Published on Web 01/22/2004

Small Molecules that Induce Cardiomyogenesis in Embryonic Stem Cells

Xu Wu,[†] Sheng Ding,[†] Qiang Ding,[‡] Nathanael S. Gray,[‡] and Peter G. Schultz*[,†,‡]

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, and Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, California 92121

Received October 8, 2003; E-mail: schultz@scripps.edu

Problem: Damaged heart cannot form (many) new heart cells

Therapy: Add stem cells to damaged heart; stem cells differentiate into heart cells

Problem: Transplanted stem cells do not differentiate efficiently into heart cells

Therapy: Differentiate stem cells into heart cells before you transplant them

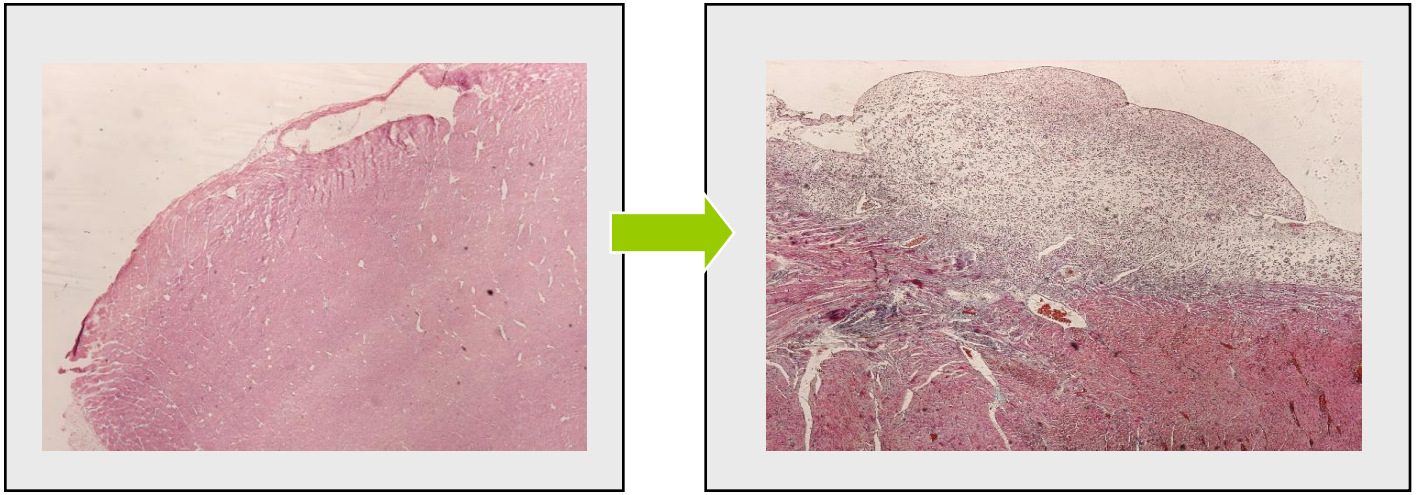
Aim: Identify molecules that can increase conversion of stem cells into heart cells

Why?

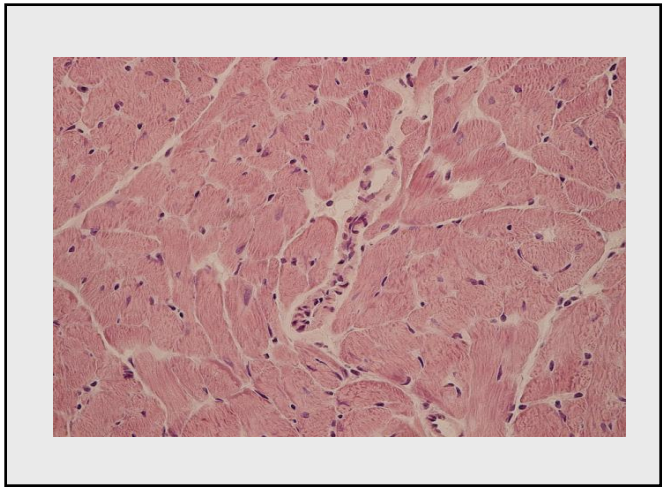
Stem cells are multipotent cells with the ability to self-renew and differentiate into specialized cells in response to appropriate signals.[1] Most tissues have endogenous stem/progenitor cells which, upon injury to the organ, can proliferate and differentiate at the damaged site. The adult heart is composed mainly of postmitotic and terminally differentiated cells. Although a subpopulation of myocardial cells with cardiac stem cell character was identified recently, their limited availability hinders therapeutic applications.[2]

