Published in final edited form as:
Trends Cardiovasc Med. 2009 August ; 19(6): 207–212. doi:10.1016/j.tcm.2009.12.006.

The Application of Phenotypic High-Throughput Screening Techniques to Cardiovascular Research

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Abstract

In traditional pure protein high-throughput drug screens, also called in vitro screens, individual compounds from a small molecule collection are tested to determine whether they inhibit the enzymatic activity or binding properties of a purified target protein. In contrast, phenotypic high-throughput drug screens, also called chemical genetic or in vivo screens, investigate the ability of individual compounds from a collection to inhibit a biological process or disease model in live cells or intact organisms. In this review, the role of phenotypic screening techniques to identify novel therapeutic agents for the treatment of cardiovascular disease will be discussed.

Introduction

A traditional screening method for the identification of pharmacological agents for the treatment of human disease involves the use of a biochemical assay with a purified target protein (Byrbaum and Sigal 1997, Crews and Splittberger 1999). In this pure protein assay, the ability of a compound from a collection to alter the enzymatic activity or binding properties of the target protein is evaluated. This traditional screening approach has been successfully applied for many target proteins, and active compounds identified by this methodology are known to alter the activity of the target in question. The ability of compounds identified in pure protein high-throughput screens to modify disease progression in human patients is not known *a priori* and may not be related to the biochemical activity of the compound *in vitro*.

To identify useful compounds for the treatment of human disease in situations where the specific enzymes responsible are unknown, methods that investigate complex phenotypes in living cells or organisms may be an attractive alternative to pure protein screens. In phenotypic screens, also called chemical genetic or in vivo screens, a biological process in live cells or intact organisms - rather than an enzymatic or binding reaction with purified protein - is assayed (Byrbaum and Sigal 1997, Crews and Splittberger 1999, Yeh and Crews 2003). The biological assay must be quantitative and reproducible: features that are characteristic of biochemical assays with pure protein, but that are not easy to achieve with biological assays. In phenotypic high-throughput screens, cells or organisms (e.g., zebrafish embryos) are placed in microtiter plates in the presence of culture or growth medium. Individual compounds from chemical libraries are pipetted into unique wells by a robotic liquid-handling device and the biological

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assay is performed. Many biological assays involve the use of fluorescent or luminescent reagents that require automated microtiter plate readers with advanced data processing. In other cases, phenotypic abnormalities of embryos or cells are evaluated by light microscopy. Active compounds are identified on the basis of their ability to modify the results of the biological assay.

Development of Phenotypic High-Throughput Screens

A variety of technical advances in biomedical research, contributed to the development of chemical biology screens, including the development of chemical libraries, robotic liquid handling devices, luminescent and fluorescent reagents and epitope tags, sensitive microtiter plate readers, and advanced data processing (Wunder and Kalthof 2008). In one early phenotypic high-throughput screen, intact A549 cells were used to identify small molecules that could affect cell cycle progression (Mayer et al. 1999). In this screen, A549 cells were seeded into microtiter plates and were treated with 16,320 small molecules from the DIVERSet™ collection (ChemBridge Corporation). After an incubation period, cell protein lysates were obtained and were examined for the presence of phospho-nucleolin, a protein that is phosphorylated at the onset of mitosis, by a high-throughput immunoassay. By use of this screening strategy, 139 cell-permeable compounds that promoted nucleolin phosphorylation were identified and many of these compounds were subsequently found to affect mitosis (see Table).

In another early phenotypic screen, zebrafish embryos were cultured in 96-well microtiter plates at a density of 3 embryos per well, and they were treated with 1,100 synthetic small molecules from the DIVERSet™ collection (ChemBridge) (Peterson et al. 2000). After an incubation period, the phenotype of the embryos was examined by visual inspection. One compound was identified that increased the size of the hindbrain ventricle. Another compound resulted in the development of 2:1 atrio-ventricular cardiac conduction block. A third compound caused abnormal cardiac development with absent blood circulation and reduced ventricular size. The authors of this study noted the laboriousness of their screening approach, and suggested that automated phenotypic analysis by use of a fluorescence-based gene expression assay would dramatically improve the efficiency of the screen. Indeed, several reagents and techniques have been developed to help better automate zebrafish high-throughput screens (Burns et al. 2005, Tran et al. 2007, Vogt et al. 2009).

