Supporting Information

Small molecules that direct mouse embryonic stem cell cardiomyocyte differentiation

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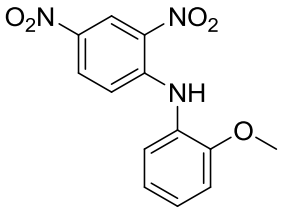
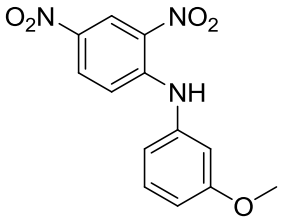
Synthesis of 2,4-dinitroanilines

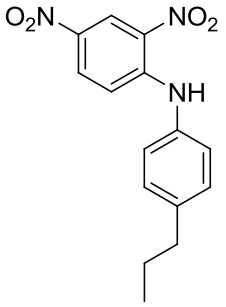
To a flask containing THF was added 1 equiv of 2,4-dinitrofluorobenzene and 1.1 equiv of the appropriate amine. The reaction mixture was stirred at room temperature overnight. After stirring overnight the solvent was concentrated *in vacuo* to give a crude solid that was recrystallized from ethanol or purified by flash chromatography (silica gel 19:1 v/v DCM/MeOH).



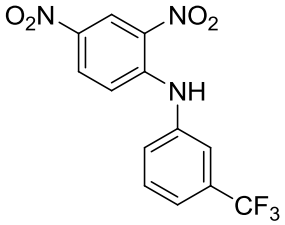
***N*-(2-Methoxyphenyl)-2,4-dinitrobenzenamine 4.** ^{1}H NMR (300 MHz $CDCl_3$); δ 3.87 (3 H, s), 7.03 – 7.08 (2 H, m), 7.11 (1 H, d, *J* = 9.6 Hz), 7.32 – 7.36 (2 H, m), 8.16 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.18 (1 H, d, *J* = 2.5 Hz), 9.88 (1 H, bs).



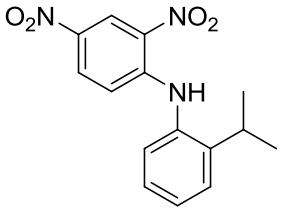
***N*-(3-Methoxyphenyl)-2,4-dinitrobenzenamine 5.** ^{1}H NMR (300 MHz $CDCl_3$); δ 3.85 (3 H, s), 6.83 – 6.93 (3 H, m), 7.22 (1 H, d, *J* = 9.6 Hz), 7.37 – 7.43 (1 H, m), 8.17 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.18 (1 H, d, *J* = 2.5 Hz), 9.94 (1 H, bs).



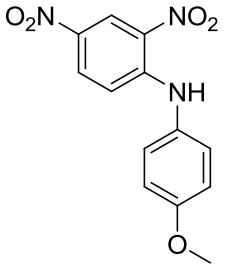
***N*-(4-*n*-Propylphenyl)-2,4-dinitrobenzenamine 6.** ^{1}H NMR (300 MHz $CDCl_3$); δ 0.98 (3 H, t, *J* = 7.2 Hz), 1.69 (2 H, m), 2.65 (2 H, t, *J* = 7.2 Hz), 7.14 (1 H, d, *J* = 9.6 Hz), 7.20 (2 H, d, *J* = 8.2 Hz), 7.30 (2 H, d, *J* = 8.2 Hz), 8.15 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.18 (1 H, d, *J* = 2.5 Hz), □9.95 (1 H, bs).



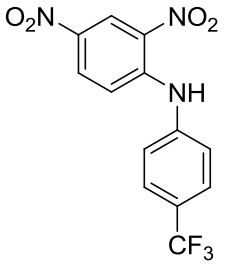
***N*-(3-Trifluoromethylphenyl)-2,4-dinitrobenzenamine 7.** ^{1}H NMR (300 MHz $CDCl_3$); δ 7.18 (1 H, d, *J* = 9.6 Hz), 7.51 – 7.66 (4 H, m,), 8.24 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.19 (1 H, d, *J* = 2.5 Hz), 9.99 (1 H, bs).



