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VUT-MK142 : a new cardiomyogenic small molecule promoting the differentiation of pre-cardiac mesoderm into cardiomyocytes

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Abstract

Intra-cardiac cell transplantation is a new therapy after myocardial infarction. Its success, however, is impeded by the limited capacity of donor cells to differentiate into functional cardiomyocytes in the heart. A strategy to overcome this problem is the induction of cardiomyogenic function in cells prior to transplantation. Among other approaches, recently, synthetic small molecules were identified, which promote differentiation of stem cells of various origins into cardiac-like cells or cardiomyocytes. The aim of this study was to develop and characterise new promising cardiomyogenic synthetic low-molecular weight compounds. Therefore, the structure of the known cardiomyogenic molecule cardiogenol C was selectively modified, and the effects of the resulting compounds were tested on various cell types. From this study, VUT-MK142 was identified as the most promising candidate with respect to cardiomyogenic activity. Treatment using this novel agent induced the strongest up-regulation of expression of the cardiac marker ANF in both P19 embryonic carcinoma cells and C2C12 skeletal myoblasts. The activity of VUT-MK142 on this marker superseded CgC; moreover, the novel compound significantly up-regulated the expression of other cardiac markers, and promoted the development of beating cardiomyocytes from cardiovascular progenitor cells. We conclude that VUT-MK142 is a potent new cardiomyogenic synthetic agent promoting the differentiation of pre-cardiac mesoderm into cardiomyocytes, which may be useful to differentiate stem cells into cardiomyocytes for cardiac repair. Additionally, an efficient synthesis of VUT-MK142 is reported taking advantage of continuous flow techniques superior to classical batch reactions both in yield and reaction time.

Introduction

Heart failure is one of the major causes of death worldwide and particularly prominent in industrialized nations. Unlike other tissues and organs, the adult heart cannot effectively regenerate after degeneration or injury. Consequently, new therapy approaches to achieve myocardial regeneration have gained much attention. The success of current intra-cardiac stem cell transplantation attempts to regenerate operational heart tissue is, however, severely restricted by poor donor cell survival, as well as by the limited capacity of the donor cells to trans-differentiate into functional cardiomyocytes.¹ During the last few years, intense efforts have been undertaken to overcome these problems directing cells of various types to form cardiomyocytes. In the course of these investigations it was shown that adult somatic cells (*e.g.* fibroblasts) can be reprogrammed to “induced pluripotent stem (iPS) cells” capable to differentiate into cardiomyocytes.² Moreover, even direct reprogramming to cardiomyocytes was reported;³ this was achieved by ectopic expression of only a few defined transcription factors. Although these inventions hold considerable promise as research tools, their clinical application is hindered by the use of viral transduction to deliver the required exogenous transcription factors. It is therefore of great importance to replace the use of viruses by alternative strategies to induce cardiomyogenesis.

In recent years, synthetic small molecules (SySMs) were identified which exhibit a remarkable capacity to trans-differentiate cells into other cell types. For example, the SySM reversine induces lineage reversal of skeletal myoblasts to become multipotent progenitor cells, which can re-differentiate into osteoblasts and adipocytes.⁴ An early report of SySMs with a cardiomyogenic effect is provided in ref. 5. These authors demonstrated that cardiogenols induce embryonic stem cells to develop into beating cardiomyocytes. Since this seminal disclosure, other small molecules with cardiomyogenic activity on embryonic stem cells were identified (gefitinib,⁶ Shz1-6,⁷ CHIR99021,⁸ XAV939 (ref. 9)). The mode-of-action of these SySMs is still unclear; some established lead compounds originate from protein kinase inhibitor libraries; other compounds capable to only stepwise cardiomyocyte specification interact with various signalling pathways. Among other recently identified cardiomyogenic compounds (Fig. 1; for reviews see Willems *et al.*¹⁰), SySMs of the sulfonyl hydrazone type were shown to trigger cardiac protein expression in a variety of embryonic and adult stem cell types.⁷ Remarkably, these authors also reported that sulfonyl hydrazone-treatment enhanced myocardial repair by stem cells in a rat model, thereby highlighting the great potential of cardiomyogenic SySMs to promote myocardial regeneration.

