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Wnt inhibition correlates with human embryonic stem cell cardiomyogenesis: A Structure Activity Relationship study based on inhibitors for the Wnt response

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Abstract

Human embryonic stem cell-based high content screening of 550 known signal transduction modulators showed that one “lead” (**1**, a recently described inhibitor of the proteolytic degradation of Axin) stimulated cardiomyogenesis. Because Axin controls canonical Wnt signaling, we conducted an investigation to determine whether the cardiogenic activity of **1** is Wnt dependent, and developed a structure activity relationship to optimize the cardiogenic properties of **1**. We prepared analogs with a range of potencies (low nanomolar to inactive) for Wnt/ β -catenin inhibition and for cardiogenic induction. Both functional activities correlated positively ($r^2 = 0.72$). The optimal compounds induced cardiogenesis 1.5-fold greater than **1** at 30-fold lower concentrations. In contrast, no correlation was observed for cardiogenesis and modulation of TGF β /SMAD signaling, that prominently influences cardiogenesis. Taken together, these data show that Wnt signaling inhibition is essential for cardiogenic activity and that the pathway can be targeted for the design of drug-like cardiogenic molecules.

Keywords

Human Embryonic Stem Cell; Wnt inhibition; SAR; cardiomyogenesis; IWR-1; inhibitors

Introduction

The proposal to produce human heart muscle cells from stem cells has attracted much attention because of the potential for promising applications in medicine and drug discovery, ranging from cell transplantation to *in vitro* pharmacological testing. Use of human stem cell-derived cardiomyocytes as tools for drug discovery and development (i.e., high throughput assays, specific disease models, target identification and validation or toxicity assessment) has many advantages over current assays that rely on a non-cardiomyocyte setup^{1–3}. Cardiomyocytes, the key cells for cardiac safety evaluation at the preclinical stage,

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Supporting Information Available The contents of Supporting Information includes the following: (1) analytical and spectral characterization data for all compounds obtained from Schemes 2–4, (2) detailed assay procedures for the hESC assay, (3) cell viability/toxicity data for selected compounds, (4) data for TGF β inhibition for selected compounds, (5) chemical stability data for select compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

are hard to come by. Scientists are usually limited to animal-derived cells or tissues or human engineered cells (cell lines heterologously expressing human cardiac ion channels, cardiac cell cultures, isolated tissue preparations, and perfused animal hearts) that have limited predictivity in human. For example, overexpression of the hERG channel in fibroblasts is commonly used in drug development as a model to evaluate cardiotoxicity of novel drugs. One major advantage of cardiomyocytes derived from hES cells is that they are of human origin and can be maintained in culture for extended time periods without losing their characteristics. Theoretically, they represent an unlimited source for human cardiomyocytes for *in vitro* testing. Moreover, the information gained from developing small molecules for stem cell-derived cardiomyocyte differentiation *in vitro* may lead to the development of drugs capable of mobilizing endogenous cardiac progenitor cells to regenerate damaged muscle in the adult heart⁴.

Cell differentiation is a complex and still poorly understood process. As for other tissue types, the development of human myocardial cells requires close temporal control of inducing factors to stimulate the stepwise progression from pluripotent cells to uncommitted progenitor, to committed precursor and finally to myocardial cells including cardiomyocytes^{4,5}. Current approaches to stimulate stem cell differentiation have included naturally occurring factors, including the introduction of lentiviral vectors carrying transcription factors, the addition of growth factors and the use of small molecule signaling pathway modulators¹. The approach described herein focuses on the latter, because use of small molecules as differentiation reagents overcomes the inherent high cost of biological factors or reagents for *in vitro* applications and can be developed into drug candidates for *in vivo* applications for therapeutic development.

We recently described a human Embryonic Stem Cell (hESC)-based high content screen (HCS) of about 550 known pathway modulators (i.e., InhibitorSelect and StemSelect, both from EMD Chemicals Inc.) and used these compounds to identify key signaling pathway(s) that control differentiation of uncommitted cardiac progenitors to form cardiomyocytes⁶. Only one small molecule (i.e., **1** (IWR-1))^{7,8}, Fig. 1) was identified as a “lead” from this library screen. The abbreviation IWR stands for Inhibitor of the Wnt Response. Wnt is a hybrid name of Int, a gene active in mouse mammary tumors, and Wingless, a gene essential for wing development in *Drosophila*. The Wnt pathway has been studied in a wide range of organisms and is implicated in mammalian development and cancer. Compound **1** was recently reported as an inhibitor of the oncogenic canonical Wnt response at the Axin level^{7,8}. Besides being involved in cancer⁹, Wnt and Wnt inhibition regulate crucial processes during embryonic development¹⁰. Wnt signaling plays a multiphasic role in heart development and Wnt inhibition is critical to form committed progenitor cells^{11,12}. This has led to the use of the natural Wnt inhibitor Dickkopf-1 (DKK-1) to enhance cardiogenesis in hESC culture¹³ and the discovery of small molecule tool compounds².

Herein, we examined the effect of the previously reported Wnt inhibitors **1–5** on human ESC-mediated cardiomyocyte-differentiation in parallel with a step-wise process to improve the pharmacological properties of **1**. Compound **1** was optimized for its ability to inhibit the Wnt pathway and stimulate cardiogenesis. Newly designed compounds **10**, **29** and **34** showed decreased IC₅₀ values for inhibition of the Wnt pathway as well as increased cardiomyogenesis potency. Compared to **1**, compounds **10**, **29** and **34** showed improved functional activity and improved physiochemical properties, thus rendering them more attractive for *in vitro* and possibly, *in vivo* studies.

Chemical Synthesis

The synthesis of **1** and certain analogs was recently described in papers reporting new Wnt inhibitors for cancer^{7,8}. From a structural perspective and as previously described by Lu et al.,⁸ compound **1** can be divided into three regions (i.e., A, B, C, Fig. 1). The SAR of all three regions of **1** was explored.

Regions A and C were modified using synthetic protocols described by Chen et al.,⁷ (Schemes 1 and 2). Briefly, the desired amino acid **6** was heated with anhydride **7** in DMF and afforded intermediates **8a–e** in good yields. The carboxylic acid moiety of **8a–e** was then activated in the presence of thionyl chloride and treated with the appropriate amine (i.e., R₃NH₂) to give compounds **9–39**, **53–56** (Scheme 1). Compounds **41**, **42c–f**, **43g–k**, **44l–p** were prepared by treating the acid chloride of *para*-nitrobenzoic acid with the appropriate amine, R₃NH₂. After hydrogenation of the nitro group, intermediate **40** was subjected to a reductive amination step or acylation or treatment with an anhydride to give the desired products **41–44** (Scheme 2).

The SAR of Region B was explored using various synthetic approaches. Compound **45** (Table 1) was prepared following the same sequence of steps as in Scheme 1 but by replacing **6** by 3-aminobenzoic acid. Compound **46** (Table 1) was prepared following Scheme 2 by replacing 4-nitrobenzoic acid with 5-nitro-2-furoic acid. Compound **48**, was prepared in three steps from 4-nitrophthalic anhydride (Scheme 3). Condensation of 5-nitroisobenzofuran-1,3-dione with 8-aminoquinoline followed by reduction of the nitro group afforded **47**. Condensation of **47** with *cis-endo*-dihydrocarbic anhydride using conditions described in Scheme 1 afforded **48**. For the reduced version **50** (Table 1), intermediate **49** was obtained in two steps by reductive amination of 8-aminoquinoline with 4-nitrobenzaldehyde followed by reduction of the nitro group with sodium dithionite. Compound **49** was treated with *cis-endo*-dihydrocarbic anhydride under conditions described in Scheme 1 to afford compound **50** (Scheme 4). Finally, the reverse amide **52** (Table 1) was synthesized using intermediate **51**. Treatment of 4-nitroaniline with carbic anhydride followed by hydrogenation gave **51** and following addition of freshly prepared quinoline-8-carbonyl chloride, treatment with aniline provided the desired reverse amide (**52**, Scheme 5).

