

# Discovering Small Molecules that Promote Cardiomyocyte Generation by Modulating Wnt Signaling

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## SUMMARY

We have developed a robust *in vivo* small-molecule screen that modulates heart size and cardiomyocyte generation in zebrafish. Three structurally related compounds (Cardionogen-1 to Cardionogen-3) identified from our screen enlarge the size of the developing heart via myocardial hyperplasia. Increased cardiomyocyte number in Cardionogen-treated embryos is due to expansion of cardiac progenitor cells. In zebrafish embryos and murine embryonic stem (ES) cells, Cardionogen treatment promotes cardiogenesis during and after gastrulation, whereas it inhibits heart formation before gastrulation. Cardionogen-induced effects can be antagonized by increasing Wnt/ $\beta$ -catenin signaling activity. We demonstrate that Cardionogen inhibits Wnt/ $\beta$ -catenin-dependent transcription in murine ES cells and zebrafish embryos. Cardionogen can rescue Wnt8-induced cardiomyocyte deficiency and heart-specific phenotypes during development. These findings demonstrate that *in vivo* small-molecule screens targeting heart size can reveal compounds with cardiomyogenic effects and identify underlying target pathways.

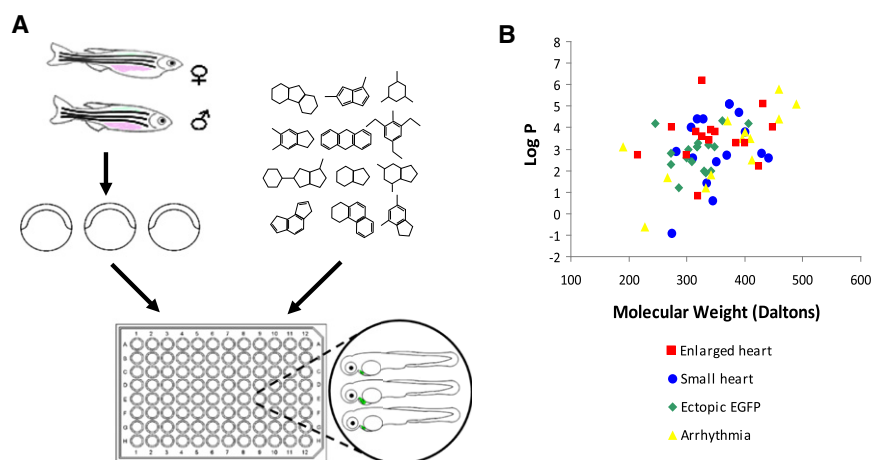
## INTRODUCTION

Dysregulation of heart development and growth is a hallmark of most cardiovascular diseases (Olson, 2004). Screening for small molecules that regulate cardiomyocyte generation will further our understanding of cardiac developmental mechanisms and aid in discovering novel therapeutics for heart diseases. The zebrafish has emerged as a model organism used in multiple steps of the drug discovery process through their use in *in vivo* phenotypic screens (Lehár et al., 2008; Murphey and Zon,

2006). Whole-embryo screens offer considerable advantages in drug discovery by evaluating target cell populations and organs, as well as other pleiotropic effects. Established heart-specific zebrafish transgenic lines permit visualization of green fluorescent proteins in developing hearts. These features make zebrafish particularly well suited for discovering small-molecule regulators of cardiac development and growth.

Despite advances in modern medicine, management of myocardial infarction and heart failure remains a major challenge. Developing therapies that can stimulate cardiomyocyte regeneration in areas of infarction would have an enormous medical and economic impact. Both embryonic and adult stem cells have received considerable attention as donor cells for therapeutic applications. Use of pluripotent embryonic stem (ES) cells is largely limited by ethical issues and concerns of their tumorigenic potential (Behfar et al., 2002), whereas recent trials featuring adult donor stem cells have demonstrated only modest clinical benefits (Lunde et al., 2006; Schächinger et al., 2006). These findings demonstrate a limited capacity of donor stem cells to differentiate into cardiomyocytes, and they highlight the need to develop small molecules that induce differentiation of exogenous and endogenous stem cells toward cardiac cell lineages.

Zebrafish cardiac development begins during the early stages of embryogenesis. Generation of the required number of cardiomyocytes involves both production of cardiac progenitor cells and proliferation of embryonic cardiomyocytes (Stainier, 2001). The size of the embryonic heart primarily reflects cardiac cell number and cell size (Jia et al., 2007). Several signaling pathways, including bone morphogenic protein (BMP), Wnt, fibroblast growth factor (FGF), Notch, and retinoic acid, are implicated in the initial selection of myocardial progenitors from a multipotential stem cell population (Keegan et al., 2005; Marques et al., 2008; Reiter et al., 2001; Rones et al., 2000). Among this population, the Wnt signaling pathway has received considerable attention for its roles in development, stem cell formation, regeneration, and cancer progression (Logan and Nusse, 2004; Moon et al., 2004). Canonical Wnt signaling is mediated by binding of secreted (Wnt) proteins to specific



**Figure 1. In Vivo Small Molecule Screen Identified Bioactive Compounds that Affect Heart Development**

(A) Schematic diagram displaying the screening process for compounds that affect heart development. Three *Tg(cmlc2-EGFP)* embryos were transferred to each well in E3 buffer at the concentration of test compounds to 10  $\mu$ M.

(B) Log P values (partition coefficients between octanol and water) of all active compounds identified in the screen plotted against their respective molecular weights. The bioactive compounds have molecular weights ranging from 200 to 500 Daltons and log P values ranging from  $-1$  to  $+7$ . Positive log P value = hydrophobic. Negative log P value = hydrophilic. Bioactive compounds were subclassified by cardiac phenotypes observed with treatment (10  $\mu$ M).

Frizzled receptor complexes, and this results in inactivation of GSK-3 $\beta$ , leading to dephosphorylation and stabilization of cytoplasmic  $\beta$ -catenin.  $\beta$ -catenin then translocates into the nucleus and activates T cell factor (Tcf)/Lymphoid-enhancing-factor (Lef)-mediated transcription (Logan and Nusse, 2004; Moon et al., 2004). During zebrafish heart development, Wnt/ $\beta$ -catenin signaling regulates heart development in a temporally biphasic fashion. It induces cardiac specification before gastrulation but inhibits heart formation during and after gastrulation (Ueno et al., 2007). Although core components of the Wnt signaling pathway are clearly defined and highly conserved, tissue-specific modifiers of the pathway remain a mystery (Logan and Nusse, 2004; Moon et al., 2004).

In this study, we screened a small-molecule library for compounds using an in vivo cardiac development assay. A novel small-molecule family containing three structurally related compounds (Cardionogen-1, -2, and -3) was identified based on the ability of its compounds to selectively enlarge the size of the embryonic heart. We show that Cardionogen is a biphasic modulator of cardiogenesis, either promoting or inhibiting heart formation, depending on the stage of treatment. Cardionogen treatment also promotes murine ES cells to differentiate into beating cardiomyocytes, demonstrating that the bioactivity of this small-molecule family is functionally conserved in mammalian cells. We indicate that Cardionogen inhibits Wnt/ $\beta$ -catenin-dependent transcriptional activity in murine ES cells ( $EC_{50}$  of  $\sim 23$  nM) and zebrafish embryos. Furthermore, Cardionogen can rescue cardiac cell and chamber deficiency induced by Wnt8 after gastrulation and reverse cardiac cell expansion caused by Wnt8 overexpression before gastrulation. These findings indicate that Cardionogen interferes with Wnt signaling during cardiac development and growth.

