Wnt inhibitor screen reveals iron dependence of β-catenin signalling in cancers

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ABSTRACT

Excessive signalling from the Wnt pathway is associated with numerous human cancers. Using a high throughput screen designed to detect inhibitors of Wnt/β-catenin signalling, we identified a series of acyl hydrazones that act downstream of the β-catenin destruction complex to inhibit both Wnt-induced and cancer-associated constitutive Wnt signalling via destabilization of β-catenin. We found that these acyl hydrazones bind iron in vitro and in intact cells and that chelating activity is required to abrogate Wnt signalling and block the growth of colorectal cancer cell lines with constitutive Wnt signalling. Additionally, we found that multiple iron chelators, desferrioxamine, deferasirox and ciclopirox olamine (CPX) similarly blocked Wnt signalling and cell growth. Moreover, in AML patients administered CPX, we observed decreased expression of the Wnt target gene AXIN2 in leukemic cells. The novel class of acyl hydrazones would thus be prime candidates for further development as chemotherapeutic agents. Taken together, our results reveal a critical requirement for iron in Wnt signalling and they demonstrate that iron chelation serves as an effective mechanism to inhibit Wnt signalling in humans.
INTRODUCTION

Inappropriate activation of the canonical Wnt/β-catenin signalling pathway contributes to the development of a numerous human cancers (1-3). In colorectal cancers, loss-of-function mutations in the tumor suppressor, Adenomatous Polyposis Coli (APC) are common. APC is an integral part of the destruction complex that controls cytoplasmic β-catenin levels by promoting ubiquitin-mediated degradation of β-catenin. Cancer-associated mutations in APC, thus lead to constitutively high levels of β-catenin and the concomitant expression of Wnt/β-catenin target genes that are important in cell growth, survival and metastasis (1-3). In other cancers, constitutive Wnt signalling is achieved by a variety of means, such as increases in Wnt ligand or decreases in secreted inhibitors (2). Deregulation of the Wnt/β-catenin pathway has been reported in acute myeloid leukemias (AML) where expression of β-catenin is correlated with poor prognosis (4, 5). In addition, Wnt/β-catenin signalling has been shown to be required for the development of the highly-proliferative leukemia stem cells (LSC), that are thought to maintain leukemias (6). The widespread deregulation of the Wnt pathway in diverse cancers makes it an attractive therapeutic target (7, 8) and while several chemical inhibitors of the Wnt pathway have been identified (7-11), there remains the need for effective small molecule inhibitors appropriate for therapeutic development.

Cancer cells have an increased demand for iron to maintain robust cell proliferation, yet use of iron chelators for cancer treatment has only recently been rigorously considered (12-15). Here, we used a mammalian cell-based screen and identified a class of acyl hydrazones that block constitutive Wnt signalling and cell growth through their activity as iron chelators and thereby
suggest that iron chelation-based therapies may be an effective means to target Wnt signalling in cancers.
MATERIALS AND METHODS

Compounds.

Compound 21H7 was purchased from Ryan Scientific, USA, Indirubin-3’-monoxime (I3M), Desferioxamine (DFO) and Dimethyloxallyl Glycine (DMOG) from Sigma-Adrich, Canada, Deferasirox (DFX) and ciclopirox olamine (CPX) from ChemPacific Corporation, USA, M-110 (16), kindly provided by Jan Jongstra (TWRI, Toronto) was synthesized by Sundia MediTech Company (Shanghai, China). OICR142 and OICR623 were synthesized by the OICR Medicinal Chemistry Group.

β-catenin-Firefly Luciferase stabilization assay and high throughput screen.

HEK293 cells, stably expressing a Flag-β-catenin-Firefly luciferase fusion protein were plated into 96-well dishes at 1250 cell/well. The next day, cells were incubated 1 h in MEM with 0.2% FCS, 1 h with 1.2 μM compounds and then Wnt3A or control conditioned media, prepared as described (17, 18) was added for 16 h. Luciferase activity was measured using Bright-Glo (Promega, USA) 10 min after addition, using the CLIPR plate reader (Molecular Devices, USA). Screens were conducted at the SMART Robotics Facility at the Samuel Lunenfeld Research Institute. Hits were identified by B-score analysis. For validation assays, Flag-β-catenin-FFluciferase was immunoprecipitated from cell lysates with anti-Flag antibody and collected using Protein G beads.
Transcriptional reporter, EMSA and cell growth assays.