Results and Discussion

Because **1**, the lead compound from screening, was reported to inhibit canonical Wnt signaling at the Axin level⁷, we compared **1** to four mechanistically different Wnt inhibitors (**2** (XAV939), **3** (IWP-3), **4** (iCRT-5) and **5** (pyrvinium), Fig. 2A) in a Wnt inhibition assay (Fig. 2B) and a hESC cardiogenesis assay (Fig. 2C) to examine the relation between Wnt inhibition and cardiogenesis and to narrow down and identify the part of the pathway blocked in the signaling cascade that influenced cardiogenic activity. To ensure that the SAR analysis was not confounded by cell toxicity, the five Wnt inhibitors (**1–5**) and certain analogs of **1** were tested in parallel for cytotoxicity (see Supporting Information, Fig. S2). Compound **1** blocks the Wnt pathway by stabilizing the Axin protein complex via a direct interaction with Axin⁷. Compound **2** stabilizes Axin by inhibiting the poly-ADP-ribosylating enzymes tankyrase 1 and 2¹⁴. Compound **3** inhibits the production of Wnt by preventing the palmitoylation of Wnt proteins by Porcupine⁷. Compound **4** has been reported to inhibit the interaction between β -catenin and TCF4 within the cell nucleus¹⁵. Compound **5** activates CK1 α activation in the Wnt pathway and promotes degradation of β -catenin and inhibited Axin degradation¹⁶. In our hands, **5** was a very modest Wnt inhibitor (IC₅₀ = 592 nM) and also quite cytotoxic, so it was not tested in the cardiac assay. Compounds **3** and **4** were similarly modest Wnt inhibitors (i.e., IC₅₀ = 538 and 728 nM,

respectively), but were not toxic to HEK293T cells at concentrations up to 20 μ M (See Suppl. Info Fig. S2). Compound **2**, described to induce cardiac differentiation in mouse ESCs¹⁷, had an IC_{50} of 51 nM in the Wnt assay and did not induce cell toxicity. Compound **1** had a similar profile (i.e., IC_{50} = 26 nM and was not toxic to HEK293T cells). As we showed previously⁶, **1**, **2** and **3** induced cardiogenesis by ca. 1000-fold at their maximum efficacious concentrations (i.e., 4, 2 and 2.5 μ M respectively, Fig. 2C) over a DMSO control but differed in their potencies. Compound **2** was about 1.5-fold less potent than **1** and **3**. Compound **4** was weakly cardiogenic but only at a higher concentration (50 μ M). Compound **3** was not selected as a lead candidate because its mode-of-action in inhibiting Wnt production is reported to be at an upstream position compared to **1**. Taken together, these data suggested that **1** was the most appealing structure as a lead candidate. It was potent both as a Wnt inhibitor and as a cardiogenic agent in the hESC studies and was not cytotoxic to the cells examined.

Wnt Inhibition

Initially, a structurally diverse library of ca. 90 analogs of **1** was synthesized and tested for Wnt inhibition at a standard concentration (i.e., 1 μ M). Compounds that showed inhibition above 50% at 1 μ M were re-evaluated for dose-dependent inhibition. Next, more focused libraries were prepared to improve potency and physicochemical features of promising analogs.

Using synthetic strategies depicted in Schemes 1–5, variations on the central region (Region B) were prepared and evaluated for Wnt inhibition (Table 1). Lu et al., previously showed that the phenyl ring of **1** could be replaced by a *trans*-cyclohexyl moiety and still maintain Wnt inhibition potency⁸. As shown in Table 1, an increase in Wnt inhibition potency was observed with the completely saturated *trans*-cyclohexyl analog **10** (i.e., IC_{50} = 4 nM). Because a cyclohexyl ring can adopt both *cis* and *trans* configurations it was of interest to examine the stereoselectivity of Wnt inhibition. The *cis* analog **11** was prepared and found to be potent (i.e., IC_{50} = 57 nM). It is possible that the potency of the *cis*-cyclohexyl moiety can be explained by **11** adopting a boat conformation. Next, modifications of the amide function revealed important information about Wnt inhibition SAR. Reduction of the amide function (**50**, 45% inhibition at 1 μ M), changing the amide position to the *meta*-position (**45**, 0% inhibition at 1 μ M) or replacing the central aryl substituent by a furan group (**46**, 0% inhibition at 1 μ M) decreased the potency of the Wnt inhibition and showed that both the amide bond and the 1,4-substitution pattern for the central B region were essential for maximal inhibitory potency. A rigidified version (**48**) and a reverse-amide version (**52**) were also prepared. Reversing the amide as in **52** was tolerated but showed some loss of Wnt inhibitory potency (i.e., IC_{50} = 119 nM) while the rigid analog **48** was inactive (i.e., 15% inhibition at 1 μ M). Compounds **9** (i.e., central phenyl ring) and **10** (i.e., central *trans*-cyclohexyl ring) possessed similar IC_{50} values (i.e., 24 and 4 nM, respectively) that suggested that the central ring itself likely seems to be a simple placeholder and does not contribute to a discernible biological interaction.

A systematic study of the effect of the position of the nitrogen atom in the quinoline heterocycle on Wnt inhibition was done with compounds possessing a *trans*-cyclohexyl ring in the central position. The results summarized in Tables 2 and 3 showed that the 8-aminoquinoline isomer was the most potent quinoline (i.e., **10**, IC_{50} = 4 nM). Potency decreased when the distance between the quinoline nitrogen and the amide function increased suggesting either an intramolecular interaction between the amide hydrogen and the quinoline nitrogen or alternatively, a specific interaction with a biological target. Lu et al., showed that by replacing the quinoline with a phenyl ring substituted at various positions by halogens or a trifluoromethyl group usually led to a significant loss of potency⁸. The

optimal aryl substitution pattern was for a 4-bromophenyl and 2-methoxyphenyl (i.e., IC_{50} = 100 nM for both compared to 200 nM for **1**)⁸. Incorporation of electron donating groups (2-hydroxyl compound **19**, 2- and 4-methoxy compounds **20** and **21**, 4-dimethylamino compound **22**, with 33% inhibition at 1 μ M, 50% inhibition at 1 μ M, IC_{50} = 215 nM, 25% inhibition at 1 μ M, respectively) or electron withdrawing groups (4-cyano compound **23**, 4-acetyl compound **24**, with IC_{50} values of 317 nM and 41% inhibition at 1 μ M, respectively) didn't lead to a clear picture of the effect of electronic substituents on Wnt inhibition (Table 3). On the other end, the presence of a methoxy group at the *ortho*-position (compound **21** with an IC_{50} of 215 nM) or a *para*-methoxy substituent (**22** with 50% inhibition at 1 μ M) suggested that the electronic nature of the substituent at the aryl ring was not critical for Wnt inhibition. It is likely that shape rather than electronic properties and the position of the substituent affected potency. A carbonyl-containing functionality at the *ortho* position afforded the most potent analogs examined and apparently mimicked the role of the quinoline nitrogen to afford potent Wnt inhibition. A methyl ester at the 2-position (**27**) had an IC_{50} of 67 nM and the 2-methylketone (**28**) had an IC_{50} of 6 nM. The tetralone analog **29** was similarly potent (i.e., IC_{50} = 7 nM). When increased steric bulk was introduced using 9H-fluoren-9-one (i.e., **30**, IC_{50} = 194 nM), potency decreased suggesting steric hindrance in this region may have a negative effect on Wnt inhibition (Table 3).

A number of unsubstituted heterocycles (i.e., 2-pyrimidine, 2-pyrazine, 3-(1,2,4-triazole), 2-thiazole, 2-pyridine, 3-pyridine, 4-pyridine) were synthesized as replacements for the quinoline substituent. While the unsubstituted 2-, 3- and 4-pyridines (i.e., compounds **31–33**, Table 3) showed IC_{50} values in the 100 nM range (i.e., IC_{50} = 109, 120 and 80 nM, respectively), none of the other heterocycles described above were functionally active (i.e., % inhibition below 50% at 1 μ M, data not shown). Substituted pyridines were then prepared by combining the optimal substituents identified from the data of Table 3 with 3- and 4-pyridine derivatives. Compounds combining the 3-pyridine with an acetyl group at the 2- or 6-positions were prepared and tested. Remarkably, the 2-acetyl-3-pyridine analog **34** gave an IC_{50} value of 7 nM while the 6-acetyl-3-pyridine isomer **35** was significantly less potent (IC_{50} = 590 nM).

As reported (but not exemplified) by Lu et al.,⁸ Region A (Fig. 1) was very sensitive to chemical modifications. In our hands, any attempts to modify this region either by replacing the bicycle by bulky R groups (i.e., compounds **41**, **42c–f**, Scheme 2), or replacing the entire carbic moiety by various groups (i.e., compounds **43g–k** and **44l–p**, Scheme 2) led to a complete loss of potency (i.e., inhibition < 32% at 1 μ M). Only minor structural alterations such as saturation of the double bond (i.e., **9**, IC_{50} = 24 nM) were well-tolerated and two-carbon-bridge analogs (i.e., **36**, IC_{50} = 412 nM and **37**, IC_{50} = 314 nM, Table 4) and oxa-analog (i.e., **39**, IC_{50} = 605 nM) were >10-fold less potent. Adding a methyl group to the carbic moiety (i.e., **38**, IC_{50} = 1176 nM) also resulted in significant loss of potency highlighting the sensitivity of this region to substitution.

Chemical Stability

Certain selected compounds, as well as **1** for comparison purposes, were evaluated for chemical stability in buffer before being tested for their cardiogenic properties. hESC cardiogenesis studies were done in a cell-based assay at pH 7.4 and biological activity was presumably required for at least 24 hours after compound addition four days after initiating hESC differentiation⁶. Therefore, we determined the aqueous stability of key compounds at pH 7.4 for at least 24 hours. Representatives of the various subseries, (i.e., compounds **1**, **10**, **28**, **32** and **52**), were incubated at 10 μ M in phosphate buffer (pH 7.4) for up to 30 hours. No degradation was observed as determined by HPLC for any of the compounds except for **52** whose half-life was 3.8 hours (Supplemental Information, Table S1).

