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(54) Title: THERAPEUTIC INHIBITORS OF VASCULAR SMOOTH MUSCLE CELLS

(57) Abstract

Methods are provided for inhibiting stenosis following vascular trauma or disease in a mammalian host, comprising administering to the host a therapeutically effective dosage of a therapeutic conjugate containing a vascular smooth muscle binding protein that associates in a specific manner with a cell surface of the vascular smooth muscle cell, coupled to a therapeutic agent dosage form that inhibits a cellular activity of the muscle cell. Methods are also provided for the direct and/or targeted delivery of therapeutic agents to vascular smooth muscle cells that cause a dilation and fixation of the vascular lumen by inhibiting smooth muscle cell contraction, thereby constituting a biological stent.

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THERAPEUTIC INHIBITOR OF VASCULAR SMOOTH MUSCLE CELLS

Field of the Invention

This invention relates generally to therapeutic methods involving surgical or intravenous introduction of binding partners directed to certain target cell populations, such as smooth muscle cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and effector cells of the immune system, particularly for treating conditions such as stenosis following vascular trauma or disease, cancer, diseases resulting from hyperactivity or hyperplasia of somatic cells and diseases that are mediated by immune system effector cells. Surgical or intravenous introduction of active agents capable of altering the proliferation or migration or contraction of smooth muscle proteins is also described. The invention also relates to the direct or targeted delivery of therapeutic agents to vascular smooth muscle cells that results in dilation and fixation of the vascular lumen (biological stenting effect). Combined administration of a cytocidal conjugate and a sustained release dosage form of a vascular smooth muscle cell inhibitor is also disclosed.

Background of the Invention

Percutaneous transluminal coronary angioplasty (PTCA) is widely used as the primary treatment modality in many patients with coronary artery disease. PTCA can relieve myocardial ischemia in patients with coronary artery disease by reducing lumen obstruction and improving coronary flow. The use of this surgical procedure has grown rapidly, with 39,000 procedures performed in 1983, nearly 150,000 in 1987, 200,000 in 1988, 250,000 in 1989, and over 500,000 PTCAs per year are estimated by 1994 (1, 2, 3). Stenosis following PTCA remains a significant problem, with from 25% to 35% of the patients developing restenosis within 1 to 3 months. Restenosis

results in significant morbidity and mortality and frequently necessitates further interventions such as repeat angioplasty or coronary bypass surgery. No surgical intervention or post-surgical treatment (to date) has proven effective in preventing restenosis.

The processes responsible for stenosis after PTCA are not completely understood but may result from a complex interplay among several different biologic agents and pathways. Viewed in histological sections, restenotic lesions may have an overgrowth of smooth muscle cells in the intimal layers of the vessel (3). Several possible mechanisms for smooth muscle cell proliferation after PTCA have been suggested (1, 2, 4, 5).

Compounds that reportedly suppress smooth muscle proliferation *in vitro* (4, 6, 7) may have undesirable pharmacological side effects when used *in vivo*. Heparin is an example of one such compound, which reportedly inhibits smooth muscle cell proliferation *in vitro* but when used *in vivo* has the potential adverse side effect of inhibiting coagulation. Heparin peptides, while having reduced anti-coagulant activity, have the undesirable pharmacological property of having a short pharmacological half-life. Attempts have been made to solve such problems by using a double balloon catheter, i.e., for regional delivery of the therapeutic agent at the angioplasty site (e.g., 8; U.S. Pat. No. 4,824,436), and by using biodegradable materials impregnated with a drug, i.e., to compensate for problems of short half-life (e.g., 9; U.S. Pat. No. 4,929,602).

Verrucarins and Roridins are trichothecene drugs produced as secondary metabolites by the soil fungi *Myrothecium verrucaria* and *Myrothecium roridium*. Verrucarin is a macrocyclic triester. Roridin is a macrocyclic diester of verrucarol (10). As a group, the trichothecenes are structurally related to sesquiterpenoid mycotoxins produced by several species of fungi and characterized by the 12,13-epoxytrichothec-9-ene basic structure. Their cytotoxic

activity to eukaryotic cells is closely correlated with their ability to bind to the cell, to be internalized, and to inhibit protein and macromolecular synthesis in the cell.

At least five considerations would, on their face, appear to preclude use of inhibitory drugs to prevent stenosis resulting from overgrowth of smooth muscle cells. First, inhibitory agents may have systemic toxicity that could create an unacceptable level of risk for patients with cardiovascular disease. Second, inhibitory agents might interfere with vascular wound healing following surgery and that could either delay healing or weaken the structure or elasticity of the newly healed vessel wall. Third, inhibitory agents killing smooth muscle cells could damage surrounding endothelium and/or other medial smooth muscle cells. Dead and dying cells also release mitogenic agents that might stimulate additional smooth muscle cell proliferation and exacerbate stenosis. Fourth, delivery of therapeutically effective levels of an inhibitory agent may be problematic from several standpoints: namely, a) delivery of a large number of molecules into the intercellular spaces between smooth muscle cells may be necessary, i.e., to establish favorable conditions for allowing a therapeutically effective dose of molecules to cross the cell membrane; b) directing an inhibitory drug into the proper intracellular compartment, i.e., where its action is exerted, may be difficult to control; and, c) optimizing the association of the inhibitory drug with its intracellular target, e.g, a ribosome, while minimizing intercellular redistribution of the drug, e.g. to neighboring cells, may be difficult. Fifth, because smooth muscle cell proliferation takes place over several weeks it would appear *a priori* that the inhibitory drugs should also be administered over several weeks, perhaps continuously, to produce a beneficial effect.

As is apparent from the foregoing, many problems remain to be solved in the use of inhibitory drugs, including cytotoxic agents, to effectively treat smooth muscle cell

proliferation. It would be highly advantageous to develop new methods for inhibiting stenosis due to proliferation of vascular smooth muscle cells following traumatic injury to vessels such as occurs during vascular surgery. In addition, delivery of compounds that produce inhibitory effects of extended duration to the vascular smooth muscle cells would be advantageous. Local administration of such sustained release compounds would also be useful in the treatment of other conditions where the target cell population is accessible by such administration.

Summary of the Invention

In one aspect of the invention, new therapeutic methods and therapeutic conjugates are provided for inhibiting vascular smooth muscle cells in a mammalian host. The therapeutic conjugates contain a vascular smooth muscle binding protein or peptide that binds in a specific manner to the cell membranes of a vascular smooth muscle cell or an interstitial matrix binding protein/peptide that binds in a specific manner to interstitial matrix (e.g., collagen) of the artery wall, coupled to a therapeutic agent that inhibits the activity of the cell. In one embodiment, inhibition of cellular activity results in reducing, delaying, or eliminating stenosis after angioplasty or other vascular surgical procedures. The therapeutic conjugates of the invention achieve these advantageous effects by associating with vascular smooth muscle cells and pericytes, which may transform into smooth muscle cells. The therapeutic conjugate may contain: (1) therapeutic agents that alter cellular metabolism or are inhibitors of protein synthesis, cellular proliferation, or cell migration; (2) microtubule and microfilament inhibitors that affect morphology or increases in cell volume; and/or (3) inhibitors of extracellular matrix synthesis or secretion. In one representative embodiment, the conjugates include a cytotoxic therapeutic agent that is a sesquiterpenoid mycotoxin such as a verrucarin or a roridin.

Other embodiments involve cytostatic therapeutic agents that inhibit DNA synthesis and proliferation at doses that have a minimal effect on protein synthesis such as protein kinase inhibitors (e.g., staurosporin), suramin, and nitric oxide releasing compounds (e.g., nitroglycerin) or analogs or functional equivalents thereof. In addition, therapeutic agents that inhibit the contraction or migration of smooth muscle cells and maintain an enlarged luminal area following, for example, angioplasty trauma (e.g., the cytochalasins, such as cytochalasin B, cytochalasin C, cytochalasin D or the like) are also contemplated for use in accordance with the present invention. Other aspects of the invention relate to vascular smooth muscle binding proteins that specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a vascular smooth muscle cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments the vascular smooth muscle binding protein binds to a CSPG target on the cell surface with an association constant of at least 10^{-4}M. In another preferred embodiment, the vascular smooth muscle binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal antibody NR-AN-01 or functional equivalents thereof.

Other aspects of the invention include methods for inhibiting stenosis, e.g., following angioplasty in a mammalian host, by administering to a human or animal subject in need of such treatment a therapeutically effective dosage of a therapeutic conjugate of the invention. In one representative embodiment, the dosage of therapeutic conjugate may be administered with an infusion catheter, to achieve a 10^{-3} M to 10^{-12}M concentration of said therapeutic conjugate at the site of administration in a blood vessel.

The present invention also contemplates therapeutic methods and therapeutic dosage forms involving sustained release of therapeutic agent to target cells. Preferably, the

target cells are vascular smooth muscle cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that are accessible by local administration of the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting vascular smooth muscle cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, contraction, migration or the like) but does not kill the cell and a vascular smooth muscle cell binding protein. Also, the methods and dosage forms of this aspect of the present invention are useful for inhibiting target cell proliferation or killing such target cells, employing a therapeutic agent that inhibits proliferation or is cytotoxic to the target cells and a target cell binding protein. In addition, the methods and dosage forms of this aspect of the present invention are useful for delivering cytostatic, cytocidal or metabolism modulating therapeutic agents to target cells, such as effector cells of the immune system, that are accessible by local administration of the dosage form, employing a target cell binding protein. Finally, dosage forms of the present invention are useful to reduce or eliminate pathological proliferation or hyperactivity of normal tissue (i.e., somatic cells).

The dosage forms of the present invention are preferably either non-degradable microparticulates or nanoparticulates or biodegradable microparticulates or nanoparticulates. More preferably, the microparticles or nanoparticles are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning. A particularly preferred structure is formed of a mixture of thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components. The lactide/glycolide structure has the added advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

Preferable therapeutic agents dispersed within the microparticulates or nanoparticulates are those exhibiting inhibition of a therapeutically significant target cell activity without killing the target cell, or target cell killing activity. For treatment of restenosis of vascular smooth muscle cells, useful therapeutic agents inhibit target cell activity (e.g., proliferation or migration) without killing the target cells. Preferred therapeutic moieties for this purpose are protein kinase inhibitors (e.g., staurosporin or the like), smooth muscle migration and/or contraction inhibitors (e.g., the cytochalasins, such as cytochalasin B, cytochalasin C, cytochalasin D or the like), suramin, and nitric oxide- releasing compounds, such as nitroglycerin, or analogs or functional equivalents thereof. In cancer therapy, useful therapeutic agents inhibit proliferation or are cytotoxic to the target cells. Preferred therapeutic moieties for this purpose are Roridin A and *Pseudomonas exotoxin*, or analogs or functional equivalents thereof. For treatment of immune system-modulated diseases, such as arthritis, useful therapeutic agents deliver cytostatic, cytocidal or metabolism-modulating therapeutic agents to target cells that are accessible by local administration of the dosage form. Preferred therapeutic moieties for this purpose are Roridin A, *Pseudomonas exotoxin*, suramin and protein kinase inhibitors (e.g., staurosporin), sphingosine, or analogs or functional equivalents thereof. For treatment of pathologically proliferating normal tissues (e.g., proliferative vitreoretinopathy, corneal pannus and the like), anti-proliferative agents or antimigration agents are preferred (e.g., cytochalasins, taxol, somatostatin, somatostatin analogs, N-ethylmaleimide, antisense oligonucleotides and the like).

The dosage forms of the present invention are targeted to a relevant target cell population by a binding protein or peptide. Preferred binding proteins/peptides of the present invention are vascular smooth muscle cell binding protein,

tumor cell binding protein and immune system effector cell binding protein. Preferred vascular smooth muscle cell binding proteins specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a vascular smooth muscle cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments, the vascular smooth muscle binding protein binds to a CSPG target on the cell surface with an association constant of at least 10^{-4} M. In other preferred embodiments, the vascular smooth muscle binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal antibody NR-AN-01 or functional equivalents thereof. Other preferred binding peptides useful in this embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among vascular smooth muscle cells. Preferred binding peptides of this type are specifically associated with collagen, reticulum fibers or other intercellular matrix compounds. Preferred tumor cell binding proteins are associated with surface cell markers expressed by the target tumor cell population or cytoplasmic epitopes thereof. Preferred immune system-modulated target cell binding proteins are associated with cell surface markers of the target immune system effector cells or cytoplasmic epitopes thereof. Binding peptides/proteins of the present invention also target pathologically proliferating normal tissues.

The present invention also provides therapeutic methods and therapeutic dosage forms involving administration of free (i.e., non-targeted or non-binding partner associated) therapeutic agent to target cells. Preferably, the target cells are vascular smooth muscle cells and the therapeutic agent is an inhibitor of vascular smooth muscle cell contraction, allowing the normal hydrostatic pressure to dilate the vascular lumen. Such contraction inhibition may be achieved by actin inhibition, which is preferably

achievable and sustainable at a lower dose level than that necessary to inhibit protein synthesis. Consequently, the vascular smooth muscle cells synthesize protein required to repair minor cell trauma and secrete interstitial matrix, thereby facilitating the fixation of the vascular lumen in a dilated state near its maximal systolic diameter. This phenomenon constitutes a biological stenting effect that diminishes or prevents the undesirable recoil mechanism that occurs in up to 25% of the angioplasty procedures classified as successful based on an initial post-procedural angiogram. Cytochalasins (which inhibit the polymerization of G- to F-actin which, in turn, inhibits the migration and contraction of vascular smooth muscle cells) are the preferred therapeutic agents for use in this embodiment of the present invention. Free therapeutic agent protocols of this type effect a reduction, a delay, or an elimination of stenosis after angioplasty or other vascular surgical procedures. Preferably, free therapeutic agent is administered directly or substantially directly to vascular smooth muscle tissue. Such administration is preferably effected by an infusion catheter, to achieve a 10^{-3}M to 10^{-12}M concentration of said therapeutic agent at the site of administration in a blood vessel.

Another embodiment of the present invention incorporates administration of a cytocidal targeted conjugate to destroy proliferating vascular smooth muscle cells involved in vascular stenosis. The mitogenic agents released after this biological arteromyectomy are prevented from stimulating the remaining viable vascular smooth muscle cells to proliferate and restenose the vessel by administration of the anti-contraction (anti-migration) or anti-proliferative sustained release agents of the present invention.

Description of the Drawings

FIGURE 1 is a photomicrograph of vascular smooth muscle cells in an artery of a 24-year-old male patient with vascular

smooth muscle binding protein bound to the cell surface and membrane. The patient received the vascular smooth muscle binding protein by i.v. administration 4 days before the arterial tissue was prepared for histology.

FIGURE 2 depicts a first scheme for chemical coupling of a therapeutic agent to a vascular smooth muscle binding protein.

FIGURE 3 depicts a second scheme for chemical coupling of a therapeutic agent to a vascular smooth muscle binding protein.

FIGURE 4A graphically depicts experimental data showing rapid binding of vascular smooth muscle binding protein to marker-positive test cells *in vitro*.

FIGURE 4B graphically depicts experimental data showing rapid binding of vascular smooth muscle binding protein to vascular smooth muscle cells *in vitro*.

FIGURE 5A presents graphically experimental data showing undesirable cytotoxicity of even low levels of therapeutic conjugate (i.e., RA-NR-AN-01), and the free RA therapeutic agent, when vascular smooth muscle cells were treated for 24 hours *in vitro*.

FIGURE 5B graphically presents experimental data showing the effects of RA-NR-AN-01 therapeutic conjugate on metabolic activity of marker-positive and -negative cells. The data show undesirable nonspecific cytotoxicity of the conjugate for all these cells in a 24 hour treatment *in vitro*. The non-specificity results from extracellular hydrolysis of the coupling ligand which exposes the tested cells to free drug.

FIGURE 6A graphically depicts experimental data showing undesirable nonspecific cytotoxicity of PE-NR-AN-01 therapeutic conjugate for marker-positive and marker-negative test cells after 24 hours of treatment *in vitro*, even though the 24 hour treatment was followed by an overnight recovery period prior to testing the metabolic activity.

FIGURE 6B depicts experimental data showing nonspecific cytotoxicity of the free PE therapeutic agent on marker-

positive and -negative test cells after 24 hours of treatment *in vitro*.

FIGURE 7A graphically presents experimental data showing that a short 5 minute "pulse" treatment, i.e., instead of 24 hours, followed by exposure to [3H]leucine, with free RA therapeutic agent being nonspecifically cytotoxic, i.e., for control HT29 marker-negative cells, but, in contrast, the RA-NR-AN-01 therapeutic conjugate is not cytotoxic in this "pulse" treatment.

FIGURE 7B presents graphically experimental data showing that free RA therapeutic agent is nonspecifically cytotoxic for control HT29 marker-negative cells, even in a 5' "pulse" treatment followed by a 24 hour recovery period prior to [3H]leucine exposure, but, in contrast, the RA-NR-AN-01 therapeutic conjugate is not cytotoxic to cells.

FIGURE 7C presents graphically results of experiments showing that "pulse" treatment of cells *in vitro* with the RA-NR-AN-01 therapeutic conjugate inhibits cellular activity in marker-positive A375 cells, as measured by protein synthesis.

FIGURE 7D presents graphically experimental data showing that "pulse" treatment of cells *in vitro* with the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity in marker-positive cells, since protein synthesis in A375 cells was not inhibited when the cells were allowed an overnight recovery period prior to testing *in vitro*.

FIGURE 8A presents graphically experimental data showing that while a "pulse" treatment of cells *in vitro* with free RA therapeutic agent was non-specifically cytotoxic, the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity in vascular smooth muscle cells, as evidenced by metabolic activity in BO54 cells that were allowed a 48 hour recovery period prior to testing.

FIGURE 8B graphically depicts experimental data similar to those presented in FIGURE 8A, above, but using a second marker-positive cell type, namely A375, the data show that

"pulse" treatment with the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity, as measured by metabolic activity in A375 cells that were allowed a 48 hour recovery period prior to testing.

FIGURE 8C graphically depicts results similar to those presented in FIGURE 8A and FIGURE 8B, above, but using a marker-negative control cell type, namely HT29. The results show that the "pulse" treatment with the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on the cellular activity of marker-negative control cells, as measured by metabolic activity in HT29 cells that were allowed a 48 hour recovery period prior to testing.

FIGURE 9A shows stenosis due to intimal smooth muscle cell proliferation in a histological section of an untreated artery 5 weeks after angioplasty in an animal model.

FIGURE 9B shows inhibition of stenosis in a histological section of an artery treated with therapeutic conjugate at 5 weeks after angioplasty in an animal model.

FIGURE 10A graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of suramin with respect to vascular smooth muscle cells.

FIGURE 10B graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of staurosporin with respect to vascular smooth muscle cells.

FIGURE 10C graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of nitroglycerin with respect to vascular smooth muscle cells.

FIGURE 11 shows a tangential section parallel to the inner surface of a smooth muscle cell which is magnified 62,500 times and is characterized by numerous endocytic vesicles, several of which contain antibody coated gold beads in the process of being internalized by the cell in vitro.

FIGURE 12 shows a smooth muscle cell which is magnified 62,500 times and is characterized by a marked accumulation of gold beads in lysosomes at 24 hours following exposure of the cell to the beads in vitro.

FIGURE 13 shows a smooth muscle cell which is magnified 62,500 times and is characterized by accumulation of gold beads in lysosomes in vivo.