Stem cells derived from other tissues, such as bone marrow, have been shown to be capable of repairing heart damage in animal models,[3] but inefficient differentiation and possible fusion with somatic cells limit their use in cardiac repair.[4] Pluripotent embryonic stem (ES) cells represent a possible unlimited source of functional cardiomyocytes. However, the in vitro differentiation of ES cells into cardiomyocytes involves a poorly defined, inefficient, and relatively nonselective process.[5] Consequently, the development of new approaches for the directed differentiation of ES cells into cardiomyocytes will likely facilitate therapeutic application of ES cells in heart disease, as well as provide important tools for probing the molecular mechanism of cardiomyocyte differentiation and heart development.

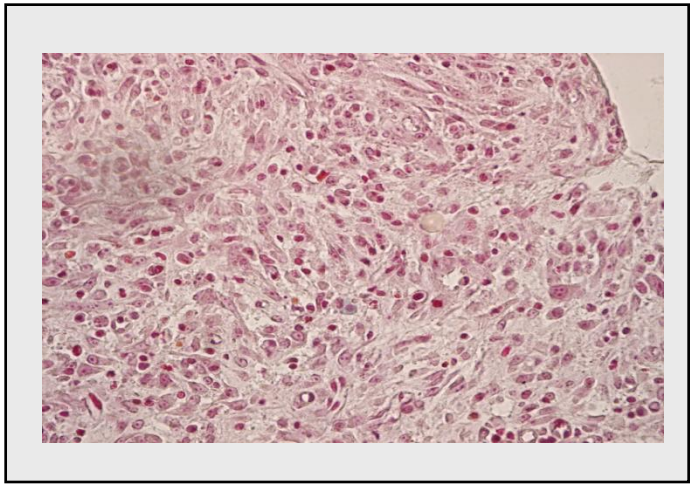
Deposition of fibrous tissue can lead to congestive heart failure