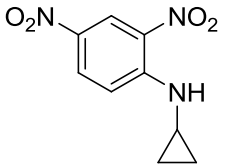
***N*-(2-Isopropylphenyl)-2,4-dinitrobenzenamine 8.** ^{1}H NMR (300 MHz $CDCl_3$); δ 1.23 (d, J = 6.9 Hz, 6 H) 3.10 (m, 1H), 6.82 (d, J = 9.6 Hz, 1H), 7.20 – 7.26 (m, 1H), 7.29 – 7.35 (m, 1H), 7.38 – 7.49 (m, 2H), 8.11 – 8.15 (m, 1H), 9.18 (d, J = 2.7 Hz, 1H) 9.85 (bs, 1H)



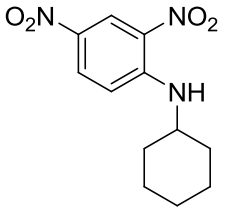
***N*-(4-Methoxyphenyl)-2,4-dinitrobenzenamine 9.** ^{1}H NMR (300 MHz $CDCl_3$); δ 3.87 (3 H, s), 7.00 – 7.03 (3 H, m), 7.22 (2 H, d, *J* = 8.8 Hz), 8.14 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.17 (1 H, d, *J* = 2.5 Hz), 9.88 (1 H, bs).



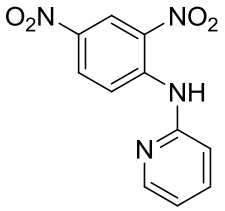
***N*-(4-Trifluoromethylphenyl)-2,4-dinitrobenzenamine 10.** ^{1}H NMR (300 MHz $CDCl_3$); δ 7.30 (1 H, d, *J* = 9.6 Hz), 7.53 (2 H, d, *J* = 8.5 Hz m), 7.76 (2 H, d, *J* = 8.5 Hz), 8.25 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 9.19 (1 H, d, *J* = 2.5 Hz), 10.01 (1 H, bs).



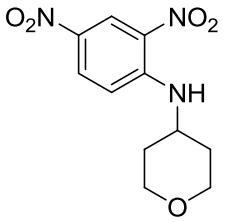
***N*-Cyclopropyl-2,4-dinitrobenzenamine 11.** ^{1}H NMR (300 MHz $CDCl_3$); δ 0.72 – 0.78 (2 H, m), 1.01 – 1.08 (2 H, m), 2.67 – 2.74 (1 H, m), 7.40 (1 H, d, *J* = 9.6 Hz), 8.31 (1 H, dd, *J* = 9.6 Hz, *J* = 2.5,), 8.56 (1 H, bs), 9.12 (1 H, d, *J* = 2.5 Hz).



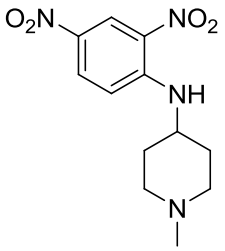
***N*-Cyclohexyl-2,4-dinitroaniline 12.** ^{1}H NMR (300 MHz CDCl3); δ 1.35 – 2.09 (10H, m), 3.61 (1H, s), 6.93 (1H, d, *J* = 9.6 Hz), 8.23 (1 H, dd, *J* = 2.8 Hz and *J* = 9.6 Hz), 8.62 (1H, bs), 9.13 (1 H, d, *J* = 2.8 Hz).



***N*-(2,4-Dinitrophenyl)pyridin-2-amine 13**. ^{1}H NMR (300 MHz CD_3OD); δ 3.93 (2 H, bs), 6.65 (1 H, bs), 6.78 (1 H, d), 6.83 – 6.88 (1 H, m), 7.55 – 7.63 (2 H, m), 7.70 – 7.74 (2 H, m), 8.25 – 8.27 (1 H, m).



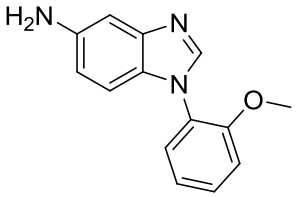
***N*-(2,4-Dinitrophenyl)tetrahydro-2H-pyran-4-amine 14**. ^{1}H NMR (300 MHz $CDCl_3$); δ 1.67 – 1.81 (2 H, m), 2.07 – 2.13 (2 H, m), 3.55 – 3.64 (2 H, m), 3.78 – 3.90 (1 H, m), 4.03 – 4.09 (2 H, m), 6.78 (1 H, d, *J* = 9.5), 8.27 (1 H, dd, *J* = 9.5 Hz and *J* = 2.5), 9.15 (1 H, d, *J* = 2.5).