Induction of Cardiogenesis

From the 180 synthetic analogs tested in the Wnt inhibition assay, 26 were selected to examine cardiogenic induction in the presence of hESCs. This subset of analogs comprised a full range of inhibitory potency in the Wnt assay and contained the most structurally diverse scaffolds for evaluation of hESC cardiogenic activity. As shown in Figure 3, analogs from the following series were selected as representative members to investigate: a) variation of the *N*-position of the quinoline C region, including phenyl- versus pyridinyl-substituents and *ortho*-carbonyl groups, b) variation of the central B region linker including aromatic versus aliphatic and *cis*- versus *trans*-configured linkers, and c) variation in the norbornene A region moiety. Figure 3A shows a list of values for the potency of Wnt inhibition in HEK293T cells and induction of cardiogenesis in hESCs. Three classes of compounds stimulated hESC cardiogenesis. The first group of compounds (i.e., **32**, **21**, **35**, **33**, **31**, **39**, **37**, **23**, **52**, **12**) was functionally inactive as inducers of cardiogenesis and possessed uniformly large IC₅₀ values in the Wnt inhibition assay (> 200 nM). A subset of these compounds (i.e., **31**, **32** and **33**), however, had IC₅₀ values in the 100 nM range as Wnt inhibitors. Further studies need to be done to explain their biological properties. Compound **52** contained a “reverse amide” and was a relatively potent Wnt inhibitor (i.e., IC₅₀ = 119 nM) but inactive as a cardiogenic inducer. The chemical half-life of **52** has been estimated to be less than five hours in the presence of assay buffer (see Supporting Information, Table S1) and this could explain its low cardiogenesis functional activity. While lack of aqueous stability would not be expected to compromise functional activity in the short timeframe of the Wnt assay, the actual concentration of **52** in the hESC assay may have declined compared to the time necessary to induce cardiogenesis and thus could explain its lack of potency as a cardiogenic stimulant. The second class (i.e., **14**, **30**, **1**, **13**, **9**, **56**, **11**, **55**, **54**, **27**, **18**, **53**) contained compounds that elicited a dose-dependent induction of cardiogenesis functional activity, without a decrease in cardiogenesis at higher doses. These compounds were also potent Wnt inhibitors based on their IC₅₀ values in the Wnt assay (20–200 nM). Compound **30** exhibited a modest Wnt inhibition (i.e., IC₅₀ = 194 nM) and solid cardiac induction (104% cardiogenesis). We suspect that the high lipophilicity of the 9-fluorene substituent causes low aqueous solubility and extensive protein binding. This could possibly explain the observed differences in these two distinct assays. The third class of compounds (i.e., **29**, **34**, **10**, **28**) were potent Wnt inhibitors and potent stimulators of cardiogenesis, even at low concentrations (i.e., < 0.15 μM). They were distinguishable from the second group of compounds by their dose-response curves (Fig. 3C). They showed a very strong cardiogenesis at the lowest concentrations followed by a constant decrease of percent cardiogenesis at increasing compound concentration in the cell-based assay. This effect could be due to anti-cardiogenic, anti-proliferative, or toxic cellular effects caused by strong Wnt inhibition. Compared to other analogs, compound **52** was substantially less stable at pH 7.4 than other analogs (see Supporting Information, Table S1) and thus its potency in the hESC assay may be possibly underestimated. Compound **18** had a negative Z score at 625 nM and thus appeared as an outlier our correlation, but it was cardiogenic at higher concentrations and reached 24% maximum cardiogenesis at 10 μM (Fig 3A).

Based on the correlation analysis (Fig. 3D), hESC cardiomyocyte induction apparently strongly paralleled Wnt inhibition and that is apparent in SAR studies. Even minor variations in the norbornene part of the molecule (Region C) led to a loss of functional activity for both Wnt inhibition and hESC cardiogenesis. For example, the two carbon-bridge analog **37** and oxa-bridge analog **39** had IC₅₀ values of 314 and 605 nM, respectively, in the Wnt inhibition assay and stimulated cardiogenesis only to the extent of less than 5%. The more potent compounds **9** (one carbon bridge analog) and **10** (saturated version) had IC₅₀ values of 24 and 4 nM, respectively, in the Wnt assay and induced cardiogenesis of 75% and 145% respectively, compared to **1**. The effect of stereochemistry

of the central portion of the molecule (Region B) on the stereoselectivity of the biological endpoints was examined. The *cis*- and *trans*-configuration of the saturated cyclohexyl linker (Region B) had a similar effect on cardiogenesis and Wnt inhibition. For example, **28** (*trans*) and **56** (*cis*) had percent cardiogenesis of 78% and 72%, respectively (Fig. 3B), and were relatively potent at Wnt inhibition (i.e., IC₅₀ values of 6 and 23 nM respectively, Table 3). A number of alternate positions of the nitrogen in the quinoline heterocycle was tolerated for functional biological activity, but complete omission of the nitrogen atom led to a dramatic decrease in induction of cardiogenic potency (i.e., **12**, 41% Wnt inhibition at 1 μM and 8% cardiogenesis). Finally, different phenyl- and pyridinyl-substituted analogs gave comparable functional activity for induction of cardiogenesis and this showed that the quinoline could be replaced while retaining cardiogenesis induction. In particular, *ortho*-substitution of phenyl- and pyridinyl-residues with carbonyl-containing groups afforded highly potent derivatives and those analogs (i.e., **28**, **29**, and **34**) were among the most potent and effective compounds tested.

Finally, we tested potent Wnt inhibitors for selective inhibition of TGFβ signaling to investigate possible off-target effects. TGFβ interacts with Wnt to modulate stem cell fate¹⁸, and inhibition of TGFβ signaling can also be cardiogenic in mouse¹⁹ and human ESCs²⁰. Therefore, ten representative structurally distinct compounds (i.e., **1**, **10**, **28**, **32**, **34**, **35**, **29**, **53**, **34** and **35**) were incubated at concentrations varying from 0.01 to 5 μM (same dose-range used in the Wnt assay) in a SMAD response element-reporter assay in HEK293T cells stimulated with human recombinant TGFβ2. No significant inhibition effect was observed compared to DMSO vehicle controls even at the highest concentrations examined (i.e., % inhibition of SMAD response <20% at 0.01, 0.1, 1 and 5 μM, respectively, see Supplemental Information Fig. S3), indicating that TGFβ inhibition is not involved in the cardiac phenotype of the IWR-1 analogs examined. Furthermore, because the functional biological activity of **1** has been attributed to Axin stabilization, these data also indicate that Axin stabilization does not inhibit TGFβ/SMAD signaling.

Conclusion

Enhancing the production of human cardiomyocytes offers a wealth of opportunities for drug research and toxicological assessment of new chemical entities. The Wnt pathway has been associated with cardiogenesis and using **1** as a “lead”, we obtained small molecule Wnt inhibitors with IC₅₀ values ranging from low nanomolar (4 nM) to the micromolar range. We showed a direct (i.e., titratable) correlation between Wnt inhibitory potency as determined by IC₅₀ values in mammalian cells and cardiogenic functional activity in a human ESC-based assay. We were also able to improve the percent of cardiogenesis to 176% with compound **29** compared to 100% for the initial “lead” **1** at much lower concentrations (i.e., 150 nM for **29** compared to 5000 nM for **1**, equivalent to 30-fold greater potency) rendering **29** more amenable to *in vitro* and *in vivo* applications. Because adult hearts contain cardiogenic progenitor cells that resemble the hESC-derived progenitor cells used in assays described herein in terms of gene expression profile and developmental potential⁴, it is possible that Wnt inhibition could promote differentiation of adult human cardiac progenitor cells. Therefore, an important next step will be to explore their utility for *ex vivo* expansion of adult heart-derived progenitors and to nominate and evaluate optimal compounds for their potential as *in vivo* regenerative compounds in animal models of myocardial infarction.

Experimental Section

General

Reagents and solvents were used as received from commercial sources. Compound **2** was purchased from Cayman Chemicals, **4** from Princeton BioMolecular Research and **5** from Sigma-Aldrich. Synthetic products were isolated using flash column chromatography system (Teledyne ISCO, CombiFlash Rf) with UV detection at 254 nm or PTLC (Preparative Thin Layer Chromatography) with UV indicator. NMR (Nuclear Magnetic Resonance) spectra were recorded at 300 MHz (¹H) on a Varian Mercury 300 or at 125 MHz (¹³C) on a Bruker AMX-500 II (NuMega Resonance Lab, San Diego, CA). Chemical shifts were reported as ppm (δ) relative to the solvent (CDCl₃ at 7.26 ppm, CD₃OD at 3.31 ppm, *d*₆-DMSO at 2.50 ppm and 3.52 ppm). S stands for singlet, d for doublet, t for triplet, q for quadruplet, m for multiplet and br for broad. Low resolution mass spectra were obtained using Hitachi M-8000 mass spectrometer with an ESI source.

