

A 2,6-Disubstituted 4-Anilinoquinazoline Derivative Facilitates Cardiomyogenesis of Embryonic Stem Cells

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Chemical approaches are widely used in directed differentiation of embryonic stem (ES) cells. In our search for novel lead compounds that could facilitate cardiomyogenesis of ES cells, we designed a two-step screening system based on P19 embryonic carcinoma and mouse ES cells. Application of this system to a quinazoline compound library including 2,3-disubstituted 8-arylamino-3*H*-imidazo[4,5-*g*]quinazolines and 2,6-disubstituted 4-anilinoquinazoline led us to the discovery of com-

pound **62**, which exhibits a stable cardiomyogenic effect on both P19 and mouse ES cells at a concentration of 0.1 μ M. An EGFR inhibition assay and molecular docking studies confirmed **62** as a potent EGFR inhibitor with a tyrosine kinase IC₅₀ value of 101 nM. However, major differences in cardiomyogenic activity were observed between iressa and **62**, indicating that other molecular events are also involved in compound **62**-induced cardiomyogenesis of ES cells.

Introduction

Appropriate screening systems for small molecules from chemical libraries have become a critical tool in basic biology research and drug discovery. Recent advances in stem cell research led us to a more comprehensive understanding of various types of stem cells, such as embryonic stem (ES) cells, adult stem cells, and induced pluripotent stem (iPS) cells. Although iPS cells have the advantage of overcoming immunologic barriers, the use of iPS cells as a renewable cell source is compromised due to safety and efficacy issues^[1,2] as well as limited differentiation capacity.^[3,4] Multiple protocols for directing the differentiation of ES cells into various cell types have been developed and analyzed.^[5,6] Directed differentiation of ES cells remains an important way of achieving reliable cell sources for regenerative medicine and drug discovery.

Chemical approaches, using small molecules to direct differentiation of ES cells, have been proven effective.^[7–9] Our previous work has also focused on small molecule-induced cardiomyogenesis of ES cells.^[10] The P19 cell line is a pluripotent cell similar to ES cells; it can differentiate into all cell lineages and is widely used in cardiomyogenesis research. P19 cells are often chosen for primary screening due to the fact that they are easy to culture, amenable to genetic manipulation, and have a low frequency of spontaneous cardiac differentiation.^[11]

Quinazoline compounds are used as drugs in various areas, particularly in anticancer treatment. Previous work identified 2,3-disubstituted 8-arylamino-3*H*-imidazo[4,5-*g*]quinazolines as a novel class of anticancer agents with stable cytotoxic effects on A549 cells.^[12,13] The target epidermal growth factor receptor (EGFR) tyrosine kinase plays an important role in tumor growth. EGFR is also detected on the embryonic cell surface at eight-cell/morula and blastocyst stages.^[14] EGF application on mouse ES cells stimulated cell proliferation.^[15] Moreover, EGF has been reported to contribute to neural differentiation of pheochromocytoma PC12 and neuronal progenitor ST14A

cells.^[16] For directed differentiation of ES cells, a specific lineage commitment is very important. Wu et al. reported the existence of a switch between cardiomyocyte and neuronal commitment.^[17] Based on the neural induction effect of EGF, we speculate that EGFR inhibitors might bias cardiomyocyte commitment of ES cells and function as a cardiomyogenesis stimulator.

By application of the P19- and mouse ES cell-based screening system on a quinazoline compound library, we hoped to identify novel cardiomyogenic compounds which could help us shed some light on the influence of the EGFR pathway in cardiomyogenesis of ES cells. Our results demonstrate the feasibility of a cardiomyocyte-specific, atrial natriuretic factor (ANF)-driven, luciferase-expressing P19 cell-based primary screen and ES cell-based secondary assays in discovering cardiomyogenic compounds.

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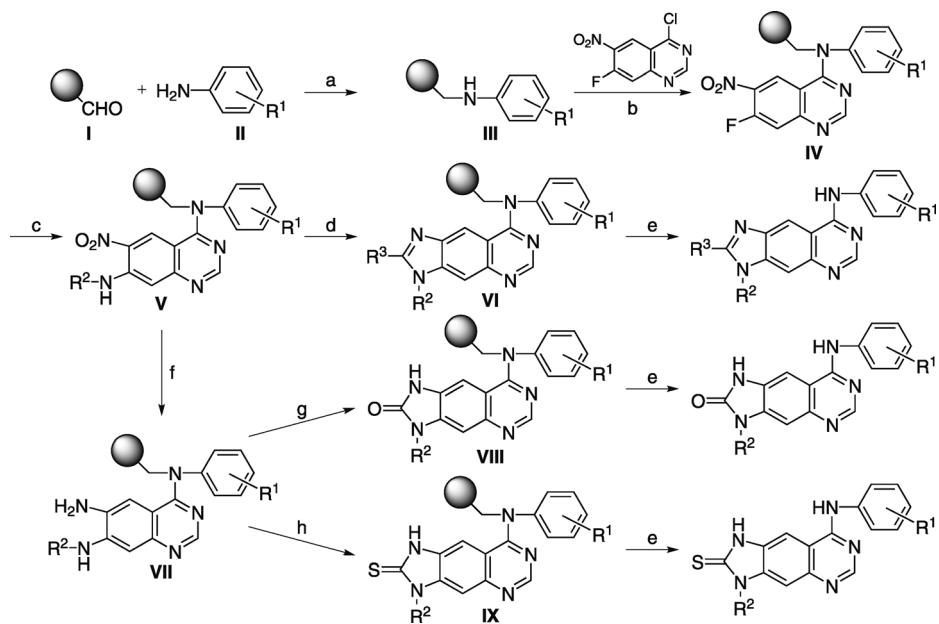
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Results

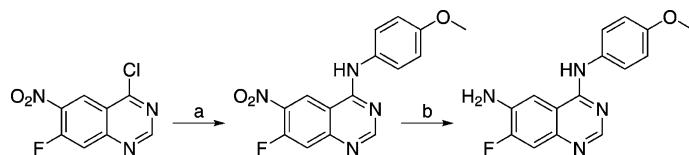
Chemistry

The compounds used for screening were synthesized according to the procedures shown in Scheme 1 and Scheme 2, and were purified using a flash column purification method. The products were characterized by electrospray LC-MS and ^1H NMR. Please see the Experimental Section below for detailed procedures.

creasing concentrations (0.1, 1, and 5 μM) were subjected to the primary screen as shown in Figure 1B and C. There is an inevitable statistical fluctuation in the screening systems. The criterion set for real effects is 1.5- to 2-fold changes, which corresponds to an effect approximate to or higher than that of retinoic acid (RA). Six compounds, numbered **62**, **165**, **195**, **51**, **D39**, and **Z34** (Figure 1A and B), were confirmed as active. Compounds **165**, **D39**, and **Z34** exhibited dose-dependent effects. Compound **62** showed the most efficient induction of cardiomyogenesis at a concentration of 0.1 μM .