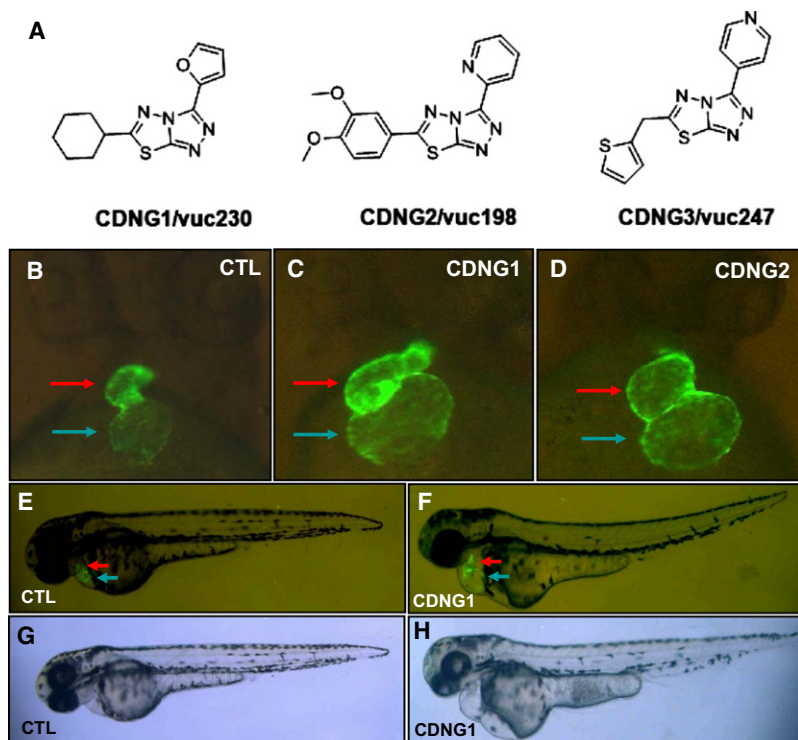
## RESULTS

### In Vivo Chemical Screens for Small-Molecule Modulators of Heart Development

The size of the zebrafish embryonic heart primarily reflects the number and size of cardiomyocytes during development (Jia et al., 2007). We hypothesized that screening for small molecules that increase heart size in zebrafish embryos may identify

compounds that induce cardiomyocyte differentiation and proliferation without causing pleiotropic effects. To test this hypothesis, we conducted an in vivo cardiac development screen using transgenic zebrafish embryos (*Tg[cmlc2:EGFP]*) in which expression of enhanced green fluorescent protein (EGFP) is under the control of the *cardiac myosin light chain 2 (cmlc2)* promoter (Burns et al., 2005). The primary focus of our screen was heart size, which was assessed by visual inspection using fluorescent microscopy. We adopted and modified a small-molecule screening procedure using zebrafish embryos (Peterson et al., 2004; Stern et al., 2005). Briefly, aliquots of test compounds were delivered into individual wells of 96-well tissue-culture plates (Figure 1A). *cmlc2-EGFP* transgenic embryos were harvested from crosses of transgenic fish and added to test wells at 5 hr postfertilization (hpf), the onset of gastrulation when cardiac progenitor cells begin to form (Stainier, 2001). We examined embryonic heart size, cardiac morphology, and contractility of treated embryos at 24, 48, and 72 hpf. In addition, overall morphologies of embryos (e.g., dorsal-ventral and anterior-posterior axes) and other organs, including the brain, eyes, notochord, and somites, were carefully examined to determine whether development of these organs was affected, providing a preliminary assessment of compound selectivity and toxicity.

A subset of the small-molecule library (4,000 compounds with diverse structures) provided by the Vanderbilt Institute of Chemical Biology was screened. Sixty-one compounds were identified as having significant effects on heart size, morphology, and contractility. Of these compounds, 15 caused an enlarged heart phenotype, 17 induced ectopic EGFP expression, 13 caused arrhythmias, and 16 delayed development at the heart tube stage. Notably, almost all bioactive compounds were hydrophobic, as indicated by positive log P values (the partition coefficient between octanol and water), suggesting that hydrophobic compounds effectively penetrate zebrafish embryos (Figure 1B). This observation is consistent with the results of previous small-molecule screens in zebrafish (Sachidanandan et al., 2008). *vuc230*, *vuc198*, and *vuc247*, named as Cardionogen (CDNG)-1, -2 and -3, were among the most potent inducers of a large-heart phenotype identified in our screen. Notably, these small molecules are structurally related and contain the same core motif, [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole



**Figure 2. Cardiogen Increases Heart Size during Zebrafish Development**

(A) Chemical structures of the Cardiogen family, including CDNG1/vuc230, CDNG2/vuc198, and CDNG3/vuc247.

(B–F) Fluorescent optics displaying untreated control heart (CTL) (B), CDNG1-treated heart (C), CDNG2-treated heart (D), untreated control embryo (CTL) (E), and CDNG1-treated embryo (F) in *Tg[cmic2:EGFP]* embryos at 60 hpf. (G and H) Light optics showing control embryos (CTL) (G) and CDNG1-treated embryos (H) at 60 hpf.

(B–D) Ventral view; (E–H) lateral view. Red arrow, ventricle; blue arrow, atrium. CDNG1 treatment (30  $\mu$ M, 5 to 60 hpf).

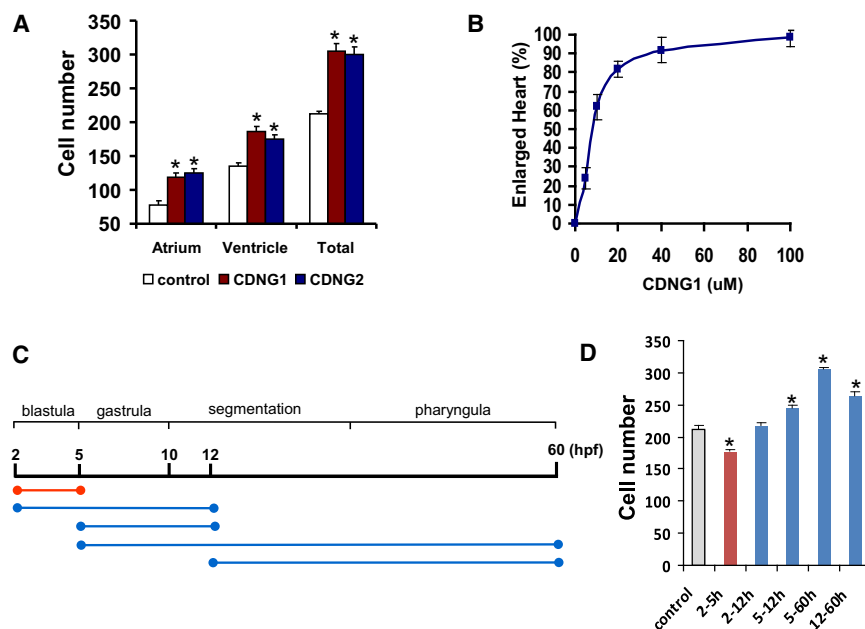
(Figure 2A). Treatment with Cardiogen-1/vuc230 (IUPAC: 6-cyclohexyl-3-furan-2-yl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole) or Cardiogen-2/vuc198 (IUPAC: 6-(3,4-dimethoxyphenyl)-3-pyridin-2-yl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole) significantly enlarged both the atrium and ventricle (Figures 2B–2D). Cardiogen-3/vuc247 (IUPAC: 3-pyridin-4-yl-6-(thiophen-2-ylmethyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole) affected heart size to a lesser extent than Cardiogen-1 and -2 (data not shown). Cardiogen treatment did not cause apparent defects of overall embryonic morphology or other organ development (Figures 2E–2H), suggesting that this class of small molecules has selective activity on heart development and growth.