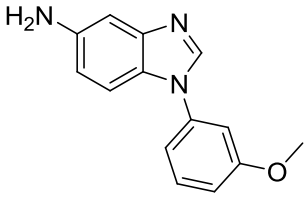
***N*-(2,4-Dinitrophenyl)-1-methylpiperidin-4-amine 15**. ^{1}H NMR (300 MHz $CDCl_3$); δ 1.72 – 1.84 (2 H, m), 2.09 – 2.15 (2 H, m), 2.25 – 2.33 (2 H, m), 2.35 (3 H, s), 2.77 – 2.89 (2 H, m), 3.59 – 3.79 (2 H, m), 6.93 (1 H, d, *J* = 9.6), 8.26 (1 H, dd, *J* = 2.8, and *J* = 9.6), 8.56 – 8.67 (1 H, m), 9.15 (1 H, d, *J* = 2.8).

Synthesis of 5-aminobenzimidazoles

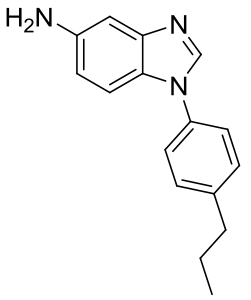
To a flask containing 1 equiv of the appropriate phenylbenzene-1,2,4-triamine was added 4M HCl and formic acid (25:1, v/v) and heated under reflux until no starting material was detected as determined by TLC. Upon completion, the reaction was cooled to 0 °C and basicified with solid NaOH until a pH of 11 was obtained. The aqueous fraction was extracted three times with ethyl acetate. The organic phases were combined, dried over sodium sulfate and concentrated. The crude benzimidazoles were purified by flash chromatography (silica gel, DCM with a gradient from 2 to 5 % methanol).



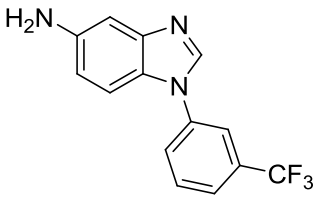
5-Amino-1-(2-methoxyphenyl)benzimidazole 17. ^{1}H NMR (300 MHz $CDCl_3$); δ 3.80 (3 H, s), 6.71 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.07 – 7.12 (3 H, m), 7.14 (1 H, d, *J* = 2.0), 7.38 – 7.45 (2 H, m,), 7.98 (1 H, s).



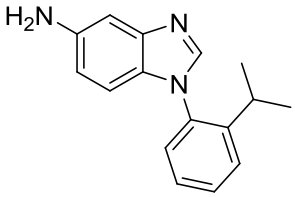
5-Amino-1-(3-methoxyphenyl)benzimidazole 18. ^{1}H NMR (300 MHz $CDCl_3$); δ 3.86 (3 H, s), 6.74 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.03 (2 H, d, *J* = 8.8), 7.14 (1 H, d, *J* = 2.0), 7.23 (1 H, d, *J* = 8.5), 7.36 (2 H, d, *J* = 8.8), 7.92 (1 H, s).



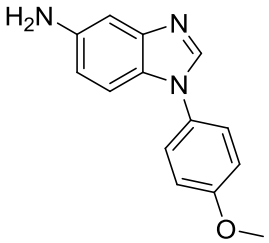
5-Amino-1-(4-*n*-propylphenyl)benzimidazole 19. ^{1}H NMR (300 MHz $CDCl_3$); δ 0.99 (3 H, t, *J* = 7.2 Hz), 1.69 (2 H, m), 2.67 (2 H, t, *J* = 7.2 Hz), 6.75 (1 H, dd, *J* = 2.2, and *J* = 8.5), 7.14 (1 H, d, *J* = 2.0), 7.31 – 7.40 (5 H, m), 7.32 (1 H, d, *J* = 8.5), 7.70 (5 H, m), 7.99 (1 H, s).