Scheme 1. Synthesis of 2,3-disubstituted 8-arylamino-3H-imidazo[4,5-g]quinazolines, 8-arylamino-1H-imidazo[4,5-g]quinazolin-2(3H)-one, and 8-arylamino-1H-imidazo[4,5-g]quinazoline-2(3H)-thione. *Reagents and conditions:* a) NaBH_3CN , DMF/AcOH, RT, 24 h; b) 4-chloro-7-fluoro-6-nitroquinazoline, THF, Et_3N , 24 h, RT, repeat; c) R_2NH_2 , CH_2Cl_2 , 24 h, RT; d) R_3CHO , $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, DMF, 50 °C, 1 h; e) TFA/ CH_2Cl_2 , 1 h, RT; f) $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, DMF, RT, 24 h; g) triphosgene, CH_2Cl_2 , 38 °C, 12 h; h) CS_2 , DIET, RT, 24 h.



Scheme 2. Synthesis of compound **62**. *Reagents and conditions:* a) $p\text{-CH}_3\text{OC}_6\text{H}_5\text{NH}_2$, CH_2Cl_2 , RT, 30 min; b) $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, MeOH, 0 °C, 30 min, then NaBH_4 , MeOH, RT, 30 min.

Primary screen using a modified P19 cell line

The P19 cell line used in the primary screen was a gift from Professor Su Zeng (College of Pharmaceutical Sciences, Zhejiang University). Luciferase gene expression in P19 cells was controlled by a cardiomyocyte-specific ANF reporter that is activated in the case of cardiomyocyte differentiation. Hit compounds were confirmed according to their abilities to enhance luciferase expression in differentiating cells. Thirty-one compounds from the quinazoline library, each in progressively in-

Secondary assay on mouse ES cells

Mouse ES cells (D-3) were differentiated under a condition favoring cardiomyogenesis as previously reported.^[10,18] Cardiomyocyte differentiation was assessed as the presence of rhythmically beating outgrowths in embryoid bodies (EBs). Four of the six confirmed compounds were subjected to a secondary assay. The commercially available EGFR inhibitor iressa (Gefitinib, ZD1839), as well as compound **193**, which was ineffective in the primary screen (Figure 1C), were compared with test compounds for EGFR-inhibiting or cardiomyogenesis-promoting effects, respectively. Retinoic acid (RA) and DMSO were used as positive and negative controls. Beating foci and beating EBs were counted simultaneously. Compound **62** demonstrated a significant cardiomyogenic effect of a 3.0 (± 0.4)-fold increase in beating EBs at its optimal concentration of 0.1 μM (Table 1). Compounds **Z34**, **D39**, and **165** were less efficient, with 1.6 (± 0.6)-, 2.7 (± 1.3)-, and 1.3 (± 0.05)-fold changes in beating EBs, respectively, at their optimal concentrations of 1 μM (Figure 2D–F). Compound **193** exhibited no cardiomyogenic effect at the tested concentrations (Figure 2C). Iressa showed a biphasic effect on

Table 1. Optimal concentrations and relative activities of compounds **62**, **D39**, **Z34**, **165**, **193**, and iressa on cardiomyogenesis of mouse ES cells.

Compd	c [μM]	Beating EBs ^[a]
iressa	0.5	1.8 ± 0.4
62	0.1	3.0 ± 0.4
D39	1.0	2.7 ± 1.3
Z34	1.0	1.6 ± 0.6
165	1.0	1.3 ± 0.05
193	1.0	1.1 ± 0.2

[a] Data are the mean \pm SD of the fold change of beating EBs.

