

Methods for Identifying Cardiovascular Agents: A Review

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Abstract: Basic and clinical investigation into many of the diverse aspects of cardiovascular drug discovery employs varied approaches aimed at determining physiologic and pathophysiologic efficacy of candidate agents for therapeutic utility with the ultimate hope of identifying those agents capable of exerting salutary influence upon cardiac and vascular tissues. Promising compounds may then be used for prophylactic cardiovascular protection and for the treatment of various disorders including hypertension, cardiomyopathy, occlusive vascular disease, and heart failure. The invention disclosed in *Methods for identifying cardiovascular agents* [1] provides screening methods which can be used to identify certain suspected cardiovascular agents that inhibit vascular smooth muscle cell (VSMC) activation and/or proliferation, functional adaptations inherent in the responses to disease or injury, or those that enhance vascular endothelial cell (VEC) activation and/or proliferation, processes thought to provide protection to jeopardized blood vessels. Additionally, these screening assays include agents that activate estrogen responsive genes in vascular cells, considering that estrogen signaling is generally suggested to serve pivotal functions in preventing many of the pathologic mechanisms contributing to occlusive vascular complications. The findings of this primary patent are directly relevant for discoveries in related inventions that disclose various provisions for cardiovascular drug discovery. This review will provide detailed synopses of these function-based screening methods capable of identifying cardiovascular protective agents for use in basic science research and clinical drug discovery.

Keywords: Cardiovascular agent, cell activation, cell culture, cell proliferation, estrogen, estrogen responsive element, gene expression, myocyte, nitric oxide synthase, receptor, reporter construct, vascular endothelial cell, vascular injury, vascular smooth muscle cell, vasoprotection.

INTRODUCTION

Cardiovascular disease remains the major cause of morbidity and mortality in American populations with an overall prevalence of approximately 34% [1]. Adding to the impact and breadth of this dreaded disease, this statistic includes only those patients *diagnosed* with high blood pressure, coronary and/or peripheral artery disease, congestive heart failure, stroke, or congenital defects and does not consider individuals with undiagnosed complications. Total direct and indirect annual economic cost for management of cardiovascular diseases including health expenditures and lost productivity is estimated at close to \$400 billion [1]. Certainly, identification of therapeutic agents for use in preventing or treating cardiovascular disorders is of critical importance for general health and longevity of all people as well as for reducing this grave financial burden. Discovery of potential agents for use in combatting cardiovascular diseases has its foundation in the outcomes from basic science and clinical research endeavors, which routinely offer candidate agents for further consideration and study. Central to the identification of cardiovascular therapeutics from these research efforts are the assays and methodologies employed. Only by utilizing the most appropriate, sound, reproducible and valid

approaches should experimental results be accepted as factual and further investigation warranted.

The principal invention under discussion, *Methods for identifying cardiovascular agents* [2], which has its foundation on several pre-existing patents by the same investigators, offers screening methods for use in determining therapeutic utility of candidate cardioprotective, vasoprotective and/or anti-hypertensive agents and provides descriptive explanations of three primary experimental approaches. In generic terms, these approaches involve culturing vascular cells in the presence or absence of a known amount of a suspected cardiovascular therapeutic agent and subsequently measuring expression and/or activity of selected outputs including cellular proliferation, nitric oxide (NO) synthesis, or specified genes or reporter constructs responsive to the hormone estrogen. Additional cohorts of non-vascular cells and null cells that do not express estrogen receptors are included as controls. These assays include methods used to identify agents which inhibit vascular smooth muscle cell (VSMC) activation and/or proliferation, agents that enhance vascular endothelial cell (VEC) activation and/or proliferation, and those that activate estrogen responsive genes in vascular cells. Vascular cell proliferation can be assessed through measuring cell number, DNA content, and/or tritiated thymidine incorporation. Cell activation can be monitored by measuring the expression of genes that are induced by activation including inducible nitric oxide synthase (iNOS) or vascular endothelial cell growth factor (VECGF) for VSMCs and endothelial nitric

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oxide synthase (eNOS) or prostacyclin (PGI₂) synthase for VECs. Expression of an estrogen responsive gene can be measured through the use of reporter constructs in which the upstream regulatory region of an estrogen responsive gene (or an estrogen responsive element) is operably linked to a readily measurable protein (i.e. luciferase). Thorough explanations of these methods as integral components of the original patent are included in this review along with pertinent background information and rationale for use, definitions of key terms, parent case text for existing and contingent patents, patent claims proper, relevancy of the assays to basic science and clinical medicine and to associated new inventions, patent limitations, and current and future directions for continued study.

Definitions

Several terms included in this invention require brief explanation to allow the reader a more complete understanding of this discussion. The comprehensive term *cardiovascular agent* refers to agents that have potential to prevent, treat, and/or ameliorate a wide variety of disorders related to the heart and/or circulation and encompasses several of the more specific examples listed below. Examples of candidate *cardiovascular agents* are those used to treat hypertension, cardiomyopathy, coronary and/or peripheral artery disease, or congestive heart failure and/or those used to maintain cardiovascular homeostasis under non-diseased eutrophic conditions. The term *vasoprotective agent* refers to agents which have potential to reduce vascular disease through anti-mitogenic effects on VSMCs and/or pro-mitogenic effects on VECs at sites of vascular trauma. Examples of *vasoprotective agents* include VSMC growth inhibitors or suppressors and VEC growth stimulators. The term *anti-hypertensive agent* refers to agents which have potential to reduce blood pressure resultant from a range of disorders including primary and secondary hypertension, migraine, renal vascular disease, Cushing's disease, pregnancy-induced hypertension and/or toxemia, and renal and/or adrenal tumors including pheochromocytoma. The term *osteoprotective agent* refers to agents that have potential to reduce osteoporosis in

patients through increased activation and/or proliferation of bone-forming osteoclasts or through inhibition of the activation and/or proliferation of bone-depleting, bone absorbing osteoclasts. The term *estrogen receptor (ER)-dependent agent* refers to an agent whose effect(s) on a cell depends on the presence and/or absence of an estrogen receptor (ER , ER , and/or ER) on that cell. These classifications are schematically illustrated in (Fig. 1).