Cell lines, from ATCC, were expanded, frozen and thawed aliquots cultured for less than 6 months. Cells were routinely tested for mycoplasma and Wnt pathway status was monitored using TOPFLASH/FOPFLASH. Cells were transfected with CMV-βgal and either 5xHRE-luciferase (provided by Dr. Michael Ohh, Toronto, Canada), TOPFLASH or FOPFLASH using calcium-phosphate (HEK293T) or Lipofectamine 2000 (SW480, DLD-1, SW620, HCT116). HEK293T cells were incubated with compounds for 1 h prior to overnight incubation with control or Wnt3A-conditioned medium. For all other cells, compounds were added 5 h post-transfection and cells were incubated for 24 h in full serum containing media. Luciferase activity, normalized to β-galactosidase activity was measured as previously (17, 18). All results were statistically significant (p < .005) using the Student’s two-tailed t-test at 5 and 10 μM of compound. IRE-binding activity of IRP was analyzed by gel shift assay as described (19). Growth inhibition was determined at 72 hours using the Sulforhodamine B (SRB) assay as published (20).

GST-E-cadherin pull down assays and immunoblotting.

For immunoblotting, cells were lysed and total protein content was measured by the Bradford assay (Bio-Rad, USA). The following antibodies were used: anti-Flag M2, rabbit anti-Ferritin and anti-actin (Sigma-Aldrich, Canada), anti-β-catenin and anti-HIF1α (BD Biosciences, Canada), anti-active β-catenin, (Millipore, Canada), and mouse anti-Transferrin Receptor (Invitrogen, Canada). For GST-pull downs, 3 μg of purified bacterially-produced GST or GST-E-cadherin (cytoplasmic tail domain) protein (21), bound to glutathione beads, was incubated
with whole cell lysates for 1 hr at 4°C to collect the free cytoplasmic pool of β-catenin. Beads were washed three times and analyzed by immunoblotting.

**Measurement of intracellular calcein-chelatable iron.**

Cells were loaded with 100 μM ferric ammonium citrate (FAC) for 24 h (22), washed twice with PBS containing 20 mM HEPES, pH 7.3, loaded with 0.25 μM calcein green acetoxyethyl ester (calcein-AM; Molecular Probes) in serum-free medium containing 20 mM HEPES, pH 7.3 for 15 min at 37°C, and then were washed and plated at 50000 cells/well in a 96-well plate (23). Intracellular fluorescence intensity of calcein (\(\lambda_{ex}=485\) and \(\lambda_{em}=520\)), a measure of the amount of labile iron (24), was determined as a function of time 1 min before and 10 min after compound addition at 37°C using FLUOstar OPTIMA (BMG Labtech) microplate reader.

**Microarray analysis and Real-Time PCR**

SW480 cells were incubated with 10 μM OICR623, 100 μM DFO or 50 μM DFX or DMSO as control for 6 h. RNA was isolated using the PureLink™ Mini Kit (Invitrogen, Canada) and cDNA samples were hybridized to the GeneChip® Human Gene 1.0 ST array (Affymetrix) and then scanned with the Affymetrix GeneChip Scanner 3000 at the Center for Applied Genomics, Toronto, Canada. Raw data was pre-normalized using RMA (robust multi-array average) algorithm, adjusted for batch effects and differentially expressed genes were identified using LIMMA (linear models for microarray data) (25). Data is available at GEO (GSE32369). Real-Time PCR was performed using SYBR Green (Applied Biosystems, USA) using validated primers (Supplementary Table 1). Gene expression was normalized to HPRT and relative
quantitation was calculated using the ΔΔCt method. Results shown are statistically significant (p < .005) using the Student’s two-tailed t-test.

Clinical trial of oral ciclopirox olamine in patients with refractory or relapsed hematologic malignancies.

