, niclosamide may serve as a negative modulator of Wnt/Frizzled1 signaling by depleting up-stream signaling molecules (i.e. Frizzled and Dishevelled), and moreover may provide a valuable means to study the physiological consequences of Wnt signaling.

Keywords

β -Catenin; Dishevelled-2; Frizzled1; LEF/TCF; Niclosamide; Receptor internalization; and Wnt signaling

The Wnt signaling pathway plays a fundamental role in the developing embryo by directing tissue patterning, in the mature organism by maintaining tissue homeostasis, and in cancer (1–3). Wnt ligands are secreted glycoproteins that exist in multiple forms. Humans express 19 different Wnt subtypes that are agonists for at least 10 different seven transmembrane Frizzled receptors (4,5). Wnt binding to Frizzled results in activation of cytosolic Dishevelled proteins, which leads to Frizzled receptor internalization (6). Downstream signaling events produced as a consequence of Wnt binding include the stabilization of cytosolic β -catenin by preventing GSK3 β phosphorylation and translocation of the stabilized β -catenin to the nucleus followed by the activation of the transcription factor LEF/TCF (3,7).

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The importance of inappropriate Wnt signaling has not been lost on the pharmaceutical industry and academia, who consider components in Wnt signaling as prime drug targets (5,8–11). Due to their accessibility, plasma membrane receptors like Frizzled are also appealing as targets of prescription drugs. However, there are currently no FDA-approved drugs or tool compounds that regulate Wnt signaling at the level of the Frizzled receptor. To address this need, we have instituted a translational small molecule screening program to identify candidate leads that affect Frizzled endocytosis. Furthermore, to expedite the development of Wnt/Frizzled signaling modulators, we searched existing libraries containing FDA-approved drugs, and we have now discovered that the anti-helminthic niclosamide promotes Frizzled1 (Fzd1) internalization, and in the presence of Wnt3A, blocks downstream β -catenin signaling.

MATERIALS AND METHODS

Plasmids, antibodies, and conditioned media

pCS2ratFrizzled1-GFP (stock #16821) and pLKO.1 (stock # 10878) were obtained from Addgene (Cambridge, MA). The reporter plasmid p8xTOPFlash was obtained from Dr. Randall Moon. A plasmid for Renilla luciferase was purchased from Promega. β_2 -adrenergic receptor-RFP (β_2 AR-RFP) was prepared similarly as described for the GFP derivative (6,12). β -catenin (sc-7963), Dishevelled-1 (sc-8025), Dishevelled-2 (sc-13974, Lot# A242), Dishevelled-3 (sc-8027, and sc-28846) and β -actin (sc-47778) antibodies were obtained from Santa Cruz. The cell lines to produce Wnt3A (CRL-2647), Wnt5A (CRL-2814), and Control (CCL-1.3) conditioned media were obtained from the ATCC. The conditioned media was generated by growing cells in DMEM plus 10% FBS according to the protocol described at “<http://www.stanford.edu/~rnusse/assays/W3aPurif.htm#assay>.”

Stable cell line generation

To obtain a Frizzled1-GFP stable cell line (Fzd1GFP-U2OS), U2OS cells were transfected with pCS2ratFrizzled1-GFP and pLKO.1 (10:1 ratio by weight), using the Nucleofection transfection protocol from Amaxa, and stable receptor expressing clones were selected using 1.5 μ g/ml puromycin in the culture medium. For generating TOPFlash stable cell lines, HEK293 cells were transfected with p8xTOPFlash, the Renilla luciferase plasmid, and pLKO.1 at a ratio by weight of 10:3:1 and stable clones selected using 1 μ g/ml puromycin in the growth medium. pLKO.1 was used to confer puromycin resistance to the stable clones.