FIGURE 14 depicts an in vivo dose response study of the effect of cytochalasin B on the luminal area of pig femoral arteries.

Detailed Description of the Invention

As used herein the following terms have the meanings as set forth below:

"Therapeutic conjugate" means a vascular smooth muscle or an interstitial matrix binding protein coupled (e.g., optionally through a linker) to a therapeutic agent.

"Target" and "marker" are used interchangeably in describing the conjugate aspects of the present invention to mean a molecule recognized in a specific manner by the matrix or vascular smooth muscle binding protein, e.g., an antigen, polypeptide antigen or cell surface carbohydrate (e.g., a glycolipid, glycoprotein, or proteoglycan) that is expressed on the cell surface membranes of a vascular smooth muscle cell or a matrix structure.

"Epitope" is used to refer to a specific site within the "target" molecule that is bound by the matrix or smooth muscle binding protein, e.g., a sequence of three or more amino acids or saccharides.

"Coupled" is used to mean covalent or non-covalent chemical association (i.e., hydrophobic as through van der Waals forces or charge-charge interactions) of the matrix or vascular smooth muscle binding protein with the therapeutic agent. Due to the nature of the therapeutic agents employed, the binding proteins will normally be associated with the therapeutic agents by means of covalent bonding.

"Linker" means an agent that couples the matrix or smooth muscle binding protein to a therapeutic agent, e.g., an organic chemical coupler.

"Migration" of smooth muscle cells means movement of these cells *in vivo* from the medial layers of a vessel into the intima, such as may also be studied *in vitro* by following the motion of a cell from one location to another (e.g., using time-lapse cinematography or a video recorder and manual counting of smooth muscle cell migration out of a defined area in the tissue culture over time).

"Proliferation," i.e., of smooth muscle cells or cancer cells, means increase in cell number, i.e., by mitosis of the cells.

"Expressed" means mRNA transcription and translation with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., CSPG synthesized by a vascular smooth muscle cell or pericyte.

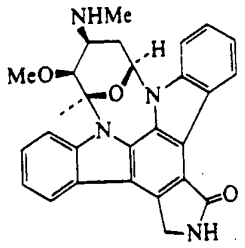
"Macrocyclic trichothecene" is intended to mean any one of the group of structurally related sesquiterpenoid macrocyclic mycotoxins produced by several species of fungi and characterized by the 12,13-epoxytrichothec-9-ene basic structure, e.g., verrucarins and roridins that are the products of secondary metabolism in the soil fungi *Myrothecium verrucaria* and *Myrothecium roridium*.

"Sustained release" means a dosage form designed to release a therapeutic agent therefrom for a time period ranging from about 3 to about 21 days. Release over a longer time period is also contemplated as a "sustained release" dosage form of the present invention.

"Dosage form" means a free (non-targeted or non-binding partner associated) therapeutic agent formulation, as well as sustained release therapeutic formulations, such as those incorporating microparticulate or nanoparticulate, biodegradable or non-biodegradable polymeric material capable of binding to one or more binding proteins or peptides to

deliver a therapeutic moiety dispersed therein to a target cell population.

"Staurosporin" includes staurosporin, a protein kinase C inhibitor of the following formula,



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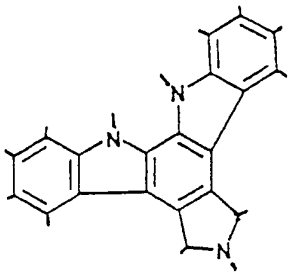
as well as diindoloalkaloids having one of the following general structures:



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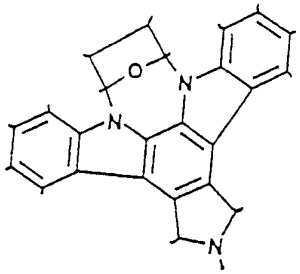

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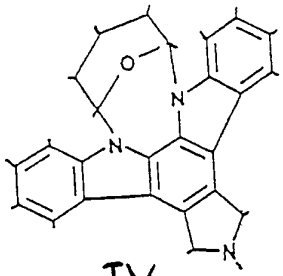

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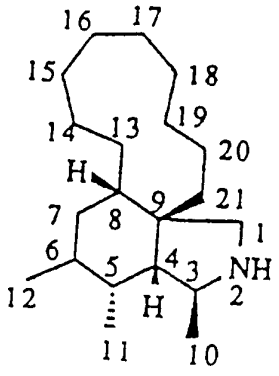
More specifically, the term "staurosporin" includes K-252 (see, for example, Japanese Patent Application No. 62,164,626), BMY-41950 (U.S. Patent No. 5,015,578), UCN-01 (U.S. Patent No. 4,935,415), TAN-999 (Japanese Patent Application No. 01,149,791), TAN-1030A (Japanese Patent Application No. 01,246,288), RK-286C (Japanese Patent Application No. 02,258,724) and functional equivalents and derivatives thereof. Derivatives of staurosporin include

those discussed in Japanese Patent Application Nos. 03,72,485; 01,143,877; 02,09,819 and 03,220,194, as well as in PCT International Application Nos. WO 89 07,105 and WO 91 09,034 and European Patent Application Nos. EP 410,389 and EP 296,110. Derivatives of K-252, a natural product, are known. See, for example, Japanese Patent Application Nos. 63,295,988; 62,240,689; 61,268,687; 62,155,284; 62,155,285; 62,120,388 and 63,295,589, as well as PCT International Application No. WO 88 07,045 and European Patent Application No. EP 323,171.

"Cytochalasin" includes fungal metabolites exhibiting an inhibitory effect on target cellular metabolism, including prevention of contraction or migration of vascular smooth muscle cells. Preferably, cytochalasins inhibit the polymerization of monomeric actin (G-actin) to polymeric form (F-actin), thereby inhibiting cell functions requiring cytoplasmic microfilaments. Cytochalasins typically are derived from phenylalanine (cytochalasins), tryptophan (chaetoglobosins), or leucine (aspochalasins), resulting in a benzyl, indol-3-yl methyl or isobutyl group, respectively, at position C-3 of a substituted perhydroisoindole-1-one moiety (Formula V or VI).



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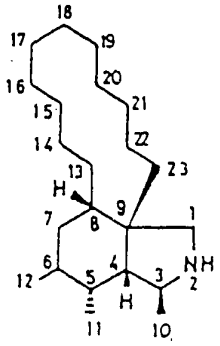


VI

The perhydroisoindole moiety in turn contains an 11-, 13- or 14-atom carbocyclic- or oxygen-containing ring linked to positions C-8 and C-9. All naturally occurring cytochalasins contain a methyl group at C-5; a methyl or methylene group at C-12; and a methyl group at C-14 or C-16. Exemplary molecules include cytochalasin A, cytochalasin B, cytochalasin C, cytochalasin D, cytochalasin E, cytochalasin F, cytochalasin G, cytochalasin H, cytochalasin J, cytochalasin K, cytochalasin L, cytochalasin M, cytochalasin N, cytochalasin O, cytochalasin P, cytochalasin Q, cytochalasin R, cytochalasin S, chaetoglobosin A, chaetoglobosin B, chaetoglobosin C, chaetoglobosin D, chaetoglobosin E, chaetoglobosin F, chaetoglobosin G, chaetoglobosin J, chaetoglobosin K, deoxaphomin, proxiphomin, protophomin, zygosporin D, zygosporin E, zygosporin F, zygosporin G, aspochalasin B, aspochalasin C, aspochalasin D and the like, as well as functional equivalents and derivatives thereof. Certain cytochalasin derivatives are set forth in Japanese Patent Nos. 72 01,925; 72 14,219; 72 08,533; 72 23,394; 72 01924; and 72 04,164. Cytochalasin B is used in this description as a prototypical cytochalasin.

As referred to herein, smooth muscle cells and pericytes include those cells derived from the medial layers of vessels and adventitia vessels which proliferate in intimal hyperplastic vascular sites following injury, such as that caused during PTCA.

Characteristics of smooth muscle cells include a histological morphology (under light microscopic examination) of a spindle shape with an oblong nucleus located centrally in the cell with nucleoli present and myofibrils in the sarcoplasm. Under electron microscopic examination, smooth muscle cells have long slender mitochondria in the juxtanuclear sarcoplasm, a few tubular elements of granular endoplasmic reticulum, and numerous clusters of free ribosomes. A small Golgi complex may also be located near one pole of the nucleus. The majority of the sarcoplasm is

occupied by thin, parallel myofilaments that may be, for the most part, oriented to the long axis of the muscle cell. These actin containing myofibrils may be arranged in bundles with mitochondria interspersed among them. Scattered through the contractile substance of the cell may also be oval dense areas, with similar dense areas distributed at intervals along the inner aspects of the plasmalemma.

Characteristics of pericytes include a histological morphology (under light microscopic examination) characterized by an irregular cell shape. Pericytes are found within the basement membrane that surrounds vascular endothelial cells and their identity may be confirmed by positive immuno-staining with antibodies specific for alpha smooth muscle actin (e.g., anti-alpha-sm1, Biomakor, Rehovot, Israel), HMW-MAA, and pericyte ganglioside antigens such as MAb 3G5 (11); and, negative immuno-staining with antibodies to cytokeratins (i.e., epithelial and fibroblast markers) and von Willdebrand factor (i.e., an endothelial marker). Both vascular smooth muscle cells and pericytes are positive by immunostaining with the NR-AN-01 monoclonal antibody.

The therapeutic conjugates and dosage forms of the invention are useful for inhibiting the activity of vascular smooth muscle cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal thickening that results from stimulation of smooth muscle cell proliferation following angioplasty, either in an animal model or in man. "Delaying" means delaying the time until onset of visible intimal hyperplasia (e.g., observed histologically or by angiographic examination) following angioplasty and may also be accompanied by "reduced" restenosis. "Eliminating" restenosis following angioplasty means completely "reducing" and/or completely "delaying" intimal hyperplasia in a patient to an extent which makes it no longer necessary to surgically intervene, i.e., to re-establish a suitable blood flow through the vessel by repeat angioplasty, atheroectomy, or coronary

artery bypass surgery. The effects of reducing, delaying, or eliminating stenosis may be determined by methods routine to those skilled in the art including, but not limited to, angiography, ultrasonic evaluation, fluoroscopic imaging, fiber optic endoscopic examination or biopsy and histology. The therapeutic conjugates of the invention achieve these advantageous effects by specifically binding to the cellular membranes of smooth muscle cells and pericytes.

Therapeutic conjugates of the invention are obtained by coupling a vascular smooth muscle binding protein to a therapeutic agent. In the therapeutic conjugate, the vascular smooth muscle binding protein performs the function of targeting the therapeutic conjugate to vascular smooth muscle cells or pericytes, and the therapeutic agent performs the function of inhibiting the cellular activity of the smooth muscle cell or pericyte.

Therapeutic dosage forms (sustained release-type) of the present invention exhibit the capability to deliver therapeutic agent to target cells over a sustained period of time. Therapeutic dosage forms of this aspect of the present invention may be of any configuration suitable for this purpose. Preferred sustained release therapeutic dosage forms exhibit one or more of the following characteristics:

- microparticulate (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, with from about 0.5 to about 2 micrometers more preferred) or nanoparticulate (e.g., from about 1.0 nanometer to about 1000 nanometers in diameter, with from about 50 to about 250 nanometers more preferred), free flowing powder structure;

- biodegradable structure designed to biodegrade over a period of time between from about 3 to about 180 days, with from about 10 to about 21 days more preferred, or non-biodegradable structure to allow therapeutic agent diffusion to occur over a time period of between from about 3 to about 180 days, with from about 10 to about 21 days preferred;

- biocompatible with target tissue and the local physiological environment into which the dosage form is being administered, including biocompatible biodegradation products;

- facilitate a stable and reproducible dispersion of therapeutic agent therein, preferably to form a therapeutic agent-polymer matrix, with active therapeutic agent release occurring through one or both of the following routes: (1) diffusion of the therapeutic agent through the dosage form (when the therapeutic agent is soluble in the polymer or polymer mixture forming the dosage form); or (2) release of the therapeutic agent as the dosage form biodegrades; and

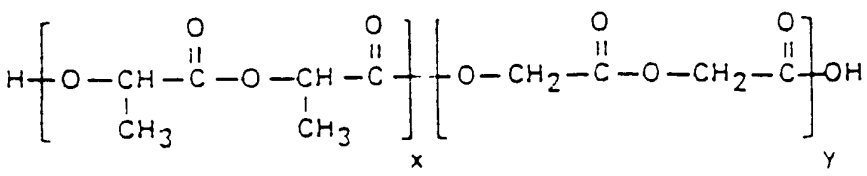
- capability to bind with one or more cellular and/or interstitial matrix epitopes, with from about 1 to about 10,000 binding protein/peptide-dosage form bonds preferred and with a maximum of about 1 binding peptide-dosage form per 150 square angstroms of particle surface area more preferred. The total number bound depends upon the particle size used. The binding proteins or peptides are capable of coupling to the particulate therapeutic dosage form through covalent ligand sandwich or non-covalent modalities as set forth herein.

Nanoparticulate sustained release therapeutic dosage forms of preferred embodiments of the present invention are biodegradable and bind to the vascular smooth muscle cells and enter such cells primarily by endocytosis. The biodegradation of such nanoparticulates occurs over time (e.g., 10 to 21 days) in prelysosomic vesicles and lysosomes. The preferred larger microparticulate therapeutic dosage forms of the present invention bind to the target cell surface or interstitial matrix, depending on the binding protein or peptide selected, and release the therapeutic agents for subsequent target cell uptake with only a few of the smaller microparticles entering the cell by phagocytosis. A practitioner in the art will appreciate that the precise mechanism by which a target cell assimilates and metabolizes a dosage form of the present invention depends on the morphology, physiology and metabolic processes of those cells.

The size of the targeted sustained release therapeutic particulate dosage forms is also important with respect to the mode of cellular assimilation. For example, the smaller nanoparticles can flow with the interstitial fluid between cells and penetrate the infused tissue until it binds to the normal or neoplastic tissue that the binding protein/peptide is selected to target. This feature is important, for example, because the nanoparticles follow lymphatic drainage channels from infused primary neoplastic foci, targeting metastatic foci along the lymphatic tract. The larger microparticles tend to be more easily trapped interstitially in the infused primary tissue.

Preferable sustained release dosage forms of the present invention are biodegradable microparticulates or nanoparticulates. More preferably, biodegradable microparticles or nanoparticles are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning to release therapeutic agent, thereby forming pores within the particulate structure.

Polymers derived from the condensation of alpha hydroxycarboxylic acids and related lactones are preferred for use in the present invention. A particularly preferred moiety is formed of a mixture of thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components, such as poly(lactide-co-glycolide). An exemplary structure, a random poly(DL-lactide-co-glycolide), is shown below, with the values of x and y being manipulable by a practitioner in the art to achieve desirable microparticulate or nanoparticulate properties.



Other agents suitable for forming particulate dosage forms of the present invention include polyorthoesters and polyacetals (Polymer Letters, 18:293, 1980) and polyorthocarbonates (U.S. Patent No. 4,093,709) and the like.

Preferred lactic acid/glycolic acid polymer containing matrix particulates of the present invention are prepared by emulsion-based processes, that constitute modified solvent extraction processes such as those described by Cowsar et al., "Poly(Lactide-Co-Glycolide) Microcapsules for Controlled Release of Steroids," Methods Enzymology, 112:101-116, 1985 (steroid entrapment in microparticulates); Eldridge et al., "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies," Infection and Immunity, 59:2978-2986, 1991 (toxoid entrapment); Cohen et al., "Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres," Pharmaceutical Research, 8(6):713-720, 1991 (enzyme entrapment); and Sanders et al., "Controlled Release of a Luteinizing Hormone-Releasing Hormone Analogue from Poly(D,L-Lactide-Co-Glycolide) Microspheres," J. Pharmaceutical Science, 73(9):1294-1297, 1984 (peptide entrapment).

In general, the procedure for forming particulate dosage forms of the present invention involves dissolving the polymer in a halogenated hydrocarbon solvent, dispersing a therapeutic agent solution (preferably aqueous) therein, and adding an additional agent that acts as a solvent for the halogenated hydrocarbon solvent but not for the polymer. The polymer precipitates out from the polymer-halogenated hydrocarbon solution onto droplets of the therapeutic agent containing solution and entraps the therapeutic agent. Preferably the therapeutic agent is substantially uniformly dispersed within the sustained release dosage form of the present invention. Following particulate formation, they are washed and hardened with an organic solvent. Water washing and aqueous non-ionic

surfactant washing steps follow, prior to drying at room temperature under vacuum.

For biocompatibility purposes, particulate dosage forms, characterized by a therapeutic agent dispersed therein in matrix form, are sterilized prior to packaging, storage or administration. Sterilization may be conducted in any convenient manner therefor. For example, the particulates can be irradiated with gamma radiation, provided that exposure to such radiation does not adversely impact the structure or function of the therapeutic agent dispersed in the therapeutic agent-polymer matrix or the binding protein/peptide attached thereto. If the therapeutic agent or binding protein/peptide is so adversely impacted, the particulate dosage forms can be produced under sterile conditions.

Release of the therapeutic agent from the particulate dosage forms of the present invention can occur as a result of both diffusion and particulate matrix erosion. Biodegradation rate directly impacts therapeutic agent release kinetics. The biodegradation rate is regulable by alteration of the composition or structure of the sustained release dosage form. For example, alteration of the lactide/glycolide ratio in preferred dosage forms of the present invention can be conducted, as described by Tice et al., "Biodegradable Controlled-Release Parenteral Systems," Pharmaceutical Technology, pp. 26-35, 1984; by inclusion of polymer hydrolysis modifying agents, such as citric acid and sodium carbonate, as described by Kent et al., "Microencapsulation of Water Soluble Active Polypeptides," U.S. Patent No. 4,675,189; by altering the loading of therapeutic agent in the lactide/glycolide polymer, the degradation rate being inversely proportional to the amount of therapeutic agent contained therein, and by judicious selection of an appropriate analog of a common family of therapeutic agents that exhibit different potencies so as to alter said core loadings; and by variation of particulate size, as described by Beck et al., "Poly(DL-Lactide-Co-Glycolide)/Norethisterone

Microcapsules: An Injectable Biodegradable Contraceptive," Biol. Reprod., 28:186-195, 1983, or the like. All of the aforementioned methods of regulating biodegradation rate influence the intrinsic viscosity of the polymer containing matrix, thereby altering the hydration rate thereof.

The preferred lactide/glycolide structure is biocompatible with the mammalian physiological environment. Also, these preferred sustained release dosage forms have the advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

Functional groups required for binding protein/peptide-particulate dosage form bonding to the particles, are optionally included in the particulate structure, along with the non-degradable or biodegradable polymeric units. Functional groups that are exploitable for this purpose include those that are reactive with peptides, such as carboxyl groups, amine groups, sulfhydryl groups and the like. Preferred binding enhancement moieties include the terminal carboxyl groups of the preferred (lactide-glycolide) polymer containing matrix or the like.