Within this work, we disclose a new synthetic compound, VUT-MK142, with promising cardiomyogenic effects on various cell types. VUT-MK142 and the other related compounds tested in this study were derived by targeted modification of the structure of the cardiomyogenic SySM Cardiogenol C (CgC) first described by Wu *et al.*⁵ Additionally, we report an efficient and easily scalable synthesis of VUT-MK142 taking advantage of continuous flow methods. This presented synthetic method is significantly superior to standard batch reactions.

Results and discussion

Chemical synthesis

Starting from CgC as the lead compound, substitution pattern modification and nitrogen replacement strategies were employed to develop preliminary SAR information for modification of the heterocyclic core structure. Upon screening of suitable heterocyclic scaffolds, 4,6-diamino substituted pyrimidines and 2,6-diamino substituted pyridines were identified as compounds with promising cardiomyogenesis inducing abilities (data not shown). Subsequently, a series of these compounds was synthesized (Scheme 1) and subjected to biological testing. Selected examples are displayed in Table 1. In the case of 4,6-diamino substituted pyrimidines, both amine residues could be introduced *via* nucleophilic substitution reactions starting from 4,6-dichloropyrimidine (Scheme 1). Initially, reactions were performed *via* the classical batch process and the intermediate was isolated after the first nucleophilic substitution step as a hydrochloride salt. Naturally, the less reactive (aromatic) amine substituent was introduced first due to electronic deactivation of the mono-substituted product for the second step. Enforced conditions (higher reaction temperature, more reactive aliphatic amine) were applied in the second substitution reaction. *Via* this method all pyrimidine products could be reliably obtained in reasonably good yields over 2 reaction steps.

A different strategy had to be applied for the synthesis of 2,6-diaminosubstituted pyridines. Nucleophilic substitution turned out to be not feasible as the first reaction step. Hence, we turned to a Pd catalysed Buchwald–Hartwig amination method following a protocol recently developed by our group¹¹ enabling facile incorporation of aromatic amines employing microwave irradiation. The aliphatic amine could then again be introduced *via* nucleophilic substitution upon extended reaction times.

In order to provide access to sufficient quantities of the most active compound in the series, VUT-MK142 was chosen for exploitation of a continuous flow-chemical process; such a methodology offers certain advantages of scalability and potential automated library synthesis of the target products.¹² Initially, we investigated both reaction steps individually utilizing the X-Cube Flash™ from Thalesnano as a reactor. Switching from batch to flow required certain adaptations (solvent: NMP; base: diisopropylethylamine; acidic conditions had to be avoided due to low solubility of the formed hydrochloride intermediate) to ensure homogeneous reaction conditions throughout the process. After optimizing the critical reaction parameters (substrate concentration [10–100 mM], temperature [150–200 °C], flow rate [0.5–1 mL min⁻¹]), the best results for the first nucleophilic substitution were obtained at 160 °C and 100 mM substrate concentration with 8 min resident time (81% yield). Similarly, the second step was optimized likewise (substrate concentration [10–100 mM], temperature [200–280 °C], flow rate [0.5–1 mL min⁻¹]) to give VUT-MK142 in 84% yield at a reaction temperature of 200 °C using 100 mM of intermediate and a tenfold excess of cyclohexylamine with a resident time of 8 min. This accounts for a total yield of 68% *via* two stages within 2 times 8 min resident time in the continuous process *vs.* 38% overall yield for the batch reactions requiring 5 hours of conversion time.