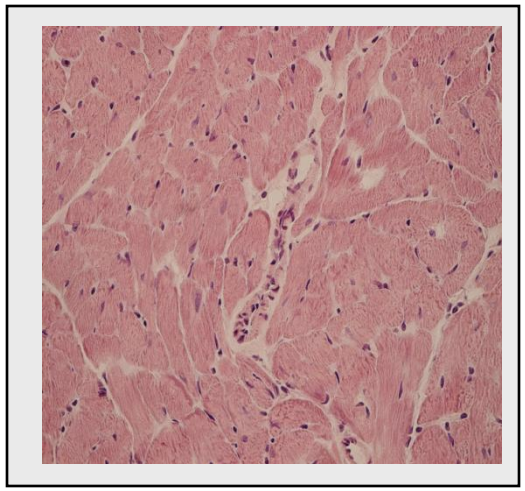


The good guys



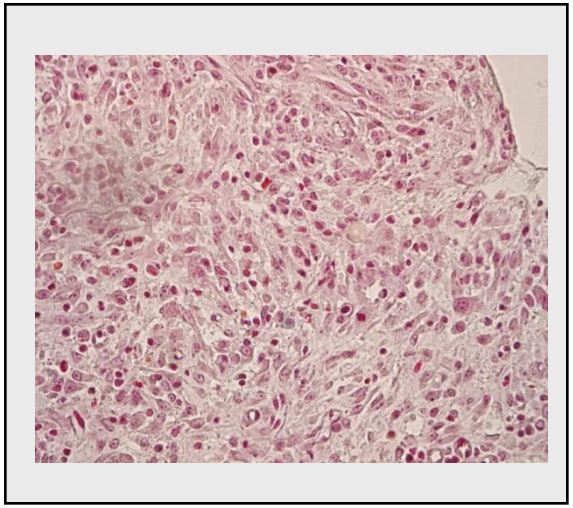
The bad guys

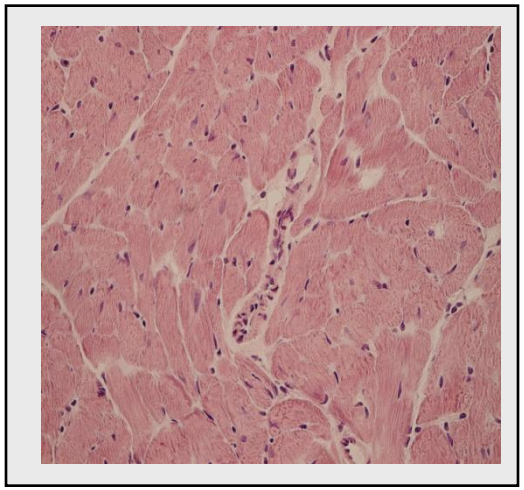




MI

Stem cells





What?

Recently, several small organic molecules identified in phenotypic cellular screens of combinatorial libraries of "privileged" heterocycles[6] were shown to be capable of inducing the selective in vitro differentiation of both adult stem cells and embryonic stem cells into osteoblasts and neurons.[7,8] We therefore decided to screen this same library for molecules that would selectively and efficiently induce the differentiation of murine ESCs to cardiomyocytes. The

How?

mouse embryonic carcinoma (EC) cell line P19 was initially chosen for high throughput screening. P19 cells, like ESCs, are pluripotent and can differentiate into cardiomyocytes after aggregation, formation of embryoid bodies (EBs), and treatment with 1% DMSO.[9] Moreover, P19 cells are easy to culture, amenable to genetic manipulation, and have a low frequency of spontaneous cardiac differentiation.

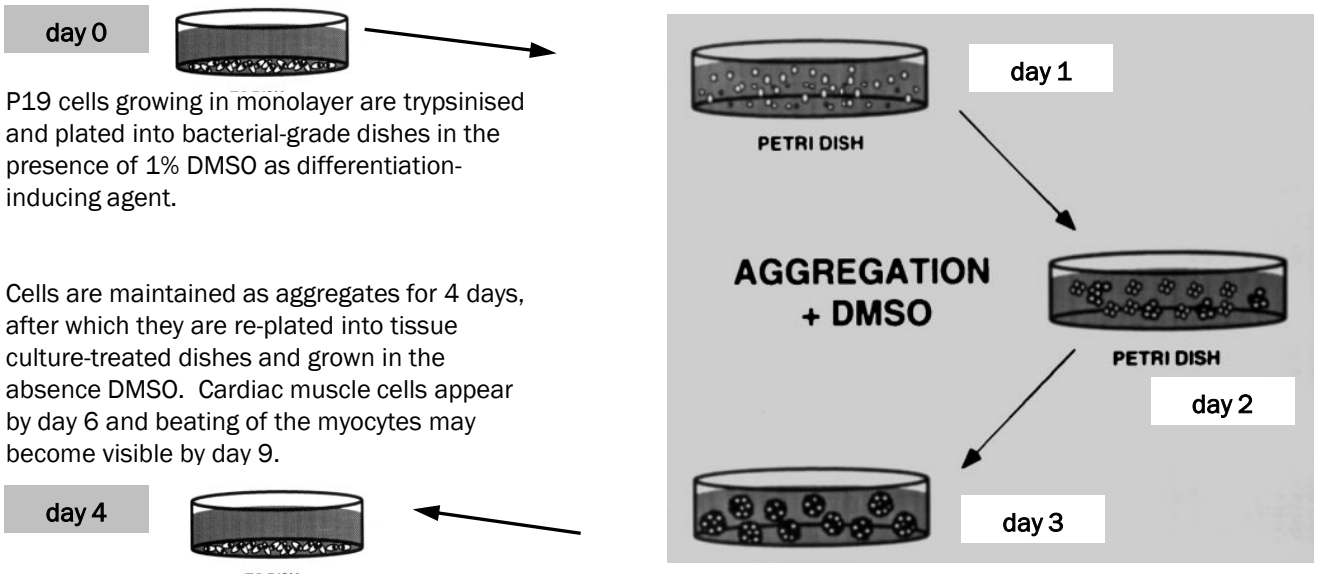
Model system

Reporter assay:
Gives you the results

To develop an appropriate reporter assay, the promoter region (~700 kb) of the rat atrial natriuretic factor (ANF) gene[10] was cloned and inserted upstream of the luciferase gene in the PGL3-BV reporter plasmid.[11] ANF is a polypeptide hormone that is synthesized primarily in cardiac myocytes and is a downstream target of several cardiomyogenesis transcriptional factors; it is considered a specific cardiomyocyte "marker" gene.[12] A stable P19

Schematic representation of P19 cell differentiation into muscle.