The use of high-throughput fluorescence microscopy was advanced in another phenotypic high-throughput screen for inhibitors of exocytosis (Feng et al. 2003). In this screen BSC1 fibroblast cells were incubated with a temperature-sensitive mutant form of vesicular stomatitis virus that was fused with a green fluorescent protein adenovirus ($VSVG^{ts}$-GFP) and plated in microtiter 384-well plates. Cells were treated with 10,240 compounds from the DIVERSet™ collection (ChemBridge) and were incubated at 40 or 32 C°. At the permissive temperature of 32 C°, the fluorescent surface glycoprotein was exported first to the Golgi apparatus and then to the plasma membrane. Compounds were evaluated by immunofluorescence microscopy for their ability to disrupt the exocytosis of the fluorescent surface glycoprotein at the permissive temperature. Thirty-two compounds were identified as hits, and they disrupted the exocytic pathway at various points between the endoplasmic reticulum and the plasma membrane.

Statistical Analysis of Phenotypic Screens

Many of the initial phenotypic high-throughput screens did not use advanced statistical methods to identify positive hits. The use of more quantitative assays, such as total fluorescence or luminescence in a well, has allowed for better statistical evaluation of these screens. Major statistical considerations related to high-throughput screening include positional effects of

wells within 96- or 384-well plates, the choice of hit threshold levels, and the vital need to reduce false-positive and false-negative rates (Malo et al., 2006).

A critical step in the analysis of any high-throughput screen is to determine the activity of each compound in the screen in a manner that corrects for plate-to-plate variability. There are many ways to achieve this goal including the “percent of control,” “normalized percent inhibition,” and the “Z score” methods (Malo et al., 2006). While the former two methods rely on direct comparisons to positive and negative control compounds within each multi-well plate, the “Z score” method assumes that most drugs are inactive and can serve as controls. In the “Z score” method, the mean score of all of the compounds on a plate are subtracted from the raw value of the compound of interest, and this difference is divided by the standard deviation of all of the values. A potentially superior analogue to the “Z score” is the “B score” method of determining a compound’s activity independent of plate-to-late variability (Brideau et al., 2003). The “B score” method minimizes measurement bias due to positional effects on multi-well plates and is resistant to statistical outliers (Malo et al., 2006).

The Use of Phenotypic High-Throughput Screens in Atherosclerosis Research

Phenotypic high-throughput screening techniques were first applied to atherosclerosis research in a project to identify inhibitors of cholesterol transfer between high density lipoprotein (HDL) and cells, a process that is mediated by the scavenger receptor class B, type I (SR-B1) (Nieland et al. 2002). Human HDL was labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) for use in the phenotypic screen. Chinese hamster ovary cells that were engineered to express high levels of murine SR-B1 were plated on 384-well microtiter plates and16,230 compounds from the DIVERSet™ collection (ChemBridge) were robotically added to individual wells. One hour later, DiI-HDL was added to the wells. Two hours after DiI-HDL was added, fluorescence was measured with a plate reader to evaluate autofluorescence and quenching. Cells were then washed four times and fluorescence was measured a second time to determine the cellular uptake of DiI-HDL. Five compounds were identified that inhibited cellular uptake of cholesterol from HDL and also the efflux of cholesterol from cells to HDL.

In a second phenotypic screen related to atherosclerosis research, agents that increased the expression of ATP-binding cassette transporter A1 (ABC1) were identified (Gao et al. 2008). ABCA1 promotes HDL particle formation and protects against the development of atherosclerosis (Singaraja et al. 2002, Joyce et al. 2002). Human hepatoma HepG2 cells were stably transfected with a plasmid encoding the promoter region of the human ABCA1 gene linked to a luciferase reporter. Transfected HepG2 cells were seeded into 96-well microtiter plates and then 2600 small molecules were added. Positive controls included thiazolidinedione compounds that are known to increase ABCA1 expression. Fourteen compounds were identified that increased luminescence by 150% or more, and 10 of these compounds were revalidated in a secondary assay. Two anthracycline antibiotics, pyrromycin and aclarubicin, and two isoflavone compounds, daidzein and pratensein, were studied further and found to promote increased ABCA1 expression in cultured HepG2 cells by quantitative real-time RT-PCR.