Useful vascular smooth muscle binding protein is a polypeptide, peptidic, or mimetic compound (as described below) that is capable of binding to a target or marker on a surface component of an intact or disrupted vascular smooth muscle cell in such a manner that allows for either release of therapeutic agent extracellularly in the immediate interstitial matrix with subsequent diffusion of therapeutic agent into the remaining intact smooth muscle cells and/or internalization by the cell into an intracellular compartment of the entire targeted biodegradable moiety, permitting delivery of the therapeutic agent. Representative examples of useful vascular smooth muscle binding proteins include antibodies (e.g., monoclonal and polyclonal affinity-purified antibodies, $F(ab')_2$, Fab', Fab, and Fv fragments and/or complementarity determining regions (CDR) of antibodies or functional equivalents thereof, (e.g., binding peptides and

the like)); growth factors, cytokines, and polypeptide hormones and the like; and macromolecules recognizing extracellular matrix receptors (e.g., integrin and fibronectin receptors and the like).

Other preferred binding peptides useful in targeting the dosage form embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among vascular smooth muscle cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the vascular smooth muscle cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and elastic fibers and other intercellular matrix material.

Preferred tumor cell binding peptides are associated with epitopes of myc, ras, bcr/Abl, erbB and like gene products, as well as mucins, cytokine receptors such as IL-6, EGF, TGF and the like, which binding peptides localize to certain lymphomas (myc), carcinomas such as colon cancer (ras), carcinoma (erbB), adenocarcinomas (mucins), breast cancer and hepatoma (IL-6 receptor), and breast cancer (EGF and TGF), respectively. Preferred immune system effector cell-binding peptides are anti-TAC, IL-2 and the like, which localize to activated T cells and macrophages, respectively. Other preferred binding proteins/peptides useful in the practice of the present invention include moieties capable of localizing to pathologically proliferating normal tissues, such as pericytes of the intraocular vasculature implicated in degenerative eye disease.

Therapeutic agents of the invention are selected to inhibit a cellular activity of a vascular smooth muscle cell, e.g., proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or secretion of extracellular

matrix materials by the cell. Preferably, the therapeutic agent acts either: a) as a "cytostatic agent" to prevent or delay cell division in proliferating cells by inhibiting replication of DNA (e.g., a drug such as adriamycin, staurosporin or the like), or by inhibiting spindle fiber formation (e.g., a drug such as colchicine) and the like; or b) as an inhibitor of migration of vascular smooth muscle cells from the medial wall into the intima, e.g., an "anti-migratory agent" such as a cytochalasin; or c) as an inhibitor of the intracellular increase in cell volume (i.e., the tissue volume occupied by a cell; a "cytoskeletal inhibitor" or "metabolic inhibitor"); or d) as an inhibitor that blocks cellular protein synthesis and/or secretion or organization of extracellular matrix (i.e., an "anti-matrix agent").

Representative examples of "cytostatic agents" include, e.g., modified toxins, methotrexate, adriamycin, radionuclides (e.g., such as disclosed in Fritzberg et al., U.S. Patent No. 4,897,255), protein kinase inhibitors (e.g., staurosporin), inhibitors of specific enzymes (such as the nuclear enzyme DNA topoisomerase II and DNA polymerase, RNA polymerase, adenyl guanyl cyclase), superoxide dismutase inhibitors, terminal deoxynucleotidyl- transferase, reverse transcriptase, antisense oligonucleotides that suppress smooth muscle cell proliferation and the like, which when delivered into a cellular compartment at an appropriate dosage will act to impair proliferation of a smooth muscle cell or pericyte without killing the cell. Other examples of "cytostatic agents" include peptidic or mimetic inhibitors (i.e., antagonists, agonists, or competitive or non-competitive inhibitors) of cellular factors that may (e.g., in the presence of extracellular matrix) trigger proliferation of smooth muscle cells or pericytes: e.g., cytokines (e.g., interleukins such as IL-1), growth factors, (e.g., PDGF, TGF-alpha or -beta, tumor necrosis factor, smooth muscle- and endothelial-derived growth factors, i.e.,

endothelin, FGF), homing receptors (e.g., for platelets or leukocytes), and extracellular matrix receptors (e.g., integrins). Representative examples of useful therapeutic agents in this category of cytostatic agents for smooth muscle proliferation include: subfragments of heparin, triazolopyrimidine (Trapidil; a PDGF antagonist), lovastatin, and prostaglandins E1 or I2.

Representative examples of "anti-migratory agents" include inhibitors (i.e., agonists and antagonists, and competitive or non-competitive inhibitors) of chemotactic factors and their receptors (e.g., complement chemotaxins such as C5a, C5a desarg or C4a; extracellular matrix factors, e.g., collagen degradation fragments), or of intracellular cytoskeletal proteins involved in locomotion (e.g., actin, cytoskeletal elements, and phosphatases and kinases involved in locomotion). Representative examples of useful therapeutic agents in this category of anti-migratory agents include: caffeic acid derivatives and nilvadipine (a calcium antagonist), and steroid hormones. Preferred anti-migratory therapeutic agents are the cytochalasins.

Representative examples of "cytoskeletal inhibitors" include colchicine, vinblastin, cytochalasins, taxol and the like that act on microtubule and microfilament networks within a cell.

Representative examples of "metabolic inhibitors" include staurosporin, trichothecenes, and modified diphtheria and ricin toxins, *Pseudomonas exotoxin* and the like. In a preferred embodiment, the therapeutic conjugate is constructed with a therapeutic agent that is a simple trichothecene or a macrocyclic trichothecene, e.g., a verrucarin or roridin. Trichothecenes are drugs produced by soil fungi of the class *Fungi imperfecti* or isolated from *Baccharus megapotamica* (Bamburg, J.R. Proc. Molec. Subcell. Biol. 8:41-110, 1983; Jarvis & Mazzola, Acc. Chem. Res. 15:338-395, 1982). They appear to be the most toxic molecules that contain only carbon, hydrogen and oxygen (Tamm, C. Fortschr. Chem. Org.

Naturst. 31:61-117, 1974). They are all reported to act at the level of the ribosome as inhibitors of protein synthesis at the initiation, elongation, or termination phases.

There are two broad classes of trichothecenes: those that have only a central sesquiterpenoid structure and those that have an additional macrocyclic ring (simple and macrocyclic trichothecenes, respectively). The simple trichothecenes may be subdivided into three groups (i.e., Group A, B, and C) as described in U.S. Patent Nos. 4,744,981 and 4,906,452 (incorporated herein by reference). Representative examples of Group A simple trichothecenes include: Scirpene, Roridin C, dihydrotrichothecene, Scirpen-4, 8-diol, Verrucarol, Scirpentriol, T-2 tetraol, pentahydroxyscirpene, 4-deacetylneosolaniol, trichodermin, deacetylcalonectrin, calonectrin, diacetylverrucarol, 4-monoacetoxyscirpenol, 4,15-diacetoxyscirpenol, 7-hydroxydiacetoxyscirpenol, 8-hydroxydiacetoxy-scirpenol (Neosolaniol), 7,8-dihydroxydiacetoxyscirpenol, 7-hydroxy-8-acetyldiacetoxyscirpenol, 8-acetylneosolaniol, NT-1, NT-2, HT-2, T-2, and acetyl T-2 toxin.

Representative examples of Group B simple trichothecenes include: Trichothecolone, Trichothecin, deoxynivalenol, 3-acetyldeoxynivalenol, 5-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, Nivalenol, 4-acetylnivalenol (Fusarenon-X), 4,15-idacetylnivalenol, 4,7,15-triacetylnivalenol, and tetra-acetylnivalenol. Representative examples of Group C simple trichothecenes include: Crotocol and Crotocin. Representative macrocyclic trichothecenes include Verrucarin A, Verrucarin B, Verrucarin J (Satratoxin C), Roridin A, Roridin D, Roridin E (Satratoxin D), Roridin H, Satratoxin F, Satratoxin G, Satratoxin H, Vertisporin, Mytoxin A, Mytoxin C, Mytoxin B, Myrotoxin A, Myrotoxin B, Myrotoxin C, Myrotoxin D, Roritoxin A, Roritoxin B, and Roritoxin D. In addition, the general "trichothecene" sesquiterpenoid ring structure is also present in compounds termed "baccharins" isolated from the

higher plant *Baccharis megapotamica*, and these are described in the literature, for instance as disclosed by Jarvis et al. (Chemistry of Alleopathy, ACS Symposium Series No. 268: ed. A.C. Thompson, 1984, pp. 149-159).

Representative examples of "anti-matrix agents" include inhibitors (i.e., agonists and antagonists and competitive and non-competitive inhibitors) of matrix synthesis, secretion and assembly, organizational cross-linking (e.g., transglutaminases cross-linking collagen), and matrix remodeling (e.g., following wound healing). A representative example of a useful therapeutic agent in this category of anti-matrix agents is colchicine, an inhibitor of secretion of extracellular matrix.

For the sustained release dosage form embodiments of the present invention, therapeutic agents preferably are those that inhibit vascular smooth muscle cell activity without killing the cells (i.e., cytostatic therapeutic agents). Preferred therapeutic agents for this purpose exhibit one or more of the following capabilities: to inhibit DNA synthesis prior to protein synthesis inhibition or to inhibit migration of vascular smooth muscle cells into the intima. These therapeutic agents do not significantly inhibit protein synthesis (i.e., do not kill the target cells) and, therefore, facilitate cellular repair and matrix production to stabilize the vascular wall lesion caused by angioplasty, by reducing smooth muscle cell proliferation.

Exemplary of such preferred therapeutic agents are protein kinase inhibitors, such as staurosporin (staurosporine is available from Sigma Chemical Co., St. Louis, Missouri) cytochalasins, such as cytochalasin B (Sigma Chemical Co.), and suramin (FBA Pharmaceuticals, West Haven, Connecticut), as well as nitroglycerin (DuPont Pharmaceuticals, Inc., Manuti, Puerto Rico) or analogs or functional equivalents thereof. These compounds are cytostatic and have been shown to exert minimal protein synthesis inhibition and cytotoxicity at concentrations where significant DNA synthesis inhibition

occurs (see Example 8 and Figs. 10A-10D). A useful protocol for identifying therapeutic agents useful in sustained release dosage form embodiments of the present invention is set forth in Example 8, for example. A practitioner in the art is capable of designing substantially equivalent experimental protocols for making such an identification for different target cell populations, such as adherent monolayer target cell types.

Other embodiments of the present invention involve agents that are cytotoxic to cancer cells. Preferred agents for these embodiments are Roridin A, *Pseudomonas exotoxin* and the like or analogs or functional equivalents thereof. A plethora of such therapeutic agents, including radioisotopes and the like, have been identified and are known in the art. In addition, protocols for the identification of cytotoxic moieties are known and employed routinely in the art.

Modulation of immune system-mediated disease effector cells can also be accomplished using the sustained release dosage forms of the present invention. Such modulation is preferably conducted with respect to diseases having an effector cell population that is accessible through local sustained release dosage form administration. Therapeutic moieties having the requisite modulating activity, e.g., cytocidal, cytostatic, metabolism modulation or like activity upon lymphorecticular cells in the treatment of arthritis (intra-articular administration), sprue (oral administration), uveitis and endophthalmitis (intra-ocular administration) and keratitis (sub-conjunctival administration), are identifiable using techniques that are known in the art. These agents can also be used to reduce hyperactivity of epithelial glands and endocrine organs that results in multiple disorders. Preferred agents for these embodiments include Roridin A, *Pseudomonas exotoxin*, suramin, protein kinase inhibitors (e.g., staurosporin) and the like, or analogs or functional equivalents thereof.

Other preferred therapeutic agents useful in the practice of the present invention include moieties capable of reducing or eliminating pathological proliferation, migration or hyperactivity of normal tissues. Exemplary of such therapeutic agents are those capable of reducing or eliminating hyperactivity of corneal epithelium and stroma, pathological proliferation or prolonged contraction of smooth muscle cells or pericytes of the intraocular vasculature implicated in degenerative eye disease resulting from hyperplasia or decreased vascular lumen area. Preferred agents for this purpose are staurosporin and cytochalasin B.

Vascular smooth muscle binding proteins of the invention bind to targets on the surface of vascular smooth muscle cells. It will be recognized that specific targets, e.g., polypeptides or carbohydrates, proteoglycans and the like, that are associated with the cell membranes of vascular smooth muscle cells are useful for selecting (e.g., by cloning) or constructing (e.g., by genetic engineering or chemical synthesis) appropriately specific vascular smooth muscle binding proteins. Particularly useful "targets" are internalized by smooth muscle cells, e.g., as membrane constituent antigen turnover occurs in renewal. Internalization by cells may also be by mechanisms involving phagolysosomes, clathrin-coated pits, receptor-mediated redistribution or endocytosis and the like. In a preferred embodiment, such a "target" is exemplified by chondroitin sulfate proteoglycans (CSPGs) synthesized by vascular smooth muscle cells and pericytes, and a discrete portion (termed an epitope herein) of the CSPG molecule having an apparent molecular weight of about 250 kD is especially preferred. The 250 kD target is an N-linked glycoprotein that is a component of a larger 400 kD proteoglycan complex (14). In one presently preferred embodiment of the invention, a vascular smooth muscle binding protein is provided by NR-AN-01 monoclonal antibody (a subculture of NR-ML-05) that binds to an epitope in a vascular smooth muscle CSPG target molecule.

The monoclonal antibody designated NR-ML-05 reportedly binds a 250 kD CSPG synthesized by melanoma cells (Morgan et al., U.S. Pat. No. 4,897,255). Smooth muscle cells and pericytes also reportedly synthesize a 250 kD CSPG as well as other CSPGs (11). NR-ML-05 binding to smooth muscle cells has been disclosed (Fritzberg et al., U.S. Pat. No. 4,879,225). Monoclonal antibody NR-ML-05 and subculture NR-ML-05 No. 85-41-4I-A2, freeze # A2106, have both been deposited with the American Type Culture Collection, Rockville, MD and granted Accession Nos. HB-5350 and HB-9350, respectively. NR-ML-05 is the parent of, and structurally and functionally equivalent to, subclone NR-AN-01, disclosed herein. It will be recognized that NR-AN-01 is just one example of a vascular smooth muscle binding protein that specifically associates with the 400 kD CSPG target, and that other binding proteins associating with this target and other epitopes in this target (14) are also useful in the therapeutic conjugates and methods of the invention. In the present case, six other murine monoclonal antibodies and two human chimeric monoclonal antibodies have also been selected, as described herein, that specifically target to the 250 kD CSPG of vascular smooth muscle cells. The antibodies also appear to be internalized by the smooth muscle cells following binding to the cell membrane. Immunoreactivity studies have also shown the binding of the murine MAbs to the 250 kD antigen in 45 human normal tissues and 30 different neoplasms and some of these results have been disclosed previously (U.S. Patent No. 4,879,225). In this disclosure and other human clinical studies, MAbs directed to the CSPG 250 kD antigen localized to vascular smooth muscle cells *in vivo*. Further, it will be recognized that the amino acid residues involved in the multi-point kinetic association of the NR-AN-01 monoclonal antibody with a CSPG marker antigenic epitope (i.e., the amino acids constituting the complementarity determining regions) are determined by computer-assisted molecular modeling and by the use of mutants having altered antibody binding affinity.

The binding-site amino acids and three dimensional model of the NR-AN-01 antigen binding site serve as a molecular model for constructing functional equivalents, e.g., short polypeptides ("minimal polypeptides"), that have binding affinity for a CSPG synthesized by vascular smooth muscle cells and pericytes.

In a presently preferred embodiment for treating stenosis following vascular surgical procedures, e.g., PTCA, selected binding proteins, e.g., antibodies or fragments, for use in the practice of the invention have a binding affinity of $>10^4$ liter/mole for the vascular smooth muscle 250 kD CSPG, and also the ability to be bound to and internalized by smooth muscle cells or pericytes.

Three-dimensional modeling is also useful to construct other functional equivalents that mimic the binding of NR-AN-01 to its antigenic epitope, e.g., "mimetic" chemical compounds that mimic the three-dimensional aspects of NR-AN-01 binding to its epitope in a CSPG target antigen. As used herein, "minimal polypeptide" refers to an amino acid sequence of at least six amino acids in length. As used herein, the term "mimetic" refers to an organic chemical polymer constructed to achieve the proper spacing for binding to the amino acids of, for example, an NR-AN-01 CSPG target synthesized by vascular smooth muscle cells or pericytes.

It will be recognized that the inventors also contemplate the utility of human monoclonal antibodies or "humanized" murine antibody as a vascular smooth muscle binding protein in the therapeutic conjugates of their invention. For example, murine monoclonal antibody may be "chimerized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Humanized vascular smooth muscle binding partners will be recognized to have the advantage of decreasing the

immunoreactivity of the antibody or polypeptide in the host recipient, which may thereby be useful for increasing the in vivo half-life and reducing the possibility of adverse immune reactions.

Also contemplated as useful binding peptides for restenosis treatment sustained release dosage forms of the present invention are those that localize to intercellular stroma and matrix located between and among vascular smooth muscle cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the vascular smooth muscle cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and elastic fibers, cytokeratin and other intercellular matrix components. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to intracellular stroma and matrix are also useful as binding peptides in this embodiment of the present invention. Such binding peptides may be identified and constructed or isolated in accordance with known techniques. In preferred embodiments of the present invention, the interstitial matrix binding protein binds to a target epitope with an association constant of at least about 10^{-4} M.

Useful binding peptides for cancer treatment embodiments of the present invention include those associated with cell membrane and cytoplasmic epitopes of cancer cells and the like. These binding peptides localize to the surface membrane of intact cells and internal epitopes of disrupted cells, respectively, and deliver the therapeutic agent for assimilation into the target cells. Minimal peptides, mimetic organic compounds and human or humanized antibodies that localize to the requisite tumor cell types are also useful as binding peptides of the present invention. Such binding peptides may be identified and constructed or isolated in

accordance with known techniques. Preferred binding peptides of these embodiments of the present invention bind to a target epitope with an association constant of at least about 10^{-6}M.

Binding peptides to membrane and cytoplasmic epitopes and the like that localize to immune system-mediated disease effector cells, e.g., cells of the lymphoreticular system, are also useful to deliver sustained release dosage forms of the present invention. The therapeutic agent is delivered to target cells for internalization therein by such sustained release dosage forms. Minimal peptides, mimetic organic compounds and human or humanized antibodies that localize to the requisite effector cell types are also useful as binding peptides of the present invention. Such binding peptides may be identified and constructed or isolated in accordance with known techniques. Preferred binding peptides of these embodiments of the present invention bind to a target epitope with an association constant of at least about 10^{-6} M.

Other preferred binding proteins or peptides useful in the practice of the present invention include moieties capable of localizing to pathologically proliferating normal tissues, such as pericytes of the intraocular vasculature implicated in degenerative eye disease. The therapeutic agent is delivered to target cells for internalization therein by such sustained release dosage forms. Minimal peptides, mimetic organic compounds and human or humanized antibodies that localize to the requisite pathologically proliferating normal cell types are also useful as binding peptides of the present invention. Such binding peptides may be identified and constructed or isolated in accordance with known techniques. Preferred binding peptides of these embodiments of the present invention bind to a target epitope with an association constant of at least about 10^{-6}M.