### Cardiogen Induces Cardiac Hyperplasia in a Biphasic Manner

To assess whether heart enlargement is due to hyperplastic and/or hypertrophic growth, we evaluated cardiac cell number, cell size, and proliferative rates after treatment with both Cardiogen-1 and -2. We quantified total cardiomyocyte number in *Tg[cmic2:DsRed-nuc]* embryos in which a gene encoding red fluorescent protein fused to a nuclear localization signal (*DsRed-nuc*) is under the control of the *cmic2* promoter (Mably et al., 2003). In these transgenic embryos, individual cardiomyocyte nuclei are marked by red fluorescence, permitting quantitative assessment of cardiomyocyte number using confocal microscopy analysis (Figure S1A). To this end, we compared Cardiogen-treated embryos versus controls in a series of flat-mount confocal sections. Cardiogen treatment caused an increase of cardiac cell numbers in both the atrium and ventricle (Figure 3A). To test whether hypertrophy of cardiomyocytes also contributed to increases in heart size, we measured cell size in *Tg[cmic2:EGFP]* embryos using confocal microscopy. No alter-

tations in cell size were observed with Cardiogen treatment (Figure S1B). Whole-mount immunohistochemistry assays ( $n = 10$  embryos) using antibodies that recognize the mitosis marker phosphohistone 3 (H3P) revealed that cardiomyocyte proliferation was not increased in Cardiogen-1-treated embryos in comparison to control embryos (data not shown). Dosage-response analyses revealed that Cardiogen exponentially reached its optimum activity at 40  $\mu$ M and maintained its peak activity at higher concentration ranges (Figure 3B). No differences in survivorship or general morphology were noted at any of the test concentrations. Taken together, our results indicate that Cardiogen enlarges heart size via cardiac hyperplasia, which is not attributed to increases in the proliferative growth of cardiomyocytes.

The results presented above suggest that Cardiogen may affect earlier stages of cardiac cell differentiation. To assess the temporal activity of Cardiogen, we quantified cardiomyocyte number in transgenic embryos (*Tg[cmic2:DsRed-nuc]*) after pulse treatments with Cardiogen-1. In the first set of pulse experiments, Cardiogen-1 was added at 2 hpf (the beginning of blastula) and washed away at progressively later stages of development, including 5 hpf (the onset of gastrula) and 12 hpf (the 5-somite stage), when *nkx2.5* starts to express (Figure 3C). Cardiac cell counts were conducted at 60 hpf. Cardiogen treatment had an inhibitory effect on cardiomyocyte generation with treatment from 2 hpf to 5 hpf ( $178 \pm 3$ ) in comparison with controls ( $212 \pm 5$ ) (Figure 3D). Extension of Cardiogen treatment to 12 hpf recovered the loss of cells ( $219 \pm 4$ ) (Figures 3C and 3D). In a second set of pulse experiments, Cardiogen-1 was added at 5 hpf and washed away at 12 hpf or 60 hpf (Figure 3C). We quantified cell number at 60 hpf and observed increased cardiac cell numbers with treatment from 5 hpf to 12 hpf ( $245 \pm 5$ ) and from 5 hpf to 60 hpf ( $305 \pm 4$ ), compared to controls ( $212 \pm 5$ ) (Figure 3D). In embryos treated from 12 hpf to 60 hpf, cardiomyocyte number was reduced from the peak but still showed a marked increase compared to controls ( $265 \pm 5$  versus  $212 \pm 5$ ) (Figures 3C and 3D). Together, these results suggest that Cardiogen has a temporally biphasic pattern of cardiomyogenic activity during development. Specifically, Cardiogen treatment promotes cardiogenesis during and after gastrulation, whereas treatment before gastrulation inhibits cardiomyocyte formation.



**Figure 3. Cardionogen Induces Cardiac Hyperplasia in a Biphasic Manner**

(A) Bar chart showing increased cardiac cell number in the atrium and ventricle in CDNG1- and CDNG2-treated embryos at 30 μM (5–60 hpf).

(B) Dose-response curve for heart enlargement with CDNG1 treatment. *Tg(cmlc2:EGFP)* (n = 30) embryos were treated at 5 hpf using CDNG1 compound at various concentrations (0–100 μM). Heart sizes were scored at 60 hpf. No differences in survivorship or general morphology were noted at any of the test concentrations.

(C) Schematic representation showing pulse treatment intervals used to define Cardionogen bioactivity period in cardiomyogenesis.

(D) Graph showing cardiomyocyte number in embryos treated by CDNG1 pulse during different stages. Three to five embryos were subjected to confocal imaging under each condition with CDNG1 treatment (30 μM), and cardiomyocyte numbers were counted using confocal microscopy analysis. All cell counts were conducted at 60 hpf. Error bars indicate the standard deviation in three independent measurements (A, B, and D). Asterisks indicate statistical significance between treated and control embryos (\*p < 0.01) (A and D). See also Figure S1.

### Cardionogen Causes Expansion of Cardiac Progenitor Cells

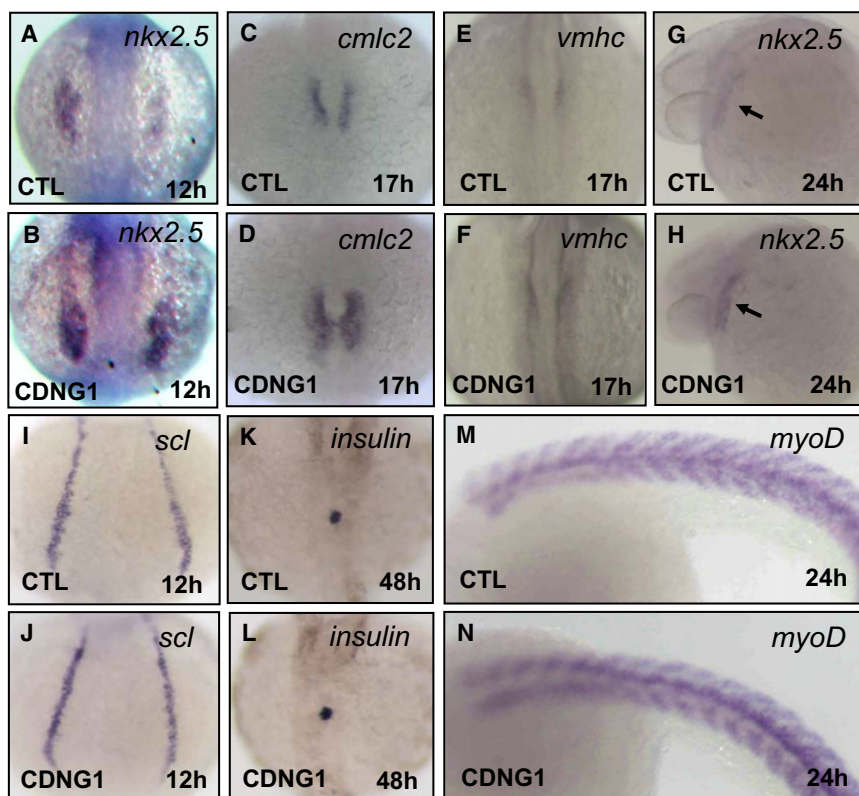
To understand how Cardionogen treatment regulates cardiogenesis, we performed in situ hybridization analyses with the cardiac progenitor marker *nkx2.5* and cardiac differentiation markers *cmlc2* and *ventricular myosin heavy chain (vmhc)* (Yelon et al., 1999). In Cardionogen-1-treated embryos, marked increases in *nkx2.5* expression were observed in the anterior lateral plate mesoderm, the definitive heart field (the heart-forming region) (Schoenebeck et al., 2007) at 12 hpf, as well as the heart tube stage at 24 hpf (Figures 4A, 4B, 4G, and 4H). Cardiomyocyte populations of Cardionogen-treated embryos were also expanded at the onset of myocardial differentiation, as evidenced by increased expression of *cmlc2* and *vmhc* at 17 hpf (Figures 4C–4F). In contrast, expression of *scl*, a hematopoietic progenitor marker in the head and the lateral mesoderm, was not affected in treated embryos compared to controls (Figures 4I and 4J). Similarly, expression of *insulin*, a pancreas marker, and *myoD*, a skeletal myoblast marker, did not appear to be affected in Cardionogen-treated embryos (Figures 4K–4N). These observations indicate that Cardionogen promotes cardiac hyperplasia via expanding the cardiac progenitor cell population.