Purity of final products was determined with a Hitachi 8000 LC-MS (Hitachi) using reverse phase chromatography (C18 column, 50 × 4.6 mm, 5 μm, Thomson Instrument Co., Oceanside, CA). Compounds were eluted using a gradient elution of 95/5 to 5/95 A/B over 5 min at a flow rate of 1.5 mL/min, where solvent A was water with 0.05% TFA and solvent B was acetonitrile with 0.05% TFA. For purity analysis, peak area percent for the TIC (Total Ion Count) at 254 nm and retention time (*t_R* in minutes) were provided. Purity of final products was 95%.

Biological Assays

Wnt Inhibition—Compounds were tested for their ability to inhibit the β-catenin-dependent canonical Wnt pathway. A Wnt assay was adapted from Chen et al.,⁷ in our labs using HEK293T cells in a 96-well format. Briefly, the commercially available Super(8x)TOPflash vector driven by a (7x)TCF-firefly luciferase response element was transiently transfected into HEK293T cells together with a TK-driven Renilla luciferase plasmid as an internal control to normalize the luminescence signal and a Wnt3A-expressing vector as the source of pathway activation. Because of the transient nature of the assay and, consequently, variations between independent experiments, **1** was included as a positive control in parallel with untreated and DMSO-treated vehicle controls in experiments conducted. Maximum inhibition of Wnt response in the assay format was around 90% with the most potent inhibitors at the highest doses examined. Nonlinear regression analysis was performed using the log(inhibitor)/normalized response equation of the Prism 5 software.

hESC Cardiogenesis—Human embryonic stem cell H9 lines carrying MYH6-mCherry reporters were used in the hESC assay as previously described in detail⁶ and is summarized in the Supplemental Information. In brief, embryoid bodies (EB) were grown until day 4, dissociated gently to single cells and transferred to 384-well plates. Concomitantly, different dilutions of small molecules were added. At day 10, media was exchanged for a serum free media (SFM) containing the thyroid hormone analog triiodothyronine T3 to increase the red signal driven by the MYH6 promoter for improved image analysis.²¹ Cells were changed to PBS at day 14 for imaging and red fluorescence images were collected on a high throughput microscope. For quantifying the level of cardiac induction, the total area and intensity of the MYH6-mCherry reporter was measured in each well. Cardiac activity was reported by stating either percent cardiogenesis normalized to **1** (set as 100% at its maximum potency) or Z scores (relative to DMSO control) (Fig. 3A and B).

Chemistry

Compounds **1** and **3** were synthesized following the procedure described in reference 7 and their ^1H NMR compared to the literature spectra⁷.

Scheme 1: Compounds 9–39, 53–56

General Procedure for Intermediates 8: Triethylamine (0.92 mL, 6.6 mmol) and the desired anhydride **7** (6 mmol) were added to a solution of 4-amino acid **6** (6 mmol) in 5 mL DMF. The solution was heated for 16 hours at 120 °C. After returning to room temperature, the solvent was evaporated. The residue was then dissolved in ethyl acetate (100 mL) and washed with 1N HCl (20 mL). The organic layer was washed with brine (25 mL), dried with anhydrous magnesium sulfate. The solution was filtered to yield the desired intermediate **8** that was then used directly or purified by liquid chromatography.

4-endo-Dihydronorbornyl benzoic acid (8a): beige solid; 84% yield; DCM/MeOH 9/1 R_f 0.5; ^1H NMR (300 MHz, CD_3OD): 1.41 (d, J = 8.4 Hz, 2H), 1.61–1.69 (m, 4H), 2.89 (br s, 2H), 3.26 (br s, 2H), 7.41 (d, J = 8.7 Hz, 2H), 8.04 (s, OH), 8.19 (d, J = 8.7 Hz, 2H).

4-(endo-Dihydronorbornyl)-trans-cyclohexanecarboxylic acid (8b): white solid; 35% yield; DCM/MeOH 9/1 R_f 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.49 min; 246.02 [M–COOH], 291.75 [M+1H]; ^1H NMR (300 MHz, CDCl_3): 1.25 (d, J = 8.4 Hz, 2H), 1.46–1.69 (m, 8H), 2.13 (d, J = 11.7 Hz, 2H), 2.21–2.44 (m 3H), 2.74 (br s, 2H), 2.99 (br s, 2H), 3.98 (tt, J = 12.1 and 3 Hz, 1H), 8.01 (s, OH).

4-(endo-Dihydronorbornyl)-cis-cyclohexanecarboxylic acid (8c): white solid; 73% yield; Hex/EtOAc 1/1 R_f 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.49 min; 246.02 [M–COOH], 291.75 [M+1H]; ^1H NMR (300 MHz, CDCl_3): 1.23 (d, J = 8.4 Hz, 2H), 1.46–1.62 (m, 8H), 2.28–2.45 (m, 5H), 2.71 (bs, 2H), 2.97 (bs, 2H), 3.96 (tt, J = 12.1 and 3 Hz, 1H), 8.01 (s, 1H).

4-cis-endo-(3a,4,7,7a-Tetrahydro-1H-4,7-ethanoisindole-1,3(2H)-dion-2-yl)-benzoic acid (8d): off-white solid; 99% yield; DCM/MeOH 9/1 R_f 0.2; ^1H NMR (300 MHz, DMSO): 1.61–1.38 (m, 4H), 2.16 (s, 2H), 2.92 (s, 2H), 7.41 (d, J = 8.4 Hz, 2H), 8.05 (d, J = 8.7 Hz, 2H), 13.15 (br s, 1H).

4-cis-endo-(Hexahydro-1H-4,7-ethanoisindole-1,3(2H)-dion-2-yl)-benzoic acid (8e): white solid; 12% yield; DCM/MeOH 9/1 R_f 0.3; ^1H NMR (300 MHz, CDCl_3): 1.68–1.61 (m, 4H), 2.16 (s, 2H), 2.92 (s, 2H), 3.25–3.23 (m, 4H), 8.06–8.03 (m, 2H), 7.29–7.26 (m, 2H).

General procedure for compounds 9–39, 53–56: Compounds were prepared in a library fashion. One gram of the appropriate intermediate **8** was heated for 16 hours in 5 mL thionyl chloride at 70 °C. TLC (DCM/MeOH 9/1) of a reaction aliquot diluted in methanol showed complete conversion to the methyl ester. Excess thionyl chloride was removed to afford the acid chloride. The acid chloride was dissolved in 20 mL of DCE. 0.3 mL of the DCE solution was added to 30 mg of the appropriate amine and 0.1 mL of pyridine. Solutions were heated at 50 °C for 16 hours and the crude material was purified by liquid chromatography.

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(quinolin-8-yl)benzamide (9): white solid; 51% yield; Hex/EtOAc 3/7 R_f 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.79 min; 411.95 [M+1H] 96.3% at 254 nm; ^1H NMR (300 MHz, CDCl_3): 1.49 (d, J = 8.4 Hz, 2H), 1.58–1.79 (m, 4H), 2.90 (br s, 2H), 3.29 (br s, 2H), 7.47–

7.63 (m, 5H), 8.16–8.23 (m, 3H), 8.84 (dd, J = 4.2 and 1.5 Hz, 1H), 8.92 (dd, J = 6.9 and 1.8 Hz, 1H), 10.79 (br s, NH).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(quinolin-8-yl)-trans-cyclohexyl carboxamide (10): off white solid; 55% yield; Hex/EtOAc 1/1 Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 5.43 min; 418.02 [M+1H] 96.3% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.28 (d, J = 8.4 Hz, 2H), 1.53–1.64 (m, 4H), 1.71–1.85 (m, 4H), 2.21 (br d, J = 12.3 Hz, 2H), 2.42 (dr q, J = 12.6 and 3.6 Hz, 2H), 2.56 (tt, J = 12 and 3.3 Hz, 1H), 2.76 (br s, 2H), 3.01 (br s, 2H), 4.09 (tt, J = 12.3 and 3.9 Hz, 1H), 7.43–7.55 (m, 3H), 8.15 (dd, J = 8.1 and 1.5 Hz, 1H), 8.76 (dd, J = 6.9 and 2.1 Hz, 1H), 8.81 (dd, J = 3.9 and 1.5 Hz, 1H), 9.92 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(quinolin-8-yl)-cis-cyclohexyl carboxamide (11): transparent oil; 50% yield; Hex/EtOAc 1/1 Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.35 min; 418.02 [M+1H] 97.4% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.4 Hz, 2H), 1.46–1.59 (m, 4H), 1.71–1.85 (m, 4H), 2.18 (br d, J = 12.3 Hz, 2H), 2.34–2.75 (m, 5H), 2.94 (br s, 1H), 3.00 (br s, 1H), 3.98–4.09 (m, 1H), 7.43–7.55 (m, 3H), 8.13 (dd, J = 8.1 and 1.5 Hz, 1H), 8.72–8.82 (m, 2H), 9.89+10.07 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(naphthalen-1-yl) benzamide (12): off white solid; 56% yield; Hex/EtOAc 1/1 Rf 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.81 min; 410.88 [M+1H] 95.3% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.41–1.51 (m, 2H), 1.58–1.79 (m, 4H), 2.89 (br s, 2H), 3.28 (br s, 2H), 7.46–7.61 (m, 5H), 7.77 (d, J = 8.7 Hz, 1H), 7.86–7.95 (m, 2H), 8–8.12 (m, 3H), 8.24 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(isoquinolin-1-yl)-trans-cyclohexylcarboxamide (13): white solid; 51% yield; DCM/MeOH 9/1 Rf 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.84 min; 418.02 [M+1H] 99.6% at 254 nm 1H NMR (300 MHz, $CDCl_3+CD_3OD$): 1.22 (d, J = 8.4 Hz, 2H), 1.51–1.61 (m, 4H), 1.65–1.77 (m, 4H), 2.16 (d, J = 11.7 Hz, 2H), 2.21–2.44 (m 3H), 2.70 (br s, 2H), 2.98 (br s, 2H), 4.03 (tt, J = 12.1 and 3 Hz, 1H), 7.43 (br d, J = 5.4 Hz, 2H), 7.53–7.59 (m, 1H), 7.67 (dt, J = 6.9 and 0.9 Hz, 1H), 7.76 (d, J = 8.1 Hz, 1H), 8.06 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(isoquinolin-4-yl)-trans-cyclohexylcarboxamide (14): yellow solid; 16% yield; DCM/MeOH 9/1 Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.10 min; 418.02 [M+1H] 99.9% at 254 nm; 1H NMR (300 MHz, $CDCl_3+CD_3OD$): 1.25 (d, J = 8.4 Hz, 2H), 1.51–1.61 (m, 4H), 1.65–1.77 (m, 4H), 2.15 (br d, J = 12.3 Hz, 2H), 2.25–2.43 (m, 3H), 2.74 (br s, 2H), 2.99 (br s, 2H), 3.99 (tt, J = 12.3 and 3.9 Hz, 1H), 7.58–7.73 (m, 3H), 7.82 (d, J = 8.4 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 8.04 (br s, 1H), 8.74 (s, NH).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(quinolin-4-yl)-trans-cyclohexyl carboxamide (15): off-white solid; 5% yield; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.75 min; 417.88 [M+1H] 99.4% at 254 nm.