Representative "coupling" methods for linking the therapeutic agent through covalent or non-covalent bonds to the vascular smooth muscle binding protein include chemical cross-linkers and heterobifunctional cross-linking compounds

(i.e., "linkers") that react to form a bond between reactive groups (such as hydroxyl, amino, amido, or sulfhydryl groups) in a therapeutic agent and other reactive groups (of a similar nature) in the vascular smooth muscle binding protein. This bond may be, for example, a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, and the like. In one illustrative example, conjugates of monoclonal antibodies with drugs have been summarized by Morgan and Foon (Monoclonal Antibody Therapy to Cancer: Preclinical Models and Investigations, *Basic and Clinical Tumor Immunology, Vol. 2*, Kluwer Academic Publishers, Hingham, MA) and by Uhr *J. of Immunol.* 133:i-vii, 1984). In another illustrative example where the conjugate contains a radionuclide cytostatic agent, U.S. Patent No. 4,897,255, Fritzberg et al., incorporated herein by reference, is instructive of coupling methods that may be useful. In one presently preferred embodiment, the therapeutic conjugate contains a vascular smooth muscle binding protein coupled covalently to a trichothecene drug. In this case, the covalent bond of the linkage may be formed between one or more amino, sulfhydryl, or carboxyl groups of the vascular smooth muscle binding protein and a) the trichothecene itself; b) a trichothecene hemisuccinate carboxylic acid; c) a trichothecene hemisuccinate (HS) N-hydroxy succinimidate ester; or d) trichothecene complexes with poly-L-lysine or any polymeric carrier. Representative examples of coupling methods for preparing therapeutic conjugates containing a trichothecene therapeutic agent are described in U.S. Patents No. 4,906,452 and 4,744,981, incorporated herein by reference. Other examples using a hydrazide for forming a Schiff base linkage between binding proteins and trichothecenes are disclosed in pending U.S. patent application No. 07/415,154, incorporated herein by reference.

The choice of coupling method will be influenced by the choice of vascular smooth muscle binding protein or peptide, interstitial matrix binding protein or peptide and therapeutic

agent, and also by such physical properties as, e.g., shelf life stability, and/or by such biological properties as, e.g., half-life in cells and blood, intracellular compartmentalization route, and the like. For example, in one presently preferred therapeutic conjugate, hemisuccinate conjugates of the Roridin A therapeutic agent have a longer serum half-life than those of Verrucarin A, and this increased stability results in a significantly increased biological activity.

The sustained release embodiment of the present invention includes a therapeutic agent dispersed within a non-biodegradable or biodegradable polymeric structure. Such dispersion is conducted in accordance with the procedure described by Cowsar et al., "Poly(Lactide-Co-Glycolide) Microcapsules for Controlled Release of Steroids," Methods Enzymology, 112:101-116, 1985; Eldridge et al., "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies," Infection and Immunity, 59:2978-2986, 1991; Cohen et al., "Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres," Pharmaceutical Research, 8(6):713-720, 1991; and Sanders et al., "Controlled Release of a Luteinizing Hormone-Releasing Hormone Analogue from Poly(D,L-Lactide-Co-Glycolide) Microspheres," J. Pharmaceutical Science, 73(9):1294-1297, 1984.

The physical and chemical character of the sustained release dosage form of the present invention is amenable to several alternative modes of attachment to binding proteins or peptides. Dosage forms (sustained release-type) of the present invention are capable of binding to binding proteins/peptides through, for example, covalent linkages, intermediate ligand sandwich attachment, or non-covalent adsorption or partial entrapment. When the preferred poly-lactic/glycolic acid particulates are formed with the therapeutic agent dispersed therein, the uncharged polymer

backbone is oriented both inward (with the quasi lipophilic therapeutic agent contained therein) and outward along with a majority of the terminal carboxy groups. These surface carboxy groups may serve as covalent attachment sites when activated by, for example, a carbodiimide) for nucleophilic groups of the binding protein/peptide. Such nucleophilic groups include lysine epsilon amino groups (amide linkage), serine hydroxyl groups (ester linkage) or cysteine mercaptan groups (thioester linkage). Reactions with particular groups depend upon pH and the reduction state of the reaction conditions.

For example, poly-lactic/glycolic acid particulates having terminal carboxylic acid groups are reacted with N-hydroxybenztriazole in the presence of a water soluble carbodiimide of the formula R-N=C=N-R' (wherein R is a 3-dimethylaminopropyl group or the like and R' is an ethyl group or the like). The benztriazole-derivatized particulates (i.e., activated imidate-bearing moieties) are then reacted with a protein/peptide nucleophilic moiety such as an available epsilon amino moiety. Alternatively, p-nitrophenol, tetrafluorophenol, N-hydroxysuccinimide or like molecules are useful to form an active ester with the terminal carboxy groups of poly-lactic/glycolic acid particulates in the presence of carbodiimide. Other binding protein/peptide nucleophilic moieties include hydroxyl groups of serine, endogenous free thiols of cysteine, thiol groups resulting from reduction of binding protein/peptide disulfide bridges using reducing agents commonly employed for that purpose (e.g., cysteine, dithiothreitol, mercaptoethanol and the like) and the like. Additionally, the terminal carboxy groups of the poly lactic/glycolic acid particulates are activatable by reaction with thionyl chloride to form an acyl chloride derivatized moiety. The derivatized particulates are then reacted with binding peptide/protein nucleophilic groups to form targeted dosage forms of the present invention.

Direct sustained release dosage form-binding protein or peptide conjugation may disrupt binding protein/peptide target cell recognition. Ligand sandwich attachment techniques are useful alternatives to achieve sustained release dosage form-binding protein/peptide attachment. Such techniques involve the formation of a primary peptide or protein shell using a protein that does not bind to the target cell population. Binding protein/peptide is then bound to the primary peptide or protein shell to provide the resultant particulate with functional binding protein/peptide. An exemplary ligand sandwich approach involves covalent attachment of avidin or streptavidin to the particulates through functional groups as described above with respect to the "direct" binding approach. The binding protein or peptide is derivatized, preferably minimally, with functionalized biotin (e.g., through active ester, hydrazide, iodoacetal, maleimidyl or like functional groups). Ligand (i.e., binding peptide or protein/functionalized biotin) attachment to the available biotin binding sites of the avidin/streptavidin primary protein shell occurs through the use of a saturating amount of biotinylated protein/peptide.

For example, poly-lactic/glycolic acid particulates having terminal carboxylic acid groups are activated with carbodiimide and subsequently reacted with avidin or streptavidin. The binding protein or peptide is reacted with biotinamidocaproate N-hydroxysuccinimide ester at a 1-3 molar offering of biotin-containing compound to the binding protein/peptide to form a biotinylated binding protein/peptide. A molar excess of the biotinylated binding protein/peptide is incubated with the avidin-derivatized particulates to form a targeted dosage form of the present invention.

Alternatively, the particulate carboxy groups are biotinylated (e.g., through carbodiimide activation of the carboxy group and subsequent reaction with amino alkyl biotinamide). The biotinylated particulates are then

incubated with a saturating concentration (i.e., a molar excess) of avidin or streptavidin to form protein coated particulates having free biotin binding sites. Such coated particulates are then capable of reaction with a molar excess of biotinylated binding protein formed as described above. Another option involves avidin or streptavidin bound binding peptide or protein attachment to biotinylated particulates.

In addition, binding protein/peptide-particulate attachment can be achieved by adsorption of the binding peptide to the particulate, resulting from the nonionic character of the partially exposed polymer backbone of the particulate. Under high ionic strength conditions (e.g., 1.0 molar NaCl), hydrogen and hydrophobic particulate-binding protein/peptide binding are favored.

Moreover, binding protein/peptide may be partially entrapped in the particulate polymeric matrix upon formation thereof. Under these circumstances, such entrapped binding protein/peptide provides residual selective binding character to the particulate. Mild particulate formation conditions, such as those employed by Cohen et al., Pharmaceutical Research, 8: 713-720 (1991), are preferred for this embodiment of the present invention. Such entrapped binding protein is also useful in target cell reattachment of a partially degraded particulate that has undergone exocytosis. Other polymeric particulate dosage forms (e.g., non-biodegradable dosage forms) having different exposed functional groups can be bound to binding proteins or peptides in accordance with the principles discussed above.

Exemplary non-biodegradable polymers useful in the practice of the present invention are polystyrenes, polypropylenes, styrene acrylic copolymers and the like. Such non-biodegradable polymers incorporate or can be derivatized to incorporate functional groups for attachment of binding protein/peptide, including carboxylic acid groups, aliphatic primary amino groups, aromatic amino groups and hydroxyl groups.

Carboxylic acid functional groups are coupled to binding protein or peptide using, for example, the reaction mechanisms set forth above for poly-lactic/glycolic acid biodegradable polymeric particulate dosage forms. Primary amino functional groups are coupled by, for example, reaction thereof with succinic anhydride to form a terminal carboxy moiety that can be bound to binding peptide/protein as described above. Additionally, primary amino groups can be activated with cyanogen bromide and form guanidine linkages with binding protein/peptide primary amino groups. Aromatic amino functional groups are, for example, diazotized with nitrous acid to form diazonium moieties which react with binding protein/peptide tyrosines, thereby producing a diazo bond between the non-biodegradable particulate and the binding protein/peptide. Hydroxyl functional groups are coupled to binding protein/peptide primary amino groups by, for example, converting the hydroxyl moiety to a terminal carboxylic acid functional group. Such a conversion can be accomplished through reaction with chloroacetic acid followed by reaction with carbodiimide. Sandwich, adsorption and entrapment techniques, discussed above with respect to biodegradable particulates, are analogously applicable to non-biodegradable particulate-binding protein/peptide affixation.

In a preferred embodiment, targeting is specific for potentially proliferating cells that result in increased smooth muscle in the intimal region of a traumatized vascular site, e.g., following angioplasty, e.g., pericytes and vascular smooth muscle cells. Aspects of the invention relate to therapeutic modalities in which the therapeutic conjugate of the invention is used to delay, reduce, or eliminate smooth muscle proliferation after angioplasty, e.g., PTCA, atheroectomy and percutaneous transluminal coronary rotational atheroblation.

In another preferred embodiment, targeting is specific for primary or metastatic tumor foci accessible to local administration, e.g., tumors exposed for infiltration by

laparotomy or visible for fluoroscopic or computerized tomography guiding and infusion needle administration to internal tumor foci or tumors confined to a small area or cavity within the mammal, e.g., ovarian cancer located in the abdomen, focal or multifocal liver tumors or the like. Aspects of this embodiment of the invention involve therapeutical modalities wherein the therapeutic agent is cytotoxic to the target cells or metabolically modulates the cells, increasing their sensitivity to chemotherapy and/or radiation therapy.

In another embodiment, targeting is specific for a local administration accessible effector cell population implicated in immune system-mediated diseases, e.g., arthritis, intra-ocular immune system-mediated disease or sprue. Aspects of this embodiment of the present invention involve therapeutic modalities wherein the therapeutic agent is cytotoxic or modifies the biological response of the target cells to effect a therapeutic objective.

In another embodiment, targeting is specific for a local administration accessible pathologically proliferating or hyperactive normal cell population implicated in, e.g., degenerative eye disease, corneal pannus, hyperactive endocrine glands or the like. Aspects of this embodiment of the present invention involve therapeutic modalities wherein the therapeutic agent reduces or eliminates proliferation or hyperactivity of the target cell population.

For treatment of a traumatized or diseased vascular site, the therapeutic conjugates or dosage forms of the invention may be administered to the host using an infusion catheter, such as produced by C.R. Bard Inc., Billerica, MA, or that disclosed by Wolinsky (7; U.S. Patent No. 4,824,436) or Spears (U.S. Patent No. 4,512,762). In this case, a therapeutically effective dosage of the therapeutic conjugate will be typically reached when the concentration of conjugate in the fluid space between the balloons of the catheter is in the range of about 10^{-3} to 10^{-12}M. It will be recognized from the

Examples provided herewith that therapeutic conjugates of the invention may only need to be delivered in an anti-proliferative therapeutic dosage sufficient to expose the proximal (6 to 9) cell layers of the intimal or tunica media cells lining the lumen to the therapeutic anti-proliferative conjugate, whereas the anti-contractile therapeutic dosage needs to expose the entire tunica media, and further that this dosage can be determined empirically, e.g., by a) infusing vessels from suitable animal model systems and using immunohistochemical methods to detect the conjugate and its effects (e.g., such as exemplified in the EXAMPLES below); and b) conducting suitable *in vitro* studies such as exemplified in EXAMPLES 3, 4, and 5, below).

In a representative example, this therapeutically effective dosage is achieved by preparing 10 ml of a 200 μg/ml therapeutic conjugate solution, wherein the vascular smooth muscle protein binding protein is NR-AN-01 and the therapeutic agent is Roridin A, a trichothecene drug. For treating vascular trauma, e.g., resulting from surgery or disease (e.g., see below), when the therapeutic conjugate is administered with an infusion catheter, 10 ml will commonly be sufficient volume to fill the catheter and infuse 1 to 1.5 ml into one to three traumatic lesion sites in the vessel wall. It will be recognized by those skilled in the art that desired therapeutically effective dosages of a therapeutic conjugate according to the invention will be dependent on several factors, including, e.g.: a) the binding affinity of the vascular smooth muscle binding protein in the therapeutic conjugate; b) the atmospheric pressure applied during infusion; c) the time over which the therapeutic conjugate administered resides at the vascular site; d) the nature of the therapeutic agent employed; and/or e) the nature of the vascular trauma and therapy desired. Those skilled practitioners trained to deliver drugs at therapeutically effective dosages (e.g., by monitoring drug levels and observing clinical effects in patients) will

determine the optimal dosage for an individual patient based on experience and professional judgment. In a preferred embodiment, about 0.3 atm (i.e., 300 mm of Hg) to about 3 atm of pressure applied for 15 seconds to 3 minutes directly to the vascular wall is adequate to achieve infiltration of a therapeutic conjugate containing the NR-AN-01 binding protein into the smooth muscle layers of a mammalian artery wall. Those skilled in the art will recognize that infiltration of the therapeutic conjugate into intimal layers of a diseased human vessel wall will probably be variable and will need to be determined on an individual basis.

Sustained release dosage forms of an embodiment of the invention may only need to be delivered in an anti-proliferative therapeutic dosage sufficient to expose the proximal (6 to 9) cell layers of the tunica media smooth muscle cells lining the lumen to the dosage form. This dosage is determinable empirically, e.g., by a) infusing vessels from suitable animal model systems and using immunohistochemical, fluorescent or electron microscopy methods to detect the dosage form and its effects; and b) conducting suitable *in vitro* studies.

In a representative example, this therapeutically effective dosage is achieved by determining in smooth muscle cell tissue culture the pericellular agent dosage, which at a continuous exposure results in a therapeutic effect between the toxic and minimal effective doses. This therapeutic level is obtained *in vivo* by determining the size, number and therapeutic agent concentration and release rate required for particulates infused between the smooth muscle cells of the artery wall to maintain this pericellular therapeutic dosage. The dosage form should release the therapeutic agent at a rate that approximates the pericellular dose of the following exemplary therapeutic agents: from about 0.01 to about 100 micrograms/ml nitroglycerin, from about 1.0 to about 1000 micrograms/ml of suramin, from about 0.001 to about 100

micrograms/ml for cytochalasin, and from about 0.01 to about 10^5 nanograms/ml of staurosporin.

It will be recognized by those skilled in the art that desired therapeutically effective dosages of the sustained release dosage form of the invention will be dependent on several factors, including, e.g.: a) the binding affinity of the binding protein associated with the dosage form; b) the atmospheric pressure and duration of the infusion; c) the time over which the dosage form administered resides at the target site; d) the rate of therapeutic agent release from the particulate dosage form; e) the nature of the therapeutic agent employed; f) the nature of the trauma and/or therapy desired; and/or g) the intercellular and/or intracellular localization of the particulate dosage form. Those skilled practitioners trained to deliver drugs at therapeutically effective dosages, (e.g., by monitoring therapeutic agent levels and observing clinical effects in patients) are capable of determining the optimal dosage for an individual patient based on experience and professional judgment. In a preferred embodiment, about 0.3 atm (i.e., 300 mm of Hg) to about 3 atm of pressure applied for 15 seconds to 3 minutes to the arterial wall is adequate to achieve infiltration of a sustained release dosage form bound to the NR-AN-01 binding protein into the smooth muscle layers of a mammalian artery wall. Wolinsky et al., "Direct Intraarterial Wall Injection of Microparticles Via a Catheter: A Potential Drug Delivery Strategy Following Angioplasty," Am. Heart Jour., 122(4):1136-1140, 1991. Those skilled in the art will recognize that infiltration of a sustained release dosage form into a target cell population will probably be variable and will need to be determined on an individual basis.

It will also be recognized that the selection of a therapeutic agent that exerts its effects intracellularly, e.g., on ribosomes or DNA metabolism, will influence the dosage and time required to achieve a therapeutically effective dosage, and that this process can be modeled

in vitro and in animal studies, such as those described in the Examples provided below, to find the range of concentrations over which the therapeutic conjugate or dosage form should be administered to achieve its effects of delaying, reducing or preventing restenosis following angioplasty. For example, therapeutic conjugates radiolabeled with alpha-, beta- or gamma-emitters of known specific activities (e.g., millicuries per millimole or milligram of protein) are useful for determining the therapeutically effective dosage by using them in animal studies and human trials with quantitative imaging or autoradiography of histological tissue sections to determine the concentration of therapeutic conjugate that is required by the therapeutic protocol. A therapeutically effective dosage of the therapeutic conjugate or dosage form will be reached when at least three conditions are met: namely, (1) the therapeutic conjugate or dosage form is distributed in the intimal layers of the traumatically injured vessel; (2) the therapeutic conjugate or dosage form is distributed within the desired intracellular compartment of the smooth muscle cells, i.e., that compartment necessary for the action of the therapeutic agent, or the therapeutic agent released from the dosage form extracellularly is distributed within the relevant intracellular compartment; and (3) the therapeutic agent inhibits the desired cellular activity of the vascular smooth muscle cell, e.g., proliferation, migration, increased cellular volume, matrix synthesis, cell contraction and the like described above.

It will be recognized that where the therapeutic conjugate or dosage form is to be delivered with an infusion catheter, the therapeutic dosage required to achieve the desired inhibitory activity for a therapeutic conjugate or dosage form can also be anticipated through the use of *in vitro* studies. In a preferred aspect, the infusion catheter may be conveniently a double balloon or quadruple balloon catheter with a permeable membrane. In one representative embodiment, a therapeutically effective dosage

of a therapeutic conjugate or dosage form is useful in treating vascular trauma resulting from disease (e.g., atherosclerosis, aneurysm, or the like) or vascular surgical procedures such as angioplasty, atheroectomy, placement of a stent (e.g., in a vessel), thrombectomy, and grafting. Atheroectomy may be performed, for example, by surgical excision, ultrasound or laser treatment, or by high pressure fluid flow. Grafting may be, for example, vascular grafting using natural or synthetic materials or surgical anastomosis of vessels such as, e.g., during organ grafting. Those skilled in the art will recognize that the appropriate therapeutic dosage for a given vascular surgical procedure (above) is determined in *in vitro* and *in vivo* animal model studies, and in human preclinical trials. In the EXAMPLES provided below, a therapeutic conjugate containing Roridin A and NR-AN-01 achieved a therapeutically effective dosage *in vivo* at a concentration which inhibited cellular protein synthesis in test cells *in vitro* by at least 5 to 50%, as judged by incorporation of radiolabeled amino acids.

In the case of therapeutic agents of conjugates or dosage forms containing anti-migratory or anti-matrix therapeutic agents, cell migration and cell adherence in *in vitro* assays, respectively, may be used for determining the concentration at which a therapeutically effective dosage will be reached in the fluid space created by the infusion catheter in the vessel wall.