Biological results

To screen for “cardiomyogenic activity” of the selected SySM compounds, we used an ANF promoter luciferase reporter assay as in the publication of Wu *et al.*⁵ ANF (atrial natriuretic factor) is a polypeptide hormone synthesized primarily in cardiomyocytes. The gene encoding for ANF is commonly used as a cardiac marker gene. Fig. 2 displays the effect of 7-day treatment with 1 μ M CgC significantly leading to increased luciferase signal in P19 (top) and C2C12 cells (bottom), indicating an up-regulation of the expression of ANF by CgC in both cell types compared to the control (CTL, cells treated with 0.005% DMSO). These results confirm the cardiomyogenic activity of CgC on stem cells, which was previously reported by others.⁵ Among the other related compounds tested (7-day treatment, 1 μ M), VUT-MK396 and VUT-MK093 showed a similar activity to CgC (Fig. 2). Compounds VUT-MK310 and VUT-MK431, on the other hand, did not show any activity on P19 and C2C12 cells, as their luciferase signals were similar to control. Fig. 2 (top) further shows that the luciferase signal of VUT-MK296-treated P19 cells was similar to that of control P19 cells. In contrast, VUT-MK296-treatment increased the luciferase signal in C2C12 cells to a similar extent to CgC (Fig. 2, bottom). Interestingly, this suggests that VUT-MK296 exhibits cardiomyogenic activity only on C2C12 cells, and thus in a cell type-specific manner. Finally, the compound VUT-MK142 showed a remarkable effect on both P19 and C2C12 cells. Thus, compared to CgC, VUT-MK142-treatment led to a markedly stronger up-regulation of the expression of ANF (Fig. 2). This may suggest superior cardiomyogenic activity of the compound VUT-MK142 over the original compound CgC.

When comparing the structures of the evaluated compounds, some conclusions can be drawn regarding important structural features: in all cases of 4,6-diaminosubstituted pyrimidines *p*-anisidine was used as an aromatic amine substituent and hence the influence of the alkylamine substituent can be evaluated. Our most active compounds from this series, VUT-MK142 and VUT-MK093, carry cyclohexylamine (VUT-MK142) and ethanolamine (VUT-MK093). VUT-MK093 is hence a regioisomer of CgC reported in the literature (Fig. 1). Interestingly, VUT-MK093 shows higher activity on C2C12 cells and lower activity on P19 cells as compared to CgC. VUT-MK142 is in both cases superior to the two previously mentioned compounds. Hence, it can be concluded that the OH-group present in VUT-MK093 and CgC is not required for activity since the hydrophobic cyclohexylamine in VUT-MK142 shows excellent results. This is also supported by the results of VUT-MK296 (ethylamine instead of ethanolamine) which has similar reactivity in C2C12 cells compared to CgC and VUT-MK093. The activity of VUT-MK296 in P19 cells is not as good as those of VUT-MK093 and CgC. If the aliphatic substituent is further reduced in size to methylamine as in VUT-MK431, the activity would be lost completely in both cell lines.

VUT-MK396, which is structurally very similar to VUT-MK093 and CgC, also gives good results in our assays, however, it contains a pyridine ring as a structurally distinctly different core structure. The amino substituents are identical and always require a 1,3-relationship. Since all three compounds show good activity, it can be concluded that the spatial relationship of the substituents is of higher importance than the heterocyclic scaffold. By comparing the results of VUT-MK396 and VUT-MK310 it can be concluded that steric factors play an important role. In these two examples, the only difference in the structure is

the residue in the *para*-position of the aniline substituent. In VUT-MK310 the significantly larger phenoxy group is located in this position as compared to a methoxy group in VUT-MK396. Obviously the phenoxy moiety is too sterically demanding and the biological activity is lost completely. These initial results will be exploited in the future to further optimize the biological activity of new compounds.

Based on the very promising preliminary ANF assays, VUT-MK142 was further characterised with respect to its cardiomyogenic properties. As a first step we tested if VUT-MK142 also up-regulates the expression of the cardiac key transcription factor Nkx2.5 (ref. 13) besides ANF. This was achieved using an Nkx2.5 promoter luciferase reporter assay.¹⁴ Compared to control P19 cells (treated with 0.005% DMSO only), the 7-day treatment of P19 cells with 1 μ M VUT-MK142 significantly ($p < 0.05$) increased the luciferase signal by 3.1 ± 0.3 luciferase ($n = 5$)-fold. The respective signal increase induced by VUT-MK142-treatment in C2C12 cells was 2.2 ± 0.2 ($n = 6$)-fold ($p < 0.01$). This suggests a pronounced up-regulation of the expression of the cardiac marker Nkx2.5 by VUT-MK142 in both cell types. Thus, not only ANF-expression (Fig. 2), but also Nkx2.5-expression is induced by VUT-MK142-treatment in P19 and C2C12 cells.