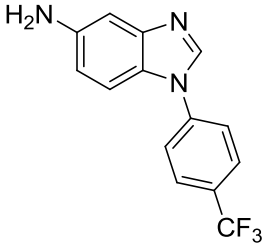
5-Amino-1-(3-trifluoromethylphenyl)benzimidazole 20. ^{1}H NMR (300 MHz $CDCl_3$); δ 6.78 (1 H, dd, *J* = 2.2, and *J* = 8.5), 7.16 (1 H, d, *J* = 2.0), 7.32 (1 H, d, *J* = 8.5), 7.70 (3 H, m), 7.77 (1 H, bs), 8.03 (1 H, s).



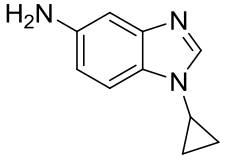
5-Amino-1-(2-isopropylphenyl)benzimidazole 21. ^{1}H NMR (300 MHz $CDCl_3$); δ 1.12 (d, *J* = 6.9 Hz, 6H), 2.63 (sept, J = 6.9 Hz, 1 H), 3.76 (bs, 2 H), 6.73 (dd, *J* = 1.9, and *J* = 8.5, 1 H), 6.91 (d, *J* = 8.5,1 H), 7.19 – 7.27 (m, 2 H), 7.31 – 7.36 (m, 1 H), 7.49 – 7.52 (m, 2H), 7.93 (s, 1 H).



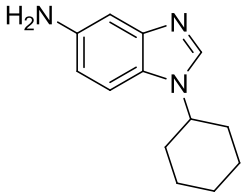
5-Amino-1-(4-methoxyphenyl)benzimidazole 22. ^{1}H NMR (300 MHz $CDCl_3$); δ 3.86 (3 H, s), 6.74 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.03 (2 H, d, *J* = 8.8), 7.14 (1 H, d, *J* = 2.0), 7.23 (1 H, d, *J* = 8.5), 7.36 (2 H, d, *J* = 8.8), 7.92 (1 H, s).



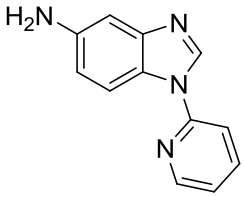
5-Amino-1-(4-trifluoromethylphenyl)benzimidazole 23. ^{1}H NMR (300 MHz $CDCl_3$); δ 6.74 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.14 (1 H, d, *J* = 2.0), 7.36 (1 H, d, *J* = 8.5), 7.63 (2 H, d, J = 8.5), 7.87 (2 H, d, J = 8.5), 8.05 (1 H, s).



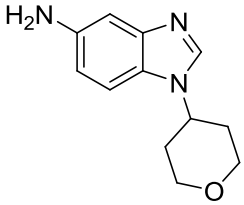
5-Amino-1-cyclopropylbenzimidazole 24. ^{1}H NMR (300 MHz $CDCl_3$); δ 1.00 – 1.10 (4 H, m), 1.00 – 1.10 (4 H, m), 3.25 – 3.35 (1 H, m), 6.74 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.06 (1 H, d, *J* = 2.0), 7.34 (1 H, d, *J* = 8.5), 7.80 (1 H, s).



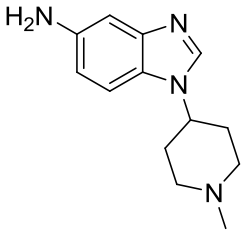
1-Cyclohexyl-1H-benzo[d]imidazol-5-amine 25. ^{1}H NMR (300 MHz $CDCl_3$); δ 1.25 – 1.39 (2 H, m) 1.43 – 1.58 (2 H, m), 1.68 – 1.85 (2 H, m), 1.95 – 2.02 (2 H, m), 2.18 – 2.23 (2 H, m), 3.62 (2 H, bs), 4.11 – 4.21 (1 H, m), 7.30 (1 H, d, *J* = 8.5 Hz), 7.39 (1H, dd, *J* = 1.6, and *J* = 8.5) 7.94 (1 H, d, *J* = 1.6 Hz), 7.97 1 H, s).