Image-based primary screening assay

A library containing approximately 1200 FDA approved drugs and drug-like tool compounds was purchased from Prestwick Chemicals Inc. U2OS cells stably expressing Frizzled1-GFP were split into glass-bottom 384 well plates (MGB101-1-2-LG, MatriCal, Spokane, WA) at a density of 6,000 cells/25 μ l media/well using a Multidrop 384 dispenser (Titertek Instruments, Huntsville, AL). The plates were incubated overnight at 37 °C in 5% CO₂. The following day, chemical compounds (5 mM in DMSO) from the Prestwick library were diluted 1:80 in culture media, 6.25 μ l of which were then added to each well of cells using a Biomek FX liquid handler configured with a 96 channel head (Beckman Coulter, San Jose, CA) to produce a 1:400 dilution overall and final compound concentration of 12.5 μ M per well. The cells were incubated with compound for 6 hrs at 37 °C prior to fixation in PBS containing 0.5% paraformaldehyde and 0.002% of the fluorescent nuclear stain DRAQ5. Plates were stored at 4 °C until analysis on an ImageXpress Ultra high throughput imaging system (Molecular Devices, Sunnyvale, CA) equipped with a 488 nm argon laser for imaging GFP and a 568 nm krypton laser for imaging DRAQ5. All imaging data were verified by visual inspection and a Z' factor of 0.44 was calculated for the robustness of the assay.

Cell surface biotinylation internalization assay

Frizzled1 internalization was assessed by a surface biotin labeling method (13). Fzd1GFP-U2OS cells were grown to confluence in a 6 cm plate, washed twice with PBS containing 10 mM HEPES, incubated at 4 °C for 1 hr with 2 ml of 1 mg/ml sulfo-NHS-S-S-biotin (Pierce, Rockford, IL), and washed three times with cold PBS containing 50 μ M Tris-HCl. To assess Frizzled1 internalization, the cells were incubated at 37 °C for 4 hrs in culture medium with or without 12.5 μ M niclosamide, returned to 4 °C, and incubated for 15 min twice with fresh glutathione cleavage solution (50 mM reduced L-glutathione, 75 mM NaCl, 10 mM EDTA, 1% BSA, and 0.075 N NaOH) in order to remove biotin remaining on the cell surface. The cells were then washed with cold PBS three times, and lysed with RIPA buffer (50 mM Tris-HCl, PH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Biotinylated proteins from the cell lysate were pulled down with Neutravidin beads (Pierce, Rockford, IL), and the beads were eluted with Laemmli SDS loading buffer/50 mM dithiothreitol for 2 hrs at room temperature. Eluted Frizzled1-GFP was identified using SDS-PAGE and anti-GFP antibody. As controls, the surface-biotinylated cells after washing with PBS/50 μ M Tris-HCl were either directly lysed with RIPA buffer to assay the total biotin-labeled Frizzled1-GFP, or immediately subjected to glutathione cleavage to monitor the efficiency of the biotin removal.

Image analysis and quantification of internalized vesicles

Confocal images were acquired with a Zeiss LSM510 confocal microscope, and analyzed using the computer program Metamorph (Universal Imaging Corporation) as described (14). To measure the number of the internalized vesicles per cell, cytosol was carefully traced to exclude cell membrane. Internalized vesicles were defined by setting the threshold of the image to 3-fold of the background intensity. The number of internalized vesicles per cell was counted by the Metamorph software. More than 30 cells per sample were analyzed to obtain statistical significance.

Transferrin endocytosis assay

Cells were serum-starved in MEM for 15 min, and then incubated with Alexa-543 conjugated Transferrin (Tf, 100 μ g/ml) together with niclosamide (12.5 μ M) for 2 hrs at 37 °C. To remove remaining surface bound Tf, the cells were exposed to unlabeled transferrin (10 mg/ml) for 2 min at room temperature. The cells were then fixed with 4% paraformaldehyde, and imaged with a LSM510 confocal microscope (Zeiss).

TOPFlash reporter assay

For the TOPFlash Luciferase assay the TOPFlash stable cells were seeded in 150 μ l growing medium/well in 96-well plates at 100% confluency. Fifty microliters of conditioned medium containing the chemical compounds to be tested or DMSO were added to each well. After an 8-hr treatment, the cells were then washed once with PBS, and lysed with 80 μ l MPER solution (Pierce, Rockford, IL). Thirty microliters of cell lysate were used for measuring luciferase activity in a 96-well plate reader (FluoStar Optima, BMG Labtech, Chicago, IL).

Detection of Cytosolic β -catenin and Dishevelled

To assay cytosolic β -catenin stabilization and Dishevelled-2 expression, U2OS cells were grown to 100% confluency, and then treated with control conditioned medium or Wnt3A conditioned medium supplemented with DMSO or varying concentrations of niclosamide for 6 hrs. After treatment, cytosolic fraction as well as cellular membrane were isolated as described (15). Immunoblots using β -catenin or Dishevelled-2 antibody were used to detect the respective protein levels in cytosol or on membrane, with β -actin immunoblots used for loading controls.