While one representative embodiment of the invention relates to therapeutic methods employing an infusion catheter, it will be recognized that other methods for drug delivery or routes of administration may also be useful, e.g., injection by the intravenous, intralymphatic, intrathecal, intraarterial, local delivery by implanted osmotic pumps or other intracavity routes. For intravenous administration, nanoparticulate dosage forms of the present invention are preferred. Intravenous administration of nanoparticulates is useful, for example, where vascular permeability is increased

in tumors for leakage, especially in necrotic areas of tumors having damaged vessels which allow the leakage of particles into the interstitial fluid, and where artery walls have been denuded and traumatized allowing the particles to enter the interstitial area of the tunica media.

Advantageously, non-coupled vascular smooth muscle cell binding protein (e.g., free NR-AN-01 antibody) is administered prior to administration of the therapeutic agent conjugate or dosage form to provide a blocker of non-specific binding to cross-reactive sites. Blocking of such sites is important because vascular smooth muscle cell binding proteins will generally have some low level of cross-reactivity with cells in tissues other than the desired smooth muscle cells. Such blocking can improve localization of the therapeutic conjugate or dosage form at the specific vascular site, e.g., by making more of the therapeutic conjugate available to the cells. As an example, non-coupled vascular smooth muscle binding protein is administered from about 5 minutes to about 48 hours, most preferably from about 5 minutes to about 30 minutes, prior to administration of the therapeutic conjugate or dosage form. In one preferred embodiment of the invention, the unlabeled specific "blocker" is a monovalent or bivalent form of an antibody (e.g., a whole antibody or an $F(ab)'_2$, Fab, Fab', or Fv fragment of an antibody). The monovalent form of the antibody has the advantage of minimizing displacement of the therapeutic conjugate or dosage form while maximizing blocking of the non-specific cross-reactive sites. The non-coupled vascular smooth muscle cell binding protein is administered in an amount effective to blocking binding of a least a portion of the non-specific cross-reactive sites in a patient. The amount may vary according to such factors as the weight of the patient and the nature of the binding protein. In general, about 0.06 mg to 0.20 mg per kg body weight or more of the unlabeled specific blocker is administered to a human.

In addition, a second irrelevant vascular smooth muscle cell binding protein may optionally be administered to a

patient prior to administration of the therapeutic conjugate or dosage form to reduce non-specific binding of the therapeutic conjugate or dosage form to tissues. In a preferred embodiment, the irrelevant binding protein may be an antibody which does not bind to sites in the patient through antigen-specific binding, but instead binds in a non-specific manner, e.g., through Fc receptor binding reticuloendothelial cells, asialo-receptor binding, and by binding to ubiquitin-expressing cells. The irrelevant "blocker" decreases non-specific binding of the therapeutic conjugate or dosage form and thus reduces side-effects, e.g., tissue toxicity, associated with the use of the therapeutic conjugate or dosage form. The irrelevant "blocker" is advantageously administered from 5 minutes to 48 hours, most preferably from 15 minutes to one hour, prior to administration of the therapeutic conjugate or dosage form, although the length of time may vary depending upon the therapeutic conjugate and route or method of injection. Representative examples of irrelevant "blockers" include antibodies that are nonreactive with human tissues and receptors or cellular and serum proteins prepared from animal sources that when tested are found not to bind in a specific manner (e.g., with a $Ka<10^3$ M^{-1}) to human cell membrane targets.

It will be recognized that the conjugates and dosage forms of the invention are not restricted in use for therapy following angioplasty; rather, the usefulness of the therapeutic conjugates and dosage forms will be proscribed by their ability to inhibit cellular activities of smooth muscle cells and pericytes in the vascular wall. Thus, other aspects of the invention include therapeutic conjugates and dosage forms and protocols useful in early therapeutic intervention for reducing, delaying, or eliminating (and even reversing) atherosclerotic plaques and areas of vascular wall hypertrophy and/or hyperplasia. Therapeutic conjugates and dosage forms of the invention also find utility for early intervention in

pre-atherosclerotic conditions, e.g., they are useful in patients at a high risk of developing atherosclerosis or with signs of hypertension resulting from atherosclerotic changes in vessels or vessel stenosis due to hypertrophy of the vessel wall.

The therapeutic conjugates and dosage forms of the invention may also be used in therapeutic modalities for enhancing the regrowth of endothelial cells in injured vascular tissues and in many kinds of wound sites including epithelial wounds. In these therapeutic modalities, the therapeutic conjugates and dosage forms of the invention find utility in inhibiting the migration and/or proliferation of smooth muscle cells or pericytes. Smooth muscle cells and pericytes have been implicated in the production of factors *in vitro* that inhibit endothelial cell proliferation, and their proliferation can also result in a physical barrier to establishing a continuous endothelium. Thus, the therapeutic conjugates and dosage forms of the invention find utility in promoting neo-angiogenesis and increased re-endothelialization, e.g., during wound healing, vessel grafts and following vascular surgery. The dosage forms may also release therapeutic modalities that stimulate or speed up re-endothelialization of the damaged vessel wall. An exemplary therapeutic agent for this purpose is vascular permeability factor.

Still other aspects of the invention relate to therapeutic modalities for enhancing wound healing in a vascular site and improving the structural and elastic properties of healed vascular tissues. In these therapeutic modalities using the therapeutic conjugate or dosage form of the invention, i.e., to inhibit the migration and proliferation of smooth muscle cells or pericytes in a vessel wall, the strength and quality of healing of the vessel wall are improved. Smooth muscle cells in the vascular wound site contribute to the normal process of contraction of the wound site which promotes wound healing. It is presently believed

that migration and proliferation of smooth muscle cells and matrix secretion by transformed smooth muscle cells may detract from this normal process and impair the long-term structural and elastic qualities of the healed vessel. Thus, other aspects of the invention provide for therapeutic conjugates and dosage forms that inhibit smooth muscle and pericyte proliferation and migration as well as morphological transformation, and improve the quality of the healed vasculature.

The present invention also provides a combination therapeutic method involving a cytocidal therapeutic conjugate and a cytostatic therapeutic agent. The cytocidal conjugate includes a binding partner (such as a protein or peptide) capable of specifically localizing to vascular smooth muscle cells and an active agent capable of killing such cells. The cytocidal conjugate is administered, preferably intravenously or through any other convenient route therefor, localizes to the target smooth muscle cells, and destroys proliferating cells involved in stenotic or restenotic events. This cellular destruction causes the release of mitogens and other metabolic events, which events generally lead, in turn, to vascular smooth muscle cell proliferation. The sustained release anti-proliferative or anti-contractile dosage forms of the present invention are next administered, preferably through an infusion catheter or any convenient dosage form therefor. The sustained release dosage form retards the vascular smooth muscle cell proliferation and/or migration and contraction, thereby maintaining luminal diameter. This treatment methodology constitutes a biological arteromyectomy useful in stenotic vessels resulting from vascular smooth muscle cell hyperplasia and the like.

The present invention also provides methods for the treatment of cancer and immune system-mediated diseases through local administration of a targeted particulate dosage form. The particulate dosage form is, for example, administered locally into primary and/or metastatic foci of

cancerous target cells. Local administration is preferably conducted using an infusion needle or intraluminal administration route, infusing the particulate dosage form in the intercellular region of the tumor tissue or in luminal fluid surrounding the tumor cells.

Primary foci introduction is preferably conducted with respect to target cells that are generally situated in confined areas within a mammal, e.g., ovarian carcinomas located in the abdominal cavity. The dosage form of the present invention binds to the target cell population and, optionally, is internalized therein for release of the therapeutic agent over time. Local administration of dosage forms of the present invention to such primary foci results in a localized effect on such target cells, with limited exposure of other sensitive organs, e.g., the bone marrow and kidneys, to the therapeutic agent.

When metastatic foci constitute the target cell population, the administered microparticles and larger nanoparticles are primarily bound to the target cells situated near the infusion site and are, optionally, internalized for release of the therapeutic agent, thereby generating a marked and localized effect on the target cells immediately surrounding the infusion site. In addition, smaller nanoparticles follow interstitial fluid flow or lymphatic drainage channels and bind to target cells that are distal to the infusion site and undergoing lymphatic metastasis.

The targeted dosage forms of this embodiment of the present invention can be used in combination with more commonly employed immunoconjugate therapy. In this manner, the immunoconjugate achieves a systemic effect within the limits of systemic toxicity, while the dosage form of the present invention delivers a concentrated and sustained dose of therapeutic agent to the primary and metastatic foci, which often receive an inadequate therapeutic dose from such "systemic" immunoconjugate administration alone, and avoids or minimizes systemic toxic effects.

Where the target cell population can be accessed by local administration, the dosage forms of the present invention are utilized to control immune system-mediated diseases. Exemplary of such diseases are arthritis, sprue, uveitis, endophthalmitis, keratitis and the like. The target cell populations implicated in these embodiments of the present invention are confined to a body cavity or space, such as joint capsules, pleural and abdominal cavity, eye and sub-conjunctival space, respectively. Local administration is preferably conducted using an infusion needle for a intrapleural, intraperitoneal, intraocular or sub-conjunctival administration route.

This embodiment of the present invention provides a more intense, localized modulation of immune system cells with minimal effect on the systemic immune system cells. Optionally, the systemic cells of the immune system are simultaneously treatable with a chemotherapeutic agent conjugated to a binding protein or peptide. Such a conjugate preferably penetrates from the vascular lumen into target immune system cells.

The local particulate dosage form administration may also localize to normal tissues that have been stimulated to proliferate, thereby reducing or eliminating such pathological (i.e., hyperactive) conditions. An example of this embodiment of the present invention involves intraocular administration of a particulate dosage form coated with a binding protein or peptide that localizes to pericytes and smooth muscle cells of neovascularizing tissue. Proliferation of these pericytes causes degenerative eye disease. Preferred dosage forms of the present invention release compounds capable of suppressing the pathological proliferation of the target cell population. The preferred dosage forms can also release compounds that increase vessel lumen area and blood flow, reducing the pathological alterations produced by this reduced blood supply.

Still another aspect of the present invention relates to therapeutic modalities for maintaining an expanded luminal volume following angioplasty or other vessel trauma. One embodiment of this aspect of the present invention involves administration of a therapeutic agent capable of inhibiting the ability of vascular smooth muscle cells to contract. Exemplary agents useful in the practice of this aspect of the present invention are those capable of causing a traumatized artery to lose vascular tone, such that normal vascular hydrostatic pressure (i.e., blood pressure) expands the flaccid vessel to or near to its maximal physiological diameter. Loss of vascular tone may be caused by agents that interfere with the formation or function of contractile proteins (e.g., actin, myosin, tropomyosin, caldesmon, calponin or the like). This interference can occur directly or indirectly through, for example, inhibition of calcium modulation, phosphorylation or other metabolic pathways implicated in contraction of vascular smooth muscle cells.

Inhibition of cellular contraction (i.e., loss of vascular tone) may operate through two mechanisms to reduce the degree of vascular stenosis. First, inhibition of cellular contraction for a prolonged period of time limits the number of smooth muscle cells that migrate from the tunica media into the intima, the thickening of which results in vascular luminal stenosis. Second, inhibition of cellular contraction causes the smooth muscle wall to relax and dilate under normal vascular hydrostatic pressure (i.e., blood pressure). Therapeutic agents, such as the cytochalasins, inhibit smooth muscle cell contraction without abolishing the protein synthesis necessary for traumatized, post-angioplasty or other surgically- or disease-damaged, smooth muscle cells to repair themselves. Protein synthesis is also necessary for the smooth muscle cells to secrete matrix, which fixes or retains the lumen in a state near its maximum systolic diameter as the vascular lesion stabilizes (i.e., a biologically-induced stenting effect).

This biological stenting effect not only results in an expanded vessel luminal area and increased blood flow rate through the vessel, but also significantly reduces elastic recoil following angioplasty. Elastic recoil is an acute closure of the vessel associated with vasospasm or early relaxation of the muscular wall, due to trauma shock resulting from vessel over-stretching by a balloon catheter during angioplasty. This spasm of the tunica media which leads to decreases in the luminal area may occur within hours, days or weeks after the balloon dilation, as restoration of vascular muscle wall tone occurs. Recent observations during microscopic examination of atheroectomy specimens suggest that elastic recoil may occur in up to 25% of angioplasty procedures classified as successful, based on the initial post-procedure angiogram. Because the biological stenting procedure relaxes the artery wall following balloon angioplasty, the clinician can eliminate over-inflation and its resultant trauma shock as a means to diminish or delay the vessel spasm or elastic recoil. Reduction or elimination of over-inflation decreases trauma to the muscular wall of the vessel, thereby reducing the determinants of smooth muscle cell proliferation in the intima and, therefore, reducing the incidence or severity of restenosis.

Biological stenting also decreases the incidence of thrombus formation. In pig femoral arteries treated with cytochalasin B, for example, the incidence of mural microthrombi was decreased as compared to the balloon traumatized arteries that were not treated with the therapeutic agent. This phenomenon appears to be a secondary benefit that may result from the increased blood flow through the traumatized vessel, said benefit being obtained through the practice of the present invention.

Cytochalasins are exemplary therapeutic agents capable of generating a biological stenting effect on vascular smooth muscle cells. Cytochalasins are thought to inhibit both migration and contraction of vascular smooth muscle cells by

interacting with actin. The cytochalasins interact with the ends of filamentous actin to inhibit the elongation of the actin filaments. Low doses of cytochalasins (e.g., cytochalasin B) also disrupt microfilament networks of actin. In vitro data indicate that after vascular smooth muscle cells clear cytochalasin B, the cells regenerate enough polymerized actin to resume migration within about 24 hours. In vivo assessments reveal that vascular smooth muscle cells regain vascular tone within 2 to 4 days. It is during this recuperative period that the lumen diameter fixation and biological stenting effect occurs.

The therapeutic agent may be targeted, but is preferably administered directly to the traumatized vessel following the angioplasty or other traumatic event. The biological stenting effect of cytochalasin B, for example, is achievable using a single infusion of the therapeutic agent into the traumatized region of the vessel wall at a dose concentration ranging from about 0.1 microgram/ml to about 1.0 micrograms/ml.

Inhibition of vascular smooth muscle cell migration (from the tunica media to the intima) has been demonstrated in the same dose range (Example 11); however, a sustained exposure of the vessel to the therapeutic agent is preferable in order to maximize these anti-migratory effects. If the vascular smooth muscle cells cannot migrate into the intima, they cannot proliferate there. Should vascular smooth muscle cells migrate to the intima, a subsequently administered anti-proliferative sustained release dosage form inhibits the intimal proliferation. As a result, the sustained release dosage form of the present invention, incorporating a cytochalasin or other anti-proliferative therapeutic agent, can be administered in combination with a free cytochalasin therapeutic agent. In this manner, the biological stenting effect, as well as an anti-proliferative or anti-migratory effect, can be achieved in a single administration protocol.

Agents useful in the protocols of the present invention are identifiable, for example, in accordance with the

following procedures. A potential agent for free agent (i.e., non-targeted) administration exhibits one or more of the following characteristics:

(i) retains an expanded luminal volume following angioplasty (e.g., PTCA, percutaneous transluminal angioplasty (PTA) or the like) or other trauma, including atheroectomy (e.g., rotoblater, laser and the like), coronary artery bypass procedures or the like; or resulting from vascular disease (e.g., atherosclerosis, eye diseases secondary to vascular stenosis or atrophy, cerebral vascular stenotic diseases or the like);

(ii) the initial increase in luminal area facilitated by the agent does not result in or accentuate chronic stenosis of the lumen;

(iii) inhibits target cell contraction or migration; and

(iv) is cytostatic.

Preferably, a therapeutic agent employed herein will have all four properties; however, the first and third are more important than the second and fourth for practice of the present invention. Cytochalasin B, for example, was evaluated to determine suitability for use in free therapeutic agent protocols. The biological stenting effect of cytochalasin B is achievable using a single infusion of the therapeutic agent into the traumatized region of the vessel wall at a dose concentration ranging from about 0.1 microgram/ml to about 1.0 micrograms/ml.

An agent useful in the sustained release embodiments of the present invention exhibits one or more of the following characteristics:

(i) retains an expanded luminal volume following angioplasty (e.g., PTCA, percutaneous transluminal angioplasty (PTA) or the like) or other trauma, including atheroectomy (e.g., rotoblater, laser and the like), coronary artery bypass procedures or the like; or resulting from vascular disease (e.g., atherosclerosis,

eye diseases secondary to vascular stenosis or atrophy, cerebral vascular stenotic diseases or the like);

(ii) inhibits target cell proliferation (e.g., following 5 minute and 24 hour exposure to the agent, in vitro vascular smooth muscle tissue cultures demonstrate a level of inhibition of ^{3}H-thymidine uptake and, preferably, display relatively less inhibition of ^{3}H-leucine uptake);

(iii) at a dose sufficient to inhibit DNA synthesis, produces only mild to moderate (e.g., grade 2 or 3 in the assays described below) morphological cytotoxic effects;

(iv) inhibits target cell contraction; and

(v) is cytostatic.

Upon identification of a therapeutic agent exhibiting one or more of the preceding attributes, the agent is subjected to a second testing protocol that involves longer exposure of vascular smooth muscle cells to the therapeutic agent.

An agent useful in the sustained release embodiments of the present invention exhibits the following characteristics:

(i) upon long term (e.g., 5 days) exposure, the agent produces the same or similar in vitro effect on vascular smooth muscle tissue culture DNA synthesis and protein synthesis, as described above for the 5 minute and 24 hour exposures; and

(ii) at an effective dose in the long term in vitro assay for DNA synthesis inhibition, the agent exhibits mild to moderate morphological cytotoxic effects over a longer term (e.g., 10 days).

Further evaluation of potential anti-proliferative agents within the present invention is conducted in an in vivo balloon traumatized pig femoral artery model. Preferably, such agents demonstrate a 50% or greater inhibition of cell proliferation in the tunica media vascular smooth muscle cells, as indicated by a 1 hour "BRDU flash labeling" prior

to tissue collection and histological evaluation. If an agent is effective for a period of time sufficient to inhibit intimal smooth muscle proliferation 50% or greater with a single exposure, it is an agent within the present invention that does not require administration in a sustained release dosage form. Agents having shorter duration activity are evaluated for sustained release if the systemic toxicity and potential therapeutic index appear to permit intravenous administration to achieve the 50% inhibition, or if the agent is amenable to local delivery to the vascular smooth muscle cells with sustained release at an effective anti-proliferative dose. Sustained release agents are evaluated in a sustained release dosage form for dose optimization and efficacy studies. Preferably, anti-proliferative agents useful in the practice of the present invention decrease vascular stenosis by 50% in balloon traumatized pig femoral arteries and, more preferably, to decrease vascular stenosis to a similar extent in pig coronary arteries. Such agents are then evaluable in human clinical trials.

Cell proliferation (i.e., DNA synthesis) inhibition is the primary characteristic for sustained release of agents. Staurosporin, for example, exhibits a differential between ^{3}H-leucine and ^{3}H-thymidine uptake such that it is cytostatic at administered doses. Longer duration cytotoxicity studies did not indicate that prolonged exposure to the therapeutic agent would adversely impact the target cells. In addition, BRDU pulsing indicated that staurosporin inhibits target cell proliferation. Any convenient method for evaluating the capability of inhibiting cell proliferation may alternatively be employed, however. Consequently, staurosporin is effective in retaining an expanded luminal volume.

The invention will be better understood by making reference to the following specific examples.