1-(Pyridin-2-yl)-1H-benzo[d]imidazol-5-amine 26. ^{1}H NMR (300 MHz DMSO-d6); δ 4.98 (2 H, bs), 6.68 (1 H, dd, *J* = 8.8 Hz, *J* = 2.2), 6.86 (1 H, d, *J* = 2.2 Hz), 7.32 – 7.37 (1 H, m), 7.85 (1 H, d, *J* = 8.5 Hz), 7.96 – 8.03 (2 H, m), 8.55 – 8.58 (1 H, m), 8.74 (1 H, s).

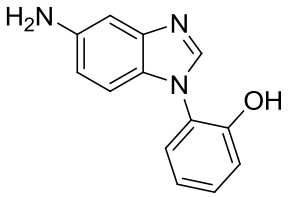


1-(Tetrahydro-2H-pyran-4-yl)-1H-benzo[d]imidazol-5-amine 27. ^{1}H NMR (300 MHz $CDCl_3$); δ 2.08 – 2.22 (4 H, m), 3.55 – 3.63 (2 H, m), 4.13 – 4.19 (2 H, m), 4.29 – 4.39 (1 H, m), 6.73 (1 H, dd, *J* = 8.5 Hz and *J* = 2.2), 7.09 (1 H, *J* = 2.2), 7.22 (1 H, d, *J* = 8.5), 7.86 (1 H, s).



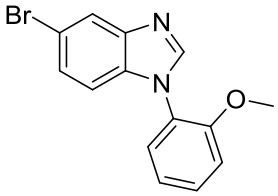
1-(1-Methylpiperidin-4-yl)-1H-benzo[d]imidazol-5-amine 28. ^{1}H NMR (300 MHz $CDCl_3$); δ 2.09 – 2.22 (6 H, m), 2.36 (3 H, s), 2.95 – 3.08 (2 H, m), 4.01 – 4.15 (1 H, m), 6.72 (1 H, dd, *J* = 2.2, and *J* = 8.5), 7.09 (1 H, d, *J* = 2.8), 7.22 (1 H, d *J* = 8.5), 7.86 (1 H, s).

Synthesis of 5-amino-1-(2-hydroxyphenyl)benzimidazole, compound 29.



To a flask containing 300 mg of **17** was added 2 mL DCM and cooled to 0 °C. To this mixture, 2.5 mL of 1 M BBr_3 in DCM was added dropwise by a syringe, warmed to 25 °C and stirred until TLC showed no **17**. The reaction was stopped by addition of saturated $NaHCO_3$ and the aqueous fraction was extracted 5 times with DCM. The organic layers were combined, dried over Na_2SO_4 and concentrated. The crude material was purified by flash chromatography (silica gel 19:1 v/v DCM/methanol). ^{1}H NMR (300 MHz $CDCl_3$); δ 6.74 (1 H, dd, *J* = 2.0, and *J* = 8.5), 7.14 (1 H, d, *J* = 2.0), 7.36 (1 H, d, *J* = 8.5), 7.63 (2 H, d, J = 8.5), 7.87 (2 H, d, J = 8.5), 8.05 (1 H, s).

