Supporting Information

VUT-MK142 - A new cardiomyogenic small molecule promoting the differentiation of pre-cardiac mesoderm to cardiomyocytes

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## Content

### Experimental Section

#### Chemistry

- **General Notes:**
  - 6-Chloro-N-(4-methoxyphenyl)-pyrimidin-2-amine 1 ................................................................. 3
  - General Procedure A: .................................................................................................................. 4
  - 6-Chloro-N-(4-methoxyphenyl)pyridin-2-amine 2 .................................................................. 4
  - General Procedure B: .................................................................................................................. 5
  - 2-[6-(4-Methoxyphenylamino)pyrimidin-4-ylamino]ethanol VUT-MK093 ............................ 5
  - N<sup>4</sup>-Cyclohexyl-N<sup>6</sup>-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK142 .......... 5
  - Flow protocol: ............................................................................................................................. 5
  - N<sup>4</sup>-Ethyl-N<sup>6</sup>-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK296 ...................... 7
  - N<sup>4</sup>-(4-Methoxyphenyl)-N<sup>6</sup>-methylypyrimidine-4,6-diamine VUT-MK431 .................. 7
  - 2-[6-(4-Phenoxyphenylamino)pyridin-2-ylamino]ethanol VUT-MK310 ...................... 16
  - 2-(6-(4-Methoxyphenylamino)pyridin-2-ylamino)ethanol VUT-MK396 ...................... 18

#### Biology

- Cell culture and treatment procedures ......................................................................................... 9
- ANF promoter luciferase reporter assay ...................................................................................... 9
- Cardiac body experiments ............................................................................................................. 10

### NMR Spectral Section

- 2-[6-(4-Methoxyphenylamino)pyrimidin-4-ylamino]ethanol VUT-MK093 ............................ 11
- N<sup>4</sup>-Cyclohexyl-N<sup>6</sup>-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK142 .......... 12
- N<sup>4</sup>-Ethyl-N<sup>6</sup>-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK296 ...................... 14
- 2-[6-(4-Phenoxyphenylamino)pyridin-2-ylamino]ethanol VUT-MK310 ............................ 16
- 2-(6-(4-Methoxyphenylamino)pyridin-2-ylamino)ethanol VUT-MK396 ............................ 18
- N<sup>4</sup>-(4-Methoxyphenyl)-N<sub>6</sub>-methylpyrimidine-4,6-diamine VUT-MK431 ................. 19
**Experimental Section**

**Chemistry**

**General Notes:**

Unless otherwise noted, chemicals were purchased from commercial suppliers and used without further purification. Microwave reactions were performed on a Biotage Initiator 60™ microwave unit or a CEM Explorer™ device. Flash column chromatography was performed on silica gel 60 from Merck (40-63μm) whereas most separations were carried out using a Büchi Sepacore™ MPLC system with a 45g column. For thin layer chromatography (TLC) aluminium backed silica gel was used. Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. HR-MS were carried out by E. Rosenberg at Vienna University of Technology, Institute for Chemical Technologies and Analytics; all samples were analyzed by LC-IT-TOF-MS in only positive ion detection mode with the recording of MS and MS/MS spectra. NMR-spectra were recorded in CDCl₃ with TMS as internal standard or in DMSO-d₆ or CD₃OD on a Bruker AC 200 (200MHz) spectrometer and chemical shifts are reported in ppm. Combustion analyses were carried out in the Microanalytical Laboratory, Institute of Physical Chemistry, University of Vienna. GC-MS runs were performed on a Thermo Finnigan Focus GC / DSQ II using a standard capillary column BGB 5 (30m x 0.32 mm ID). Reaction control and optimization of the continuous flow process was achieved by UHPLC employing a Shimadzu Nexera® device, running on a Phenomenex Kinetex® PFP (50x2.1mm, 1.7µ) column and using benzyl benzoate as internal standard. Either combustion analysis or HR-MS are provided for compounds not previously reported in the literature.