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(quinolin-5-yl)-trans-cyclohexyl carboxamide (16): white solid, 40% yield; DCM/MeOH 9/1, Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.20 min; 418.02 [M+1H] 99.5% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.25 (d, J = 8.4 Hz, 2H), 1.53–1.64 (m, 4H), 1.71–1.85 (m, 4H), 2.17 (br d, J = 12.3 Hz, 2H), 2.21–2.49 (m, 3H), 2.72 (br s, 2H), 2.99 (br s, 2H), 4.08 (tt, J = 12.3 and 3.9

Hz, 1H), 7.42 (dd, J = 8.4 and 3.9 Hz, 1H), 7.66–7.83 (m, 2H), 7.98 (d, J = 7.5 Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 8.92 (d, J = 4.5 Hz, 1H), 8.82 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(isoquinolin-5-yl)-trans-cyclohexylcarboxamide (17): yellow glassy solid; 72% yield; DCM/MeOH 9/1, R_f 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.27 min; 418.02 [M+1H] 92.9% at 254 nm; ¹H NMR (300 MHz, CDCl₃): 1.28 (d, J = 8.4 Hz, 2H), 1.53–1.64 (m, 4H), 1.71–1.85 (m, 4H), 2.22 (br d, J = 12.3 Hz, 2H), 2.24–2.58 (m, 3H), 2.71 (br s, 2H), 2.99 (br s, 2H), 4.08 (tt, J = 12.3 and 3.9 Hz, 1H), 7.55–7.64 (m, 2H), 7.79 (d, J = 8.4 Hz, 1H), 7.95 (s, 1H), 8.13 (d, J = 7.8 Hz, 1H), 8.53 (d, J = 6.3 Hz, 1H), 9.24 (s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(isoquinolin-8-yl)-trans-cyclohexylcarboxamide (18): yellow solid; 16% yield; DCM/MeOH 9/1, R_f 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.04 min; 418.02 [M+1H] 90.1% at 254 nm; ¹H NMR (300 MHz, CDCl₃): 1.27 (d, J = 6.9 Hz, 2H), 1.53–1.69 (m, 4H), 1.71–1.88 (m, 4H), 2.20 (br d, J = 12.4 Hz, 2H), 2.24–2.58 (m, 4H), 2.73 (br s, 2H), 3.00 (br s, 2H), 4.01–4.16 (m, 1H), 7.57–7.64 (m, 2H), 8.00–8.06 (m, 1H), 8.20 (br s, 1H), 8.53 (d, J = 5.8 Hz, 1H), 9.43 (s, NH).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(p-tolyl)benzamide (19): off white solid; 14% yield; Hex/EtOAc, 1/1; R_f 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.55 min; 374.88 [M+1H] 98.5% at 254 nm; ¹H NMR (CDCl₃): 1.46 (d, J = 8.4 Hz, 2H), 1.53–1.74 (m, 4H), 2.34 (s, 3H), 2.89 (br s, 2H), 3.27 (br s, 2H), 7.18 (d, J = 8.1 Hz, 2H), 7.41 (d, J = 8.7 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 7.79 (br s, 1H), 7.95 (d, J = 8.4 Hz, 2H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(4-methoxyphenyl)benzamide (20): grey solid; 20% yield; Hex/EtOAc, 1/1; R_f 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.73 min; 390.82 [M+1H] 93.4% at 254 nm; ¹H NMR (CDCl₃): 1.19–1.26 (m, 2H), 1.45–1.74 (m, 4H), 2.89 (br s, 2H), 3.27 (br s, 2H), 3.82 (s, 3H), 6.91 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H), 7.78 (s, NH), 7.95 (d, J = 8.4 Hz, 2H).

2-cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(2-methoxyphenyl)benzamide (21): white solid; 91% yield; Hex/EtOAc 3/7, R_f 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.12 min; 390.35 [M+1H] 100% at 254 nm; ¹H NMR (300 MHz, CDCl₃): 1.47 (d, J = 8.4 Hz, 2H), 1.58–1.79 (m, 4H), 2.89 (br s, 2H), 3.28 (br s, 2H), 3.92 (s, 3H), 6.92 (dd, J = 7.8 and 1.2 Hz, 1H), 6.99–7.13 (m, 2H), 7.41–7.45 (m, 2H), 7.97–8.02 (m, 2H), 8.51 (dd, J = 7.8 and 1.2 Hz, 1H), 8.52 (br s, 1H).

2-cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(4-(dimethylamino)phenyl)benzamide (22): dark green solid; 36% yield; DCM/MeOH 9/1, R_f 0.2; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.17 min; 403.95 [M+1H] 99.6% at 254 nm; ¹H NMR (CDCl₃): 1.46 (d, J = 8.4 Hz, 2H), 1.59–1.76 (m, 4H), 2.88 (br s, 2H), 2.94 (s, 6H), 3.27 (br s, 2H), 6.74 (d, J = 9.3 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 7.48 (d, J = 9.3 Hz, 2H), 7.74 (s, NH), 7.94 (d, J = 8.1 Hz, 2H).

2-cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(4-cyanophenyl)benzamide (23): white solid; 48% yield; DCM/MeOH, 95/5, R_f 0.1; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.48 min; 347.68 [M+1H] 90%; ¹H NMR (CDCl₃+CD₃OD): 1.33 (d, J = 8.4 Hz, 2H), 1.55–1.69 (m, 5H), 2.77 (br s, 2H), 3.20 (br s, 1H), 7.26 (d, J = 8.2 Hz, 2H), 7.53 (d, J = 9.1 Hz, 2H), 7.77 (d, J = 9.1 Hz, 2H), 7.90 (d, J = 8.8 Hz, 2H).

2-cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(4-acetylphenyl)benzamide (24): off white solid; 35% yield; DCM/MeOH, 95/5, Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.98 min; 402.84 [M+1H] 97.9% at 254 nm.

4-(cis-endo-1,3-dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(2-hydroxyphenyl)-trans-cyclohexylcarboxamide (25): orange solid; 43% yield; Hex/EtOAc 1/1, Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.01 min; 418 [M+1H] 99% at 254 nm; 1H NMR (300 MHz, CD_3OD): 1.23 (d, J = 8.4 Hz, 2H), 1.53–1.71 (m, 4H), 1.75–2.01 (m, 4H), 2.06 (br d, J = 12.3 Hz, 2H), 2.28–2.35 (m, 3H), 2.72 (br s, 2H), 2.98 (br s, 2H), 3.98–4.14 (m, 1H), 6.76–6.87 (m, 2H), 6.96 (dd, J = 7.98 and 1.5 Hz, 1H), 7.58 (dd, J = 7.98 and 1.5 Hz, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(2-ethoxyphenyl)-trans-cyclohexyl carboxamide (26): white solid; 73% yield; Hex/EtOAc 1/1, Rf 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.46 min; 418 [M+1H] 99% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.23 (d, J = 8.4 Hz, 2H), 1.53–1.71 (m, 10H), 2.06 (br d, J = 12.3 Hz, 2H), 2.28–2.35 (m, 4H), 2.72 (br s, 2H), 2.98 (br s, 2H), 3.98–4.14 (m, 3H), 6.83 (dd, J = 7.5 and 1.5 Hz, 1H), 6.87–7.11 (m, 2H), 7.85 (br s, NH), 8.34 (dd, J = 7.5 and 1.5 Hz, 1H).