RESULTS

In order to screen small molecule modulators of Frizzled receptor internalization and develop an assay compatible with high throughput screening, we generated a U2OS cell line stably expressing Frizzled1-GFP (Fzd1GFP-U2OS). The cellular distributions of Frizzled1-GFP chimeras were initially assessed by confocal microscopy. Frizzled1-GFP localized predominantly to the plasma membrane with almost no internalized vesicles present when the cells were not stimulated with Wnt ligands (Fig. 1A). When treated with Wnt3A conditioned medium, the cells showed a minimal internalization of receptor fluorescence (Fig. 1B), whereas cells exposed to Wnt5A conditioned medium demonstrated a moderate amount of intracellular fluorescence (Fig. 1C). These observations indicated that Frizzled1 internalization could provide a readout for agonist/ligand activity.

Over 1200 FDA-approved drug and drug-like compounds from the Prestwick Chemical Library were screened at 12.5 μ M concentration in a 384-well format. This primary screen revealed the hit niclosamide (Prestwick 01D11), which produced much more robust internalization (Fig. 2A-2C) than even Wnt3A or Wnt5A stimulation (Fig. 1B and 1C). To verify the result, the Fzd1GFP-U2OS cells were also treated with niclosamide obtained from an alternate supplier (Sigma), and similarly strong internalization of Frizzled1-GFP was observed (Fig. 2D and 2E). As a control, human EGF Receptor 2-GFP (Her2-GFP) expressed on the plasma membrane of U2OS cells (X.-R. Ren, unpublished data) did not internalize when treated with an equivalent concentration of niclosamide (Fig. 2F and 2G).

During our primary screening, we identified an additional 25 small molecule compounds (Table I) that had some effect on Frizzled1-GFP internalization, but little or no effect on Wnt signaling as assessed by the TOPFlash luciferase reporter assay (data not shown). These 25 compounds were therefore not studied further, and the small molecule hit niclosamide, which demonstrated a potential for modulating Wnt signaling (see sections below), was investigated in detail.

To assess the effect of niclosamide on Frizzled1 internalization, we employed a method independent of and complementary to our primary screening methodology using biotin labeling of the Frizzled1-GFP plasma membrane receptors. First, Fzd1GFP-U2OS cells were surface biotinylated at 4 °C to label only the cell surface receptor population. Next the labeled cells were incubated at 37 °C to allow receptor internalization in the presence of niclosamide. Receptors that internalize in this assay will have their biotin label protected from glutathione cleavage and can be visualized using anti-GFP immunoblots. In Fig. 3, Lane 1 shows the total biotin labeled cell surface Frizzled1-GFP, and the nearly complete removal of Frizzled1 bands in Lane 2 demonstrates that receptors on the cell surface (4 °C, not internalized) are susceptible to glutathione cleavage of their biotin label. In contrast, biotinylated Frizzled1-GFP receptors exposed to niclosamide at the permissible internalization temperature of 37 °C (Fig. 3, Lane 4) produce a strong immunoblot signal compared to cells not treated with niclosamide (Fig. 3, Lane 3), indicating that the niclosamide-internalized Frizzled1 receptors originate on the plasma membrane.

To determine the time-course of internalization for Frizzled1-GFP receptors we measured the accumulation of cytosolic puncta/vesicles over 6 hrs (Fig. 4A-4E). Fig. 4F is a graphical representation of the internalization time course showing a $t_{1/2}$ of 2.4 ± 0.5 hr for the niclosamide-induced Frizzled1 internalization. Fig. 5A-5G shows the dose-dependence at the 6 hr time point of Frizzled1-GFP internalization in the presence of increasing concentrations of niclosamide. Between 1 to 2 μ M niclosamide concentration we observe a significant increase in the number of internalized receptors suggesting that the potency for niclosamide induced internalization is in the low micromolar range (Fig. 5G).