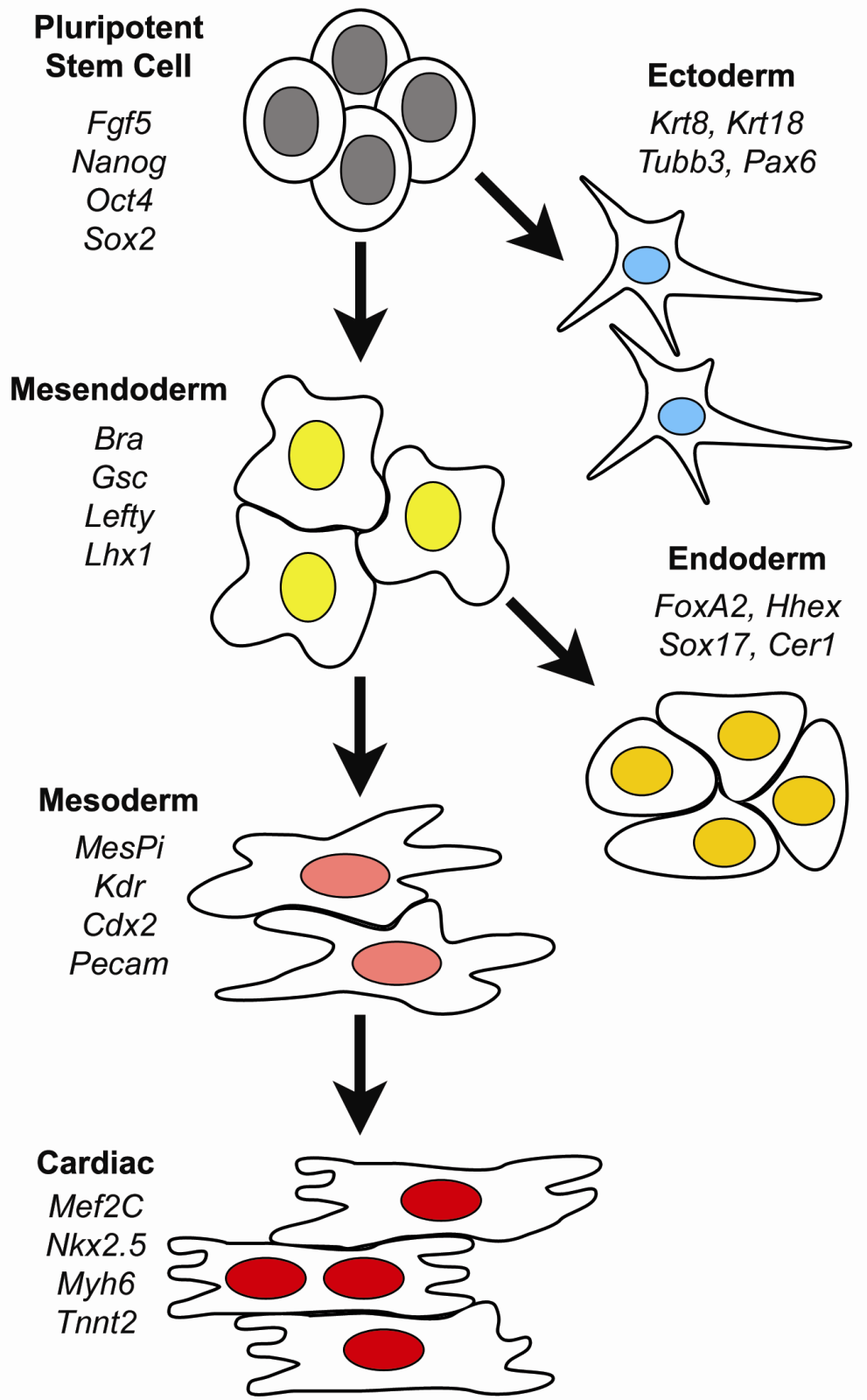
Synthesis of 5-bromo-1-(2-methoxyphenyl)-1H-benzo[d]imidazole, compound 67a.



4-bromo-*N*-(2-methoxyphenyl)-2-nitroaniline. 4-Bromo-1-fluoro-2-nitrobenzene (1.50 g, 6.82 mmol, 1.0 equiv.) was dissolved in toluene (15 mL). 2-Methoxyaniline (925 μL, 8.18 mmol, 1.2 equiv.) was added to the solution and the mixture was refluxed for 48 h. The solution was concentrated, diluted with DCM and filtered. The DCM solution was then concentrated and the crude mixture was purified by automated flash column chromatography (0-30% EtOAc/Hexanes) to yield 1.95 g (98%) of 4-bromo-*N*-(2-methoxyphenyl)-2-nitroaniline. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 3.87 (s, 3H), 6.94 – 7.03 (m, 2H), 7.09 (d, *J* = 9.2 Hz, 1H), 7.17 – 7.25 (m, 1H), 7.29 – 7.35 (m, 1H), 7.37 – 7.45 (m, 1H), 8.34 (d, *J* = 2.4 Hz, 1H), 9.38 (s, 1H).. LRMS (ESI) m/z calcd 322.00; found 324.00 $(M+1)^+$.