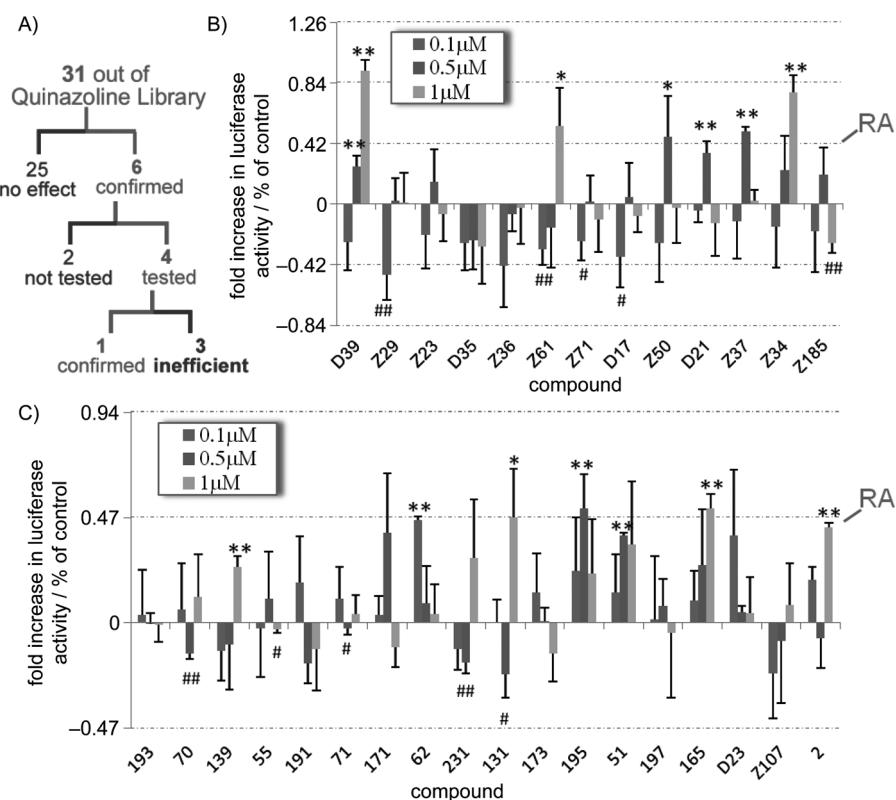


Figure 1. The two-step screening system and results from primary screening. A) Scheme for the screening system. Thirty-one compounds were selected from the quinazoline library. Six compounds were confirmed as active by primary screening; four of these were chosen due to their dose-response curves and were subjected to the secondary assay. Compound **62** was confirmed as a cardiomyogenic compound. B), C) An ANF-driven, luciferase-expressing P19 cell line was used for primary screening. Luciferase activities of P19 cells after treatment with test compounds at concentrations of 0.1, 0.5, and 1 μM , respectively, were assessed by a luciferase assay kit. Data represent the mean ($\pm \text{SD}$, $n=3$) fold increase in luciferase activity relative to DMSO control; statistical significance was set as * $p < 0.05$, ** $p < 0.01$ versus DMSO control for positive fold increase, and # $p < 0.05$, ## $p < 0.01$ versus DMSO control for negative fold increase. Retinoic acid (RA) was used as a positive control.

cardiomyocyte differentiation (Figure 2B). Iressa (0.1 μM) inhibited the appearance of beating EBs with a 0.4 (± 0.01)-fold change relative to DMSO. However, cardiomyogenesis was slightly but not significantly enhanced at a concentration of 1 μM with a 1.8 (± 0.4)-fold change in activity, which may be a statistical fluctuation and not a real effect. The change in activity remains slightly below the control group up to a concentration of 5 μM .

Sarcomeric α -actinin expression and assembly

EBs treated with compound **62** exhibited positive staining of sarcomeric α -actinin (Figure 3A); a properly assembled ladder-like sarcomeric structure can be clearly observed. This indicates that the cardiomyocytes induced by **62** have a mature cardiomyocyte phenotype. Western blot analysis showed significant upregulation of α -actinin expression within **62**-treated culture (Figure 3B). This was consistent with the increased number of beating EBs. These results further confirmed the cardiomyogenic effect of compound **62**.

EGFR inhibiting activity and molecular docking

To evaluate the EGFR-inhibiting activity of **62**, an *in vitro* EGFR tyrosine kinase (TK) assay was performed. The TK IC₅₀ values for iressa and **62** obtained from the percentage inhibition curves were 42 and 101 nM, respectively (Table 2). Molecular docking of iressa, **62**, **D39**, **Z34**, **165**, and **193** were performed using an EGFR binding model based on the EGFR crystal structure (PDB ID: 2ITY). The binding model (Figure 3A–F) showed that **62**, **193**, **D39**, **Z34**, and **165** bound well to EGFR in the region that bound iressa. Compounds **193** and **Z34** each formed one hydrogen bond with Met193 in EGFR, as did **62** (Figure 4A, Table 2). Amino acids that formed hydrogen bonds with compounds **165** and **D39** were Thr854 and Lys745, respectively. Two hydrogen bonds with Thr854 and Lys745 were present in the binding model of iressa.

Discussion

EGFR, also known as ErbB1 or Her1, plays an important role in heart development and function.^[19] Studies on Waved-2 mice, which have a mutated EGFR with 10–15% activity, demonstrate that EGFR is important for semilunar valve morphogenesis in the heart.^[20] Transactivation of EGFR is reported to be related to cardiac hypertrophy.^[21] In the present study, we used a commercially available EGFR inhibitor, iressa, on mouse ES cells to explore its influence on cardiomyogenesis. No dose-dependent response was observed in this model. Iressa at 0.1 μM ,

Table 2. *In vitro* EGFR inhibition activity and molecular docking of the test compounds.

Compd	IC ₅₀ [nM] ^[a]	Score	Hydrogen bond		
			Met 793	Thr 854	Lys 745
iressa	42	58.3	–	+	+
62	101	46.0	+	–	–
D39	–	48.0	–	–	+
Z34	–	55.8	+	–	–
165	–	57.1	–	+	–
193	–	62.9	+	–	–

[a] EGFR inhibition; where no value is given (–), compound was not evaluated in the EGFR tyrosine kinase [TK] assay.