**6-Chloro-N-(4-methoxyphenyl)-pyrimidin-2-amine 1**

4,6-Dichloropyrimidine (1.57 g, 10.6 mmol, 1.3 equiv) and p-methoxyaniline (1g, 8.10 mmol, 1 equiv) were dissolved in i-PrOH (15 mL) and HCl (37%, 1.5mL) was added. The reaction mixture was then refluxed (84°C) for approx. 2.5 hours under nitrogen atmosphere (monitored by TLC). After cooling to room temperature a precipitate was formed. The reaction mixture was kept in the freezer overnight for complete precipitation. The precipitate was collected by filtration and washed with cold i-PrOH to obtain the pure product. Yield: 56% (1.60 g, 5.88 mmol) colorless solid; mp: 121-123°C; TLC: Rf= 0.85 (EtOAc: EtOH= 10:1); ¹H NMR (DMSO-d₆, 200MHz): δ = 3.75 (s, 3H), 6.73 (s, 1H), 6.95 (d, J = 8.8 Hz, 2H), 7.49 (d, J = 8.8 Hz, 2H), 8.38 (s, 1H), 9.89 (s, 1H). ¹³C NMR (DMSO-d₆, 50MHz): δ = 55.3 (q), 104.1 (d), 114.1 (d), 122.6 (d), 131.6 (s), 155.7 (s), 157.1 (s), 158.1 (d), 161.3 (s).
**General Procedure A:**
Dihalopyridine (1 equiv), amine (1.2 equiv), K$_2$CO$_3$ (3.5 equiv), Pd(OAc)$_2$ (2 mol%), and BINAP (2 mol%) were charged into a microwave vial and dry toluene was added. The vial was then sealed, evacuated, and flushed with argon. Then the reaction mixture was irradiated at 180°C in a CEM ExplorerTM microwave unit for 30 minutes (in few cases 45 minutes) with stirring. After cooling to r.t., the solid material was removed by filtration and washed with 10mL of EtOAc or CH$_2$Cl$_2$. The organic phases were combined and the solvent was evaporated. The resulting crude product was purified by flash column chromatography.

**6-Chloro-N-(4-methoxyphenyl)pyridin-2-amine 2**
Prepared according to general procedure A starting from 2,6-dichloropyridine (100 mg, 0.68 mmol):
Column chromatography PE: EtOAc = 4:3. Yield: 72% (115 mg, 0.49 mmol). Appearance: yellow solid. Mp: 74-76°C. R$_f$ = 0.41 (PE: CH$_2$Cl$_2$= 4:3). GCMS: 219 (100), 234 (M+, 234), 221 (32), 112 (30), 233 (27). $^1$H NMR (CDCl$_3$, 200MHz): $\delta$ = 3.81 (s, 3H), 6.52 (d, $J$ = 8.2 Hz, 1H), 6.65 (d, $J$ = 7.6 Hz, 1H), 6.74 (s, 1H), 6.89 (d, J =9.4 Hz, 2H), 7.19 (d, $J$=7.2 Hz, 2H), 7.34 (t, J = 7.9 Hz, 1H).$^{13}$C NMR (CDCl$_3$, 50MHz): $\delta$ = 54.6 (q), 103.8 (d), 112.4 (d), 113.8 (d), 123.7 (d), 131.1 (s), 139.1 (d), 148.5 (s), 155.9 (s), 156.4 (s). Combustion analysis: requires C 61.41%, H 4.72%, N 11.94%; found C 61.42%, H 4.52%, N 11.78%.

**6-Chloro-N-(4-phenoxyphenyl)-pyridin-2-amine 3**
Prepared according to general procedure A starting from 2,6-dichloropyridine (100 mg, 0.68 mmol):
Column chromatography PE: EtOAc = 9:1 Yield: 77% (154 mg, 0.52 mmol); Appearance: yellow crystals. Mp: 78-81°C. R$_f$ = 0.51 (PE: EtOAc = 10: 1). GC-MS: 296 (M+, 100), 77 (80), 295 (37), 51 (33), 298 (31). $^1$H NMR (CDCl$_3$, 200MHz): $\delta$ = 6.58-6.75 (q, J= 7.2 Hz, 3H), 6.95-7.06 (m, 4H), 7.10 (d, J= 7.4 Hz, 1H), 7.21-7.31 (m, 3H), 7.32-7.36 (m, 1H), 7.37-7.44 (m, 1H). $^{13}$C NMR (CDCl$_3$, 50MHz): $\delta$ = 105.3 (d), 113.9 (d), 118.5 (d), 120.0 (d), 123.2 (d), 123.6(d), 129.8 (d), 134.8 (s), 140.0 (d), 149.7 (s), 153.5 (s’), 156.7 (s), 157.5 (s). Combustion analysis: requires C 68.81%, H 4.42%, N 9.44%; found C 68.60%, H 4.22%, N 8.92%;
General Procedure B:
The intermediate (1 equiv.) obtained after the first nucleophilic substitution step was mixed with the aliphatic amine (1.1-5 equiv) and DIPEA (2.5-5 equiv) and then dissolved in n-BuOH in a microwave vial and heated to 200°C for 45 minutes under microwave irradiation. The reaction was monitored by TLC. The crude product was either precipitated from n-BuOH or obtained by evaporating n-BuOH.