Macrophage-derived foam cells are thought to play a major role in atherosclerotic lesion formation and progression. In a recent phenotypic screen completed by our group a high throughput assay was established to evaluate the uptake of fluorescently labeled oxidized low density lipoprotein (oxLDL) by a monocye/macrophage cell line (Etzion et al. 2009). J774 macrophages were seeded into 96-well microtiter plates and compounds from the ICCB Known Bioactives Library (Biomol) were added by use of a robotic liquid-sample handler. This library

contains 480 compounds where at least one mechanism of action is known for each compound. This library therefore can provide both mechanistic and therapeutic information. DiI-labeled human oxLDL was added to each well and 2 h later total fluorescence was measured with a plate reader to evaluate autofluorescence and quenching. Cells were then washed and fluorescence was measured a second time to determine the cellular uptake of DiI-oxLDL. The 5′-NH_2 *c-jun* kinase (JNK) pathway inhibitor SP600125 was used as a positive control, because of its known ability to block oxLDL uptake by macrophages (Ricci et al. 2004). Twenty-two compounds were found to significantly alter oxLDL uptake by J774 cells. Several compounds were identified in this screen that had previously been implicated in oxLDL uptake, including the JNK pathway inhibitor SP600125, the endocytosis inhibitor ikarugamycin, the vacuolar ATPase inhibitor bafilomycin, and two Src tyrosine kinase inhibitors. Several new agents that blocked oxLDL uptake were identified, included three inhibitors of the NF-κβ signaling pathway, two different protein kinase C inhibitors and a phospholipase C inhibitor. In addition, loperamide, a μ opioid receptor agonist, was found to increase the oxLDL uptake by J774 cells. Confirmation and dose-response curves were obtained in primary peritoneal macrophages for most agents. Planned follow-up experiments include the long-term administration of identified compounds to small animal models of hypercholesterolemia and accelerated atherosclerosis.

The Use of Phenotypic High-Throughput Screens in Cardiovascular Development and Stem Cell Research

The use of zebrafish or fruitfly embryos in phenotypic high-throughput screens provides a platform for the ability of small molecules to modify cardiovascular development. In one phenotypic high-throughput screen, the gridlock mutation in zebrafish that leads to aortic coarctation, was employed (Peterson et al. 2004). Zebrafish *grl*$^{m145/m145}$ embryos were placed in 96-well microtiter plates and were exposed to 5,000 molecules from the DIVERSet™ collection (ChemBridge). Two compounds were found to restore normal circulation to the tail of zebrafish embryos. Both active compounds up-regulated the expression of vascular endothelial growth factor (VEGF) and the investigators showed that activation of the VEGF pathway was sufficient to suppress the phenotype of the gridlock mutation. When human umbilical vein endothelial cells were exposed to one of the active compounds (GS4012), tube formation was promoted.

In another zebrafish phenotypic high-throughput screen, small molecules that modify fibroblast growth factor (FGF) signaling in embryos were determined (Molina et al. 2009). In this work, transgenic zebrafish embryos were used that express destabilized green fluorescent protein in response to FGF signaling (*Tg(dusp6:EGFP)*^{pt6}). Transgenic embryos were placed in 96-well microtiter plates and were exposed to 5,000 compounds from three chemical libraries (see Table). Embryos were evaluated by immunofluorescent microscopy to determine whether agents increased FGF signaling. One molecule increased fluorescence in transgenic embryos in dose-dependent fashion. This molecule, BCI, increased FGF signaling within 2 h of addition to zebrafish embryos. BCI treatment of zebrafish embryos resulted in expansion of the pool of cardiogenic progenitor cells, demonstrated by increased expression of the cardiogenic marker genes *nkx2.5* and *gata4*. Evaluation of BCI-treated embryos at 56 h post-fertilization revealed marked expansion in cardiac tissue, and this expansion was especially noted in ventricular tissue.

Stem cell therapy for cardiac disease relies on their delivery, differentiation and integration into diseased myocardium. The differentiation of delivered stem cells may be enhanced by the administration of small molecules to stem cells. To address this possibility, two high-throughput phenotypic screen were performed with the murine embryonic carcinoma cell line P19 that is pluripotent (Wu et al. 2004, Sadek et al. 2008). The atrial natriuretic factor promoter was linked to the luciferase reporter in one screen (Wu et al. 2004), and the Nkx2.5 promoter

was linked to the luciferase reporter in the other (Sadek et al. 2008), and these constructs were transfected into P19 cells. Transfected P19 cells were plated in microtiter plates and treated with compounds from large chemical libraries, and positive hits that increased luciferase reporter activity as a marker of cardiomyocyte differentiation were identified in both screens.