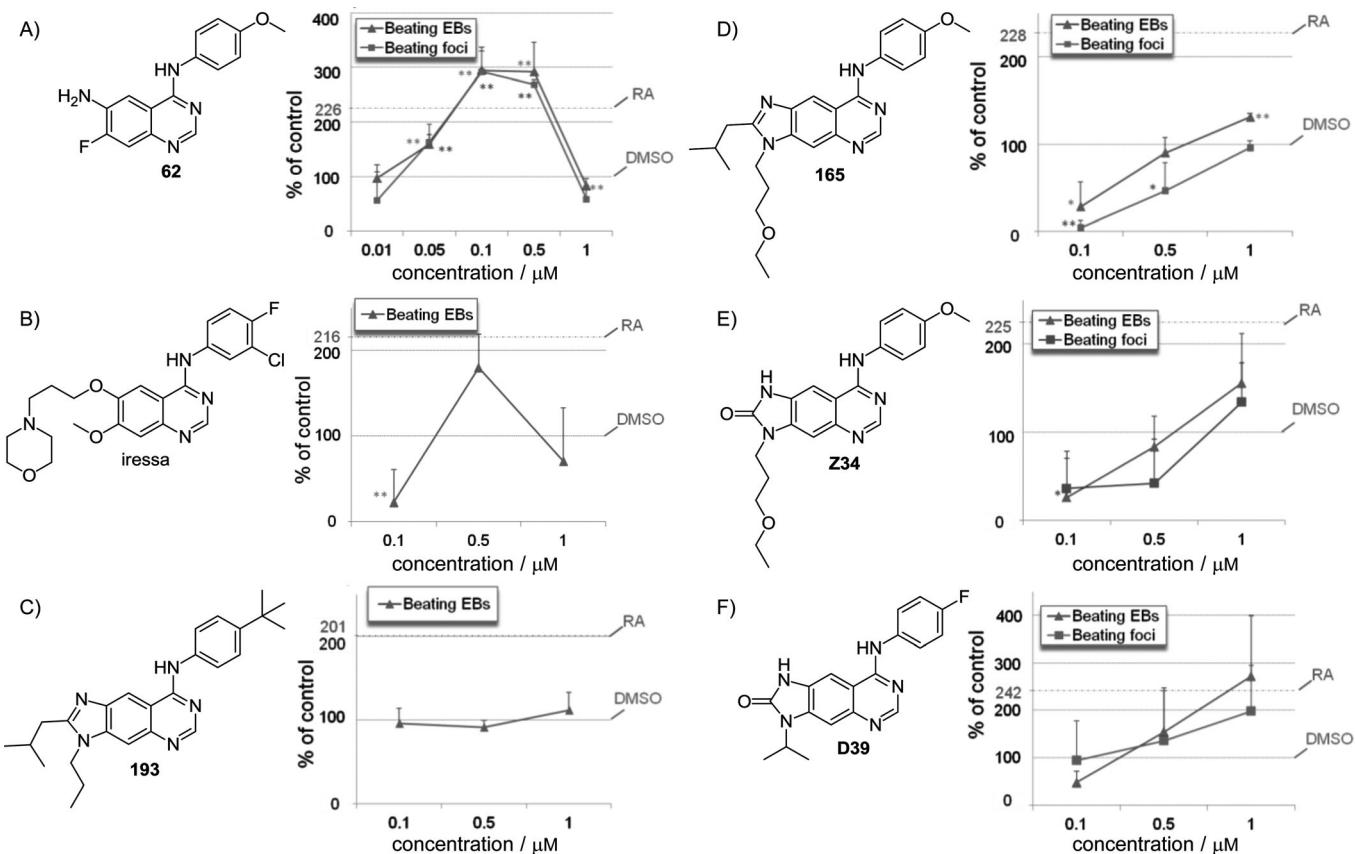


Figure 2. Secondary assay for confirmed hits in mouse ES cells. The structures of test compounds (left panel, A–F) and their effects on cardiomyogenesis of mouse embryonic stem cells (right panel, A–F). ES cells were exposed to test compounds after hanging-drop culture. After 3 days of culture in hanging drops and 2 days in suspension, the formed EBs were plated onto 24-well plates. The percentage of spontaneously beating EBs, as well as beating foci, were assessed simultaneously 7 days after plating. Data represent the mean \pm SD ($n=3$); statistical significance was set as $*p < 0.05$, $**p < 0.01$ versus DMSO control. Retinoic acid (RA) was used as a positive control.

which is ~ 2.5 -fold higher than its TK IC_{50} value, significantly decreases the number of beating EBs. It is reported that varieties of TK inhibitors targeting EGFR, such as iressa and erlotinib, are linked with cardiac toxicity,^[19] which may be the reason for this inhibitory effect. As the concentration increases, a turnover in cardiomyogenic activity is observed at $0.5 \mu\text{M}$; however, the number of beating EBs decreases to DMSO level after treatment with $1 \mu\text{M}$ iressa. This may be a toxic effect, owing to the involvement of EGFR inhibition. For **62**, a similar decrease in beating EBs was also apparent at a concentration of $0.5 \mu\text{M}$, which is roughly fivefold higher than its TK IC_{50} value. This further confirmed the cardiac toxicity of TK inhibitors at concentrations higher than their TK IC_{50} values.

In contrast to iressa, **62** exhibits dose-dependent cardiomyogenic activity at concentrations below its TK IC_{50} value (Table 2). As lower concentration of iressa, approximately equal to its TK IC_{50} value, exhibits inhibitory activity toward cardiomyocyte differentiation, it is likely that molecular pathways other than EGFR are responsible for the cardiomyogenic activity of **62**. Quinazoline derivatives are widely studied for their regulatory activity toward various molecules, such as janus-associated kinase 2 (JAK2)^[22], NF- κ B,^[23] and others. These mole-

cules are also involved in cardiomyogenesis of ES cells.^[24,25] It is possible that one or more molecular events contribute to the cardiomyogenic effect of compound **62**.

Conclusions

The results from primary and secondary screening demonstrate that compound **62** possesses a significant cardiomyogenic effect at an optimal concentration of $0.1 \mu\text{M}$. The EGFR inhibition activity assay and docking studies indicate a potent EGFR inhibitory activity of **62** with an *in vitro* TK IC_{50} value of 101 nM . According to the dose–response curves of **62** and iressa, we postulate that the decrease at higher concentrations of both **62** and iressa are due to the cardiac toxicity shared with various TK inhibitors. The cardiomyogenic effect of **62** at concentrations lower than its TK IC_{50} value may be irrelevant to its EGFR inhibitory activity. Further efforts are still needed to elucidate the cardiomyogenic mechanism of compound **62**.

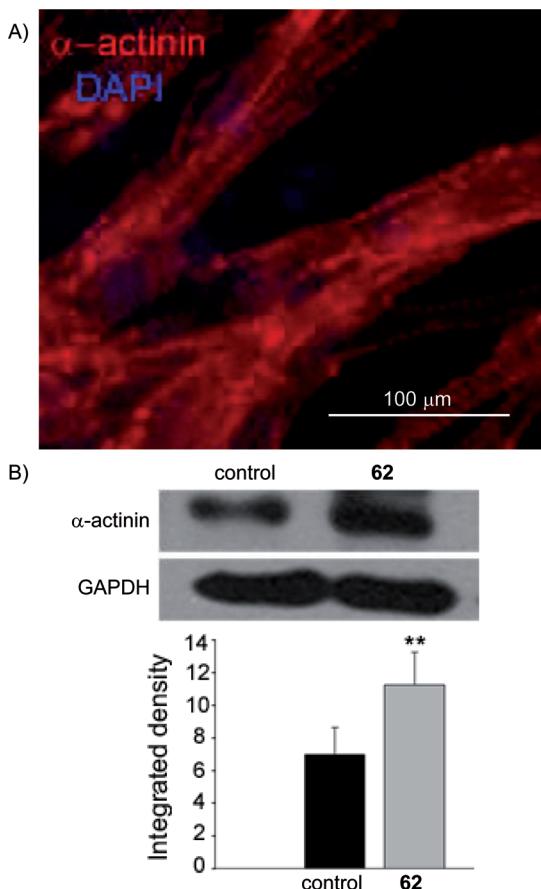


Figure 3. Effect of compound **62** on sarcomeric α -actinin assembly and protein expression in EBs. A) Sarcomeric structure observed within the **62**-induced α -actinin-positive area; scale bar = 100 μ m. B) Western blot analysis of sarcomeric α -actinin. GAPDH was used as a housekeeping protein. Data represent the mean \pm SD ($n=3$); statistical significance was set as ** p < 0.01 versus DMSO control.