Patients were treated with ciclopirox olamine at increasing concentrations from 5-80 mg/m² daily for 5 days, in a phase I clinical trial, following informed consent and REB approval by the Princess Margaret Hospital, Toronto, Canada (NCT00990587). Peripheral blood samples were obtained before, during and after drug treatment. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical) density-gradient centrifugation and AML blasts isolated by magnetic bead separation.
RESULTS

Identification of small molecule inhibitors of Wnt signalling

The deleterious effects of constitutive Wnt signalling result from abnormally high levels of β-catenin protein (1). Thus, to identify small molecule inhibitors that reverse β-catenin accumulation, we designed a homogeneous high throughput screening (HTS) assay using HEK293 cells stably expressing a fusion protein comprised of Firefly luciferase (FFluc) linked to β-catenin in which fusion protein levels were measured using a luciferase assay (Fig. 1A). Cells were treated with compounds for 1 h and then cultured with or without Wnt3A for 16 h prior to determining β-catenin-FFluc levels (Fig. 1B). Screening of 10,400 compounds from the Maybridge Collection (Figs. 1C and D) yielded 10 positive hits that were subsequently screened in a secondary assay for inhibition of the Wnt TOPFLASH reporter. One compound, 21H7, was identified as an inhibitor of the Wnt pathway that abrogated Wnt3A-dependent stabilization of β-catenin-FFluc and activation of TOPFLASH, but had no effect on FOPFLASH, a Wnt-insensitive mutant variant (Fig. 1E and F).

M-110, a structural variant of 21H7, also inhibited stabilization of β-catenin-FFluc and Wnt/β-catenin dependent activation of TOPFLASH with no effect on FOPFLASH (Figs. 1G-I) as did compound OICR623, which contains the more polar pyrimidine group and a 2-fluoro-4 chloro phenolic moiety (Fig. 1G and J). In contrast, OICR142, which lacks the ortho hydroxy group rendered the compound inactive (Fig. 1G and J). Thus, our screen identified a class of acyl hydrazones that inhibit Wnt-induced transcriptional responses.
Inhibition of Wnt/β-catenin signalling and cell proliferation in colorectal cancer cells.

Most colorectal cancer cell lines display constitutive Wnt signalling due to mutations in APC. M-110 treatment of APC-mutant DLD-1 cells (26), preferentially attenuated constitutive TOPFLASH activity as compared to FOPFLASH, and had no effect on the CMV-β–galactosidase control reporter (Fig. 2A). Real-Time PCR analysis also revealed that M-110 inhibited expression of the endogenous Wnt targets, AXIN2 and SP5 (Fig. 2B). In other APC mutant colorectal cancer cell lines, including SW480, SW620 and Colo205, M-110 similarly reduced TOPFLASH activity (Supplementary Fig. 1A) and expression of Wnt target genes (Fig. 2C). OICR623 and 21H7 also inhibited Wnt activity, whereas compound OICR142, which lacks the ortho hydroxy group did not (Fig. 2D; see also Fig. 5). The Wnt/β-catenin pathway regulates cell proliferation in colorectal cancer cells. Consistent with this, M-110, OICR623 and 21H7 inhibited the growth of DLD-1 and SW480 cells, with IC₅₀ś in the range of 0.5-1.7 μM (Fig. 2E and Supplementary Fig. 1B). Thus, this family of small molecules inhibits constitutive Wnt signalling and blocks the growth of colorectal cancer cell lines.

Acyl Hydrazones block Wnt signalling by promoting β-catenin degradation downstream of the destruction complex.