EXAMPLE 1

Binding to Vascular Smooth Muscle Cells In the Blood Vessel Wall *In Vivo*

FIGURE 1 illustrates the binding of NR-AN-01 (a murine IgG2b MAb) to the smooth muscle cells in the vascular wall of an artery in a 24-year old male patient, 4 days after the i.v. administration of NR-AN-01. FIGURE 1 is a photomicrograph of a histological section taken through the medial region of an arterial wall of the patient after NR-AN-01 administration, where the section was reacted *ex vivo* with HRP-conjugated goat anti-mouse IgG. The reaction of the HRP-conjugate with NR-AN-01 MAb was visualized by adding 4-chloro-1-naphthol or 3,3'-diaminobenzidine tetrahydrochloride as a peroxidase substrate (chromogen). The reaction product of the substrate forms an insoluble purple or dark brown precipitate at the reaction site (shown at #2, FIGURE 1). A counter stain was used to visualize collagenous extracellular matrix material (shown at #2, FIGURE 1) or cell nuclei (#1, FIGURE 1). Smooth muscle cells are visualized under microscopic examination as purple stained cells. This photomicrograph demonstrates the ability of the MAb to specifically bind to human vascular smooth muscle *in vivo*, and to be internalized by the cells and remain in the cells for extended periods.

EXAMPLE 2

Therapeutic Conjugates Containing Trichothecene Therapeutic Agents

Conjugates of NR-AN-01 and Roridin A were constructed by chemically coupling a hemisuccinate derivative of the trichothecene cytotoxin (as described below) to a monoclonal antibody designated NR-AN-01. Two conjugates were prepared, one coupled at the Roridin A 2' position and one at the 13' position. Two schemes were used in this synthesis, as depicted in FIGURE 2 and FIGURE 3. The conjugate was then purified from unreacted Roridin A by PD-10 SEPHAROSE® column chromatography (Pharmacia; Piscataway, NJ), analyzed by size

exclusion high pressure liquid chromatography, and the column fractions were characterized by SDS-PAGE and isoelectric focusing (IEF), as described below.

FIGURE 2 shows diagrammatically the first reaction scheme for synthesis of Roridin A hemisuccinyl succinimidate (RA-HS-NHS) through a two step process with reagents: succinic anhydride, triethylamine (NEt_3) and dimethyl amino pyridine (DMAP) present in dichloromethane (CH_2Cl_2) at room temperature (RT); and, N-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DCC) reagents also in CH_2Cl_2 at RT.

FIGURE 3 shows diagrammatically the second reaction scheme for synthesis of Roridin A hemisuccinyl succinimidate (RA-HS-NHS) through a five step process with reagents: t-butyl dimethyl silyl chloride (TBMS-Cl) and imidazole in dimethylformamide (DMF) at room temperature (RT); acetic anhydride, triethylamine (TEA), and diethylaminopyridine in dichloromethane (CH_2Cl_2) at RT; succinic anhydride, triethylamine (TEA) and dimethylaminopyridine (DMAP) in (CH_2Cl_2) at RT; and, N-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DCC) reagents.

Synthesis of 2' Roridin-A Hemisuccinic Acid (2):

To 0.5g (0.94 mmol) of Roridin A, 15 ml of dichloromethane was added. To this solution with stirring was added 0.104g (1.04 mmol) of succinic anhydride. To the reaction mixture, 0.2 ml of triethylamine in 5 ml dichloromethane was added. To the homogeneous reaction mixture, a catalytic amount of dimethylaminopyridine was added and stirred at room temperature for 15 hours. Completion of the reaction was followed by thin layer chromatography (CH_2Cl_2 : CH_3OH = 9.7 : 0.3 with few drops of acetic acid). At the end of the reaction, 0.3 ml of glacial acetic acid was added and the solvent removed under reduced pressure. The dried crude residue was partitioned between water and methylene chloride. The combined methylene chloride extracts (3 x 50 ml) were dried over anhydrous sodium sulfate, solvent

was removed under vacuum and dried to yield 0.575g (96%) of a crude mixture of three compounds. Preparative C18 HPLC separation of the crude mixture in 50% acetonitrile-water with 2% acetic acid yielded 0.36g (60%) of 2 as a white solid.

Synthesis of Succinimidyl 2' - Roridin A Hemisuccinate (3):

To 0.3g (0.476 mmol) of 2' Roridin A hemisuccinic acid in 30 ml dichloromethane, 0.055g (0.478 mmol) N-hydroxysuccinimide was added. To the clear reaction mixture, 0.108g (0.524 mmol) dicyclohexylcarbodiimide was added. The reaction mixture was stirred at room temperature for 6 hours. Completion of the reaction was followed by TLC (CH_2Cl_2 : CH_3OH = 9.7 : 0.3 with a few drops of acetic acid) as a developing solvent. A few drops of glacial acetic acid was added to the reaction mixture and the solvent was removed under reduced pressure. To the dried residue dichloromethane was added and the precipitated DCU was filtered. Solvent from the filtrate was removed under reduced pressure to yield a white solid. From the crude product, 0.208g (60%) of 3 was purified by preparative HPLC in 50% acetonitrile with 2% acetic acid as a mobile phase.

Synthesis of 13'-t-Butyldimethylsilyl Roridin A (4):

To 72.3 mg (0.136 mmol) of Roridin A in 0.5 ml dimethylformamide solution, 0.055g (0.367 mmol) t-butyldimethylsilyl chloride and 0.025g (0.368 mmol) of imidazole were added. The reaction mixture was stirred at room temperature for 15 hours. Completion of the reaction was followed by silica gel thin layer chromatography using 1% MeOH-$CHCl_3$ as a developing solvent. Solvent from the reaction mixture was removed in vacuo and dried. The crude product was partitioned between water and methylene chloride. Solvent from the combined methylene chloride extracts was removed under reduced pressure and dried. The crude product was purified by flash chromatography using EtOAc : Hexane (1:3)

as an eluting solvent. Solvent from the eluants was removed under reduced pressure to yield 0.66g (75%) of 4 as a solid.

Synthesis of 13'-t-Butyldimethylsilyl 2' Acetyl Roridin A (5):

To 0.1g (0.155 mmol) of 13'-t-butyldimethylsilyl Roridin A in 10 ml dichloromethane, 0.3 ml acetic anhydride, 0.2 ml triethylamine and a few crystals of dimethylaminopyridine were added and stored at room temperature for 2 hours. Completion of the reaction was followed by TLC in 1% methanol-methylene chloride as a developing solvent. Solvent was removed under reduced pressure and purified by a silica gel column using 1% methanol-chloroform as an elution solvent. Solvent from the eluants was removed under vacuum to yield 0.085g (80%) of 5 as a solid.

Synthesis of 2' Acetyl Roridin A (6):

To 0.05g (0.073 mmol) of 2' acetyl 13'-t-butyldimethylsilyl Roridin A in 5 ml tetrahydrofuran, 0.3 ml of 1 M tetrabutyl-ammonium fluoride solution in THF was added. The reaction mixture was stirred at room temperature for 2 hours. Completion of the reaction was followed by silica gel thin layer chromatography using 1% MeOH-$CHCl_3$ as the developing solvent. Solvent from the reaction mixture was removed under reduced pressure and dried. The crude product was purified on a silica gel column using 1% CH_3OH - $CHCl_3$ as an eluting solvent. Solvent from the combined eluants were removed under vacuum to yield 0.020g (48%) of 6 as a solid.

Synthesis of 2'-Acetyl 13'-hemisuccinyl Roridin A (7):

To 0.05g (0.087 mmol) of 2'-acetyl Roridin A in 1 ml of dichloromethane, 0.025g (0.25 mmol) succinic anhydride and 35 ml of triethylamine was added. A few crystals of dimethylaminopyridine was added as a catalyst. The reaction mixture was stirred at room temperature for 24 hours.

Completion of the reaction was followed by thin layer chromatography using 5% MeOH-CH_2Cl_2 as developing solvent. At the end of the reaction 30ml of glacial acetic acid was added. Solvent from the reaction mixture was removed under reduced pressure and dried. The crude product was partitioned between water and ethyl acetate. Solvent from the combined ethyl acetate fractions was removed under reduced pressure. Crude product was purified by passing through a silica gel column to yield 0.039g (66%) of 7 as a white solid.

Synthesis of Succinimidyl 2'-Acetyl 13' - Roridin A Hemisuccinate (8):

To 0.036g (0.0050 mmol) of 2'-acetyl 13'-Roridin A hemisuccinic acid in 2 ml dichloromethane, 0.009g (0.09 mmol) N-hydroxysuccinimide was added. To a stirred solution, 0.012g (0.059 mmol) dicyclohexylcarbodiimide was added. The reaction mixture was stirred at room temperature for 8 hours. Completion of the reaction was followed by silica gel thin layer chromatography using 5% MeOH-CH_2Cl_2 as a developing solvent. A few drops of glacial acetic acid was added to the reaction mixture. Solvent from the reaction mixture was removed under reduced pressure and dried. The crude product was purified on a silica gel column using 5% MeOH-CH_2Cl_2 as an eluting solvent. Solvent from the combined eluants was removed under vacuum to yield 0.025g (61%) of 8 as a white solid.

Conjugation of Succinimidyl 2'-Roridin A Hemisuccinate (3) and Succinimidyl 2'-Acetyl 13'-Roridin A Hemisuccinate (8) to NR-AN-01 Whole Antibody (MAb):

Conjugation reactions were performed at pH 8.0 in borate buffer in the presence of 25% dimethylsulfoxide (DMSO) solvent at room temperature with gentle mixing for 45 minutes prior to purification by gel permeation chromatography. The molar trichothecene drug precursor to antibody offerings were 25:1 and 40:1 for the 2' and 13' Roridin A analogues (3 and 8),

respectively. Antibody concentration was 0.9 to 1.0 mg/ml during the conjugation reaction.

A Typical 2' Analogue (3) Reaction with 25 mg of Antibody was as follows:

To 4.7 ml of 5.3 mg Ab/ml in phosphate buffered saline (i.e., PBS; 150 mM NaCl, 6.7 mM Phosphate, pH 7.3) was added 10 ml PBS and 5 ml of borate buffer (0.5 M, pH 8.0). With stirring gently to the reaction mixture, 6.3 ml of DMSO containing 1.37 mg of succinimidyl 2' Roridin A hemisuccinate (3) was then added dropwise over a 15 second period.

Purification:

To purify, one ml reaction aliquots were applied to Pharmacia PD-10 Sepharose® columns equilibrated in PBS. The eluted conjugate was collected in 2.4 to 4.8 ml fractions. The PD-10 purified conjugate aliquots were then pooled and concentrated on an Amicon PM-10 DiAflo® concentrator to 1.5 to 2.0 mg of Ab/ml; sterile filtered through a 0.2μ Gelman Acrodisc® and filled into sterile glass vials in 5 ml volume.

The 2' conjugate was quick frozen in liquid nitrogen and then stored at -70°C until use. The 13' Roridin A NR-AN-01 conjugate was stored frozen or refrigerated (i.e., 5-10°C).

Characterization of Conjugates:

Protein concentration was determined by BCA assay using the copper reagent method (Pierce Chemical Corp.).

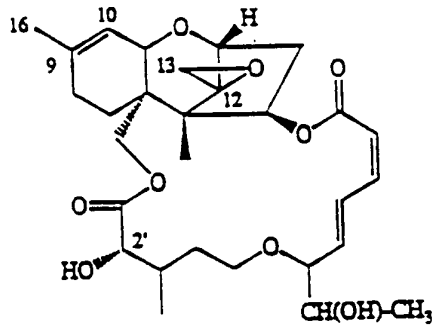
Assessment of degree of antibody derivatization was performed by first hydrolyzing an aliquot of conjugate in 0.2 M carbonate, pH 10.3 for 4 hours (at room temperature for 2' conjugate or at 37°C for the 13' conjugate) followed by filtration through a PM-30 membrane. The filtrate was then assayed for Roridin A on C-18 reverse phase HPLC using a mobile phase of 50:48:2 ratio CH_3CN:H_2O:HOAC, respectively. A 1.32 correction factor was used to correct for parallel

macrocyclic ring decomposition that gives polar products during the hydrolysis of the 13' conjugate.

Size exclusion chromatography on DuPont Zorbax® HPLC and isoelectric focusing using Serva® gel plates (pH 3 to 10) were also performed. No indication of aggregation was observed by HPLC.

Immunoassay of the Roridin A-antibody conjugates was performed by either competitive ELISA using biotinylated-Ab with Streptavidin/Peroxidase detection or by a competitive cell binding assay using ^{125}I-labeled antibody. Alternatively, immunoreactivity was measured under conditions of antigen saturation in a cell binding assay wherein antibody was first trace labeled with I-125 by the chloramine T method and then subsequently derivatized with 2' and 13' Roridin A precursors.

The structural formula of the trichothecene is shown below:



EXAMPLE 3

Kinetics of Binding to Smooth Muscle Cells

For administration by i.v. catheter, it is desirable that the therapeutic conjugates of the invention be administered in less than 3 to 5 minutes, so that blood flow can be reestablished in the patient. Therefore, studies were conducted to determine the binding kinetics of a smooth muscle binding protein with a Ka of $>10^9$ liter/mole. Because human vascular smooth muscle cells grow slowly in culture, and baboon smooth muscle cells were found to express the human

CSPG cell surface marker, BO54 baboon artery smooth muscle cells and human A375 M/M (melanoma; ATCC #CRL1619) cells bearing CSPG surface marker were used in many of the studies described in the Examples, below.

For the kinetic binding studies, A375 M/M and BO54 cells were seeded in sterile 96 well microtiter plates at 2500 cells/well. Plates were wrapped in aluminum foil, and incubated at 37°C overnight in a humidified atmosphere of 5% CO_2/95% air. After approximately 18 hr, incubation plates were removed and cells were fixed with 0.05% glutaraldehyde for 5 minutes to prevent membrane turnover. Following fixation, the plates were exhaustively washed with PBS containing 0.5% Tween-20®. Serial two-fold dilutions of an NR-AN-01 therapeutic conjugate containing Roridin A were prepared at protein concentrations of 10 mg/ml to 20 ng/ml, and each dilution was aliquoted into two wells. The plates were incubated at 4°C with the NR-AN-01 for 5, 15, 30, and 60 minutes, after which the unbound protein was removed by aspiration and 100 ml of CS buffer was added (5% chicken serum/ 0.5% Tween-20® in PBS) to each well. CS buffer was removed and the NR-AN-01 therapeutic conjugate bound to the cells was visualized by adding 100 ml of HRP-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) to each well; incubating at 4°C for 1 hr.; washing with PBS/0.05% Tween® to remove unbound goat IgG; and, adding 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) chromogenic substrate (i.e., for HRP). After incubating for 30 minutes, the amount of NR-AN-01 bound to the cells was quantified by measuring the absorbance at 415 nm and 490 nm using an ELISA plate reader equipped for data acquisition by a Compaq computer.

FIGURE 4A graphically depicts the results of *in vitro* studies in which A375m/m marker-positive cells were held at 4°C (i.e., to prevent membrane turnover) for 5 minutes (open squares, FIGURE 4A), 15 minutes (closed diamonds, FIGURE 4A), 30 minutes (closed squares, FIGURE 4A) or 60 minutes (open

diamonds, FIGURE 4A) with different concentrations of NR-AN-01 (NRAN01 μg/ml). The binding of the NR-AN-01 MAb to the A375 cells was quantified by washing to remove unbound antibody, adding HRP-conjugated goat anti-mouse IgG to react with the cell-bound MAb, washing to remove unbound goat second antibody, and adding 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate for peroxidase. Color development was monitored after 30 minutes at both 415 nm and 490 nm (ABS415,490).

FIGURE 4B graphically depicts the results of *in vitro* studies conducted in a manner similar to those described above in regard to FIGURE 4A, but using BO54 marker-positive smooth muscle cells, i.e., instead of the A375 m/m cells.

The results presented in FIGURE 4A and FIGURE 4B show significant binding of NR-AN-01 to A375 and BO54 cells within 5 minutes at 4°C, even at the lowest dose of 20 ng/ml.

EXAMPLE 4

Effects of Roridin A and RA-NR-AN-01 Conjugates

The effects of Roridin A (RA) and RA-NR-AN-01 conjugates on cellular protein synthesis (i.e., by ^{3}H-leucine incorporation) and metabolic activity (i.e., by mitochondrial MTT assay) were tested in the experiments detailed in EXAMPLE 5 and EXAMPLE 6, below. The studies in EXAMPLE 4 include experiments to determine the effects of long-term (i.e., 24 hour) treatment with the agents. The studies in EXAMPLE 5 include experiments to determine the effects of "pulse" (i.e., 5 minute) treatment on cells. In both studies, the cellular specificity of the effects were evaluated by including "target" cells (i.e., cells bearing the CSPG "marker") and non-target cells. For comparative purposes, free-RA (i.e., uncoupled) was also included in the studies. The effects on cellular protein synthesis or metabolic activity were evaluated either immediately following the treatment, or a "recovery period" was allowed (i.e., involving

incubation of the cells overnight at 37°C) to determine the long-term effects of the agents on the cell populations.

Metabolic Effects After 24 Hours Exposure:

While it is known that monoclonal antibody-drug conjugates may have a degree of specificity for cells bearing marker antigens when employed *in vivo*, it has proven more difficult in many systems to demonstrate *in vitro* specificity of action, especially with compounds that are lipophilic. Therefore, the present experiments were conducted in which the inhibitory effects of the NR-AN-01-Roridin A conjugate was tested on target and non-target cells over 24 hours. The results with RA-NR-AN-01 were compared to the effect of free Roridin A over the same 24-hour period. A modified methyl-tetrazolium blue (MTT) assay was utilized with 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (Sigma) to determine cellular metabolic activity. This assay is thought to measure cellular mitochondrial dehydrogenase activity. For some of these studies, M14 (melanoma) and BO54 (smooth muscle) cell lines were used as marker-positive target cells and HT29 cells (colon carcinoma; ATCC #HTB38) were used as the non-target specificity control. In other studies, A375 was used as a marker-positive cell. The HT29 and M14 cells were seeded in 96-well microtiter plates at a concentration of 5.0 x 10^3 cells/well, and the BO54 cells were seeded at 2.5 x 10^3 cells/well. Serial two-fold dilutions of free Roridin A and 2'RA-HS-NR-AN-01 (i.e., Roridin A coupled through a hemisuccinate (HS) coupling agent at the 2' position to NR-AN-01) were prepared in DMEM over a range of protein concentrations from 20 mg/ml to 40 pg/ml. Test agents were added (in duplicate) to microtiter wells (100 ml/well), and the plates were wrapped in aluminum foil and incubated at 37°C in a humidified atmosphere consisting of 5% CO_2/95% air for 24 hours. After 24 hours, medium was removed (by aspiration), fresh DMEM was added (100 ml/well), and the cells were returned to incubate for an additional overnight (i.e., 16-18

hours) "recovery period". At the end of the "recovery period" cellular metabolic activity was determined by adding 20 ml to each well of a 5mg/ml MTT solution. The plates were covered and incubated at 37°C for 4 hours and then the reaction was developed by adding 100 ml/well of 10% SDS/0.1 N HCl. The dark blue solubilized formazan reaction product was developed at room temperature after 16-18 hours and quantified using an ELISA microtiter plate reader at an absorbance of 570 nm.