2-[6-(4-Methoxyphenylamino)pyrimidin-4-ylamino]ethanol VUT-MK093
Prepared according to general procedure B: Compound 1 (250 mg, 0.92 mmol, 1 equiv), ethanolamine (62 mg, 1.01 mmol, 1.1 equiv), and DIPEA (296 mg, 2.29 mmol, 2.5 equiv) were dissolved in n-BuOH (5 mL) in a microwave vial and the reaction was carried out according to the general procedure B. The crude product was obtained by evaporating the solvent. The pure product was obtained by recrystallization from n-BuOH/EtOH mixture. Yield: 77% (185 mg, 0.71 mmol) colorless solid; mp: 173°C; TLC: Rf = 0.14 (EtOAc:EtOH = 10:1); 1H NMR (DMSO-d6, 200MHz): δ = 3.12-3.3.32 (m, 2H), 3.45 (q, J = 5.5 Hz, 2H), 3.70 (s, 3H), 4.71 (t, J =5.4 Hz, 1H), 5.66 (s, 1H), 6.63-6.80 (bs, 1H), 6.87 (d, J =8.9 Hz, 2H), 7.32 (d, J =9.2 Hz, 2H), 8.00 (s, 1H), 8.63 (s, 1H). 13C NMR (DMSO-d6, 50MHz): δ= 42.9 (t), 55.1 (q), 59.9 (t), 80.1 (d), 113.9 (s), 122.0 (d), 133.5 (d), 154.3 (s), 157.5 (d), 160.4 (s), 162.7 (s). Combustion analysis: requires C 59.99, H 6.20, N 21.52; found C 59.68, H 6.00, N 21.11.

N4-Cyclohexyl-N6-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK142
Prepared according to general procedure B: Substrate 1 (200 mg, 0.73 mmol, 1 equiv), cyclohexylamine (80 mg, 0.81 mmol, 1.1 equiv), and DIPEA (109 mg, 1.84 mmol, 2.5 equiv) were dissolved in n-BuOH (4mL) and reacted according to the general procedure B but for 90 minutes. The crude product was obtained by evaporating n-BuOH. Purification by MPLC (silica, PE:EtOAc = 1:3) gave the pure product VUT-MK142. Yield: 70% (230 mg, 0.77 mmol) colorless solid; mp: 218-219°C; TLC: Rf = 0.42 (PE: EtOAc = 1:3).

Flow protocol:
The continuous flow reactions were carried out using a ThalesNano X-Cube Flash reactor equipped with a 4mL Hastalloy® coil. The flow rate was adjusted to 0.5mL/min, resulting in a residence time (=reaction time) of 8 minutes, while the pressure was set to 50bar as standard value. Product collection was triggered after previous determination of the reactor dead volume. Pure solvent (NMP) was supplied via pump A, while the starting material solutions were introduced into the system via pump B. To ensure for stable reaction conditions, all parameters were set to the appropriate values and the system was started running on pure solvent until steady conditions were detected. Then, the pumps were switched and the starting material solutions were subjected to the reaction conditions. Upon completion of starting material injection, the pumps were switched again and the reactor system was...
flushed with solvent to allow for a subsequent reaction. During the optimization reactions, 4mL of starting material solutions were introduced into the system and 6mL total volume was collected (1mL pre – 4mL reaction – 1mL post).

To synthesize the intermediate, 4,6-dichloropyrimidine (190mg, 1.28mmol), p-anisidine (158mg, 1.28mmol) and DIPEA (181mg, 1.40mmol) were dissolved in NMP (10mL final volume). The starting material solution was reacted at 160°C reaction temperature. The collected product solution was mixed with ethyl acetate (60mL) and was extracted three times with saturated ammonium chloride solution (3x60mL). The combined aqueous layers were extracted once more with ethyl acetate (120mL) and the organic phases were washed five times with saturated sodium chloride solution (5x60mL). The organic phase was dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated under reduced pressure to give crude product, which was subjected to MPLC purification (SiO₂, light petroleum / ethyl acetate) to provide pure product in 81% yield (242mg, 1.03mmol).