### Cardionogen Promotes Mouse ES Cells to Differentiate into Beating Cardiomyocytes

To examine Cardionogen activity in a mammalian model, we employed a murine ES cell line (*Tg[αMHC:DsRed-Nuc]*) that was stably transfected with a *DsRed-Nuc* construct driven by the promoter of *α-myosin heavy chain (αMHC)* (Hao et al., 2008; Subramaniam et al., 1991). In this transgenic line, differentiating cardiomyocytes are marked by prominent nuclear red fluorescence (Hao et al., 2008). Embryoid bodies (EBs) were initiated (marked as day 0) in cultured ES cells. Pulse treatment of Cardionogen-1 during the initial ES cell differentiation from day 0 to day 4 failed

to induce cardiomyocyte differentiation when examined at day 12 (data not shown). Cardionogen-1 treatment from day 4 to day 10 significantly promoted ES cell differentiation into cardiomyocytes that express *DsRed* (Figures 5A–5D). We have found that Cardionogen-1 at 1 μM and 5 μM, but not at 0.1 μM, induces ES cell cardiac differentiation (Figures 5A–5D; data not shown). This treatment period coincides with the late differentiation of three germ layers in the EBs. Furthermore, cardiomyocytes generated from treated EBs formed foci that spontaneously and rhythmically contracted (Movies S1 and S2 available online). Finally, we determined the fraction of ES cell culture expressing *αMHC* using flow cytometry. Cardionogen treatment from day 4 to day 10 increased the cardiac cell percentage 4.36-fold (Figures 5E–5G). The overall cardiac differentiation percentages are consistent with previous studies using Wnt3a or GSK3β inhibitor BIO (Naito et al., 2006; Ueno et al., 2007).

To further assess the induction of cardiac cell differentiation, we examined expression of cardiac differentiation markers by real-time PCR. Treating ES cells from day 4 to day 10 with Cardionogen-1 and -2 significantly increased expression of cardiac sarcomere genes, including *βMHC*, *αMHC*, *myosin light chain-2a (MLC-2a)*, and *MLC-2v* (Figure 5H), whereas pulse treatment of ES cells with Cardionogen from day 0 to day 4 downregulated expressions of these four cardiac markers in RNA samples prepared at day 12 (Figure 5H). Furthermore, Cardionogen treatment of ES cells starting at day 4 induced the expression of cardiac progenitor cell marker *islet* (Figure 5H). Hence, Cardionogen treatment after the appearance of cells representing the different germ layers promoted cardiomyocyte differentiation, whereas treatment before this stage led to a decrease in cardiogenesis. These studies reveal a biphasic pattern of the cardiomyogenic activity of Cardionogen during murine ES cell differentiation, and the results closely mimic those obtained from our studies in zebrafish.



**Figure 4. Cardiogen Promotes Expansion of the Cardiac Progenitor Cell Population**

Expression of *nkx2.5* (A and B), *cmlc2* (C and D), *vmhc* (E and F), *nkx2.5* (G and H), *scl* (I and J), *insulin* (K and L), and *myoD* (M and N) in CDNG1-treated and control embryos was examined using in situ hybridization. Expression of *nkx2.5* [39 of 43 embryos in (A) and (B)], *cmlc2* [42 of 47 embryos in (C) and (D)], *vmhc* [41 of 45 embryos in (E) and (F)], and *nkx2.5* [38 of 41 embryos in (G) and (H)] is increased. Expression of *scl* (41 of 43 embryos), *insulin* (42 of 45 embryos), and *myoD* (39 of 42 embryos) is not altered. Images were taken at 12 hpf (A, B, I, and J), 17 hpf (C–F), 24 hpf (G, H, M, and N), and 48 hpf (K and L). Dorsal (A–F and I–L) and lateral views (G, H, M, and N) are shown. CTL, control; black arrow, heart tube. CDNG1, 30  $\mu$ M.

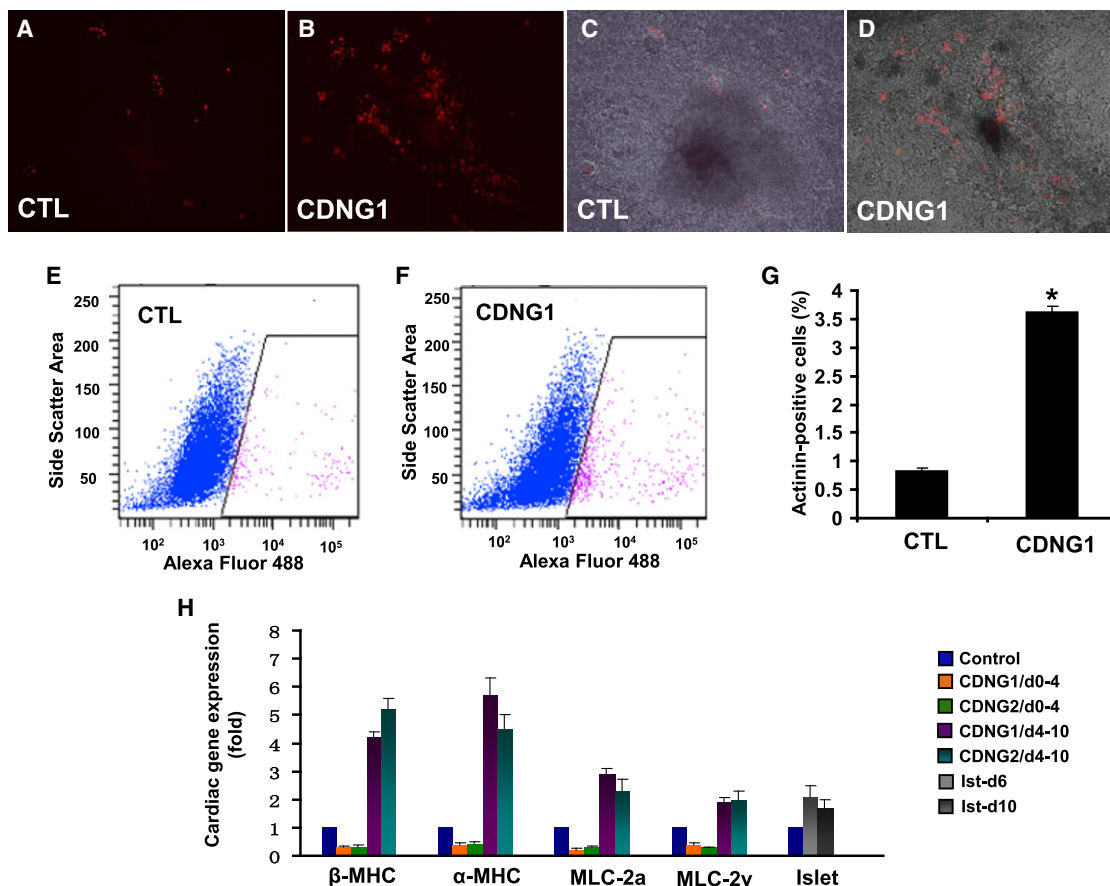
#### Cardiogen Inhibits Wnt/ $\beta$ -Catenin-Mediated Transcription