Many classes of membrane receptors internalize in clathrin coated pits. In particular, β_2 -adrenergic receptors are prototypical for clathrin-dependent internalization of G protein-coupled receptors (12,16), and Transferrin is a well-documented standard for clathrin-mediated internalization in general (17). The demonstration that internalized Frizzled1-GFP co-localizes with either β_2 -adrenergic receptor-RFP or Transferrin in intracellular vesicles would indicate Frizzled1 also internalizes in a similar manner. Fig. 6A-6C show cells expressing both β_2 AR and Frizzled1 receptors prior to activation, and under these conditions the two receptors are not intracellularly co-localized. Exposure to isoproterenol and niclosamide for 2 or 6 hrs (Fig. 6D-6I) results in multiple overlapping intracellular distributions of each receptor. Moreover, internalized Transferrin at 2 hrs has significant co-localization with internalized Frizzled1 (Fig. 6J-6L). These data suggest that niclosamide-induced Frizzled1 internalization occurs through clathrin-coated pits.

Dishevelled proteins (Dishevelled-1, 2, and 3 in mammalian cells) are intracellular molecules transducing Frizzled signaling. To assess the effect of niclosamide on Wnt signaling mechanism, we examined protein expression of Dishevelled. In U2OS cells stimulated with either control or Wnt3A conditioned medium, treatment of niclosamide for 6 hrs results in dramatic reduction of cytosolic Dishevelled-2 protein (Fig. 7A). The half maximal reduction of Dishevelled-2 occurs at a niclosamide concentration of approximately 1 μ M (Fig. 7B). No endogenous Dishevelled-2 was detected in the membrane fraction, and endogenous Dishevelled-1 and 3 were not detectable using commercial antibodies (Data not shown).

LEF/TCF transcription factor reporter (TOPFlash) assay is a general readout for canonical Wnt signaling pathways. To assess the effect of niclosamide on Wnt signaling activity, we generated an HEK293 cell line that stably expressed a LEF/TCF transcription factor reporter plasmid (TOPFlash) that responds to Wnt mediated β -catenin induction and a Renilla luciferase plasmid that serves as an internal control. The ability of niclosamide to promote Frizzled1 internalization suggests agonist or partial agonist-like behavior. Niclosamide alone does not produce a statistically significant increase in the TOPFlash (LEF/TCF) reporter signal (Fig. 8A). Upon Wnt3A stimulation, a 140 fold induction of the LEF/TCF reporter signal was observed (Fig. 8A). Remarkably, the addition of niclosamide to the Wnt3A conditioned medium blocked the increase of the reporter signal observed with Wnt3A alone (Fig. 8A), indicating niclosamide inhibits Wnt/Frizzled signaling induced by a full agonist (Wnt in this case). The inhibitory effect is dose-dependent with an IC_{50} of 0.5 ± 0.05 μ M (Fig. 8B).

The accumulation of cytosolic β -catenin is a measure of canonical Wnt signaling (15). The reporter assay indicates that Wnt inducible cytosolic β -catenin accumulation should be reduced in the presence of niclosamide. Figure 8C demonstrates that niclosamide prevents Wnt3A-stimulated cytosolic β -catenin stabilization (Compare Lane 3 and 4, upper panel, cytosol). However, the membrane-bound β -catenin levels are relatively unchanged (Fig. 8C, bottom panel, membrane), indicating the reduction in the signaling β -catenin pool is not due to a loss of β -catenin expression. The potency of niclosamide inhibition in Wnt-mediated β -catenin stabilization can be determined from the dose dependence presented in the immunoblots of Fig. 8D. The half maximal inhibition of Wnt3A signaling occurs at a niclosamide concentration of approximately 1 μ M.

In summary, our data indicate that niclosamide promotes Frizzled1 internalization, down regulates the expression of Dishevelled-2 protein, and inhibits Wnt3A-stimulated LEF/TCF (TOPflash) reporter activity and β -catenin stabilization. Therefore niclosamide functions as an inhibitor for Wnt signaling.

DISCUSSION

It has been reported that niclosamide is a salicylanilide derivative of salicylic acid. Its most common therapeutic usage is in the treatment of intestinal tapeworm infections (18), but niclosamide also has demonstrated activity against mollusks. For example, niclosamide is widely used in China as a molluscicide in an attempt to eradicate schistosome containing snails (19). More recently, niclosamide was found to be effective at low micromolar concentrations in preventing the synthesis of corona virus proteins in a tissue culture model of severe acute respiratory syndrome (SARS) (20). Its mechanism of action in this instance has not been well defined, but niclosamide can interact with DNA (20). It is believed that niclosamide uncouples oxidative phosphorylation in the tapeworm (21).