For further comparison of this VUT-MK142 activity with the parent compound CgC, Nkx2.5 promoter luciferase reporter assays were also performed on CgC-treated cells. Compared to control P19 and C2C12 cells, CgC treatment gave rise to luciferase signal increases of 1.6 ± 0.2 ($n = 5$) and 1.4 ± 0.2 ($n = 5$)-fold, respectively. Thus, compared with VUT-MK142 (see above), Nkx2.5 up-regulation by CgC was considerably less. These results are in accordance with the stronger up-regulation of the expression of ANF by VUT-MK142 compared with CgC (Fig. 2), and confirm the superior cardiomyogenic activity of VUT-MK142 *via* the parent compound. To determine whether VUT-MK142 not only promotes expression of genes involved in cardiomyogenesis but also promotes cardiomyogenesis in a way which results in the increased commitment of mesodermal precursors to the cardiomyogenic lineage or the increased differentiation of committed precursors of cardiomyocytes into rhythmically contracting adult-like cardiomyocytes, we investigated the influence of VUT-MK142 on myocardial differentiation of murine cardiovascular progenitor cells (CVPCs).¹⁵ Therefore, CVPCs were aggregated to form cardiac bodies (CBs) and cardiomyogenesis was monitored in these CBs for 20 days by determining the percentage of CBs with beating cardiomyocytes and the number of cardiomyocyte clusters per CB. The addition of VUT-MK142 at a time when CVPCs become committed to myocardial differentiation between days 0 and 5 after aggregation of cells¹⁶ resulted in a two-fold increase in the number of CBs with beating cardiomyocytes (Fig. 3A). VUT-MK431, which did not activate the ANF reporter gene (*negative control*), had no influence on cardiomyogenesis in CBs. The addition of VUT-MK142 between days 0 and 5 resulted in an earlier onset by one day and an increased degree of cardiomyogenesis, whereas the addition between days 5 and 7 had no influence on the early phase of cardiomyogenesis between days 11 and 13 but later it increased the number of cardiomyocytes (Fig. 3B).

In addition to the earlier onset of cardiomyogenesis becoming evident by the larger number of CBs with beating cardiomyocytes between days 11 and 13 (Fig. 3B) the number of

beating clusters per CB was also increased when VUT-MK142 was present at the beginning of cardiomyogenesis (Fig. 3C). Notably, when VUT-MK142 was added to the W2 embryonic stem cell line which forms only very few cardiomyocytes¹⁷ no increase in cardiomyogenesis was observed (not shown). This makes it very likely that VUT-MK142 is neither sufficient to induce cardiomyogenesis in cells lacking some essential cardiogenic factors nor that it does significantly influence the late differentiation of already committed cardiomyocytes (Fig. 3B). Thus, we may conclude that VUT-MK142 specifically promotes very early events during cardiomyogenesis in mesodermal precursors of cardiomyocytes occurring before the onset of Nkx2.5 expression during cardiomyogenesis and it might positively influence the commitment of mesodermal precursors to the cardiomyogenic fate.

To substantiate these observations we differentiated CVPCs in the presence of VUT-MK142 and determined the expression levels of early mesodermal and myocardial transcription factor genes by semi-quantitative RT-PCR (Fig. 4). Brachyury, Nkx2.5, and Mef2C expressions were upregulated in the presence of VUT-MK142, whereas Eomes, Gata4, and the late myocardial gene tropomyosin were not affected. Apart from the unexpected negative effect of VUT-MK142 on the Mesp1 expression, activation of Brachyury, Nkx2.5, and Mef2C perfectly fits to the current model of sequential activation of the cardiomyogenic program in CVPCs.¹⁸

Together our findings suggest that VUT-MK142 is a potent new cardiomyogenic synthetic small molecule promoting the differentiation of pre-cardiac mesoderm into cardiomyocytes. We are currently performing experiments to further characterise the cardiomyogenic actions of VUT-MK142, and to identify its molecular targets.