Methyl 2-(4-(cis-endo-1,3-dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-trans-cyclohexanecarboxamido)benzoate (27): white solid; 58% yield; DCM/MeOH 95/5 Rf 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.58 min; 418 [M+1H] 92.4% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.24 (d, J = 8.4 Hz, 2H), 1.51–1.72 (m, 8H), 2.14 (br d, J = 11.7 Hz, 2H), 2.26–2.41 (m, 3H), 2.73 (br s, 2H), 2.99 (br s, 2H), 3.91 (s, 3H), 4.03 (t, J = 12.6 Hz, 1H), 7.05 (t, J = 7.8 Hz, 1H), 7.51 (t, J = 8.7 Hz, 1H), 8 (dd, J = 7.8 and 1.2 Hz, 1H), 8.71 (d, J = 8.7 Hz, 1H), 11.15 (br s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(2-acetylphenyl)-trans-cyclohexyl carboxamide (28): brown solid; 38% yield; Hex/EtOAc 1/1 Rf: 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.40 min; 408.28 [M+1H] 94.7% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.4 Hz, 2H), 1.53–1.85 (m, 8H), 2.16 (br d, J = 12.3 Hz, 2H), 2.28–2.49 (m, 3H), 2.67 (s, 3H), 2.75 (br s, 2H), 3.01 (br s, 2H), 4.08 (tt, J = 12.3 and 3.9 Hz, 1H), 7.11 (t, J = 7.5 Hz, 1H), 7.55 (t, J = 8.7 and 1.5 Hz, 1H), 7.90 (dd, J = 7.8 and 1.2 Hz, 1H), 8.76 (d, J = 8.4 Hz, 1H), 11.84 (s, NH).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(8-oxo-5,6,7,8-tetrahydro naphthalen-1-yl)-trans-cyclohexylcarboxamide (29): brown oil; 51% yield; Hex/EtOAc 1/1 Rf 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.73 min; 434.48 [M+1H] 97.5% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.21 (d, J = 8.4 Hz, 2H), 1.53–1.67 (m, 8H), 2.07–2.15 (m, 4H), 2.19–2.54 (m, 3H), 2.61–2.78 (m, 4H), 2.88–3.02 (m, 4H), 3.98–4.14 (m, 1H), 6.91 (dd, J = 7.5 and 1.5 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 8.60 (dd, J = 7.5 and 1.5 Hz, 1H), 12.23 (br s, 1H).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(9H-fluoren-9-on-1-yl)-trans-cyclohexylcarboxamide (30): yellow solid; 46% yield; DCM/MeOH 95/5 Rf 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.38 min; 469 [M+1H] 99.4% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.4 Hz, 2H), 1.47–1.74 (m, 8H), 2.18 (br d, J = 11.7 Hz, 2H), 2.26–2.48 (m, 3H), 2.75 (br s, 2H), 3.00 (br s, 2H), 4.04 (t, J = 12.6 Hz, 1H), 7.15 (t, J = 7.2 Hz, 1H), 7.25–7.31 (m, 1H), 7.38–7.47 (m, 3H), 7.58 (d, J = 7.5 Hz, 1H), 8.34 (d, J = 8.1 Hz, 1H), 10.17 (br s, NH).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(pyridin-2-yl)-trans-cyclohexyl carboxamide (31): off white solid; 6% yield; DCM/MeOH 9/1 Rf 0.7; LC-MS

(1000 (+)-5.5-254-95:5) t_R 1.27 min; 367.88 [M+1H] 97.5% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.4 Hz, 2H), 1.51–1.74 (m, 9H), 2.09 (br d, J = 11.7 Hz, 2H), 2.26–2.39 (m, 2H), 2.75 (br s, 2H), 3.01 (br s, 2H), 4.01–4.17 (m, 1H), 7.01–7.05 (m, 1H), 7.69 (dt, J = 9 and 2.1 Hz, 1H), 7.99 (br s, NH), 8.21 (d, J = 8.7 Hz, 1H), 8.24–8.27 (m, 1H).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(pyridin-3-yl)-trans-cyclohexyl carboxamide (32): white solid; 32% yield; DCM/MeOH 9/1 Rf 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.46 min; 367.88 [M+1H] 97.9% at 254 nm; 1H NMR (300 MHz, $CDCl_3$ +1 drop CD_3OD): 1.28 (d, J = 8.4 Hz, 2H), 1.53–1.64 (m, 4H), 1.71–1.85 (m, 4H), 1.86 (br d, J = 12.3 Hz, 2H), 2.12–2.38 (m, 3H), 2.76 (br s, 2H), 3.01 (br s, 2H), 4.08 (tt, J = 12.3 and 3.9 Hz, 1H), 7.21 (dd, J = 8.4 and 3.6 Hz, 1H), 8.14 (br d, J = 3.6 Hz, 1H), 8.20 (br d, J = 8.4 Hz, 1H), 8.42 (d, J = 2.1 Hz, 1H)

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(pyridin-4-yl)-trans-cyclohexyl carboxamide (33): yellow solid; 8% yield; DCM/MeOH 9/1 Rf 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 1.68 min; 367.89 [M+1H] 97.3% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.25 (d, J = 8.4 Hz, 2H), 1.46–1.75 (m, 8H), 2.07 (br d, J = 12.3 Hz, 2H), 2.23–2.37 (m, 3H), 2.75 (br s, 2H), 3.02 (br s, 2H), 4.08 (tt, J = 12.3 and 3.9 Hz, 1H), 7.31 (br s, NH), 7.48 (d, J = 6.3 Hz, 2H), 8.50 (br d, J = 6.3 Hz, 2H).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(2-acetyl-pyridin-3-yl)-trans-cyclohexylcarboxamide (34): off-white solid; 17% yield; Hex/EtOAc 7/3 Rf 0.2; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.35 min; 409.28 [M+1H] 96.4% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.8 Hz, 2H), 1.51–1.78 (m, 8H), 2.18 (br d, J = 11.8 Hz, 2H), 2.31–2.52 (m, 5H), 2.76 (br s, 1H), 2.80 (s, 3H), 3.03 (br s, 1H), 3.97–4.15 (m, 1H), 7.43–7.48 (m, 1H), 8.35 (dd, J = 4.4 and 0.9 Hz, 1H), 9.09 (dd, J = 8.8 and 0.9 Hz, 1H), 11.62 (br s, NH).

4-(cis-endo-(1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl))-N-(6-acetyl-pyridin-3-yl)-trans-cyclohexylcarboxamide (35): off-white solid; 18% yield; Hex/EtOAc 7/3 Rf 0.2; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.46 min; 409.48 [M+1H] 98.6% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.28 (d, J = 8.4 Hz, 2H), 1.49–1.78 (m, 8H), 2.17 (d, J = 11.7 Hz, 2H), 2.29–2.49 (m, 5H), 2.71 (s, 3H), 2.77 (br s, 1H), 2.99 (br s, 1H), 3.98–4.11 (m, 1H), 7.64 (d, J = 5.23 Hz, 1H), 8.47 (d, J = 5.53 Hz, 1H), 10.05 (s, 1H), 11.20 (s, NH).

4-cis-endo-(3a,4,7,7a-Tetrahydro-1H-4,7-ethanoisindole-1,3(2H)-dion-2-yl)-N-(quinolin-8-yl) benzamide (36): yellow solid; 10% yield; Hex/EtOAc 1/1 Rf 0.3; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.87 min; 423.68 [M+1H] 96% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.70–1.45 (m, 4H), 3.06 (s, 2H), 3.29 (br s, 2H), 6.33–6.31 (m, 2H), 7.60–7.41 (m, 5H), 8.21–8.14 (m, 3H), 8.85–8.83 (m, 1H), 8.93–8.90 (m, 1H), 10.74 (s, 1H)

4-cis-endo-(Hexahydro-1H-4,7-ethanoisindole-1,3(2H)-dion-2-yl)-N-(quinolin-8-yl) benzamide (37): white solid; 51% yield; Hex/EtOAc 3/7 Rf 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.79 min; 411.95 [M+1H] 96.3% at 254 nm; 1H NMR (300 MHz, DMSO): 1.48–1.53 (m, 4H), 1.66–1.68 (m, 4H), 2.08–2.10 (m, 2H), 3.08–3.10 (m, 2H), 7.55 (d, J = 8.8 Hz, 2H), 7.66–7.70 (m, 2H), 7.77 (dd, J = 8.4 and 1.5 Hz, 1H), 8.17 (d, J = 8.8 Hz, 2H), 8.48 (dd, J = 8.4 and 1.5 Hz, 1H), 8.73 (dd, J = 8.4 and 1.5 Hz, 1H), 8.98–8.99 (m, 1H), 10.70 (br s, NH).