One phenotypic high-throughput screen for compounds that promote dorsalization of zebrafish embryos, resulted in the identification of a compound useful in the cardiac differentiation of embryonic stem cells (Yu et al. 2008, Hao et al. 2008). In the phenotypic screen, zebrafish embryos were plated in triplicate in 96-well plates, and 4 hours post fertilization 7,570 compounds were added to the embryos (including 5,580 from ChemBridge; 1,840 known active compounds from MicroSource Discovery Systems; and 150 known bioactive compounds from Sigma-Aldrich). Dorsalization of embryos was evaluated by visual inspection at 12, 24, and 48 hours post fertilization. One compound, named dorsomorphin, caused profound, reproducible dorsalization of zebrafish embryos (Yu et al. 2008). Dorsomorphin was subsequently found to inhibit bone morphogenetic protein (BMP) type I receptor function. In a follow-up study with mouse embryonic stem cells, addition of dorsomorphin during the first 24 hours of embryonic stem cell differentiation, robustly induced cardiomyogenesis as determined by the formation of synchronously beating cells and by the expression of marker genes (Nkx2.5, Myh6, Myl2) (Hao et al. 2008).

The Use of Phenotypic High-Throughput Screens in Heart Failure Research

The difficulty of culturing cardiomyocytes has made high-throughput phenotypic screening with this cell type somewhat problematic. However, one group took advantage of a transformed rat cardiomyocyte cell line, H9C2 cells, to perform a phenotypic high-throughput screen to look for small molecules that modified calcineurin-mediated signaling (Bush et al. 2004). Calcineurin promotes cardiomyocyte growth via its ability to activate nuclear factor of activated T cell (NFAT) transcription factors (Muslin 2009). Modulatory calcineurin-interacting proteins (MCIPs) are NFAT-inducible factors that regulate calcineurin activity. A MCIP1 promoter, luciferase reporter construct was transiently transfected into H9C2 cells. Transfected cells were seeded into 96-well microtiter plates, and 20,000 compounds from the Myogen Library were added to the plates with a robotic liquid handling device. Twenty-one compounds were identified that increased MCIP1-luciferase expression by 2-fold or greater. The strongest positive hit, pyridine activator of myocyte hypertrophy (PAMH), is a 4-aminopyridine that has some structural similarities with serotonin (5-hydroxytryptamine, 5-HT). PAMH treatment of neonatal rat cardiomyocyte induced MCIP1 expression and also caused cell growth. Furthermore, PAMH was found to be a selective agonist for the 5-$HT_{2A/2B}$ receptors, and 5-HT receptor antagonists blocked the hypertrophic effect of PAMH stimulation.

In another cardiomyocyte high-throughput phenotypic screen, H9C2 cells were treated with hydrogen peroxide to induce oxidant stress and cell viability was assayed by use of the methylthiazolyldiphenyl-tetrazolium bromide (MTT) method (Gero et al. 2007). The positive control employed was PJ34, a poly(ADP-ribose)-polymerase (PARP) inhibitor. H9C2 cells were seeded into 96-well microtiter plates, and were treated with 1,280 compounds (LOPAC 1280, Sigma-Aldrich). Cell viability was determined by the MTT assay after 3 or 24 h of incubation with hydrogen peroxide. Positive hits were defined as drugs that resulted in greater mean viability rates. Several dozen positive hits were identified including agents that interfere with DNA repair and cell cycle progression, such as PARP inhibitors, topoisomerase inhibitors, and cyclin dependent kinase inhibitors.

Conclusion

Phenotypic high-throughput drug screens provide a distinct methodology to identify small molecules that may be useful for the treatment of humans with cardiovascular disease (see Table). These screens use biological endpoints in living cells or organisms to investigate the activity of libraries of small molecules. In general, these screens provide minimal information about the biochemical target of the positive hits, but they provide a substantial amount of biological information, such as the ability of a compound to ameliorate the phenotype of a cellular or intact organism disease model. The response of an intact organism to a drug is often dependent on interactions between various cell types and tissues that are not possible to predict based on the results of a pure protein high-throughput screen. Phenotypic screens also frequently provide drug toxicity information that can either be determined by the primary assay in the screen or in a secondary assay performed concurrently (Etzion et al. 2009). Furthermore, phenotypic screens can give important information about small molecule penetration into intact cells or whole organisms. Whether phenotypic high-throughput screens can identify compounds for the treatment of human disease that would not be identified by traditional pure protein screens remains to be determined.

Many of the phenotypic screens performed to date employed a subset of the DIVERSet™ collection of 50,000+ compounds that is designed to provide the broadest pharmacophore coverage possible (ChemBridge). While this and other similar collections provide a broad sampling of small molecule structural motifs, while excluding non-drug like compounds and undesirable chemical groups, most included compounds have unknown biochemical mechanisms of action. Therefore, upon completion of the phenotypic screen, the biochemical targets of action of the positive hits remain mysterious in most cases. In contrast, when a "known bioactives" library of small molecules is employed, both biochemical and biological information can be gleaned from a single phenotypic high-throughput screen. As "known bioactives" chemical libraries grow in complexity, their utility should increase dramatically.