Estrogen-responsive gene refers to a gene whose expression can be affected by the presence of estrogen and includes a plethora of cellular, molecular, and biochemical signals (several which are mentioned below). Of the *estrogen responsive genes* whose upstream regulatory regions can be employed are the vitellogenin gene, the progesterone gene, the prolactin receptor gene, the nucleophosmin gene, and the VEGF gene. The term *estrogen receptor* indicates a protein that specifically binds an estrogen (including but not limited to 17 -estradiol and estrogen) and includes the isoforms , , and . *Estrogen receptor-dependent agents* can have one effect on an ER-expressing cell and yet a different effect on that same cell when the ER is absent, dysfunctional, or not expressed. These agents can exert supplementary non-ER-mediated effects on the cell as well. Tamoxifen, 17 -estradiol, and ICI 182,780 are all examples of *estrogen receptor-dependent agents*. An *estrogen responsive (recognition) element* refers to a DNA sequence in the upstream regulatory region of estrogen responsive genes which confers upon the gene to which it is operably linked a heightened responsiveness to the presence of estrogen. The consensus ER response element is an example of an *estrogen responsive element*. A *reporter gene* refers to a sequence containing a mammalian promoter, a sequence encoding a detectable protein (i.e. luciferase, green fluorescent protein (GFP), chloramphenicol acetyl transferase (CAT)), and usually a poly(A) signal sequence that is routinely inserted into vectors and is used to investigate upstream regulatory regions. The pGL3-Promoter Vector (Promega) is an example of a vector that contains an SV40 promoter upstream of a firefly luciferase gene (including a poly(A) tail). The term *operably* or *operatively linked* refers to a sequence element inserted upstream of a

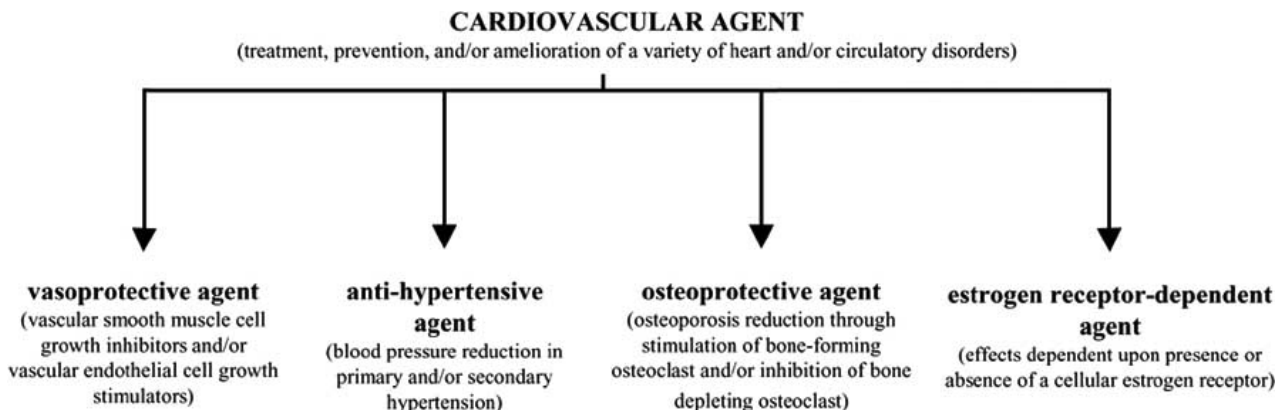


Fig. (1). Categorization of candidate agents in investigational cardiovascular drug discovery. Identification of prospective agents to be used for cardiovascular health maintenance and/or disease prevention involves examination of proliferation rates (vasoprotective agents), blood pressure responses (anti-hypertensive agents), changes in bone structure (osteoprotective agents), and/or alterations in estrogen receptor-dependent processes (estrogen receptor-dependent agents). All of these classifications can be included under agents that promote general health and prophylaxis in the cardiovascular system (cardiovascular agent).

reporter gene promoter so that it can influence expression of the protein encoded by that gene. For example an *estrogen responsive element* inserted into the polylinker upstream of the SV40 promoter in pGL3-Promoter Vector is *operably (operatively) linked* to the luciferase reporter gene. The term *vascular cell* encompasses VECs and VSMCs while the term *non-vascular cells* refers to any other non-blood vessel cell type used in these methods (discussed below). The term *myocyte* refers to muscle cells and generally encompasses cardiac muscle cells. *Null cells* refers to cells that do not express an ER, and COS-1 and COS-7 cells, CHO cells, and HEK 293 cells are examples of ER⁻ non-expressing cells.

Background

Abnormal cellular mechanisms contribute to a variety of cardiovascular disorders including high blood pressure, coronary and/or peripheral occlusive vascular disease (atherosclerosis), primary or secondary stenosis (“restenosis”) following vascular intervention (CABG, PTCA), in-stent hyperplasia, and congestive heart failure. These etiologies generally consist of VEC dysfunction (especially during the initiation phase of atherogenesis and following luminal intervention or injury) and/or VSMC dysfunction leading to altered cell proliferation and migration and an imbalance of extracellular matrix constituents with or without cell loss via necrosis and/or apoptosis. Accordingly, at sites of vascular disturbance preservation of VEC integrity and homeostasis along with maintenance of a non-activated (i.e. non-synthetic) contractile phenotype of VSMCs serves important vasoprotective roles and is a target for the experimental methods of this invention.