For the synthesis of VUT-MK142, 1 (237mg, 1.01mmol), cyclohexylamine (998mg, 10.1mmol) and DIPEA (390mg, 3.02mmol) were dissolved in NMP (10mL final volume). The starting material solution was reacted at 200°C reaction temperature. Product purification was performed as described above, replacing the ammonium chloride extraction with sodium carbonate extraction. After MPLC purification (SiO₂, light petroleum / ethyl acetate), pure product was isolated in 84% yield (252mg, 0.85mmol).

\[^{1}H\text{ NMR (CDCl}_3,\ 200MHz): \delta = 1.05-2.01 (m, 11H, 5CH}_2, \text{ CH), 3.85 (s, 3H), 4.69 (d, 1H, J = 6.5 Hz), 5.49 (s, 1H), 6.68 (s, 1H), 6.93 (d, J = 8.9 Hz, 2H), 7.16 (d, J = 8.9 Hz, 2H), 8.13 (s, 1H), 8.13 (s, 1H).}\]

\[^{13}C\text{ NMR (CDCl}_3,\ 50MHz): \delta = 24.7 (t), 25.7 (t), 33.0 (t), 49.6 (d), 55.6 (q), 80.9 (d), 114.6 (d), 125.3 (d), 131.5 (s), 156.9 (s), 158.4 (d), 161.8 (s), 162.2 (s).\ \text{HR-MS: predicted [MH]}^+ = 299.1866; \text{measured [MH]}^+ = 299.1878; \text{diff in ppm} = 0.01.\]
**N^4^-Ethyl-N^6-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK296**

Substrate 1 (100 mg, 0.37 mmol, 1 equiv), ethylamine (83 mg, 1.85 mmol, 5 equiv) and DIPEA (120 mg, 0.93 mmol, 2.5 equiv) were dissolved in n-BuOH (2mL) in a microwave vial and reacted according to the general procedure B. The crude product was obtained by evaporating n-BuOH. Purification was achieved by crystallization from n-BuOH/EtOH mixture. Yield: 71% (64mg, 0.26mmol) colorless solid; mp: 190-193°C; TLC: Rf = 0.24 (EtOAc); \(^1\)H NMR (CDCl\(_3\), 200MHz): \(\delta = 1.20\) (t, J = 6.8 Hz, 3H), \(3.07-3.25\) (m, 2H), \(3.82\) (s, 3H), \(4.83\) (bs, 1H), \(5.49\) (s, 1H), \(6.91\) (d, J = 8.6 Hz, 2H), \(7.19\) (d, J = 8.6 Hz, 2H), \(8.12\) (s, 1H, H2). \(^1\)C NMR (CDCl\(_3\), 50MHz): \(\delta = 14.3\) (q), \(36.3\) (t), \(55.4\) (q), \(80.3\) (d), \(114.6\) (d), \(125.5\) (d), \(131.3\) (s), \(157.0\) (s), \(158.2\) (d), \(162.0\) (s), \(163.0\) (s). Combustion analysis: requires C 63.91, H 6.60, N 22.93; found C 63.68, H 6.43, N 22.59.

**N^4^-{4-Methoxyphenyl}-N^6-methylpyrimidine-4,6-diamine VUT-MK431**

Substrate 1 (100 mg, 0.37 mmol, 1 equiv), methylamine solution in water (57 mg, 1.85 mmol, 5 equiv) and DIPEA (238 mg, 1.84 mmol, 5 equiv), were dissolved in n-BuOH (2mL) in a screw cap vial and heated to 120°C for 1 hour. The reaction was monitored by TLC. After 1 h the reaction was complete. The crude product was obtained by evaporating n-BuOH. Purification was conducted by recrystallization from n-BuOH/EtOH mixture. Yield: 91% (77 mg, 0.33 mmol) colorless solid; mp: 174-182°C; TLC: Rf = 0.29 (PE: EtOAc= 2:1); \(^1\)H NMR (DMSO-d\(_6\), 200MHz): \(\delta = 2.70\) (d, J = 4.7 Hz, 3H), \(3.72\) (s, 3H), \(5.58\) (s, 1H), \(6.65-6.80\) (m, 1H), \(6.87\) (d, J = 8.8 Hz, 2H), \(7.37\) (d, J = 8.8 Hz, 2H), \(8.02\) (s, 1H), \(8.67\) (s, 1H. \(^1\)C NMR (DMSO-d\(_6\), 50MHz): \(\delta = 27.4\) (q), \(55.1\) (q), \(81.9\) (d), \(113.8\) (d), \(121.8\) (d), \(133.6\) (s), \(154.3\) (s), \(157.5\) (d), \(160.5\) (s), \(163.1\) (s). HR-MS: predicted [MH]\(^+\)= 231.1240; measured [MH]\(^+\)= 231.1242; diff in ppm = 0.87.