Cardiogen promotes cardiomyocyte generation following gastrulation and inhibits heart formation prior to gastrulation. Inversely, Wnt/ $\beta$ -catenin signaling during and after gastrulation inhibits cardiogenesis, whereas signaling before gastrulation induces heart formation (Naito et al., 2006; Ueno et al., 2007). These apparently opposing activities of Cardiogen and Wnt/ $\beta$ -catenin signaling led us to hypothesize that Cardiogen modulates heart development by antagonizing Wnt/ $\beta$ -catenin activity. The canonical Wnt/ $\beta$ -catenin pathway is a highly conserved signaling pathway whereby activation of Wnt signaling causes  $\beta$ -catenin to translocate into the nucleus and bind to Tcf and Lef transcription factors, resulting in activation of downstream gene expression (Logan and Nusse, 2004; Moon et al., 2004). To determine whether Cardiogen opposes Wnt/ $\beta$ -catenin activity, we performed  $\beta$ -catenin/Tcf-mediated transcription assays. We employed a murine CGR8 embryonic stem cell line that was stably transfected with a TOPflash construct containing six copies of the Tcf/Lef binding site upstream of the thymidine kinase minimal promoter and luciferase cDNA (Ishitani et al., 1999). In this assay, Wnt3a was used to activate TOPflash luciferase activity. Cardiogen-1 inhibited Wnt3a/ $\beta$ -catenin-mediated luciferase activity in a dose-dependent manner. Effector Cardiogen concentrations for half-maximal response ( $EC_{50}$ ) and maximal response are 23 nM and 100 nM, respectively (Figure 6A). IWR1, a known Wnt inhibitor (Chen et al., 2009), reduced TOPflash activity, as a positive control (Figure 6A). In contrast, Cardiogen-1 failed to inhibit TOPflash activity induced by  $\Delta$ NLef-VP16 (Figure 6B).  $\Delta$ NLef-VP16 can

activate TOPflash activity independently of catenin, through a process in which  $\Delta$ NLef, lacking a  $\beta$ -catenin binding site, fuses with transactivation domain VP16 (Aoki et al., 1999). As a control, IWR1 also failed to reduce  $\Delta$ NLef-VP1-induced TOPflash activity (Figure 6B). Together, these findings suggest that Cardiogen inhibits  $\beta$ -catenin-dependent Wnt signaling.

We next examined whether Cardiogen disrupted Wnt/ $\beta$ -catenin signaling within responding cells. LRP6ICD, a Wnt coreceptor, can constitutively activate Wnt signaling within cells (Tahinci et al., 2007; Tamai et al., 2004). GCR8-ES cells were transfected with LRP6ICD. Cardiogen-1 treatment resulted in a reduction of LRP6ICD-induced TOPflash activity, whereas IWR1 caused loss of the same luciferase activity (Figure 6C). Thus, Cardiogen blocks Wnt/ $\beta$ -catenin signaling within responding cells rather than disrupting ligand-cell interaction or ligand production. Finally, we determined whether Cardiogen treatment altered other signaling activities. Cardiogen-1 did not reduce BMP4-induced Id2 expression, whereas Dorsomorphin (DM), a known BMP4 inhibitor, did reduce Id2 expression (Figure 6D). Furthermore, Cardiogen-1 failed to alter Notch- or SRF/MAPK-induced transcription in ES cells (Figures S2A and S2B). These findings demonstrate the Cardiogen specificity for Wnt signaling.

To test whether Cardiogen inhibits Wnt signaling in developing embryos, we examined the effects of Cardiogen on GFP expression in *Tg(TOP:GFP)* embryos, in which GFP transgene expression is under the control of four consensus Lef binding sites and a minimal cFos promoter (Dorsky et al., 2002). In *Tg(TOP:GFP)* embryos, GFP fluorescence is only observed in the midbrain using fluorescent microscopy analysis (Figures 6E and 6F) (Dorsky et al., 2002). We were unable to observe GFP fluorescence in other embryo regions, which is consistent with previous studies (Dorsky et al., 2002). Nevertheless, treating *Tg(TOP:GFP)* embryos with Cardiogen-1 reduced GFP fluorescence in the midbrain (Figures 6G and 6H). As a control, IWR1 treatment caused loss of GFP fluorescence



**Figure 5. Cardionogen Induces Murine ES Cells to Differentiate into Cardiomyocytes**

(A and B) Fluorescent optics revealing myocardial differentiation (red areas) in 0.1% DMSO-treated (CTL) and 1  $\mu$ M CDNG1-treated CGR8-ES cells (*Tg[ $\alpha$ MHC:DsRed-nuc*]).

(C and D) Bright-field pictures merged with fluorescent images of control (A) and CDNG1-treated cells (B).

(E and F) Flow-cytometry analyses revealing the fraction of ES cells expressing  $\alpha$ MHC:DsRed-nuc in 0.1% DMSO-treated and CDNG1-treated ES cells. The x axis is the intensity of Alexa Fluor 488 immunostaining; the y axis is the side scatter area.

(G) Analysis of sarcomeric  $\alpha$ -actinin by flow cytometry indicates that CDNG1 treatment enhances cardiomyocyte content 4.36-fold (from  $0.83 \pm 0.06\%$  to  $3.62 \pm 0.09\%$ ). CGR8-ES cells were treated with 0.1% DMSO and 1  $\mu$ M CDNG1 from day 4 to day 10 and analyzed at day 12.

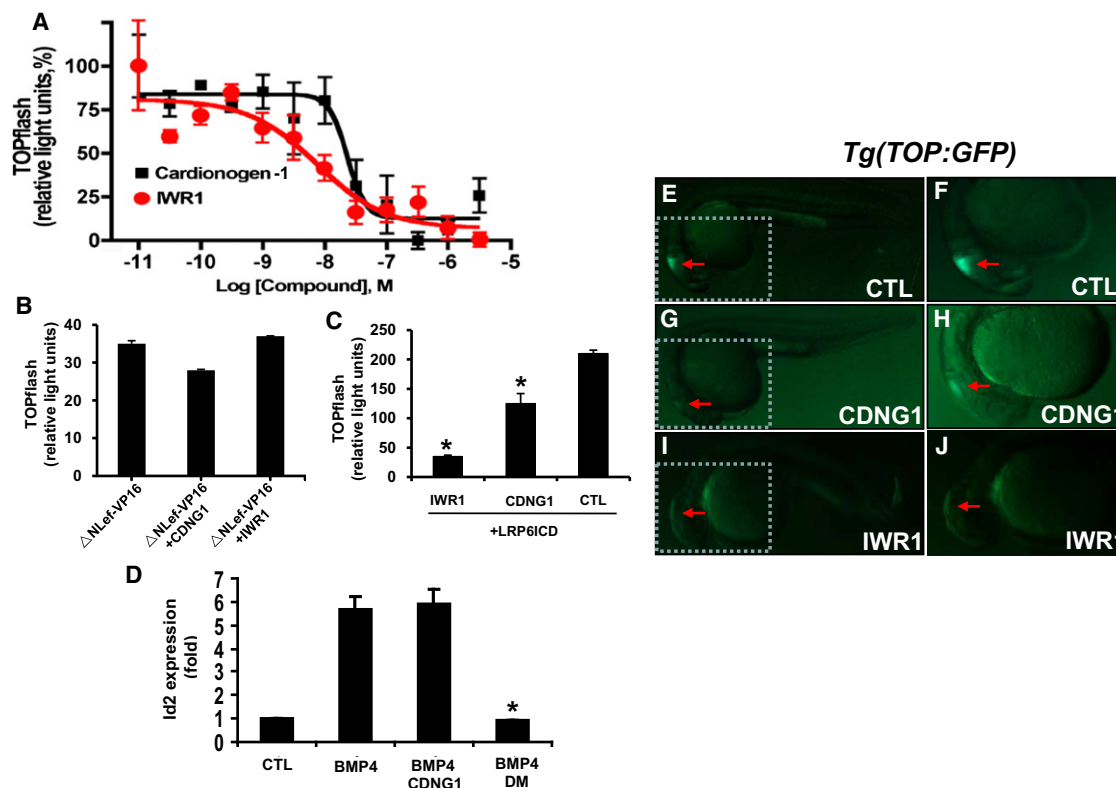
(H) Bar chart depicting relative expression folds of  $\beta$ MHC,  $\alpha$ MHC, MLC-2a, MLC-2v, and *islet* in 1  $\mu$ M CDNG1- and CDNG2-treated ES cells, compared to 0.1% DMSO-treated controls.  $\beta$ MHC,  $\alpha$ MHC, MLC-2a, and MLC-2v were examined at day 12; *islet* was examined at days 6 and 10. GAPDH was used as internal controls for normalization. CTL values were arbitrarily set to 1. Graphs (G and H) show mean  $\pm$  s.d., performed in triplicate; \*p < 0.01 compared with control. See also [Movies S1](#) and [S2](#).