Specifically related to methods included in this patent, activation and/or augmented proliferation of otherwise quiescent VSMCs contributes significantly to the underlying processes of cardiovascular disease and thus, logically, inhibiting VSMC activation and/or proliferation would serve salutary protective functions against such complications. Interestingly, activation and/or proliferation of resident VECs serves the opposite effect by reducing phenotypic switching of adjacent VSMCs and by restoring vascular stability through enhanced endothelial regrowth following denudation. Therefore, candidate agents for use in cardiovascular therapeutics would need to inhibit VSMC activation/proliferation and concomitantly exacerbate VEC activation and/or proliferation at the site of vessel disturbance. These agents should also not deleteriously impact neighboring cells or cause unwanted disruption of adjacent cellular or tissue or organ functions.

The many protective effects of estrogen signaling in cardiovascular tissues have been well documented [3-6]. Incidence of atherosclerotic disease is generally low in pre-menopausal women, increases in post-menopausal women, and is reduced to pre-menopausal levels in post-menopausal women receiving estrogen replacement therapy [7-9]. Mechanistically, estrogen has been suggested to confer vasoprotection upon cardiovascular tissues by targeting serum lipid and carbohydrate metabolism, counterbalancing androgen-mediated growth-promoting effects, reversing pro-thrombotic states, and directly impacting blood vessels via enhanced vasodilation and reduced responses to

experimental trauma. Logically, stimulation of estrogen signaling would serve protective functions in cardiovascular tissues through multiple pathways and is an approach utilized by the invention herein discussed.

Nitric oxide, a well-known diatomic gas capable of exerting a multitude of physiologic and pathophysiologic effects, is synthesized by a family of oxidoreductases termed NO synthase (NOS) during L-arginine to L-citrulline conversion. Constitutive, calcium/calmodulin-dependent (nNOS, eNOS) and inducible (iNOS) isoforms of NOS exist in cardiovascular tissues that are capable of liberating NO in low, basal levels or in large quantities following stimulation, respectively. In general, NO serves protective functions in the cardiovascular system through multiple avenues but mostly via stimulation of soluble guanylate cyclase (sGC) and production of cyclic guanosine monophosphate (cGMP) with subsequent phosphorylation of cGMP-dependent protein kinases (cGK). Through these mechanisms NO is capable of exerting significant vasodilation, atheroprotection, enhanced defense against injury, and inhibition of platelet function; however, evidence of tachyphylaxis, NO-induced cytotoxicity, and the production of NO-dependent unstable reactive oxygen species necessitate caution when utilizing this multifaceted pathway.

PARENT CASE TEXT FOR PRIMARY PATENT

This primary invention is a continuation-in-part of and claims priority from the US patent *Methods for identifying vasoprotective agents* [10] and is a divisional of US patent *Methods for identifying cardiovascular therapeutic agents* [11] by the same authors.

CLAIMS IN PRIMARY PATENT

In order to provide background on claims encompassed by the patent *Methods for identifying cardiovascular agents* [1], the invention states 12 individual claims; however, these can be consolidated and presented as “methods for evaluating whether an agent is a candidate cardiovascular therapeutic using cultured VECs or VSMCs containing endogenous (genomic) or non-endogenous eNOS, iNOS, or neuronal NOS (nNOS), and measuring NOS activity via NO production with/without known amount of said cardiovascular agent within approximately 15 minutes after administration; methods whereby cultured VECs or VSMCs are transfected with an estrogen receptor (ER) construct expressing ER⁻, ER⁺, or ER⁺.” Additional claims and numerous comprehensive embodiments are mentioned throughout the patent such as evaluation of potential osteoprotective agents, use of non-vascular and null cells as controls, and assays used to measure cell proliferation and cell activation.

SUMMARY OF PRIMARY PATENT

This synopsis will provide a highly abridged version of the methods included in the patent and their use for determining if a suspected agent can exert vasoprotection and/or can be used as a cardiovascular therapeutic agent. Specific assay criteria listed in the patent are based on growth promoting or suppressing actions, NOS expression/NO formation, and/or estrogen responsiveness. In general, such methods incorporate comparisons between the

differences in a specific endpoint between treated and non-treated cohorts in the cell type of interest (VECs, VSMCs, myocytes) versus those differences between treated and non-treated cohorts of control (non-vascular, null, non-myocytic) cells. Based on the specific endpoint analyzed (proliferation rate, expression of a growth-dependent gene, NOS activity via NO synthesis, estrogen responsiveness), an appropriate measure of the efficacy of a suspected vasoprotective or cardiovascular agent would then be determined. For example, if an agent exerts anti-mitogenic effects on VSMCs yet exerts pro-mitogenic effects on VECs, it would be considered to be a therapeutic agent. Similarly, if the agent enhances sensitivity towards estrogen and/or augmented estrogen signaling more in VECs than in VSMCs, then it would be deemed therapeutic for use in cardiovascular disorders. It is recommended that the reader perform a slow and deliberate reading of this section as it may be difficult to read and comprehend as repetition of certain phrases and similarity of text may cause confusion and lack of understanding of inherent details. Special note should also be taken of the underlined terms as these results dictate interpretation of results for each assay. Table 1 illustrates key concepts of this patent as described in this Section based on the relative ratios of the differences in a specific endpoint between treated and non-treated cells of interest (VECs, VSMCs, etc.) to those differences between treated and non-treated control cells.