There are two cellular pools of β-catenin, plasma membrane E-cadherin-associated and Wnt-modulated cytoplasmic pools (27). In mouse L-cells, where most of the β-catenin is cytoplasmic, M-110, OICR623 and 21H7 blocked Wnt-induced stabilization of total β-catenin and the active, non-phosphorylated (S37/T41) β-catenin, which mediates Wnt signalling (Fig. 3A). In SW480 cells, M-110 also decreased the levels of free cytoplasmic β-catenin as determined by E-cadherin pulldown assays, which collects β-catenin that is not pre-bound to E-cadherin, and reduced the
pool of the active, non-phosphorylated \( \beta \)-catenin (Fig. 3B). Total \( \beta \)-catenin levels, which include the E-cadherin-bound pool were not significantly affected (Fig. 3B), nor were \( \beta \)-catenin mRNA levels (Supplementary Fig. 1C). Degradation of \( \beta \)-catenin occurs in a destruction complex comprised of Axin and APC, within which CK1 and GSK3\( \beta \)-mediated phosphorylation of \( \beta \)-catenin marks it for ubiquitin-mediated degradation (1). DLD-1 and SW480 cells have a mutant APC, suggesting that the compounds act downstream of the destruction complex. Consistent with this, M-110 also blocked Wnt signalling when other destruction complex components were disrupted, including abrogation of AXIN1/2 expression using siRNAs or inhibition of GSK3\( \beta \) activity using LiCl or Indirubin-3’-monoxime (I3M) (Fig. 3C and D). The ability of M-110 to decrease the levels of active \( \beta \)-catenin was mitigated in the presence of MG132, a proteasome inhibitor, but not by chloroquine, a lysosomal inhibitor (Fig. 3E), indicating ubiquitin-mediated degradation of \( \beta \)-catenin. M-110, OICR623 and 21H7 also inhibited activation of the TOPFLASH reporter in HCT116 cells, which harbor a constitutively-active version of \( \beta \)-catenin and in HEK293T cells over-expressing versions of \( \beta \)catenin lacking the phosphorylation sites (Fig. 3F and G; Supplementary Fig. 1D). Altogether, these data demonstrate that the compounds act downstream of the destruction complex to destabilize active \( \beta \)-catenin.

**Gene expression profiling reveals compounds induce an iron chelation signature.**

To gain insights into molecular mechanisms, we used microarrays to examine changes in gene expression in SW480 cells treated for 6 h with OICR623. Analysis of results from replicate runs revealed that OICR623 significantly (\( p = <0.05 \)) upregulated the expression of 31 genes by 1.5 fold or greater (Fig. 4A and B) including several genes induced by iron chelators (28), such as DDIT4, VEGFA and NDRG1. This gene profile was next compared with that obtained using two
well-described iron chelators, Desferioxamine (DFO) and Deferasirox (DFX) in cells treated in parallel. At 6 hours of treatment, 64 and 72 genes were upregulated by greater than 1.5 fold (p = <0.05) by DFO and DFX, respectively, of which 64 overlapped (Fig. 4A). Remarkably, all 31 genes upregulated by OICR623 were found within the DFO/DFX signature. Similar results were observed in the case of significantly (1.5 fold, p = <0.05) downregulated genes, although this overlap set was comprised of only four genes (Fig. 4B). Of note, at this early time point, inhibition of Wnt target gene expression was not yet manifested. These results thus show that the OICR623-induced gene signature is entirely encompassed by that of the iron chelators, DFO and DFX.

Iron depletion inhibits the activity of iron-dependent enzymes, including prolyl hydroxylases (PHD), which promote stabilization of the hypoxia inducible transcription factor, HIF1α (29, 30). Treatment of SW480 and DLD-1 cells with OICR623, M-110 and 21H7, stabilized HIF1α and activated a HIF1α transcriptional reporter, 5xHRE-luciferase (Fig. 4C and D). Furthermore, genes induced by OICR623, DFO and DFX in the microarray analysis included the known HIF1α targets (29), VEGFA, ADM, EGLN3, and NDRG1 (Fig. 4A). Verification of microarray results by Real-Time PCR demonstrated that all three acyl hydrazones, OICR623, M-110 and 21H7, as well as the iron chelators, DFO and DFX, rapidly induced expression of the iron-dependent and/or HIF1α target genes, NDRG1, DDIT4 VEGFA and GLUT1 in SW480 cells, used for the microarray study and in DLD-1 and SW620 cells (Fig. 4E and Supplementary Fig. 2A and B).
Under conditions of iron depletion, Iron Regulatory Proteins (IRPs) recognize and bind to Iron Responsive Elements (IRE) in mRNAs such as Ferritin (Ft), to stall translation or to Transferrin Receptor I (TfRI) mRNA to enhance mRNA stability and translation (14). As expected for iron chelators, treatment of SW480 cells with M-110 or DFX resulted in enhanced IRE-IRP binding activity, in an RNA-binding gel shift assay (Fig. 4F). A concomitant reduction in Ferritin and increase in TfRI was observed by immunoblotting for M-110 (Fig. 4F) and OICR623, 21H7 and DFX (Supplementary Fig. 3A). Thus, given the gene expression profiles and characteristic cell-based responses of iron depletion, our data strongly suggest that OICR623 and related compounds act as iron chelators.