Conclusions

By targeted modification of a previously reported hit structure we were able to generate the new synthetic compound VUT-MK142 with promising cardiomyogenic effects on cells of various types. This candidate represents a high profile pharmacological probe complementing an increasing number of small molecules capable to trigger cardiomyogenesis. Further efforts are required to elucidate the mode of action of the compound and its eventual potential to promote cardiac repair by stem cells. The initial synthetic entry towards the test compounds was extended towards continuous flow-chemistry in particular for the lead VUT-MK142, enabling facile up-scaling in synthesis as well as diversity oriented library synthesis employing automation assisted flow-devices.

Experimental section

Chemistry

6-Chloro-*N*-(4-methoxyphenyl)-pyrimidin-2-amine 1—4,6-Dichloropyrimidine (1.57 g, 10.6 mmol, 1.3 equiv.) and *p*-methoxyaniline (1 g, 8.10 mmol, 1 equiv.) were dissolved in *i*-PrOH (15 mL) and HCl (37%, 1.5 mL) was added. The reaction mixture was then refluxed (84 °C) for approx. 2.5 hours under a 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: R_f = 0.85 (EtOAc–EtOH = 10: 1); ^1H NMR (DMSO- d_6 , 200 MHz): 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). ^{13}C NMR (DMSO- d_6 , 50 MHz): 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).

***N*⁴-Cyclohexyl-*N*⁶-(4-methoxyphenyl)pyrimidine-4,6-diamine VUT-MK142—**

Prepared according to the general procedure B (see Supporting Information): 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 (4 mL) and heated to 200 °C for 90 minutes under microwave irradiation. 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: R_f = 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 4 mL Hastalloy® coil. The flow rate was adjusted to 0.5 mL min^{−1}, resulting in a residence time (=reaction time) of 8 minutes, while the pressure was set to 50 bar as the standard value. Product collection was triggered after the 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 solution was subjected to the reaction conditions. Upon completion of starting material injection, the pumps were switched again and the reactor system was flushed with the solvent to allow for a subsequent reaction. During the optimization reactions, 4 mL of starting material solution was introduced into the system and 6 mL total volume was collected (1 mL pre – 4 mL reaction – 1 mL post).

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

For the synthesis of VUT-MK142, 1 (237 mg, 1.01 mmol), cyclohexylamine (998 mg, 10.1 mmol) and DIPEA (390 mg, 3.02 mmol) were dissolved in NMP (10 mL 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), the pure product was isolated in 84% yield (252 mg, 0.85 mmol).

¹H NMR (CDCl₃, 200 MHz): δ = 1.05–2.01 (m, 11H), 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). ¹³C NMR (CDCl₃, 50 MHz): δ = 24.7 (t), 25.7 (t), 33.0 (t), 49.6 (d), 55.6 (q), 80.2 (d), 114.8 (d), 125.3 (d), 131.7 (s), 156.9 (s), 158.4 (d), 161.8 (s), 162.2 (s). HR-MS: predicted [MH]⁺ 299.1866; measured [MH]⁺ = 299.1878; diff. in ppm = 4.01.

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⁻¹ penicillin, 50 μ g mL⁻¹ 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⁻¹ penicillin, 50 mg mL⁻¹ streptomycin) as in the publication of Wu *et al.*,⁵ containing 1 μ M of the respective single SySM compound to be tested. DMSO, which was used as the 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⁻¹ glucose, 4 mM L-glutamine, 50 U mL⁻¹ penicillin, 50 μ g mL⁻¹ 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 the 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 μ M) were always added at the same time when differentiation was induced, and were applied for 7 days (see above).