Vascular ECs, VSMCs, non-vascular cells (including myocytic and non-myocytic cells), and null cells each

containing a non-endogenous reporter construct comprising an *estrogen-responsive element* operatively linked to a reporter gene are cultured in the presence or absence of a pre-determined amount of the candidate agent. The agent is determined to be vasoprotective when the expression of the reporter gene in VECs in the presence of the agent compared to expression of the reporter in control non-treated VECs is greater than the expression of the reporter gene in non-vascular cells in the presence of the agent compared to expression of the reporter in control non-treated non-vascular cells. Conversely, the agent is determined to be vasoprotective when the expression of the reporter gene in VSMCs in the presence of the agent compared to expression of the reporter in control non-treated VSMCs is less than the expression of the reporter gene in non-vascular cells in the presence of the agent compared to expression of the reporter in control non-treated non-vascular cells. For experiments utilizing non-ER-expressing null cells as controls, the same theories apply. Explicitly, the agent is determined to be vasoprotective when the expression of the reporter gene in VECs in the presence of the agent compared to expression of the reporter in control non-treated VECs is greater than the expression of the reporter gene in null cells in the presence of the agent compared to expression of the reporter in control non-treated null cells. Conversely, the agent is determined to be vasoprotective when the expression of the reporter gene in VSMCs in the presence of the agent compared to expression of the reporter in control non-treated VSMCs is less than the expression of the reporter gene in null cells in the presence of the agent compared to expression of the reporter in control

Table 1. Key Concepts of Methods for Identifying Cardiovascular Agents [1]

Specific endpoint	Cell type of interest/control cell	Outcome
Gene expression for a reporter construct comprising an estrogen-responsive element	VECs/non-vascular and/or null cells	
	VSMCs/non-vascular and/or null cells	
	VECs/VSMCs	
Proliferation rate	VECs/null cells	
	VSMCs/null cells	
	VECs/VSMCs	
Gene expression for a reporter construct comprising a functional regulatory sequence from a growth-promoting gene	VECs/ non-vascular and/or null cells	
	VSMCs/ non-vascular and/or null cells	
	VECs/VSMCs	
Gene expression for a reporter construct comprising a functional regulatory sequence from a growth-arrest gene	VECs/VSMCs	
	VSMCs/non-vascular cells	
NOS activity	VECs	
	VSMCs	
Gene expression for a reporter construct comprising an estrogen-responsive gene	Myocytes/non-myocytic or null cells	

Arrows indicate result for the ratio of differences in a specific endpoint between treated and non-treated cells of interest (VECs, VSMCs, myocytes.) versus those differences between treated and non-treated control cells (non-vascular cells, null cells) and signify the outcome needed for determining an agent to be a cardiovascular therapeutic.

non-treated null cells. Moreover, in comparing effects between VECs and VSMCs, an agent is considered vasoprotective when the expression of the reporter gene in VECs in the presence of the agent compared to expression of the reporter in control non-treated VECs is greater than the expression of the reporter gene in VSMCs in the presence of the agent compared to expression of the reporter in control non-treated VSMCs.

Analysis of *cell proliferation* is another method for evaluating whether a proposed agent is considered vasoprotective. Vascular ECs, VSMCs and null cells are grown with/without a known amount of the agent and proliferation rates are measured by common techniques. An agent is identified as vasoprotective when the proliferation rate of VECs in the presence of the agent compared to the proliferation rate of control non-treated VECs is greater than the proliferation rate of nulls cells in the presence of the agent compared to control non-treated null cells. Alternatively, an agent is identified as vasoprotective when the proliferation rate of VSMCs in the presence of the agent compared to the proliferation rate of control non-treated VSMCs is less than the proliferation rate of nulls cells in the presence of the agent compared to control null cells. Moreover, in comparing effects between VECs and VSMCs, an agent is deemed vasoprotective when the proliferation rate of VECs in the presence of the agent compared to the proliferation rate of control VECs is greater than the proliferation rate of VSMCs in the presence of the agent compared to control VSMCs.

Another measure for estimating whether an agent is vasoprotective includes culturing VECs, VSMCs, non-vascular cells, and null cells in the presence or absence of the candidate agent with each cell type containing a non-endogenous reporter construct comprising functional regulatory sequences derived from a *growth-promoting gene* operatively linked to a reporter gene. An agent is considered vasoprotective when reporter gene expression in VECs in the presence of a known amount of the agent compared to reporter gene expression in control VECs is greater than reporter gene expression in non-vascular cells grown in the presence of the agent compared to reporter gene expression in control non-vascular cells. Similarly, an agent is deemed vasoprotective when reporter gene expression in VSMCs grown in the presence of the agent compared to reporter gene expression in control VSMCs is less than reporter gene expression in non-vascular cells grown in the presence of the agent compared to reporter gene expression in control non-vascular cells. Additionally, an agent is considered vasoprotective when reporter gene expression in the VECs in the presence of the agent compared to reporter gene expression in control VECs is greater than reporter gene expression in VSMCs in the presence of the agent compared to reporter gene expression in control VSMCs.

Complimenting experiments focusing on growth promoting genes, similar studies can be performed on *growth suppressing or arresting genes*. Vascular ECs, VSMCs, non-vascular cells, and null cells are cultured in the presence or absence of the candidate agent with each containing a non-endogenous reporter construct comprising functional regulatory sequences derived from a *growth*

arrest gene operatively linked to a reporter. An agent is judged vasoprotective when reporter gene expression in the VECs in the presence of the agent compared to reporter gene expression in control VECs is less than reporter gene expression in VSMCs in the presence of the agent compared to reporter gene expression in control VSMCs. Likewise, an agent is considered to be vasoprotective when reporter gene expression in the VSMCs in the presence of the agent compared to reporter gene expression in control VSMCs is greater than reporter gene expression in non-vascular cells in the presence of the agent compared to reporter gene expression in control non-vascular cells.