Compounds bind iron in vitro and in cultured cells and this activity mediates inhibition of Wnt signalling and cell growth.

The acyl hydrazone group is a characteristic metal chelating motif, thus, to test whether OICR623 can bind iron in vitro, we mixed Fe (NO₃)₃·9H₂O with OICR623 and analyzed the mixture by liquid chromatography/mass spectroscopy. An iron complex of OICR623 in a 1:2 ratio (iron : OICR623) with a molecular ion mass [M+1] of 698 was observed. Based on the reported crystal and molecular structure of Compound 311 and its iron (III) complex (31), we speculate that OICR623 is a tridentate chelator (Fig. 5A).

We next assessed the ability of the compounds to bind intracellular iron in colorectal cell lines by calcein assay. Upon cell entry, calcein-AM is cleaved into calcein, whose fluorescence is quenched upon chelation of labile iron (23). Calcein-AM loaded SW480 cells were briefly incubated with M-110, OICR623 and 21H7. Similar to the iron chelator, DFX, both M-110 and
OICR623 increased calcein fluorescence (Fig. 5B) while 21H7, yielded a more modest increase that was similar to that observed for DFO, an iron chelator with poor cell-permeability properties (32). We next tested the effect of excess iron on compound activity in cells. Analysis of TOPFLASH activity in SW480 cells treated with compounds (5 μM) co-incubated with a 2-fold molar excess (10 μM) of various divalent cations revealed that addition of FeSO₄ or FAC completely neutralized compound activity, while Mg²⁺, Mn²⁺ or Zn²⁺ salts had no effect (Fig. 5C). Moreover, in cell growth assays, addition of FeSO₄ at an equimolar ratio of iron to compound completely abrogated the inhibitory activity of the compounds in SW480 and DLD-1 cells (Supplementary Figure 3B). Treatment of DLD-1 or SW480 cells with the structurally unrelated iron chelators, DFO or DFX, similarly inhibited expression of Wnt target genes (Fig. 5D) and inhibited cell growth with IC₅₀s of 2.9-3.0 μM (Fig. 5E), roughly 5-10 fold higher than the acyl hydrazones, but similar to previously reported IC₅₀ values for these compounds (33) (Fig. 5D and E). Stabilization of HIF1α and activation of the HIF1α reporter, 5xHRE-luciferase by Dimethyloxallyl Glycine (DMOG), an inhibitor of 2-oxoglutarate dependent enzymes including PHDs, to levels (0.1 mM) comparable as that achieved by acyl hydrazones or DFX, had no effect on TOPFLASH activity, though at 10-fold higher DMOG doses, some inhibition was observed suggesting that HIF1α is unlikely to be the primary mechanism whereby iron chelators inhibit Wnt signalling (Supplementary Fig. 3C).

Altogether, our results demonstrate that acyl hydrazones are potent iron chelators in cells and that depletion of intracellular iron abrogates cell proliferation and Wnt signalling by promoting β-catenin degradation downstream of the destruction complex.
Oral administration of the iron chelator, CPX, reduces Wnt target gene expression in the leukemic cells of AML patients

Ciclopirox olamine (CPX) functions as an anti-cancer agent in both leukemic cell lines and primary Acute Myeloid Leukemia (AML) patient samples via its intracellular iron chelation activity (34). We confirmed in SW480 cells that CPX also binds intracellular iron (Fig. 5B), induces the HIF1α responsive reporter and inhibits TOPFLASH in an iron-dependent manner (Fig. 6A and B).