in the same region (Figures 6I and 6J). Consistent with EC<sub>50</sub> values of Cardionogen-1 and IWR1 using TOPflash reporter assays in murine ES cells (23 nM versus 7.5 nM, respectively), these data indicate that Cardionogen reduces Wnt/ $\beta$ -catenin signaling activity in zebrafish embryos and, further, that IWR1 is a more potent Wnt-pathway antagonist than Cardionogen. Notably, IWR1 treatment resulted in defects in the anterior-posterior body development and loss of tail structure posterior to the yolk extension (Figures 6I, S3A, and S3B). We next examined whether IWR1 affects heart development. At early stages, IWR1 treatment caused an increased expression of cardiac progenitor cell marker *nkx2.5* (Figures S3C and S3D) and cardiac differentiation marker *cmlc2*, compared to controls (Figures S3E and S3F). However, at late stages, IWR1-treated embryos displayed defects in cardiac chamber formation. Wild-type embryos form two distinct cardiac chambers (atrium and ventricle)

(Figures S3G and S3I). In contrast, IWR1-treated embryos develop only one cardiac chamber (Figures S3H and S3J). We determined the chamber identity using a ventricular marker, *vmhc*, and an atrial marker, *atrial myosin heavy chain (amhc)*. *vmhc* expression analyses indicated that the single chamber formed in IWR1-treated embryos possessed ventricular identity compared to controls (Figure S3K and S3L). *amhc* expression analyses revealed atrial myocytes aligned bilaterally in IWR1-treated embryos, resulting in failure to form the atrium, compared to controls (Figure S3M and S3N).

#### Cardionogen Reverses Wnt-Induced Cardiac Phenotypes

Since Cardionogen represses  $\beta$ -catenin/Tcf-mediated transcription in murine cells and zebrafish embryos, we evaluated whether Cardionogen can rescue Wnt-induced cardiac inhibitory



**Figure 6. Cardionogen Inhibits Wnt3a/β-Catenin-Mediated Transcription**

(A) Cardionogen-1 inhibits Wnt3a-induced TOPflash activity in ES cells. CGR8-ES cells were treated with Wnt3a (100 ng/ml)-conditioned media plus CDNG1 or IWR1 compounds in a series of concentrations. Dose-response curves represent TOPflash activities normalized to cell number (mean ± s.d.; performed in quadruplicate). The calculated EC<sub>50</sub> values for Cardionogen-1 and IWR1 are 23 nM and 7.5 nM, respectively. Graphs were made in Prism 4 (GraphPad Software) with nonlinear regression fit to a sigmoidal dose-response curve.

(B) Cardionogen does not inhibit Lef/Tcf transcription that is independent of β-catenin activity. CGR8-ES cells were transfected with ΔNLeF-VP16 and treated with 1 μM Cardionogen-1 or 1 μM IWR1. Graph represents TOPflash activities (mean ± s.d.; performed in triplicate).

(C) Cardionogen inhibits LRP6-mediated Wnt signaling. CGR8-ES cells were transfected with a constitutively active LRP6ICD and treated with 1 μM Cardionogen-1, 1 μM IWR1, or 0.1% DMSO (CTL). TOPflash activity is graphed (mean ± s.d.; performed in triplicate; \*p < 0.01).

(D) Bar chart showing relative expression fold of BMP4-induced Id2 expression (Hua et al., 2006; Nakahiro et al., 2010) in the presence of CDNG1, BMP4, BMP4+CDNG1, BMP4+DM, compared to its expression in 0.1% DMSO (CTL). Concentrations were 30 ng/ml for Bmp4, 1 μM for CDNG1, and 1 μM for DM. Id2 expression normalized to GAPDH is graphed (mean ± s.d.; performed in triplicate; \*p < 0.01). CTL values were arbitrarily set to 1.

(E–J) Images taken at 24 hpf (see also Figures S2 and S3). Red arrows indicate GFP expression.

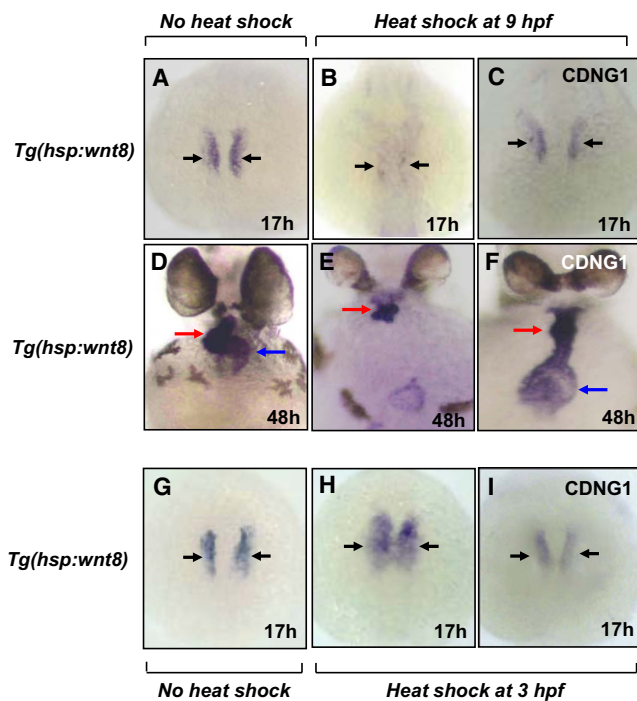
(E and F) Fluorescent optics revealing GFP fluorescence in the midbrain in *Tg(TOP:GFP)* embryos [outlined area enlarged in (F)].

(G and H) CDNG1 treatment (30 μM; 5–24 hpf) reduces GFP fluorescence in the midbrain of *Tg(TOP:GFP)* embryos [outlined area enlarged in (H)].

(I and J) IWR1 treatment (30 μM; 5–24 hpf) eliminates GFP fluorescence in the midbrain of *Tg(TOP:GFP)* embryos [outlined area enlarged in (J)].

phenotypes in zebrafish embryos. To test this possibility, we used transgenic zebrafish embryos (*Tg[hsp:wnt8-EGFP]*), in which *wnt8* fused to *EGFP* is under the control of a heat-shock promoter (Ueno et al., 2007). We heat shocked transgenic embryos to elevate *wnt8* expression at the end stage of gastrulation (9 hpf), then treated these embryos with Cardionogen-1. Although cardiomyocyte formation and *cmlc2* expression are normally inhibited in *Tg[hsp:wnt8-EGFP]* embryos after heat shock (Figures 7A and 7B), Cardionogen-1 treatment largely rescued cardiomyocyte deficiency labeled by *cmlc2* expression (Figure 7B and 7C). The same cardiac rescue effects were also observed by monitoring *nkx2.5* expression at the 8-somite stages (data not shown). Remarkably, the rescue effects persisted through late stages of heart development. Although *wnt8*-induced embryos failed to form the atrium and a large part

of the ventricle at 48 hpf (Figures 7D and 7E), Cardionogen-1 treatment completely restored the atrium and ventricle, albeit with looping defects persisting (Figures 7E and 7F). Notably, the small eye size induced by *wnt8* overexpression was not rescued in embryos with Cardionogen treatment (Figures 7D–7F), suggesting that Cardionogen may block Wnt signaling in the cardiac mesoderm but not in other tissues. In a second set of experiments, we examined whether Cardionogen could inhibit expansion of cardiac cell domains induced by *wnt8* overexpression before gastrulation. Transgenic embryos (*Tg[hsp:wnt8-EGFP]*) were heat shocked to induce *wnt8* expression at 3 hpf before gastrulation; this was followed by treatment with Cardionogen-1. Although *cmlc2* expression was expanded in the lateral plate mesoderm of heat shocked control embryos (Figures 7G and 7H), Cardionogen-1 treatment reduced the



**Figure 7. Cardionogen Rescues Wnt8-Induced Cardiac Phenotypes**

(A–C) *cmlc2* expression in non-heat-shocked embryos (A), heat-shocked embryos (B), and CDNG1-treated heat-shocked embryos (C). Of 25 embryos, 23 were rescued.