In summary, phenotypic high-throughput screens have identified compounds that are active in various cell culture-based or small animal models of cardiovascular disease. In nearly all cases, follow-up studies to test the efficacy of these compounds in mammalian models of cardiovascular disease and in human patients have not been completed. Therefore, the ultimate clinical utility of this methodology remains unproven despite its many potential scientific advantages.

Acknowledgments

The authors wish to thank the Chemical Genetics Screening Core at Washington University School of Medicine.

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Table

A summary of the phenotypic high-throughput drug screens discussed in this review. See the text for a detailed description of individual screens.

Phenomenon Investigated	Cell or Organism	Reagent and Assay	Chemical Library	Most Notable Findings	Ref.
Cell Cycle Progression	A549 Cells	Phospho-Nucleolin Immunoblotting	16,320 DIVERSet Compounds (ChemBridge)	139 Compounds Affected Mitosis	Mayer 1999
Embryonic Development	Zebrafish Embryos	Visual Inspection	1,100 DIVERSet Compounds (ChemBridge)	One Compound Caused 2:1 Atrio-Ventricular Block	Peterson 2000
Exocytosis	BSC1 Fibroblasts	Fluorescent Surface Glycoprotein Export	10,240 DIVERSet Compounds (ChemBridge)	32 Compounds Inhibited Exocytosis	Feng 2003
Cholesterol Transfer to HDL	CHO Cells Expressing SR-B1	Cell Uptake of DiI-HDL	16,230 DIVERSet Compounds (ChemBridge)	Five Compounds Inhibited HDL Uptake	Nieland 2002
Cholesterol Efflux	HepG2 Cells	ABCA1 Promoter- Luciferase Reporter Assay	2,600 Compounds	Ten Compounds Increased ABCA1 Expression (Two Anthracycline Antibiotics)	Gao 2008
Foam Cell Formation	J774 Cells	Cell Uptake of DiI- oxLDL	480 ICCB Known Bioactives (Biomol)	Inhibitors Of NF-κβ, Src Kinases, PKC, PLC, JNK, Actin Polymerization	Etzion 2009
Aortic Coarctation	Zebrafish Embryos	Gridlock Mutant Embryos, Visual Inspection	5,000 DIVERSet Compounds (ChemBridge)	Two Compounds Restored Normal Circulation By Increasing VEGF	Peterson 2004
Embryonic FGF Signaling	Zebrafish Embryos	Transgene: FGF Signaling Induced EGFP Expression	5,000 Compounds (From NCI Diversity Set, MicroSource Discovery Systems, ChemDiv)	One Compound (BCI) Increased FGF Signaling, Also Increased Cardiomyogenesis	Molina 2009
Cardiac Differentiation Of Stem Cells	Embryonic Carcinoma P19 Cells	ANF Promoter- Luciferase Reporter Assay	100,000 Compound Heterocycle Library	35 Compounds Increased ANF And MHC Expression, Cardiogenol C Most Potent	Wu 2004
Cardiac Differentiation Of Stem Cells	Embryonic Carcinoma P19 Cells	Nkx2.5 Promoter- Luciferase Reporter Assay	147,000 Compound UTSW Chemical Library	1,600 Compounds Increased Nkx2.5 Expression, Multiple Sulfonyl-Hydrazone Hits	Sadek 2008
Dorsalization Of Embryos	Zebrafish Emrbyos	Visual Inspection	7,570 Compounds (From ChemBridge, MicroSource Discovery Systems, Sigma-Aldrich)	Dorsomorphin Promoted Dorsalization, BMP Type 1 Receptor Antagonist, Promoted ES Cell Cardiomyogenesis	Yu et al. 2008
Cardiomyocyte Calcineurin Signaling	H9C2 Cells	MCIP1 Promoter- Luciferase Reporter Assay	20,000 Compounds (Myogen Library)	21 Compounds Increased MCIP1 Expression, One Compound (PAMH) With Serotonin-Like Activity	Bush 2004

Phenomenon Investigated	Cell or Organism	Reagent and Assay	Chemical Library	Most Notable Findings	Ref.
Oxidant Stress- Induced Cardiomyocyte Death	H9C2 Cells	H_2O_2-Treated H9C2 Cells, MTT Assay	1,280 LOPAC Compounds (Sigma-Aldrich)	Many Compounds Inhibited H9C2 Cell Death, Including PARP Inhibitors	Gero 2007