4-bromo-*N*-(2-methoxyphenyl)benzene-1,2-diamine. 4-bromo-*N*-(2-methoxyphenyl)-2-nitroaniline (1.10 g, 3.75mmol, 1.0 equiv.) was dissolved in EtOH (40 mL). A solution of sodium dithionite (2.0 g, 11.5mmol, 3.0 equiv.) in H_2O (5 mL) was added at room temperature with stirring. The solution was then heated to 70 °C until complete consumption of was visable by TLC (1h). After the solution was cooled to room temperature, the EtOH was removed *in-vacuo* and the aqueous was extracted three times with ethyl acetate. The organic phase was combined, dried over sodium sulfate and concentrated. The isolated product was of sufficient purity to use directly without further purification. Isolated 800 mg (81%) of 4-bromo-N1-phenylbenzene-1,2-diamine. LRMS (ESI) m/z calcd 292.02; found 293.00 $(M+1)^+$.

5-bromo-1-(2-methoxyphenyl)-1H-benzo[d]imidazole 67a. 4-bromo-*N*-(2-methoxyphenyl)benzene-1,2-diamine (800 mg, 3.04 mmol, 1.0 equiv.) was dissolved in trimethylorthoformate (5 mL) and heated to 110 °C for 1h. The reaction mixture was allowed to cool to room temperature and then stored at 4 °C for 2h during which 645 mg (78%) of **67a** as light pink needles precipitated from the solution. The solid was collected by filtration, washed with hexanes, and dried. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 3.81 (s, 3H), 7.08 – 7.19 (m, 3H), 7.34 – 7.41 (m, 2H), 7.43 – 7.51 (m, 1H), 8.00 (d, *J* = 1.9 Hz, 1H), 8.05 (s, 1H).LRMS (ESI) m/z calcd 302.01; found 303.07 $(M+1)^+$.



Supplemental Figure S1. Fate Map for pluripotent stem cells. Black arrows indicate lineage choice; genes in italics indicate gene expression unique to each lineage during differentiation.

Image Analysis:

In the primary screen, custom image analysis algorithms supported by Q3DM/Beckman (Vala Science, San Diego, CA) was conducted and used gross image subtraction of the non-specific red fluorescence from the green fluorescent image. 100% of the well area was captured with 4 images (4x4 binning) using a 4-fold objective in blue, green and red fluorescent channels.

In the primary confirmation screen and SAR screen, more refined image analysis algorithms were developed using the software package Developer Toolbox provided by GE Healthcare, (Piscataway, NJ) based on a global threshold of each image field. The algorithm can be boiled down to the following equation that was applied to the images from the green channel (eGFP):

(Source Pixel) – (Image Global Average (IGA) * Multiple (M))

where M was empirically determined to perform best in the range of 1.6 to 1.8. The mask was then used to collect data from blue, green and red fluorescent images. This resulted in very sensitive detection of rare foci, but underestimated the amount of eGFP-positive foci when a compound was very potent due to an unusually high IGA. Thus, the algorithm gathered conservative metrics for the most potent compounds. For all primary and SAR screens, the total integrated intensity was a function of both intensity and area. The red channel was captured for subtraction of non-specific broad-band fluorescent signal in the densely populated day-10 cultures. The values in the blue channel (i.e., nuclear counter-stain) were used to filter toxic and fluorescent compounds. Approximately 75% of the well area was captured with 9 images (4x4 binning) using a 10x objective in blue, green and red fluorescent channels.

Data were reported as fold-increase in relative activity relative to DMSO and standardized to 100% activity of a hydrochloride salt preparation of the parent benzimidazole derived from the primary screen (e.g., compare phenyl with 2-methoxyphenyl in compound **1**). To produce a summary metric that permitted quick evaluation of the relative small molecule potency in the SAR, the sum of fluorescent signal throughout the dilution range was obtained and the sum of squared deviations calculated accordingly. The sum of the fluorescent signal of a given small molecule was divided by the vehicle control (i.e., DMSO-treated cells) for each assay and the standard deviation was scaled accordingly. Thus, the greater the fold-increase in fluorescence signal, the more potent the compound was at induction of cardiomyogenesis.

Movie of Cardiomyogenesis

Mouse cardiomyocytes treated with 6 μM compound **1** at days 2-4 were imaged in real time on day-10 of differentiation. The movie was captured with a 10x objective over a period of 5-10 seconds. Cells with αMHC-GFP positive areas beat as expected on the basis of our experience with early stage cardiomyocytes differentiated from pluripotent stem cells.