A5 CVPCs were isolated from newborn mouse hearts and cultured in the M15 medium¹⁵ on mitotically inactivated SNL76/7 fibroblasts.¹⁶

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.⁵ 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 the 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 the luciferase activity was measured (Wallac 1420 Victor multilabel counter, Perkin Elmer) using a Promega's dual-luciferase reporter assay kit. P19 cells treated with 10 nM all-*trans* retinoic acid (RA) for 7 days served as a

positive control for the assay. In this low concentration, RA drives cardiomyogenic differentiation in embryonic stem cells.¹⁹

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)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.^{16,20} Shortly after, CVPCs were aggregated to cardiac bodies (CBs) in hanging drop cultures at a density of 900 ± 50 cells per 20 mL medium M15 (ref. 16) for 4.5 days and then plated on gelatine coated 10 cm tissue culture plates at a density of 0.9×10^4 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 CBs during the entire duration of the experiment. In addition, the borders of the disc shaped CBs 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 day 20. Sequences of primers used for semiquantitative RT-PCR are available on request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Examples of cardiogenic compounds known in the literature.

Fig. 2.

Screening for cardiomyogenesis using an ANF promoter reporter assay. P19 (top) and C2C12 (bottom) cells were treated with single SySM compounds in a concentration of 1 μ M for 7 days. The control cells (CTL) were treated with 0.005% DMSO for 7 days. The same DMSO concentration is used for the compound-treated cells. In control experiments (not shown), 0.005% DMSO-treated cells showed similar luciferase activities to untreated cells. Luciferase activities are expressed normalized to the control. Column “RA” represents P19 cells treated with 10 nM retinoic acid for 7 days, and serves as a positive control. The dotted lines allow for a direct comparison with the luciferase activity of control (lower line) and CgC-treated (upper line) cells. Datapoints represent mean values; error bars indicate standard errors of mean (SEM). *n* values for executed ANF assays (each performed on independent cell preparations): 3–10. Statistical analyses (paired two-tailed Student’s *t*-tests performed with unnormalised raw data) to test for differences between control and compound-treated cells were only performed for CgC and VUT-MK142, because only for these compounds *n* values were sufficiently high (5–10). *, **, and *** indicate a significant difference to the control with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Fig. 3.

Influence of VUT-MK142 on cardiomyogenesis in cardiac bodies. Cardiovascular progenitor cells were induced to differentiate by aggregation and cardiac bodies (CBs) were incubated from day 0 to day 5 in the presence of 0.005% DMSO (control, solvent for MKs) and in the presence of 1 μ M VUT-MK142 and VUT-MK431, respectively. (A) Cardiomyogenesis was determined as the percentage of CBs with beating cardiomyocytes from day 0 to 20 where first beating cardiomyocytes appeared between days 10 and 11. Data for control and VUT-MK142 are from 7 experiments and those for VUT-MK431 are from 3 experiments with each 80 CBs monitored. (B) VUT-MK142 was also added between days 5 to 7 to investigate an influence on the already committed cardiomyocytes. Evaluation of the extent of cardiomyogenesis at the onset of rhythmic beating between days 11 and 13 was compared to that between days 14 to 20. Each 80 CBs were monitored in 3 (VUT-MK142 from days 5 to 7), and 7 experiments (VUT-MK142 from days 0 to 5). (C) The increase in cardiomyogenic differentiation was measured by counting the number of cardiomyocyte clusters in a total of 560 CBs over a period of 10 days in 3 experiments. Error bars, standard deviation.

Fig. 4.

Semi-quantitative RT-PCR analysis of CVPCs differentiated in the absence and presence of 1 μ M VUT-MK142, with primer pairs as indicated.

Scheme 1.

(a) Conc. HCl, i-PrOH, reflux. (b) Diisopropylethylamine 2.25 equiv., n-BuOH, 200 °C. (c) $Pd(OAc)_2$ 2 mol%, (+/-)-BINAP 2 mol%, K_2CO_3 3.5 equiv., toluene, 180 °C, microwave, 30 min (d) amine as a solvent at 150 °C, 3 days.

Table 1

Selected pyrimidine- and pyridineamines subjected to biological testing

Entry	Structure	Name	Yield ^a [%]
1		VUT-MK093	55
2		VUT-MK142	38 (68) ^b
3		VUT-MK296	51
4		VUT-MK431	65
5		VUT-MK310	73
6		VUT-MK396	52

^a Isolated yield over 2 steps.^b Isolated yield with the continuous flow protocol