There are currently no radiolabeled Wnt binding assays for Frizzled, preventing a direct test of niclosamide's ability to compete for the Wnt3A binding site on Frizzled1. However, our data indicate that niclosamide can be used as a tool compound to modulate Wnt/Frizzled function in the study of cancer and regeneration at the molecular level. Niclosamide has proven safe in humans when administered for short durations. In exploring the clinical utility of niclosamide as a possible cancer treatment, its ability to block Wnt directed transcriptional activity will have to be weighed against its other biological effects. However, in cases of refractory cancers the benefits may be worth the risks. Alternatively, with structural modification, derivatives of niclosamide may eventually provide safe and effective drug therapies for patients with underlying Wnt directed cancers.

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Figure 1. Wnt-mediated Frizzled1-GFP Internalization

U2OS cells stably expressing Frizzled1-GFP were treated for 6 hrs with control conditioned medium (CTL CM) (A), Wnt3A conditioned medium (Wnt3A CM) (B), and Wnt5A conditioned medium (Wnt5A CM) (C). Cells were imaged using a LSM510 confocal microscope (Zeiss) with a 100 $\times$ objective and 488 nm excitation. Internalized vesicles are indicated by arrowheads.

Figure 2. Niclosamide-induced Frizzled1-GFP Internalization

(A-B) Fzd1-GFP stable U2OS cells (Fzd1 GFP-U2OS) were exposed to compounds taken from the Prestwick Chemical Library for 6 hours at 37 °C. (A) Depicts vehicle (DMSO) treated cells and (B) cells exposed to the hit niclosamide (Prestwick 01D11, 12.5 μ M). Internalized vesicles are indicated by arrowheads. Images were acquired on an ImageXpress Ultra high throughput confocal imaging system using a 40X objective and 488 nm excitation. (C) Chemical structure of niclosamide. (D) Vehicle treated Frizzled1-GFP cells, and (E) Cells treated with niclosamide (12.5 μ M) obtained from a secondary source (Sigma). Vesicles are indicated by arrowheads. (F-G) Confocal images of U2OS cells stably expressing Her2-GFP (Her2GFP-U2OS) treated with DMSO (F) or niclosamide (12.5 μ M) (G). Cells in (D-G) were imaged using a Zeiss LSM510 confocal microscope using a 100X objective and 488 nm excitation.

Figure 3. Measurement of Frizzled1 Receptor Internalization by Biotinylation

Fzd1-GFP stable U2OS cells were subjected to cell-surface biotin labeling at 4 $^{\circ}$ C as described in Materials and Methods. The upper panel shows an immunoblot (IB) for biotin-labeled Frizzled1-GFP enriched using a Neutravidin bead pull down and anti-GFP antibody for Frizzled1-GFP detection. β -actin in the lower panel serves as loading control to ensure that equal amount of cell lysate were used for the Neutravidin pull down. The treatments of surface biotinylation, glutathione, niclosamide, and temperature are indicated above the blots.

Figure 4. Time Course of Niclosamide-stimulated Frizzled1-GFP Internalization
 (A-E) Fzd1-GFP stable U2OS cells were treated with niclosamide (12.5 μ M) for indicated times (0, 1, 2, 4 and 6 hrs). (F) Internalization was assessed by counting the number of Frizzled1-GFP containing vesicles in the cytosol using the computer program Metamorph, and the results are presented in the graph for the given times (0 hr, n = 51 cells; 1 hr, n = 69; 2 hr, n = 32; 4 hr, n = 55, and 6 hr, n = 61). Paired data against time 0 hr were analyzed by Students t-test with a significance of $P \leq 0.0005$, ***.

Figure 5. Dose-dependence of Niclosamide Stimulated Frizzled1-GFP Internalization
(A-F) Fzd1-GFP stable U2OS cells were treated with vehicle containing DMSO (0 μM) or niclosamide (0.47 μM, 0.94 μM, 1.88 μM, 3.75 μM, and 7.5 μM) for 6 hrs. (G) Vesicles per cell were quantified as described above in Fig. 4 for the respective number of cells corresponding to an individual dose: n = 51, 42, 75, 64, 55, and 57. Data were analyzed paired against 0 μM sample using Students t-test with a significance of $P \leq 0.0005$, ***.