FIGURE 5A graphically depicts the results of *in vitro* studies in which BO54 marker-positive smooth muscle cells were incubated with different concentrations of RA-NR-AN-01 (NRAN01-RA; open squares, FIGURE 5A) or free Roridin A (Free RA; closed diamonds, FIGURE 5A) for a period of 24 hours, washed, and then returned to culture for an additional 16-18 hour overnight (o/n) recovery period prior to testing metabolic activity in an MTT assay. The concentrations of Free RA and RA-NR-AN-01 are expressed as the calculated concentration of Roridin A (in mg/ml plotted on a log scale) in the assay (i.e., rather than the total mg/ml of NR-AN-01 protein in the assay), so that direct comparisons could be made. The metabolic activity of the cells in the MTT assay is presented as the percentage of the metabolic activity measured in a control untreated culture of cells (i.e., % control).

FIGURE 5B graphically depicts the results of *in vitro* studies conducted in a manner similar to those described above in regard to FIGURE 5A, but comparing the effects of only RA-NR-AN-01 (NRAN01-RA) on three different cell types: namely, BO54 marker-positive smooth muscle cells (BO54-NRAN01-RA; open squares, FIGURE 5B); HT29 marker-negative control cells (HT29-NRAN01-RA; closed diamonds,FIGURE 5B); and, M14 marker-positive cells (M14-NRAN01-RA; closed squares, FIGURE 5B). As described above in regard to FIGURE 5A, the concentrations in the present experiment are expressed in terms of ug/ml of Roridin A. Metabolic activity of the cells is expressed in

a manner similar to that in FIGURE 5A, i.e., as the percentage of activity measured in an untreated control culture of cells (% control).

The results presented in FIGURE 5A and FIGURE 5B show that metabolic activity measured in the MTT assay was significantly decreased in all populations of test cells, even 16-18 hours after a 24-hour incubation in either free Roridin A or the 2' or 13' RA-NR-AN-01 conjugates. The effects of the RA-NR-AN-01-conjugates appeared to be non-specifically inhibitory for both target (BO54 and M14) and non-target (HT29) cells (FIGURES 5A and 5B). The inhibitory effects were observed at a free Roridin A or RA-conjugate concentration of >10 ng/ml.

For comparative purposes, a second study was conducted in which the effects of *Pseudomonas* exotoxin (PE) conjugates on cells were evaluated in a similar protocol. For these studies, target and non-target cells were treated with PE or PE-NR-AN-01 for 24 hours, and then allowed a "recovery period" (as above) before metabolic activity was tested in an MTT assay.

FIGURE 6A graphically depicts the results of *in vitro* studies conducted in a manner similar to those described above in regard to FIGURE 5A, but designed to study the metabolic effects of PE-NR-AN-01 (NRAN01-PE) on cells, i.e., rather than RA-NR-AN-01. Three different cell types were utilized: namely, BO54 marker-positive smooth muscle cells (BO54; open squares, FIGURE 6A); HT29 marker-negative control cells (HT29; closed diamonds, FIGURE 6A); and, M14 maker-positive cells (MT14; closed squares, FIGURE 6A). In this study, the concentration of conjugate is expressed in μg/ml NR-AN-01 protein (plotted on a log scale), and the metabolic activity is expressed as the percentage of the MTT activity measured in an untreated control culture (% control).

FIGURE 6B graphically depicts the results of *in vitro* studies conducted in manner similar to those discussed above in regard to FIGURE 6A, but designed to compare the effects

obtained with free PE (PE) to those obtained above, i.e., in FIGURE 6A, with PE-NR-AN-01. The cells, culture conditions, calculations, and presentation of the results are the same as in FIGURE 6A, above.

The results presented in FIGURE 6A and FIGURE 6B show that 24 hours exposure to PE-NR-AN-01 or free PE was non-specifically inhibitory to cells at concentrations of >100 ng/ml.

While this type of non-specific inhibition was judged to be of potential value for biological atheroectomy, it was not considered desirable for treatment of restenosis following angioplasty where dead and dying cells may release factors that stimulate smooth muscle proliferation.

EXAMPLE 5

Effects of Pulse-Treatment on Cellular Activity

Additional studies were conducted to evaluate the effects of a short-term, i.e., 5 minute, exposure to a Roridin A-containing therapeutic conjugate on cells. In these studies, both metabolic activity (measured in MTT assays) and cellular protein synthesis (measured by ^{3}H-leucine incorporation) were evaluated.

Effects After 5 Minutes of Exposure: Protein Synthesis

The effects of a 5-minute exposure to free Roridin A (RA) or a therapeutic conjugate were evaluated. Roridin A-NR-AN-01 coupled through a hemisuccinyl (HS) at either the 2' position (2'RA-HS-NR-AN-01) or the 13' position (13'RA-HS-NR-AN-01) were employed. (In the case of 13'RA-HS-NR-AN-01, the 2' position of Roridin A was also acetylated.) The RA, 2' or 13'RA-NR-AN-01 conjugates were diluted two fold in sterile DMEM over a range of concentrations from 400 ng/ml to 780 pg/ml of Roridin A. (The test samples were all normalized to Roridin A, so that direct comparisons could be made of the effects at comparable doses.) Samples were aliquoted (in

duplicate) into duplicate microtiter plates at 100 ml/well and incubated at room temperature for five minutes.

Both short-term and long-term effects of the test samples on marker-positive A375 and marker-negative HT29 cells were determined. For studying the short-term effects, 100 ml/well of [^{3}H]-leucine (0.5 mCi/ml) was added immediately after the 5-minute treatment with conjugate (or RA) and protein synthesis was evaluated over a four-hour period. For determining the long-term effects, the cells were treated for 5 minutes, washed, and then returned to culture for a 24-hour "recovery" period in DMEM medium containing either 5% NBS/5% Serum Plus® (i.e., for A375 or HT29 cells) or 10% FBS (i.e., for BO54 cells). At the end of the "recovery" period, the incubation medium was removed (i.e., by aspiration) and ^{3}H-leucine was added (as above). In both cases (i.e., whether short-term or long-term), protein synthesis of the cells was evaluated by incubating the cells with the ^{3}H-leucine for 4 hours at 37°C in a humidified chamber (as above), and all results are calculated by comparison with non-treated cells (i.e., 100% control). After 4 hours the ^{3}H-leucine was removed, the cells were removed from the substrata by trypsin-treatment, aspirated (using a PHD™ cell harvester (Cambridge Technology, Inc., Cambridge, MA)) and collected by filtration on glass fiber filters. The glass fiber filters were dried and radioactivity quantified by liquid scintillation spectroscopy in a Beckman liquid scintillation counter.

FIGURE 7A graphically depicts the results of *in vitro* studies conducted to investigate the effects on control HT29 marker-negative cells of a 5 minute exposure to different concentrations of Roridin A (Free RA; open squares, FIGURE 7A), or 2'RA-NR-AN-01 (2'RA-NRAN01; closed squares, FIGURE 7A), or 13'RA-NR-AN-01 (13'RA-NRAN01; closed triangles, FIGURE 7A) conjugates. The concentrations of Free RA, 2'RA-NR-AN-01 or 13'NR-AN-01 are expressed as the calculated concentration of Roridin A in the assay (in μg/ml plotted on a log scale),

i.e., rather than the total μg/ml of NR-AN-01 protein, so that direct comparisons of the results can be made. For these studies, the cells were treated for 5 minutes, washed, and then returned to culture for 4 hours, during which time cellular protein synthesis was evaluated by adding 0.5 mCi/ml of ^{3}H-leucine to the culture medium. At the end of the 4 hour period, cellular proteins were collected and radioactivity was determined. The results are expressed as the percentage of the radioactivity recorded in a control (non-treated) HT29 cell culture (i.e., %control).

FIGURE 7B graphically depicts the results of *in vitro* studies investigating the effects on control HT29 marker-negative cells of a 5 minute expose to different concentrations of Free RA (open squares,FIGURE 7B), 2'RA-NRAN01 (closed squares, FIGURE 7B), or 13'RA-NRAN01 (closed triangles, FIGURE 7B), as described above in regard to FIGURE 7A, but in the present experiments the cells were incubated for a 16-18 hour recovery period (i.e., overnight; o/n) prior to testing protein synthesis in a four hour ^{3}H-leucine protein synthesis assay. The results are presented in a manner similar to those above in FIGURE 7A.

The results presented in FIGURE 7A and FIGURE 7B show the short-term and long-term effects, respectively, of RA, 2'RA-HS-NR-AN-01, and 13'RA-HS-NR-AN-01 on protein synthesis by HT29 control cells. The results show a dose-response inhibition of cellular protein synthesis by the free Roridin A, but not by RA-NR-AN-01, in HT29 cells. The inhibition triggered by RA during the 5 minutes of incubation was still manifest after the 16-18 hours recovery period (FIGURE 7B). In contrast, treatment of non-target HT29 cells with 2'RA-HS-NR-AN-01 or 13'RA-HS-NR-AN-01 did not result in detectable inhibition of protein synthesis. Thus, these results (in contrast to those obtained above over 24 hours) seem to suggest a surprising degree of specificity to the *in vitro* action of the NR-AN-01-conjugates when treatment was delivered in a 5-minute "pulse". However, it was also

possible that the NR-AN-01-conjugate was inactive, and so additional experiments were conducted to evaluate the effect of the conjugates on target cells.

FIGURE 7C graphically depicts the results of *in vitro* studies investigating the effects on A375m/m marker-positive cells of a 5 minute exposure to different concentrations of Free RA (open squares, FIGURE 7C), 2'RA-NR-AN-01 (closed squares, FIGURE 7C) or 13'RA-NR-AN-01 (closed triangles, FIGURE 7C), as described above in regard to FIGURE 7A. In the present studies, the A375 cells were incubated for 5 minutes in the test agent, washed, and tested for protein synthesis over the next 4 hours by adding 0.5 mCi/ml ^{3}H-leucine to the culture medium. The results of the experiments are plotted in a manner similar to those described, above, in regard to FIGURE 7A.

FIGURE 7D graphically depicts the results of *in vitro* studies investigating the effects on A375 m/ml marker-positive cells of a 5 minute exposure to different concentrations of Free RA (open squares ,FIGURE 7D), 2'RA-NRAN01 (closed squares, FIGURE 7D), 13'RA-NRAN01 (closed triangles, FIGURE 7D), as described above in regard to FIGURE 7B. In the present studies, the A375 cells were incubated for 5 minutes in the test agent, washed, and then returned to culture for a 16-18 hour recovery period (i.e., overnight; o/n Recovery), after which time protein synthesis was evaluated during a 4 hour ^{3}H-leucine protein synthesis assay. The results of the experiments are plotted in a manner similar to those described above in regard to FIGURE 7A.

The results presented in FIGURES 7C and FIGURE 7D show the short-term and long-term effects, respectively, of RA, 2'RA-HS-NR-AN-01 and 13'-RA-HS-NR-AN-01 on protein synthesis by A375 target cells. Treatment of target cells with either the 2' or 13'RA-NR-AN-01 therapeutic conjugate resulted in a short-term inhibition of protein synthesis, i.e., observed immediately after the 5-minute pulse treatment (FIGURE 7C). These findings, when combined with the findings in FIGURE 7A

and FIGURE 7B, above, suggest that the RA-NR-AN-01 conjugates were active and that they were specifically inhibitory for target cells but not non-target cells. Interestingly, when "pulse" treated target cells were returned to culture no long-term inhibitory effects were observed (FIGURE 7D). The results presented in FIGURES 7C and FIGURE 7D again show that Roridin A is non-specifically inhibitory to test cells (i.e., in a manner similar to FIGURE 7A and FIGURE 7B, above) and that its effect on the cells is manifest even after a 16-18 hour recovery period. Thus, the specific effects of the RA-NR-AN-01 conjugates on target cells during a "pulse" treatment appear to be a property of the NR-AN-01 binding protein.

The results obtained with BO54 arterial smooth muscle cells were similar to those obtained with the A375 cells, above, i.e., free Roridin A showed a dose-response inhibition of protein synthesis in the short-term equated to be 60%, 66%, and 90% of control at 200 ng/ml, 100 ng/ml, and 50 ng/ml; and in long-term the effects on protein synthesis were equated to be 27%, 46%, and 98% of control at the same dosages. In contrast, the 2' or 13'RA-NR-AN-01 showed only 10-20% inhibition for short- or long-term effects on protein synthesis (i.e., >80% of control).

Thus, the results show a short-term specific reversible effect of Roridin A-conjugated NR-AN-01 on target cells when delivered as a "pulse" treatment. However, since only protein synthesis was evaluated in these experiments, it was possible that cellular metabolic activity might be affected in the cells as a result of the "pulse" treatment. Therefore, additional studies were conducted in which cellular metabolic activity was evaluated following "pulse" treatment.

Effects After 5 Minutes of Exposure: Metabolic Activity

MTT assays were conducted at 48 hours following a 5-minute exposure of target and non-target cells to RA or RA-NR-AN-01 conjugates. Target cells in these studies

included BO54 and A375, and non-target cells included HT29 cells. Sterile 96 well microtiter plates were seeded with 2500 cells/well, wrapped in aluminum foil and incubated in a humidified chamber containing 5% CO_2/95% air for 16-18 hours. Serial two-fold dilutions of Roridin A (RA), 2'RA-HS-NR-AN-01 and 13'RA-HS-NR-AN-01 were prepared from 400 ng/ml to 780 pg/ml, and 100 ml aliquots of the dilutions were dispensed into duplicate wells. After 5 minutes exposure to the test samples, the cells were washed to remove the test samples, and fresh medium was added. The cells were allowed 48 hours of recovery prior to testing: i.e., plates were incubated for 48 hours, and then cellular metabolic activity was determined by adding 20 ml/well of a 5 mg/ml MTT solution. The plates were covered and incubated at 37°C for 4 hours and then the reaction was developed as described above (see EXAMPLE 4, above). The dark blue solubilized formazan reaction product was developed at room temperature after a 16-18 hour incubation. The samples were quantified using an ELISA microtiter plate reader at an absorbance of 570 nm.

FIGURE 8A graphically depicts the results of *in vitro* studies investigating the effects on BO54 marker-positive smooth muscle cells of a 5 minute exposure to different concentrations of Roridin A (open squares, FIGURE 8A), 2'RA-NR-AN-01 (NRAN01-2'RA; closed diamonds, FIGURE 8A), or 13' RA-NR-AN-01 (NRAN01-13'RA; closed squares, FIGURE 8A). The experiments were conducted in a manner similar to those described above in regard to FIGURE 7B, but metabolic activity was assayed by MTT assay, i.e., rather than protein synthesis as in FIGURE 7B, and cells were also given 48 hours to recover (rather than 24 hours, as in FIGURE 7B). The results of the experiments are plotted in a manner similar to those described (above) in regard to FIGURE 7A .

FIGURE 8B graphically depicts the results of *in vitro* studies investigating the effects on A375 m/m marker-positive cells of a 5 minute exposure to different concentrations of Roridin A (open squares, FIGURE 8B), 2'RA-NR-AN-01 (NRAN01-

2'RA; closed diamonds, FIGURE 8B), 13'RA-NR-AN-01 (NRAN01-13'RA; closed squares, FIGURE 8B). The experiments were conducted (and the results plotted) in a manner similar to those described above in regard to FIGURE 8A.

FIGURE 8C graphically depicts the results of *in vitro* studies investigating the effects on HT29 marker-negative cells of a 5 minute exposure to different concentrations of Roridin A (open squares, FIGURE 8C), 2'RA-NR-AN-01 (NRAN01-2'RA; closed diamonds, FIGURE 8C), 13'RA-NR-AN-01 (NRAN01-13'RA; closed squares, FIGURE 8C). The experiments were conducted (and the results plotted) in a manner similar to those described above in regard to FIGURE 8A.

The results presented in FIGURES 8A-8C show slight differences between the different RA-NR-AN-01 conjugates at the highest doses, but at the lower doses the 2' and 13'RA-NR-AN-01 did not significantly inhibit target cell (i.e., BO54 and A375) or non-target cell (i.e., HT29) metabolic activity over the long-term (i.e., 48 hours). Thus, the results suggest that the short-term inhibition of target cell protein synthesis (FIGURES 7C-7D, above) does not result in long-term metabolic effects on the cells, as measurable in MTT assays. That these assays were able to detect metabolic alterations in cells resulting from a 5 minute exposure is evidenced by the results obtained with free Roridin A. In this case, free Roridin A was non-specifically inhibitory to target and non-target cell types, even when the cells were exposed to the agent for only 5 minutes and then returned to culture for the 48-hour recovery period (FIGURES 8A-8C).

Thus, the findings with free Roridin A suggest that the MTT assay was capable of detecting metabolic alterations induced during a 5-minute exposure. Taken together these finding suggest that RA-NR-AN-01 conjugates can specifically inhibit target cell activity (i.e., protein synthesis) when administered in a "pulse" treatment, and that these effects were reversible without significant long-term effects on either protein synthesis or cellular metabolic activity (as

measured in an MTT assay). These *in vitro* properties of the RA-NR-AN-01 conjugates were judged to be highly useful for inhibition of smooth muscle cell activity *in vivo*. Therefore, animal model studies were next conducted to evaluate the effects of these therapeutic conjugates *in vivo*.

EXAMPLE 6

Determination of Infusion Conditions in an Animal Model

The therapeutic conjugates of the invention are useful for inhibiting stenosis following vascular trauma or disease. In an illustrative example, vascular trauma that is induced during angioplasty is treated during the surgical procedure by removing the catheter used to perform the angioplasty, and inserting a balloon infusion catheter into the vessel. The infusion catheter is positioned with the instillation port (or, alternatively, a permeable membrane region) in the traumatized area of the vessel, and then pressure is applied to introduce the therapeutic conjugate. For example, an infusion catheter with two balloons may be used, and when one balloon is inflated on either side of the trauma site a fluid space is created that can be filled with a suitable infusion fluid containing the therapeutic conjugate. It has been reported previously that infusion of a horseradish peroxidase (HRP) marker enzyme at a pressure of 300 mm Hg over 45 seconds in dog or human coronary arteries resulted in penetration of the HRP into the vessel wall (6). However, HRP is a smaller molecule than NR-AN-01 and human and dog coronary arteries are also considerably smaller than the carotid or femoral arteries in the present domestic pig model system. Experiments were therefore conducted to determine, in a domestic pig model system, the infusion conditions suitable for delivery of a therapeutic conjugate to the vascular smooth muscle cells in carotid and femoral arteries. Delivery conditions were monitored by evaluating the penetration of the therapeutic conjugate into the vascular wall, and specific binding of the

therapeutic conjugate to the vascular smooth muscle cells in the vessel wall.

Using an infusion catheter, the coronary and femoral arteries of domestic pigs or non-human primates were infused with NR-AN-01 for 45 seconds to 3 minutes at multiple pressures in the range of about 0.4 atmospheres (300 mm Hg) to 3 atmospheres. After infusion, the vessels were flushed with sterile saline and prepared for immunohistochemistry using HRP-conjugated goat anti-mouse IgG to detect the NR-AN-01 mouse IgG in the vessel wall. It was determined that full penetration was achieved of NR-AN-01 into these vessel walls at a pressure of 3 atmospheres after 3 minutes.

Immunohistology was also used to determine which animal model systems expressed the target antigen for NR-AN-01. Vascular tissue sections from readily available experimental animal species were exposed to NR-AN-01, washed, and reacted with HRP-conjugated goat anti-mouse IgG. Only non-human primates and swine were found to share the 250kD NR-AN-01 target antigen with man.