Mutations in Wnt pathway components have not been reported in AML, but expression of β-catenin is correlated with poor prognosis (5). Thus, to determine if iron chelators modulate Wnt signalling in vivo, we assessed the effect of systemic administration of CPX to patients with refractory hematologic malignancies. Expression of AXIN2 in AML patients taking orally administered CPX as part of an on-going Phase I dose escalation trial was determined in isolated leukemic blasts. Analysis of samples from 9 patients with relapsed AML receiving CPX doses between 5-80 mg/m² daily for 5 days revealed that 7 of 9 patients displayed a decrease in AXIN2 levels. For 4 patients (A-D), marked decreases were detected within 1 day of CPX administration (Day 2) that became more pronounced from Day 3-5 (Fig. 6C and D). In 3 patients (E-G), decreases in AXIN2 levels were transient, while in 2 patients (H and I) there was no reduction (Fig. 6C and D). Excluding the 2 patients (H and I) that showed no changes, the average reduction in AXIN2 levels from Day 2-5 ranged from 60 to 74% (median) or 40 to 71 % (mean; Fig. 6D). Of note, hematopoietic cells isolated from two CPX-administered patients (J-K) with myeloma or MDS, but lacking circulating malignant cells showed no reduction in AXIN2 (Fig. 6E), suggesting that leukemic cells may be more susceptible to iron chelation than non-
malignant hematopoietic cells. Although only a small, short-term study, these results show that administration of iron chelators can decrease the expression of Wnt target genes in patients with hematologic malignancies and further indicate that iron chelators might be of therapeutic value for Wnt-pathway driven tumors.
DISCUSSION

Stabilization of β-catenin is an invariant feature of cancers with excessive Wnt signalling (1, 2). Our screen, designed to identify small molecules that destabilize β-catenin, identified a compound series that inhibits β-catenin stabilization and blocks Wnt-induced transcription. The compounds were shown to function downstream of the β-catenin destruction complex and were effective in blocking Wnt signalling and growth in APC and β-catenin mutant colorectal cancer cells with constitutive Wnt signalling. This is consistent with studies showing that down regulation of β-catenin inhibits proliferation of colon cancer cell lines grown in vitro or as xenografts (35, 36). Given the frequent occurrence of excessive Wnt signalling through a range of mechanisms, these inhibitors which act downstream of the destruction complex to control β-catenin levels could have widespread therapeutic utility in a diverse range of Wnt-driven cancers.

Investigation of the mechanism whereby the acyl hydrazones block the Wnt pathway showed that the compounds bind iron and that this iron chelation activity is essential for compound activity. Iron loading of colorectal cancer cells has been reported to promote Wnt signalling and cell proliferation (37) consistent with our observations that iron depletion can attenuate the pathway. Our conclusions were further supported by the observation that a series of structurally unrelated iron chelators, including DFO, DFX and CPX also inhibit Wnt signalling and that AML patients taking CPX, show a marked reduction in the expression of the Wnt target gene, AXIN2. Our work along with a recent study (38) demonstrate that iron depletion attenuates Wnt signalling, but which of the plethora of iron-dependent proteins, are most relevant for inhibiting Wnt signalling remains to be determined.
Iron is essential for cell growth and metabolism and cancer cells in particular, have an increased demand for iron to maintain cell proliferation having acquired diverse alterations to ensure increased iron accumulation (12-15). In breast cancer, decreases of the intracellular iron exporter, ferroportin, is associated with reduced metastasis-free survival, while ferroportin overexpression decreases breast cancer cell growth in an orthotopic mouse model (39). Expression levels of ferroportin are correlated with levels of intracellular iron, indicating that altering iron levels can modulate tumor growth in vivo. It is thus tempting to speculate that an increase in iron accumulation promotes widespread increases in the pro-proliferative Wnt signalling pathway to promote tumorigenesis. Testing the utility of iron chelators, such as the acyl hydrazones identified herein, for cancer therapeutics is an important area for future investigation.
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REFERENCES


FIGURE LEGENDS

Figure 1. Identification of a Wnt/β-catenin inhibitor.