Experimental Section

Biology

Materials: Retinoic acid (RA), dimethyl sulfoxide (DMSO), β -mercaptoethanol (β -ME), and 4,6-diamidino-2-phenylindole (DAPI), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media (α -MEM and DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Burlington, ON, Canada). Newborn calf serum was purchased from Sijiqing (Hangzhou, China). Non-essential amino acids (NEAA) stock solution was purchased from Hyclone (Logan, UT, USA). Recombinant mouse leukemia inhibitory factor (LIF) was purchased from Chemicon (Ramona, CA, USA). Primary antibody against α -actinin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies were purchased from Multi-sciences.

Primary screen of quinazoline compound library in P19 cells: The transfected mouse teratocarcinoma cell lines P19 cells were cultured in growth medium consisting of α -MEM supplemented with 10% FBS at 37 °C in 5% CO₂. For induction of differentiation, cells were trypsinized and suspended in growth medium in the presence or absence of test compounds. Approximately 10 000 cells were plated onto 96-well plates. After 3 days, culture medium was changed to growth medium without test compounds. Cells were

cultured for an additional 4 days in growth medium. Luciferase activity was assessed after 7 days using a luciferase assay kit (BioVision, San Francisco, CA, USA) according to the manufacturer's protocol.

Mouse ES cell culture and differentiation: A mouse ES-D3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in high glucose DMEM, supplemented with 10% FBS, 0.1 mM β -ME, 1% NEAA, and 1 \times 10⁶ UL⁻¹ LIF on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) cells. Upon differentiation, ES cells were trypsinized to form a single cell suspension in differentiating medium consisting of high glucose DMEM, supplemented with 20% FBS, 0.1 mM β -ME, and 1% NEAA, with and without test compounds. Approximately 600 cells were cultivated in hanging drops to form EBs for 3 days, then kept in suspension in the same medium for another 2 days. After 5 days, EBs were plated onto 24-well plates (1 EB per well) in the same medium with only 10% FBS. Spontaneously beating EBs and beating foci were detected 7 days after plating.

Western blot analysis of cardiac α -actinin: Embryoid bodies were washed with PBS, harvested 7 days after plating, and lysed on ice in extraction buffer containing Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), EDTA-2 Na (20 mM), NP-40 (1%), sodium deoxycholate (0.1%), DTT (1 mM), NaF (20 mM), Na₃VO₄ (1 mM), and deoxycholic acid sodium salt (0.5%) supplemented with PMSF (1 mM), leupeptin (10 g mL⁻¹), and aprotinin (30 g mL⁻¹). Lysates were centrifuged at 4 °C and 13 000 g for 15 min. The supernatant was aliquoted, and the protein concentration was determined using the Bio-Rad protein assay kit. Protein (20 g) was loaded onto a SDS-PAGE gel and subjected to electrophoresis. Proteins were subsequently transferred onto PVDF membrane and blocked with 5% milk at room temperature for 1 h. The blot was incubated with anti- α -actinin antibody (1:1000 dilution, Sigma-Aldrich) at 4 °C overnight. After washing three times with TTPS, the blot was further incubated with HRP-conjugated secondary antibody (1:5000) at room temperature for 1 h. After successive washing, protein bands were detected by an enhanced chemiluminescent substrate (ECL).

Immunofluorescence analysis of cardiac α -actinin: Immunofluorescence staining was performed on EBs exposed to compound **62** for 5 + 7 days. EBs were fixed in pre-cooled MeOH at -20 °C for 15 min. After washing in PBS, EBs were permeabilized in 0.1% Tween 20 for 30 min and blocked in 10% newborn calf serum for 60 min at room temperature. Anti- α -actinin antibodies (1:50) were diluted in PBS with 10% newborn calf serum and incubated with EBs at 4 °C overnight. After washing with PBS three times in 10 min intervals, EBs were then incubated with Daylight 549-conjugated secondary antibodies (1:200 in 10% newborn calf serum) at room temperature for 1 h.

EGFR protein tyrosine kinase assay: The EGFR protein tyrosine kinase (PTK) assay was carried out as previously reported.^[12] A modified PTK-101 kit from Sigma-Aldrich was used to measure TK inhibition. The assay was carried out according to manufacturer's instructions. The results are expressed as percentages of the control (T/C%), and IC₅₀ values were calculated from these inhibition curves using Graph-Pad Prism software.

Molecular modeling

Molecular docking of compound **62** into the three-dimensional EGFR complex structure (2ITY, downloaded from the RCSB Protein Data Bank) was conducted according to our previous report.^[26] The starting structures of compounds were constructed and optimized