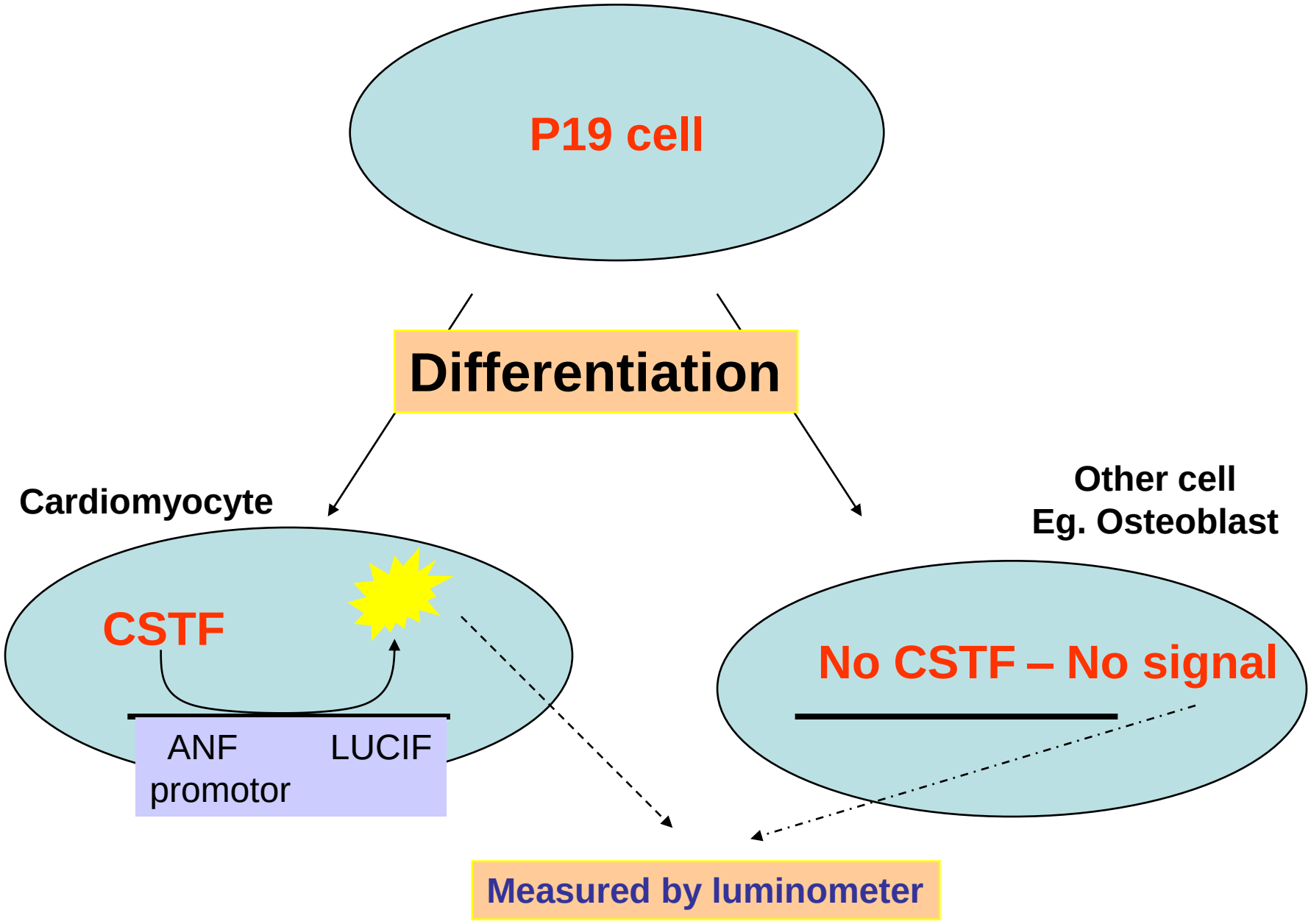
Differentiation is initiated by allowing the cells to aggregate in suspension in the presence of DMSO. Cells are plated into bacterial-grade dishes which prevent adherence of cells to the dish and promote aggregation. Aggregates are re-plated into tissue culture-treated dishes after 4 days and allowed to differentiate into cardiac muscle cells.