Evaluation of *NOS activity via NO production* is an alternate method for determining whether a candidate agent can be used as a cardiovascular therapeutic. For these methods VECs or VSMCs containing a NOS gene (eNOS, iNOS, and/or nNOS) will be cultured in the presence or absence of a suggested agent and NOS activity will be measured via NO production approximately 15 minutes after administration of the agent. An agent is identified as a cardiovascular agent when NOS activity in the VECs or VSMCs in the presence of the agent is greater than NOS activity in control non-treated VECs or VSMCs, respectively. In an alternate embodiment of this approach, these cells may be co-transfected with an estrogen receptor expression construct expressing an ER , ER , and/or ER , and similar endpoints analyzed.

Use of non-vascular cells is a necessary component of these methods considering the microenvironment within cardiovascular tissues and the intimate relationship between cells of many different types and origins. In these approaches myocytes, non-myocytes, and null cells containing an endogenous, genomic or a non-endogenous *estrogen responsive gene* are cultured in the presence or absence of the proposed cardiovascular agent. Estrogen responsive genes whose expression can be affected by the presence of estrogen include the NOS isoforms eNOS, iNOS, and/or nNOS, endothelin-1, matrix metalloproteinases, E-selectin, transforming growth factor (TGF)- 1, coagulation system proteins including PDGF, tissue factor, and tissue plasminogen activator, ion channel proteins, a variety of lipoproteins, factors of the renin-angiotensin II cascade, hormones, a wealth of growth factors including insulin-like growth factor, TGF- , and VEGF, vasoactive peptides including PGI₂ and PG synthases, early oncogenes such as c-fos and egr-1, various adhesion molecules, and assorted channels and receptors. A proposed agent is determined to be a cardiovascular agent when the ratio of reporter construct expression of the *estrogen responsive gene* in the myocytes grown in the presence of the agent to the reporter construct expression in myocytes grown in the absence of the agent is greater than the ratio of reporter construct expression in the non-myocyte cells or the null cells grown in the presence of the agent to the reporter construct expression in the non-myocytes or null cells in the absence of the agent. In another embodiment these cells may be co-transfected with an estrogen receptor expression construct expressing the ER , ER , and/or ER . In other embodiments these approaches utilizing NOS activity analyses may be used in conjunction with any other assay used to estimate the vasoprotective ability of a candidate cardiovascular agent.

DETAILS OF MATERIALS AND METHODS IN PRIMARY PATENT

Summary

Details are provided in the patent for the various methods used for screening candidate agents to identify cardiovascular, vasoprotective, and/or anti-hypertensive therapeutic agents. As mentioned, these methods rely on examining the effect(s) of candidate agents on various indices of growth activation or suppression and employ direct and indirect approaches for examining these phenomena. Direct measurement of cell growth involves monitoring cell activation and/or proliferation rates via measurement of cell number, DNA content, and/or tritiated thymidine incorporation. Indirect approaches involve monitoring gene expression for cell growth or cell arrest factors through use of reporter constructs in which all or part of the upstream regulatory region is operably linked to a sequence encoding a readable and measurable protein. For analyses of expression of estrogen responsiveness in these cells, the estrogen responsive gene can be naturally-occurring or synthetic and can be examined directly (through monitoring mRNA and/or protein expression of the naturally-occurring estrogen responsive gene itself) or indirectly (via measuring expression of a reporter construct operably linked to a readily detectable protein).

Cell Types

As mentioned, the approaches described in this invention are to be used in vascular and non-vascular cells from either fresh tissues (primary or low passaged cells) or from established cell lines. Cells may also be derived from genetically-altered animals using primary tissue or cell cultures. The vascular cells of interest are VECs and VSMCs which may be cultured independently or co-cultured for certain experiments. Vascular cells of special interest in this application include WB572 cells (spontaneously transformed human saphenous vein SMCs) and SV-E6 cells (human saphenous vein cells stably transfected with the E6 viral oncogene). Additional vascular cells that may be used in these methods include ECV304 cells or HUV-EC-C cells (human umbilical vein ECs), T/G HA-VSMCs (human VSMCs), and a wide variety of rat and bovine cells of vascular origin. The use of non-vascular cells (i.e. myocytes, tumor cells) in this application is essential considering the cellular milieu in which vascular cells are normally present within the body environment. Vascular and non-vascular cells co-exist within given tissues and are in intimate, communicative, and dependent relationship with one another. Co-dependency between these cells is vital for proper cellular function(s) and tissue homeostasis. Certain regulatory signals and "cross-talk" between vascular and non-vascular cells control various aspects of cell signaling and ultimately cell growth, activation, migration, and/or death. Specific examples pertinent to the current invention include cell-specific gene expression that may be regulated by the existence of co-factors (i.e. SRC-1), repressors (i.e. NCOR/SMRT), or expression modulating proteins secreted from neighboring or endocrine non-vascular cells. In this patent, non-vascular cells of particular interest include cardiomyocytes, various bone cell lines (UMR-106 cells from rat osteogenic sarcoma, HOS cells from human

osteosarcoma), and cells that do not express ER including COS-1 and COS-7 cells, CHO cells, and HEK 293 cells. Additional non-vascular cells useful for these methods include a plethora of human cancer cell lines. All cells and cell lines (with the exception of primary cultures) can be obtained from ATCC (Bethesda, MD).