(A, B) Wnt/β-catenin pathway screen. The levels of a Flag-β-catenin/FFluc fusion protein stably expressed in HEK293 cells was measured by luciferase assay. (C) Results from duplicate 96-well plates in the presence and absence of added Wnt3A. B-score analyses identified compound 21H7. (D and G) Structures of the screen hit, 21H7 (D) and structural variants, M-110, OICR623 and OICR142 (G). (E and H) Inhibition of β-catenin stabilization. HEK293T cells stably expressing Flag-β-catenin/FFluc were treated overnight with 5 μM 21H7 (E), 5 μM M-110 (H) or DMSO (-) with or without Wnt3A. Following anti-Flag immunoprecipitation, β-catenin-FFluc levels were measured by luciferase assay. (F, I and J) Inhibition of Wnt3A-dependent transcription. HEK293T cells were transfected with TOPFLASH or FOPFLASH and cultured overnight with or without Wnt3A and the indicated compounds. Promoter activity was measured by luciferase assay and is shown as the mean of three replicates +/- standard deviation.

Figure 2. Compounds inhibit constitutive Wnt activity and growth in colon cancer cells.

(A) Inhibition of TOPFLASH by M-110 in DLD-1 cells. Cells were transfected with TOPFLASH or FOPFLASH and treated with the indicated concentration of M-110. Promoter activity was measured by luciferase assay normalized to β-galactosidase activity (bars). β-galactosidase activities alone are plotted as lines. Data is shown as the mean of three replicates +/- standard deviation. (B and C) M-110 decreases expression of Wnt target genes. Cells were treated with M-110 overnight and the expression of Wnt target genes was measured by Real-Time PCR. Relative gene expression is plotted as the average of three PCR replicates +/- the
range in DLD-1 cells (B) or as fold change represented by colours (C). (D) Inhibition of TOPFLASH by M-110, OICR623, 21H7 and the inactive variant, OICR142 was determined in SW480 and DLD-1 cells as in panel A. (E) IC$_{50}$ values for growth inhibition.

Figure 3. Compounds block Wnt signalling downstream of the destruction complex.

(A) Compounds inhibit Wnt3A-induced stabilization of β-catenin in mouse L-cells. Total lysates from cells incubated overnight with DMSO (-) or compounds and then stimulated with Wnt3A for 3 hours were subjected to immunoblotting (IB) to detect total β-catenin, active β-catenin or actin. (B) M-110 decreases the level of active β-catenin in colorectal cancer cells. SW480 cells were incubated overnight with 10 μM M-110 and the levels of cytoplasmic (free) β-catenin was determined by GST-E-cadherin (E-Cad) pulldown followed by anti-β-catenin IB. Levels of total and active β-catenin in total cell lysates is shown (input). Full-length blots of these cropped images are presented in Supplementary Fig. 4A and B. (C-D) Inhibition of TOPFLASH downstream of destruction complex components by M-110. HEK293T cells were transfected with TOPFLASH (C-D), siControl (siCtl) or siAXIN1/2 (C) and treated with M-110 with or without 25 mM LiCl or Indirubin-3’-monoxime (I3M; D). (E) The proteasome inhibitor, MG132 but not chloroquine blocks the M-110-mediated decrease in the level of active β-catenin in SW480 cells as determined by E-cadherin pull-down assays and immunoblotting as in panel B. (F-G) Compounds inhibit TOPFLASH activity in β-catenin mutant, HCT116 cells and in HEK293T cells over-expressing murine wild type (WT) or Ser33/Ser37/Thr41/Ser45 to Ala mutant (mut) β-catenin-6xmyc.
Figure 4. Microarray analysis demonstrates that compounds display an iron chelation gene signature.