**2-[6-(4-Phenoxyphenylamino)pyridin-2-ylamino]ethanol VUT-MK310**

Compound 3 (130 mg, 0.44 mmol, 1 equiv.) was mixed with ethanolamine (214 mg, 3.52 mmol, 8 equiv.) and heated in a screw cap vial under argon at 150°C in a heating block for 3 days. After cooling to r.t. water (10 mL) was added and the mixture was extracted with EtOAc (10 mL) for three times. The EtOAc layer was then washed with brine and subsequently dried over Na\(_2\)SO\(_4\). Evaporating the EtOAc layer gave the crude product which was purified by flash column chromatography (PE: EtOAc = 2:1). Yield: 73% (102 mg, 0.32 mmol). Appearance: beige oil. Rf = 0.14 (PE: EtOAc = 1:1). \(^1\)H NMR (CDCl\(_3\), 200MHz): \(\delta = 3.27\) (t, J = 5 Hz, 2H), \(3.75\) (t, J = 5 Hz, 2H), \(5.76\) (d, J = 8.4 Hz, 1H), \(5.95\) (d, J = 8.4 Hz, 1H), \(6.85-7.35\) (m, 1H), \(9.00\) (bs, 3H). \(^1\)C NMR (CDCl\(_3\), 50MHz): \(\delta = 45.2\) (t), \(60.4\) (t), \(94.3\) (d), \(94.4\) (d), \(118.7\) (d), \(119.7\) (d), \(123.3\) (d), \(125.2\) (d) \(129.7\) (d), \(133.1\) (s), \(143.7\) (d), \(152.7\) (s), \(153.7\) (s), \(154.6\) (s), \(157.1\) (s), \(177.4\) (s).
2-(6-(4-Methoxyphenylamino)pyridin-2-ylamino)ethanol VUT-MK396

Compound 2 (190 mg, 0.82 mmol, 1 equiv.) was mixed with ethanolamine (397 mg, 6.49 mmol, 8 equiv.) and heated in a screw cap vial under argon at 150°C in a heating block for 3 days. After cooling to r.t. water (5 mL) was added and the mixture was extracted with EtOAc (5 mL) for three times. The EtOAc layer was then washed with brine and subsequently dried over Na₂SO₄. Evaporating the EtOAc layer gave the crude product which was purified by flash column chromatography (PE: EtOAc = 3:1). Yield: 73% (153 mg, 0.59 mmol). Appearance: beige oil. Rᵣ = 0.37 (PE: EtOAc = 3:1).

¹H NMR (CDCl₃, 200MHz): δ = 3.35 (t, J = 4.8 Hz, 2H), 3.80 (s, 3H), 3.84 (t, J = 4.6 Hz, 2H), 5.80 (d, J = 7.8 Hz, 1H), 5.92 (d, J = 9.4 Hz, 1H), 6.89 (d, J = 8.9 Hz, 2H), 7.17 (d, J = 8.2 Hz, 2H), 7.32 (t, J = 7.6 Hz, 1H).

¹³C NMR (CDCl₃, 50MHz): δ = 45.3 (t), 55.5 (q), 61.0 (t), 94.4 (d), 114.7 (d), 125.7 (d), 131.0 (s), 143.0 (d), 153.4 (s), 154.6 (d), 157.3 (s), 177.7 (s). HRMS: requires [MH]⁺ = 260.1394; found 260.1389; diff in ppm = -1.0.
Biology

Cell culture and treatment procedures
P19 mouse embryonic carcinoma cells (embryonic stem cell model; American Type Culture Collection, ATCC) were cultured in minimum essential medium (MEM)-alpha containing 4 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 7.5% new born calf serum, and 2.5% fetal bovine serum at 37 °C in 5% CO₂. For SySM-treatment, the cells were incubated in an “induction medium” (MEM-alpha, 5% fetal bovine serum, 4 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin) as in the publication of Wu et al., containing 1 µM of a respective single SySM compound to be tested. DMSO, which was used as solvent for all the SySMs, was added in equal amounts (0.005%) to “control cells”. SySM-treatment lasted 7 days, whereby fresh media were supplied twice during this time period.