Reporter Construct Experiments

Reporter constructs are used to indirectly monitor the effects of a proposed therapeutic agent on the activation and/or proliferation of cells and on expression of estrogen responsive genes. Suitable reporter constructs can be prepared by placing a sequence encoding a readily detectable protein of choice (luciferase, GFP, CAT) under the control of all or part of the upstream transcription control region of an estrogen responsive gene. In general, any gene that is responsive to an ER can serve as the basis for a reporter construct. This includes a plethora of cellular, biochemical, and molecular factors responsible for a variety of divergent cellular functions. A listing of candidate vascular genes of interest are included in the patent as well as in several published references by the same authors [3,5].

If the estrogen responsive gene is a growth-related gene (i.e. AP-1 (fos/jun), c-myc, or c-myb for growth promotion; p21, p27, p53, gas, gax, or Rb for growth suppression), then altered expression as an indicator of cardiovascular therapeutic utility depends on the specific cell type (VEC vs. VSMC). More specifically, preferred agents for cardiovascular therapy will exert growth inhibitory functions upon VSMCs (evidenced by decreased expression of growth-promoting genes and/or enhanced expression of growth-suppressing genes) while conversely exerting growth stimulation upon VECs (evidenced as enhanced expression of growth-promoting genes or diminished expression of growth inhibiting genes). Several examples of agents that could serve as potential cardiovascular therapeutics would be demonstration of diminished expression of genes for collagen, elastin, epidermal GF receptor, neu, egr-1, vascular adhesion molecules, VSMC calcium channels, ryanodine receptor, and FGF receptor in both VECs and VSMCs, augmented expression of the genes for prostaglandin cyclooxygenase and synthase, NOS isozymes, progesterone receptor, VEGF, and ER isoforms in both VECs and VSMCs, and discordant gene expression for c-fos, heat shock protein 27, and FLT4 receptor tyrosine kinase in VECs and VSMCs. Consequently, analyses of the effects of various estrogen responsive genes in terms of their utility as a cardiovascular agent must take into account the cellular milieu in which gene expression occurs.

A recommended reporter construct for use in studies on cardiovascular therapeutics is pmVEGF-Luc. This construct consists of a 1.6 kB fragment of genomic DNA (which includes the transcription start site, a 1.2 kB sequence upstream of the start site, and a 0.4 kB downstream sequence) upstream of the murine VEGF gene that is inserted into the pGL2-Basic luciferase reporter plasmid (Promega). Similar constructs can be created using upstream regulatory sequences from the human VEGF gene, and useful variants can be made by deleting a portion of the murine or human VEGF control region.

To initiate experiments utilizing such reporter constructs, a selected estrogen responsive reporter is introduced into VSMCs, VECs, non-vascular cells (preferably derived from reproductive tissue such as breast or uterus), and perhaps null cells (non-ER-expressing COS, HeLa, or HEK 293 cells) via stable transfection (although transient transfection can be used for rapid testing of new cell lines to determine their suitability for further testing). Human, murine, and/or rat cells are recommended for use in these methods. There are three preferred formats for assays based on estrogen responsiveness, and all three involve use of at least two different cell types that contain identical estrogen responsive reporters. These recommended assays use vascular cells and non-vascular cells, vascular cells and null cells, and/or VECs and VSMCs, although other combinations may also be used as appropriate.

Co-Culturing Methods

At the investigators discretion, co-culturing methods can be used with these procedures that employ any VEC cell line incubated with any VSMC cell line. These can be performed by culturing a heterogeneous cell population or by use of a Transwell.RTM system (Costar) with separate inserts for each cell type. In these assays, only a single cell type (the cell type of particular interest) contains the reporter construct while the second cell line is simply used to mimic *in vivo* conditions.

In Vivo Assays

Several whole animal models can employ the methods of this invention in order to examine the viability and feasibility of suspected cardiovascular agents under whole animal *in vivo* conditions. These models employ various approaches to induce mechanical injury to the blood vessel, and resulting neointimal and/or mural remodeling responses to the injury are examined. These responses, similar to the pathogenesis of various cardiovascular diseases, involve dysfunction and/or loss of luminal VECs and augmented proliferation, activation, and migration of nearby medial and adventitial VSMCs. Specifically listed in the patent, these methods can be utilized in the mouse carotid artery wire injury denudation model [12] or in the porcine femoral artery injury model [13,14], although alternate animal vascular injury models may also be used.

Agents Used in Screening Assays

Agents used for examination of cardiovascular potential and study can be derived from various sources, albeit libraries of synthetic and/or natural compounds are recommended by the authors. Synthetic compound libraries are available from Maybridge Chemical Company (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). Natural compound libraries can be obtained from Pan Laboratories (Bothell, WA) and Mycosearch (NC). Published scientific and medical references are also valuable resources to obtain potential candidates for further investigation.

SPECIFIC EXAMPLES

Several examples from basic science and clinical medicine are provided in this patent and briefly discussed here for illustrative purposes to demonstrate practicality of the methods included in this patent. In these examples cells have been stably transfected with a luciferase reporter construct, and these assays are performed in 96-well plates. Alternate practices (transient transfection for rapid testing of new cell lines, co-transfection of an estrogen receptor expression construct, use of reporter proteins other than luciferase, use of various sized culture wells or plates, etc.) may be substituted in these examples as desired. Given the formatting of the key concepts of this patent (Table 1), examples are given here for vascular cells/non-vascular cells, vascular cells/null cells, and vascular cells/vascular cells. Moreover, the reporter assays described in this patent have been utilized in many published reports examining growth factor activation of the vascular cell ER [15], a role for the progesterone receptor in the response to experimental vascular injury [16], and the involvement of ER in vascular function [17]. Excellent and comprehensive reviews of studies utilizing estrogen and similar reporter assays have been recently published by authors of this invention [5, 18-20].