4-(cis-endo-8-Methyl-1,3-dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(quinolin-8-yl) benzamide (38): orange solid; 64% yield; Hex/EtOAc 1/1 Rf 0.4; 1H NMR (300 MHz, DMSO): 1.47 (d, J = 8.4 Hz, 1H), 1.58–1.79 (m, 1H), 1.83 (s, 3H), 3.28 (br s, 2H), 3.92 (s,

2H), 5.87 (s, 1H), 7.49–7.55 (m, 2H), 7.52–7.77 (m, 3H), 8.13 (d, J = 8.8 Hz, 2H), 8.45 (dd, J = 8.53 and 0.9 Hz, 1H), 8.69 (dd, J = 8.53 and 0.9 Hz, 1H), 8.95 (dd, J = 4.4 and 0.9 Hz, 1H).

4-(Hexahydro-1H-4,7-epoxyisoindole-1,3(2H)-dion-2-yl)-N-(quinolin-8-yl)benzamide (39): 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride was prepared according to literature procedures²² beige solid; 68% yield; DCM/MeOH 9/1 Rf 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.23 min, 414 [M+1H] >99% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.68–1.75 (m, 2H), 1.94–1.98 (m, 2H), 3.11 (s, 2H), 5.05 (m, 2H), 7.47–7.63 (m, 5H), 8.16–8.19 (m, 2H), 8.21 (br d, J = 1.67 Hz, 1H), 8.85 (dd, J = 1.67, 4.21 Hz, 1H), 8.91 (dd, J = 1.89, 7.08 Hz, 1H), 10.75 (s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(4-bromo-2-methoxyphenyl)-trans-cyclohexylcarboxamide (53): off-white solid; 11% yield; DMC/MeOH 9/1 Rf 0.8; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.98 min; 474.48–476.48 [M+1H] 90.3% at 254nm; 1H NMR (300 MHz, $CDCl_3$): 1.11 (d, J = 8.4 Hz, 2H), 1.45–1.77 (m, 6H), 2.04 (br d, J = 12 Hz, 4H), 2.08–2.41 (m, 4H), 2.74 (br s, 2H), 2.99 (br s, 2H), 3.87 (s, 3H), 4.04 (t, J = 12.6 Hz, 1H), 7 (s, 1H), 7.07 (d, J = 7 Hz, 1H), 7.87 (br s, NH), 8.24 (d, J = 7 Hz, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(5-hydroxyl-pyridin-3-yl)-trans-cyclohexylcarboxamide (54): beige solid, 21% yield; DCM/MeOH, 9/1; Rf 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 1.47 min; 383.88 [M+1H] 97.2% at 254nm; 1H NMR (300 MHz, $CDCl_3+CD_3OD$): 1.06 (d, J = 8.4 Hz, 2H), 1.39–1.59 (m, 6H), 1.82 (br d, J = 11.7 Hz, 2H), 2.06–2.38 (m, 3H), 2.58 (br s, 2H), 2.88 (br s, 2H), 4.04 (t, J = 12.6 Hz, 1H), 7.59 (s, 1H), 7.65 (s, 1H), 7.87 (s, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(2-chloropyridin-3-yl)-trans-cyclohexylcarboxamide (55): pale yellow solid, 35% yield; DCM/MeOH, 9/1; Rf 0.6; LC-MS (1000 (+)-5.5-254-95:5) t_R 2.91 min; 401.68 [M+1H] 98.7% at 254nm; 1H NMR (300 MHz, $CDCl_3$): 1.26 (d, J = 8.4 Hz, 2H), 1.51–1.74 (m, 6H), 2.18 (br d, J = 11.7 Hz, 2H), 2.26–2.48 (m, 3H), 2.73 (br s, 2H), 3.00 (br s, 2H), 3.92–4.02 (m, 1H), 7.25 (dd, J = 8.4 and 4.9 Hz, 1H), 7.69 (br s, NH), 8.10 (dd, J = 4.9 and 1.8 Hz, 1H), 8.73 (dd, J = 8.4 and 1.8 Hz, 1H).

4-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(2-acetylphenyl)-cis-cyclohexyl carboxamide (56): beige wax; 40% yield; Hex/EtOAc 1/1 Rf 0.5; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.06 min; 408.42 [M+1H] 96.2% at 254nm; 1H NMR (300 MHz, $CDCl_3$): 1.24 (d, J = 8.4 Hz, 2H), 1.48–1.79 (m, 7H), 2.29–2.46 (m, 5H), 2.64 (s, 3H), 2.72 (br s, 2H), 2.98 (br s, 2H), 3.97–4.08 (m, 1H), 7.07–7.13 (m, 1H), 7.54 (tt, J = 8.7 and 1.5 Hz, 1H), 7.89 (td, J = 8.1 and 1.5 Hz, 1H), 8.76 + 8.87 (dd, J = 8.7 and 0.9 Hz, 1H), 11.80 + 11.96 (br s, NH).

3-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisoindol-2-yl)-N-(quinolin-8-yl)benzamide (45): Compound **45** was synthesized according to procedures described for compounds **9–39**, **53–56** but replaced 4-aminobenzoic acid by 3-aminobenzoic acid. white solid; 96% yield; Hex/EtOAc 1/1; Rf 0.4; LC-MS (1000 (+)-5.5-254-95:5) t_R 3.91 min; 411.82 [M+1H] 98.9% at 254 nm; 1H NMR (500 MHz, d_6 -DMSO): 1.32–1.37 (m, 2H), 1.57–1.64 (m, 3H), 1.70–1.72 (m, 1H), 2.70–2.72 (m, 2H), 3.31–3.33 (m, 2H, overlaps with the residual H_2O signal), 7.53–7.55 (m, 1H), 7.66–7.70 (m, 2H), 7.75–7.79 (m, 2H), 7.88–7.89 (m, 1H), 8.10–8.12 (m, 1H), 8.47 (dd, J = 8.4 and 1.5 Hz, 1H), 8.69 (dd, J = 8.4 and 1.5 Hz, 1H), 8.96–8.97 (m, 1H), 10.68 (br s, NH, 1H).

5-(cis-endo-1,3-Dioxooctahydro-2H-4,7-methanoisindol-2-yl)-N-(quinolin-8-yl)furan-2-carboxamide (46): Compound **46** was synthesized according to procedures described for compounds **41–44** but replaced 4-nitrobenzoic acid by 5-nitrofuroic acid. orange oil; 20% yield; DCM/MeOH 9/1 Rf 0.7; LC-MS (1000 (+)-5.5-254-95:5) t_R 4.12 min; 401.82 [M+1H]; 90% at 254 nm; 1H NMR ($CDCl_3$): 1.2–1.82 (m, 5H), 2.92 (br s, 2H), 3.33 (br s, 2H), 7.36 (d, J = 3.6 Hz, 1H), 7.43–7.48 (m, 3H), 7.54–7.58 (m, 1H), 8.17 (dd, J = 8.25 and 1.65 Hz, 1H), 8.76 (dd, J = 6.9 and 2.1 Hz, 1H), 8.87 (dd, J = 3.9 and 1.5 Hz, 1H), 10.74 (br s, NH).

Scheme 5, Step a: N-(4-Nitrophenyl)-(3aR,4S,7R,7aS)-1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isindol (51)—Compound **51** was synthesized according to the procedure described for intermediate **8** but starting from 1.64 g carbic anhydride (10 mmol), 1.67 mL triethylamine and 1.52 g 4-nitroaniline. Compound **51** was isolated as a yellow solid with 52% yield (DCM/MeOH, 9/1; Rf 0.6). LC-MS (1000 (+)-5.5-254-95:5) t_R 3.35 min; 96.8 % at 254 nm. 1H NMR (300 MHz, $CDCl_3$): 1.62–1.68 (m, 1H), 1.80–1.86 (m, 1H), 3.49 (dd, J = 1.63, 3.09 Hz, 2H), 3.55 (m, 2H), 6.27 (br t, J = 1.80 Hz, 2H), 7.40–7.44 (m, 2H), 8.26–8.31 (m, 2H).

Scheme 5, Step b: N-(4-Aminophenyl)-(3aR,4S,7R,7aS)-1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isindol—300 mg of **51** was dissolved in 30 mL of EtOH and 40 mg of Pd/C (10%) added. The mixture was stirred at room temperature for 16 hours under a hydrogen atmosphere. The solution was filtered through a pad of Celite and concentrated to dryness. The crude product was purified by flash chromatography (Hex/EtOAc 1/1 Rf 0.2) to afford 180 mg (67% yield). LC-MS (1000 (+)-10.0-254-95:5) t_R 3.14 min, 257 [M+1H]; 98.8% at 254 nm. 1H NMR (300 MHz, $CDCl_3$): 1.43–1.72 (m, 6H), 2.85 (m, 2H), 3.21 (m, 2H), 3.81 (br s, 2H, NH_2), 6.71–6.75 (m, 2H), 6.98–7.01 (m, 2H).