(D–F) Rescue of heart formation labeled by *cmlc2* expression in CDNG1-treated heat-shocked embryos (F) compared to heat-shocked embryos (E) and non-heat-shocked embryos (D). Of 26 embryos, 25 were rescued.

(G–I) Inhibition of cardiac expansion labeled by *cmlc2* expression in CDNG1-treated heat-shocked embryos (I), compared to heat-shocked embryos (H) and non-heat-shocked embryos (G). Of 24 embryos, 21 were inhibited. Heat shock was administered at 38.5°C for 15 min at 3 hpf (H and I) and 9 hpf (B, C, E, and F). Black arrow, cardiomyocytes; red arrow, ventricle; blue arrow, atrium.

expansion of the *cmlc2* domain (Figures 7H and 7I). Together, these findings demonstrate that Cardionogen reverses Wnt8-induced heart-specific phenotypes.

## DISCUSSION

In this study, we describe an *in vivo* small-molecule screen in zebrafish that is capable of identifying chemical modulators of cardiac development. To our knowledge, we have identified a family of novel small molecules (named Cardionogen) that enlarges the size of the embryonic heart by promoting cardiomyocyte formation. Cardionogen either induces or inhibits the expansion of cardiac progenitor cells, depending on the timing and stages of treatment. Importantly, we have linked Cardionogen to the Wnt signaling pathway. Cardionogen inhibits Wnt/ $\beta$ -catenin signaling activity in murine ES cells ( $EC_{50}$  of  $\sim 23$  nM) and zebrafish embryos. Cardionogen can rescue Wnt8-induced cardiomyocyte deficiency and heart-specific phenotypes during development. These findings demonstrate that a complex but sensitive development screen targeting organ size can identify both active small molecules and their target pathways.

Embryonic heart size primarily reflects cardiac-cell number and size. However, dysregulation of myocardial patterning and morphology may also cause alterations in overall heart size. As such, failure of concentric myocardial growth in *heart of glass*, *santa*, and *valentine* mutants leads to an enlarged-heart phenotype without changing cardiomyocyte number (Mably et al., 2006; Mably et al., 2003). Therefore, it is critical to determine whether bioactive small molecules enlarge heart size by modulating cardiomyocyte number, cell size, and/or myocardial patterning. Notably, only a few zebrafish genetic mutants have been identified as affecting cardiac cell number, which suggests that certain limitations (e.g., functional redundancy) may prevent identification of relevant genes and pathways in cardiomyocyte generation through mutagenesis studies. Small-molecule screens may overcome these obstacles by identifying compounds that interact with these important pathways. We observed that Cardionogen induces murine ES cells to differentiate into cardiomyocytes at 1  $\mu$ M and 5  $\mu$ M, but not at 0.1  $\mu$ M, indicating that a higher concentration is required to promote cardiac differentiation in EBs than the concentration (0.1  $\mu$ M) used to block Wnt signaling in undifferentiated ES cells. This might be due to the fact that these two assays are conducted at two distinct points of ES cell differentiation, each marked with stage-specific expression of various Wnt ligands (Schulz et al., 2009). From the TOPflash dose-response analyses, IWR1 is a more potent Wnt inhibitor than Cardionogen in murine ES cells. These findings are consistent with activities of Cardionogen and IWR1 in zebrafish *Tg(TOP:GFP)* embryos, in which GFP fluorescence in the midbrain is reduced in CDNG1-treated embryos but eliminated in IWR1-treated embryos. It is noted that the Cardionogen dose-response curve is steep compared to the IWR1 dose curve (Figure 6A), suggesting cooperative effects of Cardionogen in inhibiting TOPflash activity. Multiple Cardionogen molecules may bind to one target protein to reduce TOPflash activity quickly, resulting in a rise in the slope of the dose-response curve. However, to demonstrate this effect will require detailed molecular studies of the interaction of Cardionogen with its potential binding proteins in the Wnt pathway.

Wnt signaling is a key regulator of a variety of developmental processes, including primitive streak formation, mesoderm and endoderm induction and patterning, anterior-posterior (AP) axis development, neural differentiation, and heart formation (Kimelman, 2006; Logan and Nusse, 2004; Moon et al., 2004). Blocking Wnt signaling in zebrafish development after gastrulation with *dickkopf homolog 1* (*dkk1*) induces cardiac progenitor cell formation but truncates the posterior axis at late stages (Ueno et al., 2007). Similarly, we observed that IWR1 treatment increased the expression of cardiac progenitor cell marker *nkx2.5* but caused AP axis defects and disruption of atrium formation. Failure to form the atrium might be due to overall embryonic defects (i.e., AP axis abnormality in combination with other mesoderm defects) in IWR1-treated embryos. Notably, Cardionogen treatment induces cardiac progenitor cell formation without causing tail truncation and atrium disruption. In addition, Cardionogen rescued the Wnt8-induced heart defects but not the Wnt8-induced small-eye phenotypes. This demonstrates the benefits of a whole-organism-based chemical screen. We believe that the phenotypic differences between zebrafish treated with IWR1 and those treated with Cardionogen are



more likely due to differences in their mechanisms of Wnt inhibition rather than “off-target” effects, although the latter could be a possibility. Cardionogen might affect only a select population of Wnt-responding cells in embryos, considering that Cardionogen does not cause overall Wnt-dependent embryonic phenotypes but affecting Wnt signaling in the heart and the midbrain. The facts that IWR1 inhibits Wnt signaling in both HEK and ES cells and can cause typically Wnt-dependent embryonic phenotypes suggest that IWR1 antagonizes Wnt signaling in broader cell types and tissues compared to Cardionogen. Importantly, we have not observed any phenotype independent of Wnt signaling defects in embryos treated with IWR1, suggesting that the off-target effect is unlikely, or that if there is one, it is minimal. We wondered whether Cardionogen reduces Tcf/Lef-mediated luciferase activities in human embryonic kidney (HEK) cells. Notably, Cardionogen-1 does not inhibit Wnt3a-induced Topflash activity in HEK cells, whereas IWR1 does (Figure S2C). Thus, we propose that Cardionogen selectively reduces Wnt/ $\beta$ -catenin signaling activity in certain cell types and tissues (i.e., heart and others) during development. We speculate that Cardionogen may inhibit tissue (heart)-specific modifiers that activate Wnt signaling. Alternatively, Cardionogen might interfere with interactions between Wnt pathway components and tissue (heart)-specific transcriptional factors.

The core components in the Wnt signaling pathway have been clearly defined and studied. However, tissue-specific modifiers of the pathway remain unknown (Logan and Nusse, 2004; Moon et al., 2004). Identifying *in vivo* binding factors of Cardionogen may provide novel insights into the mechanisms related to tissue-specific modifiers of the Wnt signaling pathway. Recent studies have established that inhibition of the canonical Wnt pathway after the germ layer cell formation is necessary to promote cardiomyocyte differentiation from human cardiovascular stem cells (Yang et al., 2008). Our findings further support these human ES cell studies, as treatment of murine ES cells with Cardionogen after the germ layer cell formation promotes cardiac differentiation. Thus, evaluating the potential of Cardionogen on human adult and ES cells, along with other known cardiogenic small molecules (Hao et al., 2008; Sadek et al., 2008; Wu et al., 2004), is the next logical step in defining therapeutic regimens to enhance repopulation of infarcted myocardium and restore function in diseased hearts.