Figure 6. Colocalization of niclosamide-stimulated Frizzled1-GFP with the β_2 -adrenergic Receptor or Transferrin

Confocal images of unstimulated U2OS cells containing both β_2 -adrenergic receptor-RFP (β_2 AR-RFP) (A) and Fzd1-GFP (B). (C) Shows the merged image. (D-F) and (G-I) shows the images of the receptors in cells stimulated by 0.1 μ M isoproterenol (Iso) and 12.5 μ M niclosamide at 37 °C for 2 and 6 hrs, respectively. The merged images are presented in (F) and (I). (J-L) depicts similar studies in cells expressing the Fzd1-GFP and exposed to Alexa-546 Transferrin (Tf) at 100 μ g/ml and 12.5 μ M niclosamide for 2 hrs at 37 °C. The merged image is presented in (L). Arrowheads indicate co-localized vesicles.

Figure 7. Niclosamide inhibits the cytosolic expression of endogenous Dishevelled-2

(A) In U2OS cells, 12.5 μM Niclosamide treatment for 6 hrs reduced the cytosolic level of endogenous Dishevelled-2 as demonstrated by immunoblotting. The positions of molecular weight standards are indicated at the right. β-actin serves as a loading control. (B) is an immunoblot demonstrating that the inhibitory effect of niclosamide on Dishevelled-2 expression occurs over a range of 1–7.5 μM of niclosamide.

Figure 8. Niclosamide is an Inhibitor of Wnt3A Signaling

(A-B) HEK293 cells stably expressing TOPFlash luciferase reporter and Renilla luciferase were treated with control conditioned medium or Wnt3A conditioned medium in the presence of DMSO (vehicle) or niclosamide (niclo). The inhibitory effect on Wnt3A stimulated TOPFlash reporter activity by 12.5 μM niclosamide is shown in (A). ns, not significant ($P > 0.05$); ***, $P < 0.0001$, t-tests. CTL CM and Wnt3A CM depict control and Wnt3A conditioned medium, respectively. (B) demonstrates that the inhibition is dependent on the niclosamide concentration. The average TOPFlash reporter activity of Wnt3A plus DMSO treatment was set as 100%, and the relative reporter activity of Wnt3A plus the indicated concentration of niclosamide was calculated and plotted. (C-D) Niclosamide inhibits Wnt3A-

stimulated cytosolic β -catenin stabilization in U2OS cells as demonstrated by immunoblotting of cytosolic β -catenin. β -actin serves as a loading control. (C) shows the effect of 7.5 μ M niclosamide treatment, whereas (D) demonstrates that this inhibitory effect occurs over a range of 1–7.5 μ M niclosamide (n=3).

Table I
Summary of Frizzled1-GFP Internalization Screening

The internalization of Frizzled1 was scored visually. The names and molecular weights of the chemical compound hits were listed.

Internalization Score (+ to +++, low to high) (Number of hits)	Compound Hit Name (Molecular Weight, dalton)
+++ (4 hits)	Niclosamide (327.12571); Quinacrine dihydrochloride dehydrate (508.92067); Lasalocid sodium salt (612.78651); Tetrandrine (622.76824)
++ (3 hits)	Perhexiline maleate (393.57158); Fendiline hydrochloride (351.92337); Amiodarone hydrochloride (681.78455);
+	(19 hits) Triflupromazine hydrochloride (388.8857); Cyproheptadine hydrochloride (323.86919); Alverine citrate salt (473.57135); Chlorpromazine hydrochloride (355.33235); Perphenazine (403.97787); Dicyclomine hydrochloride (345.95727); Clomipramine hydrochloride (351.32253); Amodiaquin dihydrochloride dehydrate (464.82346); Metixene hydrochloride (363.95332); Hycanthone (356.49048); Acetopromazine maleate salt (442.53807); Clomiphen citrate (Z,E) (598.09862); Bepidil hydrochloride (403.01235); Flupentixol dihydrochloride cis-(Z) (507.44964); Prenylamine lactate (417.59665); Nitrazine dihydrochloride (380.36429); Monensin sodium salt (692.87077); Zuclopenthixol hydrochloride (437.43532); Thiethylperazine malate (667.80337)