C2C12 mouse skeletal myoblasts (ATCC) were cultured in growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose, 4 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20% fetal calf serum. The cells were incubated at 37 °C and 5% CO₂, and when about 50–70% confluence was reached, differentiation was induced by serum reduction. For this purpose, myoblasts were incubated in differentiation medium that was identical to the growth medium, except that it contained 2% horse serum instead of 20% fetal calf serum. SySM compounds to be tested (concentration, 1 µM) were always added at the same time as differentiation was induced, and were applied for 7 days (see above).

A5 CVPCs were isolated from newborn mouse hearts and cultured in M15 medium on mitotically inactivated SNL76/7 fibroblasts.

ANF promoter luciferase reporter assay
For the assay, a fragment containing the rat ANF promoter region was amplified and then subcloned into the PGL3-BV luciferase reporter plasmid. P19 or C2C12 cells were transiently transfected with this plasmid (a kind gift from P.G. Schultz), and with the pRL-SV40 Renilla luciferase control reporter vector (Promega). For transfections (always one day before start of SySM-treatment), lipofectamine plusTM Reagent (Invitrogen) was used according to the manufacturer’s protocol. The expression of Renilla luciferase was used as an internal control for normalization of experimental variations such as differences in cell densities and transfection efficiencies. After 7 days of compound treatment (or 0.005% DMSO-treatment for control cells), cells were harvested, and luciferase activity was measured (Wallac 1420 Victor multilabel counter, Perkin Elmer) using Promega’s dual-luciferase reporter assay kit. P19 cells treated with 10 nM all-trans retinoic acid (RA) for 7 days served as positive control for the assay. In this low concentration, RA drives cardiomyogenic differentiation in embryonic stem cells.

For several experiments to further test the cardiomyogenic activity of the SySM compound VUT-MK142, an Nkx2.5 promoter luciferase reporter plasmid (NKE24, Promega pGL3b plasmid (E1751)
with an insert of the nkx2.5 promoter region\(^\text{14}\) was used. The experimental procedure applied was identical to that described above for the ANF reporter assay.

**Cardiac body experiments**

In vitro differentiation, analysis of the phenotype, and RT-PCR of CVPCs were essentially performed as with ESCs. Shortly, CVPCs were aggregated to cardiac bodies (CBs) in hanging drop cultures at a density of 900±50 cells per 20 \(\mu\)l medium M15 for 4.5 days and then plated on gelatine coated 10 cm tissue culture plates at a density of 0.9 ± 0.1 CBs/cm\(^2\). 15 CBs were manually distributed over the entire surface of the plate so that the majority of these CBs did never contact the neighboring CB during the entire duration of the experiment. In addition, the borders of the disc shaped CBs were always were composed mainly of endothelial cells connected via tight junctions to each other which could be easily identified under phase contrast illumination. Cardiomyogenesis becomes apparent by spontaneously beating clusters of cardiomyocytes and was monitored in individual CBs for at least 20 days. Data obtained from the differentiation experiments lasting 25 days were normalized to the control (DMSO) for each day to get rid of the oscillation caused by the feeding protocol and the time dependent increase in differentiation of cardiomyocytes and are presented as the mean increase in cardiomyogenesis from day 11 to 20. Sequences of primers used for semiquantitative RT-PCR are available on request.
NMR Spectral Section

2-[6-(4-Methoxyphenylamino)pyrimidin-4-ylamino]ethanol VUT-MK093

VUT-MK093 1H NMR in d6-DMSO

VUT-MK093 13C NMR in d6-DMSO
$N^4$-Cyclohexyl-$N^6$-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK142

VUT-MK142 1H NMR in CDCl$_3$

![1H NMR Spectrum](image1)

VUT-MK142 13C NMR in CDCl$_3$

![13C NMR Spectrum](image2)
VUT-MK142 DEPT in CDCl$_3$
$N^4$-Ethyl-$N^6$-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK296

VUT-MK296 1H in CDCl$_3$

VUT-MK296 13C in CDCl$_3$
VUT-MK296 DEPT in CDCl$_3$
2-[6-(4-Phenoxyphenylamino)pyridin-2-ylamino]ethanol VUT-MK310
VUT-MK310 1H in CDCl₃

VUT-MK310 13C in CDCl₃
VUT-MK310 DEPT in CDCl₃
2-(6-(4-Methoxyphenylamino)pyridin-2-ylamino)ethanol VUT-MK396

VUT-MK396 1H in CDCl₃

VUT-MK396 13C in CDCl₃ in CDCl₃
$N^4$-(4-Methoxyphenyl)-$N^6$-methylpyrimidine-4,6-diamine VUT-MK431

VUT-MK431 1H in d6-DMSO

VUT-MK431 13C in d6-DMSO