Vascular Cell/Non-Vascular Cell Format

Following standard cell culture techniques, cells are plated in 96 well plates in complete medium at approximately 70% confluence and allowed to adhere undisturbed for ~4-6 hours. Each well is rinsed twice with 200 μ l warm serum-free media and then re-fed with 200 μ l serum-free media containing pre-determined amounts of the candidate agent for testing. Generally each well is treated with three different concentrations of the agent (using a log-scale) or to a vehicle control, and each treatment (or control) is performed in triplicate. The cells are allowed to incubate in a humidified 37°C chamber for 48 hours, after which cells are lysed *in situ* with 50 μ l lysis buffer (make sure lysis buffer is compatible with the luciferase assay). Luciferase activity is then measured spectrophotometrically directly in the wells with a microplate reader or after collecting cell lysates from the microplates in separate tubes which are then read spectrophotometrically. In this example agents that increase luciferase activity in the vascular cells over control conditions and do not alter luciferase activity in the non-vascular cells are determined to be vasoprotective agents.

Vascular Cell/Null Cell Format

Following the guidelines listed in the example above for cell culture and the same conditions thereof, a VSMC line, WB 572 cells, and a null cell line, COS cells, are stably transfected with the estrogen responsive reporter pERE-Luc (a luciferase reporter construct). After appropriate growth and treatment (as above), luciferase expression is measured. Any increase in luciferase activity of the treated WB 572 cells compared to control non-treated cells is compared to the change in luciferase activity of treated COS cells compared to control COS cells. An agent that increases luciferase expression preferentially in the WB 572 cells (over that of the COS cells) can be deemed a candidate cardiovascular agent.

Vascular Cell/Vascular Cell Format

Again, following the same guidelines listed in the examples above, a VSMC line and a VEC line are stably transfected with the estrogen responsive reporter pERE-Luc, grown, and treated. Agents that increase luciferase expression in both cell types are considered candidate cardiovascular agents.

REVIEW COVERAGE OF ASSOCIATED PATENTS

Several patents on cardiovascular agents and drug discovery have been founded over the past two years that correlate with the findings in the primary patent discussed in this review [1]. In this section brief synopses and discussions of these patents will be provided in relation to the primary patent.

The invention entitled *Upregulation of type III endothelial cell nitric oxide synthase by agents that disrupt actin cytoskeleton organization* [21] offers approaches for pharmacologically inducing eNOS-derived NO through disruption of the cellular actin cytoskeleton. As mentioned earlier, NO serves manifold salutary functions in the cardiovascular system such as protection against inflammation, platelet and vascular smooth muscle cell activation, and vasoconstriction, and dysregulation of NO signaling is directly implicated in numerous cardiac and vascular pathologies. Similar to approaches utilized in the primary patent [2] that use changes in NOS activity to indicate therapeutic utility of candidate agents, this invention involves use of agents that have capacity to disrupt cytoskeletal organization and, in turn, to upregulate eNOS activity and NO synthesis and to serve as salutary cardiovascular therapeutics. Therefore, these agents, chosen from a group including myosin light chain kinase inhibitors, myosin light chain phosphatases, protein kinase N inhibitors, phosphatidylinositol 4-phosphate 5-kinase inhibitors, cytochalasin D, angiotensin-converting enzyme inhibitors, or estrogen agonists, are especially useful in pathologies arising from NO deficiency secondary to eNOS dysfunction. These disorders include brain injury resulting from stroke or cerebrovascular accident, heart failure, progressive renal disease, cellular and/or tissue ischemia, and particularly pulmonary hypertension. Similar to the primary patent in this report [2], this new invention offers categories of prospective agents that may be used as cardiovascular therapeutics based on their influence on NO biology. A related invention, *Increasing cerebral bioavailability of drugs* [22], provides methods and compositions for increasing the cerebral bioavailability of blood-borne compositions through elevations of brain NO levels by eNOS-dependent and eNOS-independent mechanisms. This approach would be especially useful under conditions of impaired cerebral blood flow (i.e., stroke) whereby enhanced NO signaling would serve to improve circulation through vascular dilation. This improved circulation from augmented NO would confer enhancement of blood-based delivery of therapeutic agents under conditions of stroke or cerebrovascular dysfunction. Such agents to be used include eNOS-dependent L-arginine and eNOS cofactors NADPH or tetrahydrobiopterin, and eNOS-independent factors that increase cerebral blood flow. Another similar recent discovery, *Compounds for the treatment of ischemia* [23], offers an alternate route for

enhancing blood flow via use of adenosine A₃ receptor agonists which are particularly useful for reducing tissue damage resulting from ischemia or hypoxia. All of these inventions offer prospective agents that could be used to serve salutary functions in cardiac and vascular tissues based on key concepts of cardiovascular drug discovery as described in *Methods for identifying cardiovascular agents* [2].