(A) Clustering of gene expression profiles of individual runs for all common genes up- or down-regulated (>1.5 fold, p < 0.05) by OICR623, DFO and DFX are depicted as a heat map matrix. Reported (28, 29) iron-regulated (*) and HIF1α target genes (°) are bolded. (B) A Venn diagram indicating the number of genes upregulated by OICR623, DFO or DFX by >1.5 fold (p < 0.05) is shown. (C) SW480 or DLD-1 cells were incubated overnight with 10 μM M-110 and HIF1α protein levels were determined by immunoblotting of total cell lysates. Full-length blots of these cropped images are presented in Supplementary Figure 4C. (D) Cells transfected with the 5xHRE-luciferase reporter were treated overnight with M-110 and luciferase activity determined. (E) Compounds induce expression of iron-responsive target genes. Cells were treated overnight with 5 μM M-110, OICR623, 21H7, 50 μM DFX or 100 μM DFO and relative expression of target genes, measured by Real-Time PCR is plotted as the average of three PCR replicates +/- the range. (F) SW480 cells were incubated with 10 μM M-110, 50 μM DFX or 100 μM FAC and aliquots of cell lysates were split and analyzed by RNA gel shift assays using an [32P]-human Ferritin H chain IRE probe (EMSA) and by immunoblotting to detect Ferritin and TfRI proteins.

Figure 5. Iron chelators block Wnt signalling and colorectal cancer cell growth.

(A) A modelled structure of the iron-OICR623 complex. (B) Compounds bind intracellular iron. SW480 cells were loaded with calcein-AM and the fluorescence intensity change, after and before the addition of 5 μM M-110, OICR623, 21H7, CPX, 100 μM DFO or 50 μM DFX is plotted. (C) Excess iron blocks compound-mediated inhibition of constitutive Wnt signalling.
SW480 cells were transfected with TOPFLASH and treated with 5 μM M-110, OICR623 or 21H7 in the absence or presence of 10 μM of the indicated salts. Promoter activity is shown as the mean of three replicates +/- standard deviation. (D) Iron chelators decrease the expression of Wnt target genes. SW480 and DLD-1 cells were treated with 5 μM M-110, OICR623, 21H7, 100 μM DFO or 100 μM DFX overnight and relative gene expression is plotted as the average of three PCR replicates +/- the range. (E) IC₅₀ values for growth inhibition.

Figure 6. CPX inhibits Wnt signalling in cultured cells and in vivo.

(A) CPX inhibits Wnt signalling and activates a HIF1α-dependent reporter. SW480 cells transfected with TOPFLASH or 5xHRE-luciferase reporters were treated overnight with CPX or M-110. (B) Excess iron blocks CPX-mediated inhibition of constitutive Wnt signalling in SW480 cells. Cells were transfected with TOPFLASH and treated with 5 μM CPX in the absence or presence of 10 μM of the indicated salts. (C-E) CPX administration decreases AXIN2 expression in leukemic blasts isolated from AML patients. The expression of AXIN2 on each day of treatment for individual patients was measured by Real-Time PCR and is calculated relative to levels prior to CPX administration. Data for individual patients are plotted as a bar graph (C, E) or scatter plot (D). The horizontal lines indicate the median (dashed line) or mean (solid line) for patients A-G (filled symbols). Results for patients H and I are indicated by open symbols. Probabilities were determined using Student’s paired, one-tailed t-test: ***, p = .008, **, p = .05, *, p = .02. (E) AXIN2 expression in two patients lacking circulating malignant cells.
Figure 1
Figure 2

A. TOPFLASH and FOPFLASH activity assays show the relative luciferase activity in response to M-110 concentrations.

B. Real-Time PCR analysis for Axin2 and Sp5 expression levels after various M-110 concentrations.

C. Heatmaps for different cell lines (SW480, SW620, Colo205) showing the fold change in expression for Axin2, Sp5, Nkd1, and TNFRSF19 after M-110 concentrations.

D. BAR charts for SW480 and DLD-1 cells showing relative luciferase activity for M-110, OICR623, and 21H7 treatments.

E. IC50 values for M-110, OICR623, and 21H7 in SW480 and DLD-1 cell lines.
Figure 3
Figure 4
Figure 5
Wnt inhibitor screen reveals iron dependence of β-catenin signaling in cancers

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