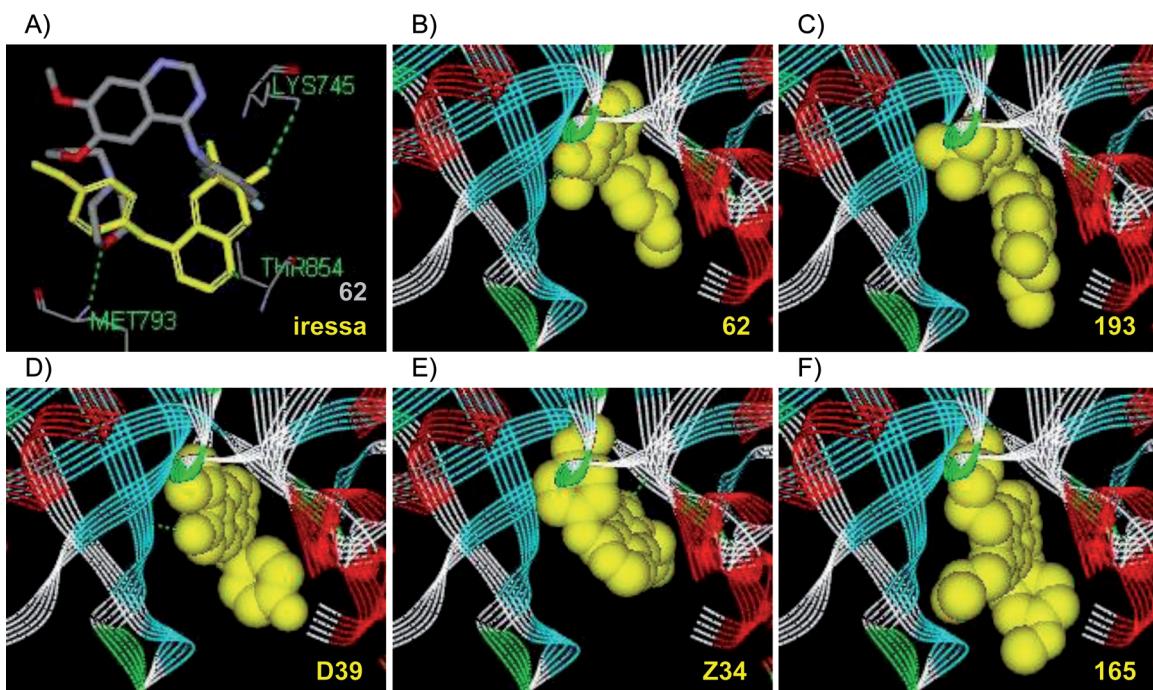


Figure 4. Molecular docking of confirmed hits with an EGFR crystal structure. A) hydrogen bonds formed between the EGFR crystal structure and iressa, as well as compound **62**. Binding model of energy-minimized compounds B) **62**, C) **193**, D) **D39**, E) **Z34**, and F) **165**.

using the DS CHARMM force field. All conformers were minimized to an RMSD value of $0.01 \text{ kcal mol}^{-1}$. The lowest-energy conformer was used for docking. The receptor docking site was defined based on the binding position of iressa in the receptor and specified as all atoms within a radius of 12.0 \AA of iressa using an “eraser” algorithm. The Monte Carlo method was employed to carry out a conformational search of the ligands. The LigandFit method was used to analyze the ligand–site match and the orientation/permuation during docking; docked ligands were evaluated by Dock-Score. All modeling work was carried out using Discovery Studio Modeling software package (Accelrys, San Diego, CA, USA) run on a Dell Power-Edge 2600 Server with default setting values excepting those explicitly stated.

Chemistry

General: ^1H NMR data were recorded on Avance500 or DPX-400 with CDCl_3 or DMSO as solvents and TMS as the internal standard. J values are given in Hertz (Hz). Chemical shifts are expressed in ppm downfield from internal TMS. The following abbreviations are used to designate the multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. LC-MS (ESI) spectra were recorded on a Finnigan Mat LCQ mass spectrophotometer at 214 nm using a Betasil C_{18} ($3 \mu\text{m}$, 100 \AA , $3 \times 50 \text{ mm}$) column. Purity of the final products was analyzed by an Agilent 1100 series HPLC (mobile phase MeOH/aqueous potassium dihydrogen phosphate (0.02 mol L^{-1}) solution, 60:40; flow rate: 1 mL min^{-1} , wavelength for detection set at 214 nm). 4-(4-Formyl-3-methoxyphenoxy)butyryl AM resin (70–90 mesh) batch: A31603, 1% DVB, substitution: 0.88 mmol g^{-1} was purchased from NovaBiochem (Merck KGaA, Darmstadt, Germany). Flash chromatography was performed using 200–300 mesh silica gel. DMF was treated with CaH_2 , and THF was distilled from sodium and benzophenone before use. Other reagents were used directly without further purification.

Procedure for the synthesis of 7-fluoro-N-(4-methoxyphenyl)quinazoline-4,6-diamine (62): 4-Chloro-7-fluoro-6-nitroquinazoline was synthesized as previously reported^[27] and was dissolved in $200 \text{ mL} \text{ CH}_2\text{Cl}_2$. A solution of aniline (10.5 g, 55 mmol) in EtOH (50 mL) was added and the resulting mixture was stirred at room temperature for 15 min until a precipitate of hydrochloride product formed. After a further 15 min, hexane was added in a quantity sufficient to ensure complete precipitation, and the solid was collected by filtration, washed with petroleum ether, and dried to give pure product. 7-fluoro-N-(4-methoxyphenyl)-6-nitroquinazolin-4-amine (1 mmol) and nickel(II) chloride hexahydrate (2 mmol) were dissolved in MeOH (30 mL). The solution was cooled to 0°C , and NaBH_4 (4 mmol) was added portionwise while stirring and cooling for 30 min, then stirring was continued for 30 min at room temperature (20°C). The dark precipitate was filtered, and the solution was evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel, eluting with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (10:1) (yield: 92%); ^1H NMR (500 MHz, DMSO) $\delta = 9.36$ (s, 1 H), 8.28 (s, 1 H), 7.65 (d, $J = 9.0 \text{ Hz}$, 2 H), 7.58 (d, $J = 9.0 \text{ Hz}$, 1 H), 7.38 (d, $J = 12.5 \text{ Hz}$, 1 H), 6.94 (d, $J = 9.0 \text{ Hz}$, 2 H), 5.67 (s, 2 H), 3.76 ppm (s, 3 H); ^{13}C NMR (125 MHz, $[\text{D}_6]\text{DMSO}$) $\delta = 156.52$, 155.9, 151.9, 143.6, 143.5, 137.1, 137.1, 133.0, 125.1, 124.4, 114.0, 113.7, 112.1, 111.9, 105.0, 105.0, 55.6 ppm; MS (ESI) $m/z: 285.2 [M + H]^+$.