Several additional patents have been recently described that compliment the primary patent in this review in terms of cardiovascular drug utility. *Cardioprotective phosphonates and malonates* [24] provide pharmacologic compositions and methods for pyridoxine phosphonate- and pyridoxine malonate-based treatment of cardiovascular (and related) diseases including hypertension, heart failure, arrhythmia, systemic and myocardial infarct, diabetes, and platelet-based disorders. *Pyridoxal analogues and methods of treatment* [25] is a coupled patent by the same inventor that offers additional avenues for cardiovascular utility in related diseases through pyridoxal-5'-phosphate and its analogues. Pyridoxal-5'-phosphate, the end-product of vitamin B₆ metabolism and also its biologically active form, serves as cofactor in many enzymatic reactions and confers protection against an array of cardiovascular disorders. Another recent discovery, *Compounds and therapies for the prevention of vascular and non-vascular pathologies*, is based on the immunosuppressive, anti-mitogenic, and anti-migratory roles of the pleiotropic factor TGF- in the cardiovascular system [26]. Certain cardiac and vascular pathologies including atherosclerosis are characterized by reduced TGF- levels, and therapeutic modalities (i.e., aspirin, tamoxifen) whereby TGF- levels are maintained or increased could provide much needed therapeutic intervention under such conditions. Beneficial roles for pharmacological inhibition of HMG-CoA reductase in reducing cholesterol biosynthesis, modulating blood serum lipids such as lowering LDL and/or increasing HDL, and in treating hyperlipidemia, hypercholesterolemia, hypertriglyceridemia, and atherosclerosis are disclosed in *HMG-CoA reductase inhibitors and method* [27]. This invention outlines pharmaceutical compositions for use in lowering lipids and cholesterol that would serve as important candidate cardiovascular agents.

RELEVANCY TO BASIC SCIENCE AND CLINICAL MEDICINE

The comprehensive methods included in this invention offer redundant and scientifically sound approaches for identifying agents for use in a broad range of cardiovascular therapeutics through analyses of cell proliferation and activation and estrogen responsiveness. Through use of vascular, non-vascular, and null cells critical and duplicative controls are provided. The assays described in this patent are directly relevant for use in basic science and clinical research endeavors as the initial processes of identifying candidate agents for continued study. Relevancy to intact organs and the whole animal environment is offered via utility in *ex vivo* models utilizing vascular tissue bioassays and through various animal *in vivo* models of vascular injury and repair. Direct relevancy to clinical development and regulatory review for novel cardiovascular drugs is also provided and includes pharmaceutical compositions, routes of

administration, dosing guidelines, suitability of combination therapies, and recommended usage of proposed agents. A number of stipulations and caveats of the embodiments of this application are also given to ensure appropriate and stringent use of these applications; nonetheless, several additional considerations are discussed below.

LIMITATIONS OF PRIMARY PATENT

As mentioned, authors of the primary invention in this report include several limitations and special considerations for the methods of their patent. Brief discussion of additional stipulations of these applications is provided here for the readers' information.

Estrogen exerts a multitude of physiologic and patho-physiologic actions yet long-term genomic adaptation to estrogen therapy, specifically cardiovascular gene and protein expression, may elicit unwanted or deleterious side-effects unrelated to the disorder at hand. These potential drawbacks include an increased risk of tumorigenesis and especially formation of breast and/or endometrial cancers, hormone-related feminization, venous thrombosis and thromboembolism, and endometrial hemorrhage [5]. Moreover, estrogen stimulates a variety of effects on both vascular and non-vascular cells, and relative selectivity for the vascular cells has not yet been achieved with currently used estrogens. Possible clinical manifestations of estrogen metabolites, phyto-estrogens, and local conversion of testosterone to estradiol all warrant continued investigation and elucidation. Estrogen has also been associated with a complex mixture of pro-fibrinolytic and pro-coagulant activities in addition to its established vasoprotective effects [28,29]. Untoward effects of estrogen replacement on indices of inflammation are complex and divergent [30] and require further study. Perhaps most importantly though for matters of this invention, recent controlled clinical trials of estrogen administration for primary and secondary prevention of cardiovascular disease in postmenopausal women have yielded negative results [31-33], indicating that alternative interventions for cardiovascular disease prophylaxis and/or treatment in postmenopausal women must be explored. Logically then, these provisions of estrogen signaling in cardiovascular tissues necessitate prudence when utilizing estrogen responsiveness as an indicator of vasoprotection; however, in conjunction with the alternate approaches included in this patent (cellular activation and proliferation, NOS expression), estrogen responsiveness is still considered a viable and significant endpoint for cardiovascular therapeutic utility.

A general caveat for this and associated patents is the production of reactive metabolites that might contribute to the toxicity of the candidate therapeutic agent. Toxicity of proposed therapeutic agents accounts for significant attrition during exploratory development. Interestingly, a recent patent outlines a high throughput method for identifying drug candidates that might produce undesirable reactive metabolites, which is especially relevant to cardiovascular drug discovery [34]. In this invention, glutathione conjugation is the high throughput method used to identify prospective agents that produce potentially deleterious reactive metabolites. This screening tool could serve to eliminate compounds with potential toxicity early in the drug

discovery process and would enhance identification of candidate cardiovascular agents with favorable therapeutic indices.

CURRENT AND FUTURE DEVELOPMENTS

Cardiovascular drug discovery remains a pivotal and "rate-limiting" step in the protracted process of clinical development and legislative review and regulation of novel therapeutics. The methods outlined in this patent offer rigorous complimentary approaches for exploration of new and potentially beneficial and clinically acceptable agents for use in preventing and/or treating a wide array of cardiovascular disorders. Based on well founded scientific parameters, these methods are currently routinely used and will continue to be used in the future to screen prospective compounds for their cardiovascular utility and study. Future directions for research based largely on the discoveries included in this invention and considering the caveats listed above should focus on alternate more physiologically-acceptable and safe replacements for estrogen signaling in cardiac and vascular tissues. Vascular cell-specific interventions without the many side-effects of untoward manifestations of estrogen would enable more direct visualization of the protective nature of candidate agents on the cardiovascular system and would serve to provide more viable prospects for cardiovascular drug discovery.

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