## SIGNIFICANCE

**There is intense interest in developing chemical probes or drug leads to influence cardiac differentiation from stem cells, as well as enhance regenerative capacities in the heart. Unfortunately, very few cardiogenic small molecules have been described. The zebrafish has emerged as an important animal model in multiple steps of drug discovery process. We have established an exciting approach to discover small molecules in inducing cardiomyocyte production in zebrafish embryos. Three compounds, Cardionogen 1–3, with the same core structure have been discovered. Characterization of these compounds revealed that the enlarged heart size in treated embryos is due to the expansion of cardiac progenitor cell population. Cardionogen induces murine ES cells to differentiate into beating**

**cardiomyocytes. We show that Cardionogen blocks Wnt/ $\beta$ -catenin-dependent transcriptional activity in both embryos and ES cells but not in HEK cells, suggesting that Cardionogen selectively reduces Wnt signaling activity associated with embryogenesis. Cardionogen treatment induces cardiogenesis without causing AP axis defects in zebrafish embryos. However, IWR1 treatment promotes cardiac differentiation but results in tail truncation and atrium disruption. Identifying *in vivo* binding factor of Cardionogen may provide novel insights into the mechanisms related to tissue (heart)-specific modifiers of Wnt signaling pathway. Our findings demonstrate that Cardionogen functions as a Wnt inhibitor for cardiomyogenesis. This may ultimately aid in design of therapeutic approaches for cardiac regeneration and repair.**

## EXPERIMENTAL PROCEDURES

### Zebrafish Strains and Maintenance

Zebrafish strains used in this study were raised according to standard procedures (Westerfield, 2000). Transgenic lines *Tg(cmlc2:EGFP)*, *Tg(cmlc2:DsRed-nuc)*, and *Tg(hsp:wnt8-EGFP)* have been previously described (Burns et al., 2005; Ueno et al., 2007) (Mably et al., 2003).

### Cardiac Phenotype-Based Small-Molecule Screen

Experimental compounds were obtained from Vanderbilt Institute of Chemical Biology. Aliquots (1  $\mu$ L) of the diluted chemical compounds (1 mM) were delivered into each of the wells using a Labcyte Echo 550. Immediately after aliquot addition, E3 buffer (69  $\mu$ L) was delivered into each well. Well12A served as a negative control (1% DMSO). Embryos were generated by crossing homozygous *Tg(cmlc2:EGFP)* transgenic fish. Harvested embryos were placed in E3 and incubated at 28.5°C. When embryos reached 5 hpf (50% epiboly), 3 embryos were transferred to each well in a 30- $\mu$ L aliquot of E3 buffer, bringing the final compound concentration to 10  $\mu$ M. Plates were incubated at 28.5°C and screened using fluorescent microscopy at 24, 48, and 72 hpf.

### Partition Coefficient Analysis

Log P values and molecular weights of bioactive compounds were obtained from the National Center for Biotechnical Information (<http://pubchem.ncbi.nih.gov>)

### Cardionogen Synthesis

Synthesis of the Cardionogen series (vuc198 and vuc230) was designed and conducted by Vanderbilt Chemical Synthesis Core. Compound identities were confirmed by  $^1\text{H}$  NMR.

### Analyses of Cardiac Cell Number, *In Situ* Hybridization, and Immunohistochemistry

The transgenic zebrafish lines *Tg(cmlc2:DsRed-nuc)* and *Tg(cmlc2:EGFP)* were employed to assess changes in cardiomyocyte number and size using confocal microscopy (Jia et al., 2007; Mably et al., 2003). Whole-mount *in situ* hybridizations were carried out as described (Zhong et al., 2001), using antisense ribonucleotide probes for *nkx2.5*, *cmlc2*, *vmhc*, *scl*, *myoD*, and *insulin*. Immunofluorescence was performed as described (Jia et al., 2007), using primary anti-phosphorylated histone H3 antibody (1:100; Santa Cruz Biotechnology) and secondary antibody Alexa Fluor 555 donkey anti-rabbit conjugate (1:200; Molecular Probes).

### Murine ES Differentiation Assay

Murine CGR8-ES cells were stably transfected with the  $\alpha$ -MHC-*DsRed-Nuc* plasmid, maintained, and differentiated as described (Hao et al., 2008). Cells were cultured on gelatin-coated culture plates with Glasgow Minimum Essential Medium (GMEM; Sigma) containing 10% heat-inactivated FBS, 20  $\mu$ M L-Glutamine, 50  $\mu$ M  $\beta$ -Mercaptoethanol, and 100 units/ml Leukemia Inhibitory Factor (ESGRO-Chemicon). CGR8-ES cells were differentiated in Iscove's

Modified Dulbecco's Medium (IMDM; GIBCO) containing 20% heat-inactivated FBS, 16  $\mu$ M L-Glutamine, nonessential amino acids, and 80  $\mu$ M  $\beta$ -Mercaptoethanol. EBs were initiated (day 0) and grown in hanging drops for two days. Each EB initially consisted of 500 cells in 20  $\mu$ l of IMDM differentiation medium. Formed EBs were washed down into an uncoated Petri dish and suspended in IMDM differentiation medium for two more days. On day 4, EBs were transferred to gelatin-coated plates, allowed to attach, and incubated in differentiation medium until the analyses on day 12. The medium was replaced every 48 hr, and differentiating cell cultures were microscopically examined for the presence of contractile cardiomyocytes marked by red fluorescence. Cells were harvested at day 12 and RNAs were isolated using RNeasy kit (QIAGEN) for real-time RT-PCR (see [Supplemental Experimental Procedure](#)).

#### Fluorescence-Activated Cell Sorting

EBs created with CGR8-ES cell line were washed in 1X PBS and dissociated with collagenase (20mg/ml) in 1X PBS. These cells were centrifuged for 3.5 min at 3500 rpm. Supernatant was removed and cells were washed with fluorescence-activated cell sorting buffer (1X PBS and 5% Fetal bovine serum). Washed cells were fixed in 2% PFA/PBS for 10 min, and probed using the first  $\alpha$ -Actinin monoclonal antibody (Sigma; 1:2,000 dilution), then the secondary antibody goat anti-mouse IgG Alexa Fluor 488 (Invitrogen; 1:200 dilution). Cells were sorted on LSR-II (BD Biosciences) and analyzed using FACS Diva v6.1.3 software.

#### Reporter Assay

Murine CGR8-ES cells were transfected with constructs of pTOPflash reporter (Upstate Biotechnology Inc., now Millipore), RBP-Jk reporter, or SRF/MAPK reporter (SABiosciences), using Lipofectamine 2000 transfection reagent (Invitrogen). These transfected cells were grown in feeder-free conditions as monolayers in GMEM medium supplemented with 10% FBS, 100 units/ml LIF, 2 mM L-glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol in a humidified 5% CO<sub>2</sub> atmosphere at 37°C, as described (Meyer et al., 2000). HEK293 cells were maintained in DMEM, 10% FBS, and antibiotics. Cells were then lysed with 1X Passive Lysis Buffer (Promega). Luciferase activity was measured by Steady-Glo Luciferase Assay (Promega) on a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) and normalized to viable cell number using the CellTiter-Glo Assay (Promega). Graphs were made in Prism 4 (GraphPad Software) with nonlinear regression fit to a sigmoidal dose-response curve.

#### Wnt8-Induced Cardiac Phenotype Assays

*Tg(hsp:wnt8-EGFP)* fish were out-crossed to the wild-type AB line. Progeny embryos were all heterozygous for the transgene. Embryos at 3 hpf or 9 hpf were placed in 15-ml Falcon tubes with E3 buffer. The tubes were then submerged in a 38.5°C water bath for 15 min for heat shock, while non-heat-shock control embryos were maintained at 25°C. After heat shock, embryos were washed with E3 at room temperature. Heat-shocked embryos were either treated with CDNG1 (90  $\mu$ M) or returned to E3 buffer as controls. All embryos were incubated at 25°C until fixation at 17 hpf or 48 hpf for in situ hybridization analyses.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two movies, and Supplemental Experimental Procedures, and can be found with this article online at doi:10.1016/j.chembiol.2011.09.015.

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