General procedure A for the synthesis of 2,3-disubstituted 8-arylamino-3*H*-imidazo[4,5-*g*]quinazolines: These compounds were synthesized according to our previous report.^[28] Arylamine II (10 equiv, 0.1 M) and NaBH_3CN (10 equiv, 0.1 M) in anhydrous DMF/AcOH (99:1) were added to the 4-(4-formyl-3-methoxyphenoxy)butyryl AM resin I (220 mg, 0.2 mmol), sealed within a polypropylene mesh packet. The mixture was shaken for 24 h at room temperature. The resin was then washed three each with DMF, CH_2Cl_2 , and MeOH. The resulting resin-bound compound (III) was coupled with 4-chloro-7-fluoro-6-nitroquinazoline (10 equiv, 0.1 M) using Et_3N

(10 equiv, 0.1 M) in anhydrous THF at room temperature for 24 h. The resin was washed three times each with DMF, CH₂Cl₂, and MeOH. This procedure was repeated, then resin-bound compound **IV** was reacted with an alkylamine (20 equiv, 0.2 M) in CH₂Cl₂ for 24 h at room temperature. The resin was washed three times each with DMF, CH₂Cl₂, and MeOH to afford resin-bound compound **V**. Reduction and cyclization reactions of resin-bound compound **V** were carried out using an alkylaldehyde (10 equiv, 0.1 M) and SnCl₂·2H₂O (2 M) in DMF to afford resin-bound compound **VI**. After washing three times each with DMF, CH₂Cl₂, and MeOH, the resin-bound tricyclic quinazoline **VI** was treated with TFA/CH₂Cl₂ (1:1) at room temperature for 1 hour before removing the solvent under reduced pressure. The pure product was obtained after flash column purification with eluent EtOAc/EtOH (97:3).

General procedure B for the synthesis of 8-arylamino-1*H*-imidazo[4,5-*g*]quinazolin-2(3*H*)-one: Reduction of resin-bound compound **V** was carried out using SnCl₂·2H₂O (2 M) in DMF for 24 h at room temperature to afford resin-bound compound **VII**. The resin was washed three times each with DMF, CH₂Cl₂, and MeOH. Resin-bound compound **VII** was reacted with triphosgene (10 equiv, 0.1 M) in CH₂Cl₂ for 12 h at reflux to afford resin-bound compound **VIII**. After washing three times each with DMF, CH₂Cl₂, and MeOH, the resin-bound tricyclic quinazoline **VIII** was treated with TFA/CH₂Cl₂ (1:1) at room temperature for 1 h, then the solvent was removed under reduced pressure. The pure product was obtained after flash column purification with eluent EtOAc/EtOH (97:3).

General procedure C for the synthesis of 8-arylamino-1*H*-imidazo[4,5-*g*]quinazoline-2(3*H*)-thione: Reduction of resin-bound compound **V** was carried out using SnCl₂·2H₂O (2 M) in DMF for 24 h at room temperature to afford resin-bound compound **VI**. The resin was washed three times each with DMF, CH₂Cl₂, and MeOH. Resin-bound compound **VII** was reacted with CS₂ (10 equiv, 0.1 M) and DIET (10 equiv, 0.1 M) in THF at room temperature for 24 h to afford resin-bound compound **IX**. After washing three times each with DMF, CH₂Cl₂, and MeOH, the resin-bound tricyclic quinazoline **IX** was treated with TFA/CH₂Cl₂ (1:1) at room temperature for 1 h, then the solvent was removed under reduced pressure. The pure product was obtained after flash column purification with eluent EtOAc/EtOH (97:3).

N-(4-(tert-butyl)phenyl)-2-isobutyl-3-propyl-3*H*-imidazo[4,5-*g*]quinazolin-8-amine (193): General procedure A was followed to give **193** (yield: 92%, HPLC purity: 91%): ¹H NMR (400 MHz, CDCl₃) δ = 8.65 (1 H, s), 8.39 (1 H, s), 8.25 (1 H, brs), 7.77 (1 H, s), 7.65–7.67 (2 H, m), 7.40–7.42 (2 H, m), 4.04–4.08 (2 H, t, J = 7.4 Hz), 2.74–2.76 (2 H, d, J = 7.1 Hz), 2.32–2.38 (1 H, m), 1.82–1.87 (2 H, m), 1.33 (9 H, s), 1.01–1.02 (6 H, d, J = 6.0 Hz), 0.958–0.995 ppm (3 H, t, J = 7.4 Hz); ¹³C NMR (100 MHz, CDCl₃) δ = 160.0, 158.4, 152.8, 147.6, 144.1, 142.4, 139.9, 135.7, 125.9, 121.9, 111.1, 109.7, 105.6, 45.6, 36.6, 34.4, 31.4, 27.8, 22.8, 22.6, 11.3 ppm; LC-MS (ESI) m/z: 416.7 [M + H]⁺.

8-(4-methoxyphenyl)amino-3-(3-methoxypropyl)-1*H*-imidazo[4,5-*g*]quinazolin-2(3*H*)-one (Z34): General procedure B was followed to give **Z34** (yield: 94%, HPLC purity: 89%): ¹H NMR (400 MHz, [D₆]DMSO) δ = 11.56 (1 H, brs), 9.71 (1 H, brs), 8.44 (1 H, s), 8.06 (1 H, s), 7.65–7.67 (2 H, m), 7.38 (1 H, s), 6.95–6.97 (2 H, m), 3.93–3.96 (2 H, t, J = 6.6 Hz), 3.77 (3 H, s), 3.36–3.41 (2 H, m), 3.22 (3 H, s), 1.90–1.93 ppm (2 H, m); LC-MS (ESI) m/z: 380.7 [M + H]⁺.

8-((4-fluorophenyl)amino)-3-isopropyl-1*H*-imidazo[4,5-*g*]quinazolin-2(3*H*)-one (D39): General procedure B was followed to give **D39** (yield: 30%, HPLC purity: 74%): ¹H NMR (400 MHz, [D₆]DMSO) δ = 11.52 (1 H, brs), 9.65 (1 H, brs), 8.45 (1 H, s), 8.05 (1 H, s), 7.83–

7.86 (2 H, m), 7.47 (1 H, s), 7.18–7.23 (2 H, m), 4.66–4.70 (1 H, m), 1.50–1.52 ppm (6 H, d, J = 6.8 Hz); LC-MS (ESI) m/z: 338.6 [M + H]⁺.