Scheme 5, Step c: N-[4-((3aR,4S,7R,7aS)-1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isindol-2-yl)phenyl]-quinoline-8-carboxamide (52)—Quinoline-8-carbonyl chloride was freshly prepared by treating 135 mg (0.78 mmol) of quinoline-8-carboxylic acid with an excess of thionyl chloride (622 μ L) for three hours at 60–70 °C. After returning to room temperature, excess thionyl chloride was removed by evaporation. The mixture was added dropwise to a solution of 100 mg of the aniline precursor from step b (0.39 mmol) and 5 equivalents of triethylamine (1.95 mmol) in 7 mL of dry acetonitrile. The mixture was stirred at room temperature for three days, concentrated in a vacuum and purified by flash chromatography (toluene/EtOAc/acetone 6/3/1 Rf 0.5). The product was obtained as an off-white solid with 87% yield (140 mg). LC-MS (1000 (+)-5.5-254-95:5) t_R 3.45 min, 412 [M+1H]; >99% at 254 nm; 1H NMR (300 MHz, $CDCl_3$): 1.48–1.75 (m, 6H), 2.88 (m, 2H), 3.25 (m, 2H), 7.27–7.32 (m, 2H), 7.56 (dd, J = 4.3, 8.3 Hz, 1H), 7.74 (dd, J = 7.45, 7.50 Hz, 1H), 7.98–8.03 (m, 2H), 8.02 (br d, J = 1.6 Hz, 1H), 8.34 (dd, J = 1.8, 8.4 Hz, 1H), 8.96 (dd, J = 1.6, 7.4 Hz, 1H), 9.01 (dd, J = 1.8, 4.3 Hz, 1H).

Supplementary Material

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Acknowledgments

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Abbreviations

hESC	Human Embryonic Stem Cell
HCS	High Content Screen
HEK293T	Human Embryonic Kidney 293T
IWR	Inhibitor of Wnt Response

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Figure 1. Screening “lead” **1**. Regions A, B, C indicate the different areas of the molecule that were systematically modified for lead optimization.

Figure 2.

Inhibition of the Wnt pathway with reported inhibitors **1–5**. **A)** Structures of compounds **1–5**; **B)** Dose-response curves and IC₅₀ values for Wnt inhibition for **1–5** representing different classes of Wnt inhibitors. Data is the mean ± std. dev. of at least three independent experiments; **C)** Maximum cardiogenesis in the presence of Wnt inhibitors in hESCs (data from Willems et al.)⁶; human recombinant DKK-1 = 200 ng/mL (7.75 nM). ^a Cardiogenesis corresponds to MYH6-mCherry expression levels as fold induction over DMSO vehicle control (=1-fold).

Figure 3.

Cardiogenic activity and Wnt inhibition of selected analogs. **A)** Table shows data set for analogs tested in cardiac and Wnt assays; IC₅₀ (nM) were measured in the Wnt inhibition assay. Red highlighted are “outlier” compounds. **B)** Maximum cardiogenic activity for each compound tested relative to the maximum induction for **1** (set at 100%). **C)** Representative dose-response curves of IWR analogs in hESCs for cardiogenesis. **D)** Correlation of cardiogenesis (based on the Z score at 625 nM) and Wnt inhibition (IC₅₀ values) in a double-logarithmic plot; data points (in red) indicate “outlier” derivatives and were excluded from the correlation (r^2 is highlighted in red). The correlation coefficient r^2 (in black) includes all data.

^a % cardiogenesis represents normalized data for most effective (cardiac) concentration to the maximum induction of **1** (100% at 2.5 μM); ^b Z score at a dose of 625 nM; ^c maximum cardiac activity = normalized to **1**, no cardiac activity = Z-score of 0;

Scheme 1. Synthesis of analogs **9-39**, **53-56**.

a) DMF, 120 °C, 16h. b) thionyl chloride, DMF, 80 °C, 16h. c) R₃NH₂, Py, DCE, r.t. to 50 °C, 16h.

Scheme 2. Synthesis of compounds **41–44**.a) thionyl chloride, reflux, 2h. b) R₃NH₂, Pyridine, reflux, 3h. c) H₂, Pd/C, EtOH, r.t., 16h.d) anhydride, TEA, DMF, 120 °C, 16h. e) R₄COCl, TEA, DCE, 70 °C, 16h. f) R₄CHO,NaBH(OAc)₃, AcOH, MgSO₄, DCE, 50 °C, 72h.

Scheme 3. Synthesis of **48**.

a) DMF, 120 °C, ovn.; b) H₂, Pd/C, EtOH, r.t., ovn.; c) *cis*-endo-dihydrocarbic anhydride, DMF, 120 °C, 10h.

Scheme 4. Synthesis of **50**.

a) NaBH_3CN , AcOH, EtOH, r.t., 16h. b) $\text{Na}_2\text{S}_2\text{O}_4$, EtOH/ $\text{NaHCO}_3\text{aq.}$, r.t., 16h. c) *cis-endo*-dihydrocarbic anhydride, DMF, 120 °C, 16h.

Scheme 5.**Synthesis of **52**.**

a) *cis*-endo-carbic anhydride, DMF, 120 °C, 16h. b) H_2 , Pd/C, EtOH, r.t. c) quinoline-8-carbonyl chloride, TEA, 3d, r.t.

Table 1

SAR for the Central Region (B) of the molecule for Wnt inhibition.

Cpd#	% inhibition (1 μ M)	IC ₅₀ (nM)
9	93 $\pm$ 1	24 $\pm$ 4
10	89 $\pm$ 2	4 $\pm$ 2
11	90 $\pm$ 1.4	57 $\pm$ 5
45	0	NM ^a
46	0	NM
48	15 $\pm$ 2	NM
50	45 $\pm$ 3	NM
52	91 $\pm$ 1	119 $\pm$ 9

^aNM: not measured

Table 2

SAR for Region C: A systematic study of the effect of the nitrogen position of the quinoline heterocycle on potency for Wnt inhibition.

	Cpd#	% inhibition (1 μ M)	IC ₅₀ (nM)
Naphtalen-1-yl ^a	12	41 $\pm$ 13	1590 $\pm$ 280
isoquinolin-1-yl	13	88 $\pm$ 1	95 $\pm$ 3
isoquinolin-4-yl	14	89 $\pm$ 2	55 $\pm$ 8
quinolin-4-yl	15	90 $\pm$ 1	109 $\pm$ 7
quinolin-4-yl	16	68 $\pm$ 4	604 $\pm$ 90
isoquinolin-5-yl	17	87 $\pm$ 1	161 $\pm$ 16
isoquinolin-8-yl	18	91 $\pm$ 1	93 $\pm$ 4
quinolin-8-yl	10	89 $\pm$ 2	4 $\pm$ 2

^a**12** has a phenyl central ring instead of the *trans*-cylcohexyl.

Table 3

SAR of Region A of the molecule: Effects of phenyl and pyridinyl substituents on the potency for Wnt inhibition.

		Cpd #	% inhibition (1 μ M)	IC ₅₀ (nM)
2-hydroxy-phenyl	19	33 $\pm$ 5	NM ^a
	4-methoxy-phenyl	20	50 $\pm$ 2	NM
	2-methoxy-phenyl	21	84 $\pm$ 2	215 $\pm$ 18
	4-dimethylamino-phenyl	22	25 $\pm$ 25	NM
	4-cyano-phenyl	23	70 $\pm$ 2	317 $\pm$ 8
	4-acetylphenyl	24	41 $\pm$ 13	NM
2-hydroxy-phenyl	25	76 $\pm$ 3	484 $\pm$ 196
	2-ethoxyphenyl	26	78 $\pm$ 2	190 $\pm$ 46
	2-CO ₂ Me-phenyl	27	86 $\pm$ 16	67 $\pm$ 10
	2-COMe-phenyl	28	91 $\pm$ 1	6 $\pm$ 3
	8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl	29	92 $\pm$ 13	7 $\pm$ 1
	(9H-fluorene-9-one)-1-yl	30	88 $\pm$ 2	194 $\pm$ 20
	2-pyridinyl	31	82 $\pm$ 5	109 $\pm$ 4
	3-pyridinyl	32	89 $\pm$ 1	120 $\pm$ 32
	4-pyridinyl	33	84 $\pm$ 1	80 $\pm$ 10
	2-acetyl-3-pyridinyl	34	79 $\pm$ 1	7 $\pm$ 2
	6-acetyl-3-pyridinyl	35	56 $\pm$ 3	590 $\pm$ 153
	2-methoxy-4-bromophenyl	53	82 $\pm$ 5	37 $\pm$ 12
	5-hydroxy-pyridin-3-yl	54	86 $\pm$ 2	152 $\pm$ 25
	2-chloro-pyridin-3-yl	55	13 $\pm$ 1	169 $\pm$ 45
2-COMe-phenyl	56	91 $\pm$ 1	23 $\pm$ 5

^aNM: not measured

Table 4

SAR for Region A of the molecule for Wnt inhibition.

Cpd #	9	36	37	38	39
% inh @ 1 μ M	93 $\pm$ 1	83 $\pm$ 4	87 $\pm$ 1	76 $\pm$ 1	49 $\pm$ 4
IC ₅₀ (nM)	24 $\pm$ 4	412 $\pm$ 26	314 $\pm$ 19	1176 $\pm$ 260	605 $\pm$ 260