Low frequency differentiation

Reporter assay

- Atrial naturetic factor (ANF) promotor
  - ANF is cardiac specific
- Luciferase gene
  - When activated it makes cell glow
  - Can be picked up with a luminometer
  - Will only be activated in heart cells that activate the upstream ANF promotor via expression of cardiomyocyte-specific transcription factors



CSTF = Cardiomyocyte-Specific Transcription Factor

considered a specific cardiomyocyte "marker" gene.[12] A stable P19 clone harboring the reporter plasmid afforded a 5- to 7-fold increase (Figure 1) in luciferase signal upon standard cardiomyogenesis differentiation conditions for P19 cells (EB formation and treatment with 1% DMSO). This cell line was used to screen a 100 000 compound heterocycle library in a monolayer format, and approximately 80 compounds were identified that up-regulated luciferase activity >4-fold in the absence of EBs.

Blue bars: Control – no induction of cardiomyogenesis

Purple bars: induced with 1% DMSO and EB formation

35 000/40 000

Blue vs Purple = 5-7 fold increase

600/ 7000

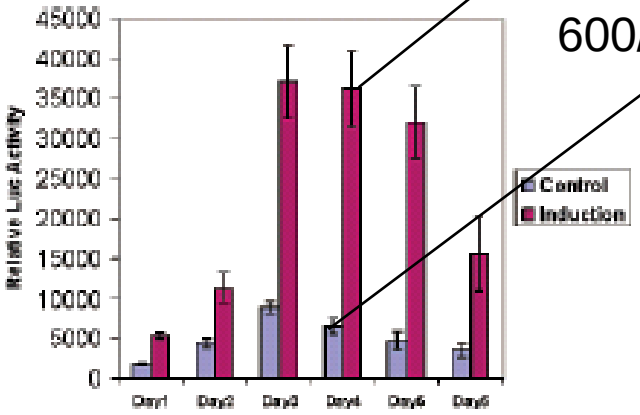


Figure 1. A high throughput screen for cardiomyogenesis using an ANF-promoter reporter assay.

P19 cells usually need to form EB for differentiation

luciferase activity >4-fold in the absence of EBs. Sarcomeric myosin heavy chain (MHC) is one of the essential motor proteins responsible for cardiac muscle contractibility[5] and was used as a secondary assay for differentiation. Thirty-five of the 80 compounds also induced MHC expression in P19CL6 cells[13,14] as determined by immunostaining with anti-MHC antibody (MF20) (Figure 2F).

Are you sure? What about ESC?

A-E: ESC
F: P19 cells

Use immunohistochemistry as secondary assay

A & F: Myosin heavy chain: green
B: GATA-4: red
C: MEF2: red
D: Nkx2.5: red
E: MHC (green) and MEF2 (red)