3-(3-ethoxypropyl)-2-isobutyl-N-(4-methoxyphenyl)-3*H*-imidazo[4,5-*g*]quinazolin-8-amine (165): General procedure A was followed to give **165** (yield: 90%, HPLC purity: 93%): ¹H NMR (400 MHz, CDCl₃) δ = 8.66 (1 H, s), 8.32 (1 H, s), 7.93 (1 H, s), 7.78 (1 H, s), 7.58–7.60 (2 H, m), 6.93–6.95 (2 H, m), 4.32–4.36 (2 H, t, J = 6.8 Hz), 3.78 (3 H, s), 3.30–3.40 (4 H, m), 2.82–2.84 (2 H, d, J = 7.6 Hz), 2.32–2.38 (1 H, m), 1.98–2.01 (2 H, m), 1.12–1.15 (3 H, t, J = 7.0 Hz), 1.03–1.05 ppm (6 H, d, J = 7.2 Hz); ¹³C NMR (125 MHz, CDCl₃) δ = 160.0, 158.6, 156.8, 153.5, 145.5, 142.4, 139.7, 131.5, 124.4, 114.3, 111.3, 109.4, 106.4, 66.4, 66.3, 55.5, 40.9, 36.2, 29.4, 27.8, 22.6, 15.2 ppm; LC-MS (ESI) m/z: 434.6 [M + H]⁺.

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- [1] R. M. Kanashiro-Takeuchi, I. H. Schulman, J. M. Hare, *J. Mol. Cell. Cardiol.* **2011**, *51*, 619–625.
- [2] K. Okita, S. Yamanaka, *Philos. Trans. R. Soc. London Ser. B* **2011**, *366*, 2198–2207.
- [3] K. Seiler, M. Soroush Noghabi, K. Karjalainen, M. Hummel, F. Melchers, M. Tsuneto, *Stem Cells Dev.* **2011**, *20*, 1131–1142.
- [4] O. Bar-Nur, H. A. Russ, S. Efrat, N. Benvenisty, *Cell Stem Cell* **2011**, *9*, 17–23.
- [5] S. C. Desbordes, D. G. Placantonakis, A. Ciro, N. D. Soccia, G. Lee, H. Djabballah, L. Studer, *Cell Stem Cell* **2008**, *2*, 602–612.
- [6] J. P. Saxe, H. Wu, T. K. Kelly, M. E. Phelps, Y. E. Sun, H. I. Kornblum, J. Huang, *Chem. Biol.* **2007**, *14*, 1019–1030.
- [7] J. A. Efe, S. Ding, *Philos. Trans. R. Soc. London Ser. B* **2011**, *366*, 2208–2221.
- [8] A. J. Firestone, J. K. Chen, *ACS Chem. Biol.* **2010**, *5*, 15–34.
- [9] S. Zhu, H. Wurdak, J. Wang, C. A. Lyssiotis, E. C. Peters, C. Y. Cho, X. Wu, P. G. Schultz, *Cell Stem Cell* **2009**, *4*, 416–426.
- [10] L. Ding, X. G. Liang, Y. Hu, D. Y. Zhu, Y. J. Lou, *Stem Cells Dev.* **2008**, *17*, 751–760.
- [11] X. Wu, S. Ding, G. Ding, N. S. Gray, P. G. Schultz, *J. Am. Chem. Soc.* **2004**, *126*, 1590–1591.
- [12] Z. Chen, X. Huang, H. Yang, W. Ding, L. Gao, Z. Ye, Y. Zhang, Y. Yu, Y. Lou, *Chem. Biol. Interact.* **2011**, *189*, 90–99.
- [13] Y. Zhang, Z. Chen, Y. Lou, Y. Yu, *Eur. J. Med. Chem.* **2009**, *44*, 448–452.
- [14] B. C. Paria, S. K. Dey, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 4756–4760.
- [15] J. S. Heo, Y. J. Lee, H. J. Han, *Am. J. Physiol. Cell Physiol.* **2005**, *290*, C123–133.
- [16] R. Gallo, F. Zazzeroni, E. Alesse, C. Mincione, U. Borello, P. Buanne, R. D'Eugenio, A. R. Mackay, B. Argenti, R. Gradini, M. A. Russo, M. Maroder, G. Cossu, L. Frati, I. Screpanti, A. Gulino, *J. Cell Biol.* **2002**, *158*, 731–740.
- [17] J. Wu, J. Kubota, J. Hirayama, Y. Nagai, S. Nishina, T. Yokoi, Y. Asaoka, J. Seo, N. Shimizu, H. Kajihara, T. Watanabe, N. Azuma, T. Katada, H. Nishina, *Stem Cells Dev.* **2010**, *19*, 1723–1734.
- [18] D. Y. Zhu, Y. J. Lou, *Acta Pharmacol. Sin.* **2006**, *27*, 311–320.
- [19] P. Sanchez-Soria, T. D. Camenisch, *Semin. Cell Dev. Biol.* **2010**, *21*, 929–935.
- [20] N. C. Luetke, H. K. Phillips, T. H. Qiu, N. G. Copeland, H. S. Earp, N. A. Jenkins, D. C. Lee, *Genes Dev.* **1994**, *8*, 399–413.
- [21] Y. Li, H. Zhang, W. Liao, Y. Song, X. Ma, C. Chen, Z. Lu, Z. Li, Y. Zhang, *Am. J. Physiol. Heart Circ. Physiol.* **2011**, *301*, H1941–1951.

- [22] S. H. Yang, D. B. Khadka, S. H. Cho, H. K. Ju, K. Y. Lee, H. J. Han, K. T. Lee, W. J. Cho, *Bioorg. Med. Chem.* **2011**, *19*, 968–977.
- [23] R. S. Giri, H. M. Thaker, T. Giordano, J. Williams, D. Rogers, K. K. Vasu, V. Sudarsanam, *Bioorg. Med. Chem.* **2010**, *18*, 2796–2808.
- [24] K. Foshey, G. Rodriguez, B. Hoel, J. Narayan, G. I. Gallicano, *Stem Cells* **2005**, *23*, 530–543.
- [25] W. K. Jones, M. Brown, X. Ren, S. He, M. McGuinness, *Cardiovasc. Toxicol.* **2003**, *3*, 229–254.
- [26] Z. Q. Wang, N. Weber, Y. J. Lou, P. Proksch, *ChemMedChem* **2006**, *1*, 482–488.
- [27] G. W. Rewcastle, B. D. Palmer, A. J. Bridges, H. D. Showalter, L. Sun, J. Nelson, A. McMichael, A. J. Kraker, D. W. Fry, W. A. Denny, *J. Med. Chem.* **1996**, *39*, 918–928.
- [28] Y. Zhang, C. Xu, R. A. Houghten, Y. Yu, *J. Comb. Chem.* **2007**, *9*, 9–11.

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