Nuclei are blue

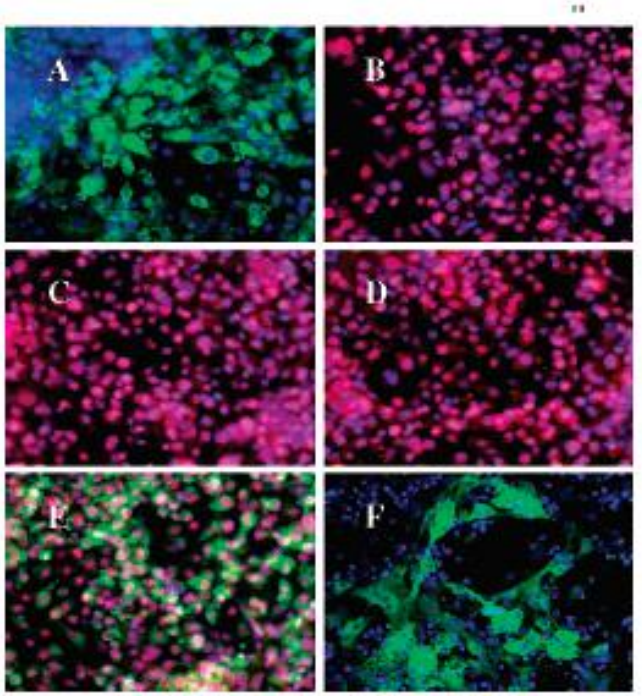


Figure 2. Immunostaining of cardiac muscle markers in ESCs (A–E) and P19CL6 cells (F) treated with 0.25 μM cardiogenol C: (A) and (F) myosin heavy chain (green); (B) GATA-4 (red); (C) MEF2 (red); (D) Nkx2.5 (red); and (E) myosin heavy chain (green) and MEF2 (red). Cell nuclei were stained with DAPI (blue).

Cardiogenol A-D = Pyrimidine analogues

Among the latter compounds, four diaminopyrimidines, cardiogenol A−D (Table 1), were the most potent in inducing MHC expression. These compounds share significant structural similarities. All have a 2-hydroxylamino substitution at the C-4 position, and all have bulky, hydrophobic groups at the C-2 position, suggesting that they function by a common mechanism.

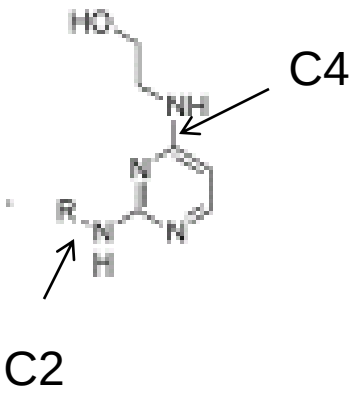
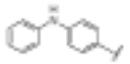
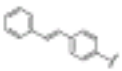


Table 1. Chemical Structures and Biological Activities of Cardiogenols

	R	EC_{50}	Optimal Activity
Cardiogenol A		1 μM	++
Cardiogenol B		0.5 μM	+++
Cardiogenol C		0.1 μM	++++
Cardiogenol D		0.1 μM	++++

++: 10-25% cells are positive for MHC after 7 days.
+++: 25-40% cells are positive for MHC after 7 days.
++++: 40-55% cells are positive for MHC after 7 days.

Potency and toxicity

Cardiogenol C,[18] which has a *p*-methoxy aniline substituent at the pyrimidine C2 position, is the most potent compound with an EC_{50} of 0.1 μM for inducing the differentiation of MHC positive cardiomyocytes from ESCs. Cardiogenol C showed significant cellular toxicity only at concentrations greater than 25 μM; after treating R1 cells 0.25 μM compound for 3 days and further culturing in medium without compound for 4 days, more than 50% of the cells stained positive for MHC and more than 90% of the cells are positive for GATA-4, consistent with the previous observation that GATA-4 is expressed earlier than MHC.[5] Moreover, there were many beating areas in R1 cells treated with cardiogenol C, suggesting that these MHC positive cells can form functional cardiac muscle. These results indicate that the majority of the cell population was induced by cardiogenol C to differentiate into cardiac lineage (in the absence of aggregation and EB formation). This is in contrast

Cardiogenol C is most potent. Effective concentration (EC_{50}) is 0.1uM for inducing differentiation.

That means that 0.1uM gets 50% of the cells to differentiate into heart cells (cardiomyocytes)

Toxicity only evident at 25uM (much higher than EC_{50})

Looked at MHC and GATA-4 as well as beating of cells.

So, how is this different to other work and why is it important?

muscle. These results indicate that the majority of the cell population was induced by cardiogenol C to differentiate into cardiac lineage (in the absence of aggregation and EB formation). This is in contrast to the current standard method of inducing cardiomyogenesis of ESCs by aggregation and formation of EBs, which results in only 5% of the cell population forming cardiomyocytes.[5]

No aggregation required
Differentiation more efficient

Novel and better mechanisms for differentiation and better therapies for heart disease

We are currently carrying out biochemical and genomics experiments to identify the molecular targets of cardiogenol C. These experiments may reveal novel molecular mechanisms related to cardiomyogenesis, and ultimately facilitate the application of ESCs to the repair of damaged myocardium in acute heart diseases.