To determine whether NR-AN-01 could bind in a specific manner to its target antigen *in vivo*, the coronary and femoral arteries of domestic pigs were infused with therapeutic conjugates using an infusion catheter, the infusion sites were flushed with sterile saline, the surgical sites were then closed, and the animals were maintained for an additional 3-5 days. At the end of this time, the vascular infusion sites were excised and prepared for immunohistology, once again using goat anti-mouse IgG to identify NR-AN-01. NR-AN-01 was identified in the vessel wall of swine coronary and femoral arteries 3-5 days after surgery, and the NR-AN-01 appeared to be associated only with vascular smooth muscle cells. These findings suggest that NR-AN-01 is capable of specifically binding to its target antigen *in vivo*.

EXAMPLE 7

Inhibition of Vascular Smooth Muscle Cells *In Vivo*

Intimal smooth muscle proliferation that follows balloon catheter-induced trauma is a good model to evaluate the therapeutic efficacy of conjugates for inhibiting smooth muscle cell activity *in vivo* in response to vascular trauma, including restenosis following angioplasty. Domestic pigs were used to study the effects of NR-AN-01 (i.e., termed vascular smooth muscle binding protein or simply VSMBP in these studies; and therapeutic conjugates with Roridin A are termed VSMBP - RA). The events which normally follow balloon angioplasty in the porcine artery have been described previously (12). In these studies, dilation of the carotid artery using an oversized balloon (balloon: artery ratio approximately 1.5:1) resulted in complete endothelial denudation over an area of 1.5-2 cm in length. Although this length of traumatic injury was selected in an attempt to minimize thrombosis, there was still marked platelet deposition and thrombus formation. The procedure also resulted in dissection through the internal elastic lamina into the arterial media and necrosis of medial smooth muscle cells. Intimal thickening due to smooth muscle proliferation was apparent 7 days after injury and reached a mean maximum thickness of 85 mm at 14 days. The histological appearance of this neointima is very similar to the proliferative neointimal tissue of human restenosis (13).

A single dose test protocol was conducted in domestic pigs with NR-AN-01-Roridin A conjugates. Localized administration of the test conjugates, i.e., through a catheter into a region of traumatized vessel confined by temporary slip ligatures, was designed to reduce systemic toxicity while providing a high level of exposure for the target smooth muscle cells. This intra-artery route of administration in animal model studies simulates the proposed route in human coronary arteries. The test protocol was designed as an initial *in vivo* screening of intra-arteriolar,

site specific, catheter administered, vascular smooth muscle binding protein (VSMBP) conjugates. Toxicity of free drug was also evaluated, i.e., for pathobiological effects on arteriolar smooth muscle cells. The therapeutically effective dosage of the Roridin A-NR-AN-01 conjugate was determined by *in vitro* studies, and the proper intra-arteriolar administration pressure was determined by administering free MAb and MAb conjugates to animals, as described above in Example 7.

Six domestic crossbred swine (Duroc X), weanling feeder pigs of approximately 30 pounds body weight, were used in the experiment. The animals were randomly assigned to the following treatment regimen where each pig has four different treatments divided between the right and left carotid and femoral arteries, one of which is a PBS control (Tables 1-3, below).

Table 1

GROUP NO.	TREATMENT GROUP	MATERIAL DESCRIPTION
1	CONTROL, VSMBP	VSMBP, 200 μg/ml in PBS, pH 6.5
2	CONTROL, PBS	PBS, pH 6.5, in injection sterile water
3	CONTROL, DRUG	Roridin A, 2.5 μg/ml in PBS, pH 6.5
4	TEST, CONJUGATE	VSMBP-RA2' (200 μg/ml VSMBP & 2.5 μg/ml RA)
5	TEST, CONJUGATE	VSMBP-RA13' (200 μg/ml VSMBP & 3.1 μg/ml RA)
6	TEST, CONJ+RA	VSMBP-RA2' (200 μg/ml VSMBP & 2.5 μg/ml RA) PLUS free Roridin A (2.5 μg/ml)
7	TEST, CONJ+RA	VSMBP-RA13' (200 μg/ml VSMBP & 3.1 μg/ml RA) PLUS free Roridin A (2.5 μg/ml)

Surgical Procedure:

Test conjugates and control compounds were administered as a single intra-artery infusion at the site of endothelial denuding and trauma induced by a balloon catheter. Both the carotid and femoral arteries were abraded over 1 cm to 2 cm of endothelium by intraluminal passage of a 23 cm, size 3 (femoral) and size 4 (carotid) Uresil Vascu-Flo® silicone occlusion balloon catheter (Uresil Technology Center, Skokie, IL), sufficiently distended with saline to generate slight resistance. This technique produced slight distension of the artery. Following this treatment, proximal and distal slip ligatures, 3-0 silk, were placed near the ends of the abraded region, and a size 8 French, Infant Feeding Catheter (Cutter-Resiflex, Berkeley, CA) attached to an Inflation Pro® (USCI, C.R. Bard, Inc., Billerica, MA) pressure syringe was used to administer the test conjugates and control compounds directly to the denuded segment at a pressure of three atmospheres for three minutes. The slip ligatures were removed after the three minute exposure period and arterial blood flow was re-established. In these studies, branches of the femoral or carotid arteries were ligated with 00 silk suture as required to attain pressurized infusion in the treated region. The largest distal branch of the femoral artery (the saphenous artery) was incised and used as an entry site for the catheters which were then passed into the main femoral artery. Following this catheterization procedure in the main femoral artery, the secondary branch was ligated. In these cases, ligation or incision was used to allow entry of the catheters and the opening was then closed with 3 to 4 sutures of 5-0 monosilamen polybutester (Novafil, D & G Monofil Inc., Monati, PR).

Follow-up Procedures:

Following surgery, the pigs were kept in 3 X 5 foot indoor runs with cement floors during the quarantine and surgical recovery periods. They were then transferred to

indoor/outdoor pens for the remainder of the five week healing period prior to collection of tissues for histology.

The animals recovered normally from surgery with no evidence of hemorrhage or inflammation at the surgical sites. All six animals were examined 5 to 6 days after treatment with a doppler stethoscope, and all arteries in each of the animals were patent. Post treatment all animals had normal appetite, activity and weight gain.

Gross Pathology and Histological Evaluation:

Five weeks following the traumatization and treatment of the arteries, the animals were sedated with 0.6 ml Telazol® (tiletamine hydrochloride; A.H. Robins Co., Richmond, VA) and 0.5 ml xylazine (Lloyd Laboratories, Shenandoah, IA) per 30 lb body weight by intramuscular injection, heparinized (i.v. 2 ml sodium heparin, 1000 units/ml), and euthanized by i.v. pentobarbital. Both the right and left carotid and femoral arteries were removed with normal vessel included both proximal and distal to the treated segment. The arteries were measured and the location of ligatures and gross abnormalities noted. The arteries were transected at 2 mm intervals and arranged in order in cryomolds with O.C.T. (optimum cutting temperature) compound (Tissue Tek®, Miles Laboratories Inc., Elkhart, IN) and frozen in liquid nitrogen. The blocks were sectioned at 5 microns and stained with H&E, Massons Trichrome and Movats Pentachrome for morphological studies. Sections were also used for immunohistological staining of vascular smooth muscle.

Histological examination of the step sections of the arteries revealed marked inhibition of intimal smooth muscle proliferation in the regions traumatized and treated with RA-NR-AN-01 conjugates (Table 2). This inhibition was evident even at sub-gross evaluation of the vessels. The inhibition of intimal smooth muscle cell proliferation was produced with minimal or no histological evidence of smooth muscle cell

death in the artery wall. A cross-sections of one such traumatized artery is provided in FIGURES 9A and 9B.

Table 2

INTIMAL SMOOTH MUSCLE PROLIFERATION IN TRAUMATIZED AND TREATED PORCINE ARTERIES

TREATMENT	NO. ARTERIES EVALUATED	INTIMAL SMC HYPERTROPHY* ave. (range)
Control, MAB	4	3.75 (3-4)
Control, PBS	4	4 (4)
Control, RA	2	4 (4)
Test, 2'RA (High pressure)	1	1 (1)
(Low pressure)	1	3 (3)
Test, 13'RA (High pressure)	1	1 (1)
(Low pressure)	1	1 (1)

*Intimal SMC Hypertrophy: intimal smooth muscle cell hypertrophy scored on a scale from 1+ (minimal) to 4+ (maximal).

The results presented in FIGURE 9A show (at 160x magnification) a cross-sectional of an untreated artery 5 weeks after angioplasty. Dominant histological features of the artery include displacement of the endothelium (see #1 in FIGURE 9A) away from the internal elastic lamina (see #2, FIGURE 9A), apparently due to intimal smooth muscle proliferation (see #3, FIGURE 9A).

The results presented in FIGURE 9B show (at 160x magnification) a cross-section of a treated artery 5 weeks after angioplasty and infusion of the RA-NR-AN-01 therapeutic conjugate. The vessel in this section was subjected to greater mechanical stresses than the vessel shown in FIGURE 9A, with multiple sites where the external elastic membrane was ruptured and associated proliferation of smooth muscle cells in the outer layers of the media was observed (i.e., see #4 in FIGURE 9B). Treatment with therapeutic conjugate inhibited intimal hypertrophy, as evidenced by the lack of displacement of the endothelium (see #1, FIGURE 9B) from the internal elastic lamina (see #2, FIGURE 9B). Surprisingly, this inhibitory effect on intimal smooth muscle cells was accomplished without inhibiting hypertrophy of medial smooth muscle cells in the areas where the external elastic membrane was ruptured (see #4, FIGURE 9B).

This is a highly fortunate result because wound healing proceeds in the treated vessel without the adverse consequences of intimal hyperplasia and stenosis, or necrosis of smooth muscle cells in the media.

In these histological studies, comparisons were also made of the effectiveness of both the 2' and the 13' - Roridin A conjugate with the finding that the 13' conjugate (i.e., 13'RA-HS-NR-AN-01) appeared to be more active in inhibiting intimal hyperplasia of smooth muscle cells than the 2' conjugate (i.e., 2' RA-HS-NR-AN-01). In this study, low pressure infusion of the 13' conjugate appeared to inhibit smooth muscle proliferation more effectively than high pressure and the 13' conjugate also appeared to be more effective than the 2' conjugate.

In FIGURE 9B, therapeutic conjugate administered at the site following angioplasty resulted in approximately 95% inhibition of the smooth muscle hypertrophy that restricted the lumen of the untreated vessel (FIGURE 9A). Significantly, the therapeutic conjugate exerted its effects on the smooth muscle cells migrating from the medial smooth muscle layers

into the intima, without affecting either endothelium, or producing any signs of necrosis (i.e., cell death) in the smooth muscle cells in the medial layers of the arterial wall. Studies also failed to show any histological signs of mononuclear infiltration or fibrosis such as might result from toxic effects on the vessel wall. Also, visible signs of healing were observed in the intimal layers of treated vessels and with re-growth of endothelium observed, i.e., endothelial cells growing over the thin layer of smooth muscle cells in the intima that lie between the endothelium and internal elastic lamina (i.e., #1 and #2, FIGURE 9B). These combined histological observations suggest the highly desirable features of wound healing, re-growth of endothelium and improved vascular strength following treatment with a therapeutic conjugate that inhibits smooth muscle hyperplasia in the intimal layers of the vessel.

EXAMPLE 8

Vascular Smooth Muscle Cell In Vitro DNA and Protein Synthesis Inhibition

The ability of various therapeutic agents to inhibit DNA synthesis and protein synthesis in vascular smooth muscle cells was tested. ^{3}H-leucine and ^{3}H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

5 minute exposure; ^{3}H-leucine uptake: Vascular smooth muscle cells at 40,000 cells/ml were seeded in sterile 24 well plates at 1 ml/well. The plates were incubated overnight at 37°C, 5% CO_2, 95% air in a humidified atmosphere (saturation). Log dilutions of the therapeutic agent of interest were incubated with the vascular smooth muscle cells for 5 minutes or 24 hours. Samples of the therapeutic agents were diluted in DMEM:F-12 medium (Whittaker Bioproducts, Walkersville, Maryland) with 5% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and 5% Serum Plus® (JRH Biosciences,

Lenexa, KS). Following therapeutic agent incubation, the solution was aspirated, and 1 ml/well of 0.5 microcurie/ml ^{3}H-leucine in leucine-free DMEM (Dulbecco's Modified Eagle's Medium) with 5% Serum Plus® was added. The plates were re-incubated overnight at 37°C, 5% CO_2 in a humidified atmosphere. The cells were visually graded using an inverted microscope using a scoring scale to determine viability and cell number. The 1 to 3 grade is based upon percent of cell viability and number compared to control wells, with 3=100%, 2=70%-100% and 1=0%-70%. A record of this scoring assisted in determining the immediate cytotoxic effect of the therapeutic agents. The medium was then aspirated, and the cells were washed twice with cold 5% TCA. 400 microliters of 0.2M NaOH was added per well, and the plates were incubated for two hours at room temperature on a rotating platform. 200 microliters per well of the cell solution was transferred into plastic scintillation vials (Bio-Rad Laboratories), and 4 milliliters of Bio-Safe® II liquid scintillation fluid (Research Products InterCorp., Mount Prospect, IL) was added prior to vortexing. Vials were counted on a Beckman LS2800 liquid scintillation counter interfaced with Beckman "Data Capture" software for conversion to a Lotus 1-2-3® file and analysis using Lotus 1-2-3®.

5 minute exposure; ^{3}H-thymidine uptake: Vascular smooth muscle cells were incubated in complete medium with 5% FBS (Gibco) overnight at 37°C in a humidified, 5% CO_2 environment in sterile 24 well plates. The medium was aspirated from the wells and serum free medium supplemented with growth factors (DMEM: F-12 basal medium supplemented with growth factor cocktail, catalog number I1884, which contains insulin (5 micrograms/ml), transferrin (5 micrograms/ml) and sodium selenite (5 nanograms/ml), available from Sigma Chemical, St. Louis, Missouri) was added. Cells were incubated in this medium for 24 hours. For a 5 minute therapeutic agent exposure, log dilutions of the therapeutic agent were

incubated with the cells in complete medium. After 5 minutes and medium aspiration, 1 ml/well of 1.0 microcurie/ml ^{3}H-thymidine dispersed in complete medium was added. The 24 hour exposure involved incubation of the cells with 1 ml/well of 1.0 microcurie/ml of ^{3}H-thymidine dispersed in complete medium and log dilutions of the therapeutic agent being tested. In both exposure trials, the cells were then incubated overnight at 37°C in a humidified, 5% CO_2 environment. The cells were visually scored for viability and cell number. Cells were washed and prepared for transfer into plastic scintillation vials as described for the ^{3}H-leucine protocol. Vials were counted on a Beckman LS2800 liquid scintillation counter interfaced with Beckman "Data Capture" software for conversion to a Lotus 1-2-3® file and analysis using Lotus 1-2-3®.

These protocols are amenable to use with other target cell populations, especially adherent monolayer cell types.

Morphological Cytotoxicity Evaluation-Pulsed Exposure: Vascular smooth muscle cells were seeded at 4.0 x 10^4 cells/ml medium/well on a commercially prepared four well slide (Nunc, Inc., Naperville, Illinois). Enough slides were seeded to accommodate two pulsed exposure lengths (5 minutes and 24 hours) and prescribed increment evaluation points (24 hours to 128 hours). All slides were run in duplicate to reveal any assay anomalies. The therapeutic agent was diluted in the same medium used in the ^{3}H-leucine and ^{3}H-thymidine assays. Each four well slide was concentration bracketed to one log greater concentration (well "B"), one log lower concentration (well "D") of the minimal effective concentration (well "C"), as determined by the 3H-leucine and 3H-thymidine assays described above. As a control for normal morphology, one well (well "A") was left untreated (medium only). Incubation took place in a 37°C, 5% CO_2 humidified incubator. After each of the two (5 minutes and 24 hours) exposure points, the therapeutic agent medium was aspirated from each well, including the untreated well. One milliliter of fresh medium

was then added to replace the aspirated medium. Re-incubation followed until each of the incremented evaluation points were achieved. At those points, the medium was aspirated and subsequently replaced with 1 ml of 10% neutral buffered formalin for one hour to allow for proper fixation. These fixed slides were stained by hematoxylin (nuclear) and eosin (cytoplasmic) for morphologic evaluation and grading.

Results: The results of the 24 hour ^{3}H-leucine protein inhibition assay and the 24 hour ^{3}H-thymidine DNA synthesis inhibition assay are shown in Figs. 10A-10D for suramin, staurosporin, nitroglycerin and cytochalasin B, respectively. All of the tested compounds showed an available therapeutic range (area under the curve of ^{3}H-leucine assay is greater than that resulting from the ^{3}H-thymidine assay), indicating usefulness in the practice of sustained release dosage form embodiments of the present invention. More specifically, the compounds inhibited the ability of vascular smooth muscle cells to undergo DNA synthesis in the presence of 5% FBS to a greater extent than they inhibited protein synthesis of vascular smooth muscle cells. The protein and DNA synthesis inhibitory effects of suramin, staurosporin, nitroglycerin and cytochalasin B during a 5 minute and 24 hour pulsed exposure are shown in Figure 10 A-D, respectively.

EXAMPLE 9

Specific Binding and Internalization of Targeted Particles by Vascular Smooth Muscle Cells

The ability of vascular smooth muscle cells to bind and internalize particles coated with binding protein or peptide was demonstrated with monoclonal antibody (NR-AN-01) coated gold beads both *in vitro* and *in vivo*. The vascular smooth muscle cell tissue cultures (BO54), an antigen positive control cell line (A375) and an antigen negative control cell line (HT29) were incubated with 10 nm gold beads, with one group coated with NR-AN-01 and a second, uncoated control

group. The cells were exposed to the beads as monolayer and cell suspension cultures, and were examined at six time points (i.e., 1 minute, 5 minutes, 15 minutes, 30 minutes, 60 minutes and 24 hours) for binding and internalization by electron microscopy.

Table 3 shows the results of the experimentation, indicating that the binding to the cell surface is specific. The relative grading system used throughout Table 3 represents a subjective assessment of particle binding, wherein 0 = none; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked. If aggregates of particles settled on the monolayer surface of both the smooth muscle cells and the control cells, the particles were nonspecifically internalized by macro and micro phagocytosis. When the cells were maintained in a cell suspension, non-specific internalization was minimal or absent. Non-specific adherence of gold beads devoid of NR-AN-01 to surface mucin produced by HT29 cells was observed, resulting in modest non-specific internalization thereof. Vascular smooth muscle cell uptake of NR-AN-01 targeted gold beads was highly specific in cell suspension cultures.

Table 3

Time	Grid	Product	Cell Line	Cell Surface	Primary vessicle micro/macro phagostasis pinocytosis	coated pit	secondary vessicle	lysosome	golgi	endoplasmic reticulum
				Cell Monolayer						
1 min	Aa	05(G)	A375	2	0	0	0	0	0	0
	Ba	05(G)	HT29	0	0	0	0	0	0	0
	C	05(G)	B054	2	1	0	0	0	0	0
	Da	(G)	A375	0	0	0	0	0	0	0
	Eb	(G)	HT29	0	0	0	0	0	0	0
	F	(G)	B054	0	0	0	0	0	0	0
5 min	Ac	05(G)	A375	4	1	0	0	0	0	0
	Bb	05(G)	HT29	0	0	0	0	0	0	0
	Ca	05(G)	B054	3	0	0	0	0	0	0
	Dc	(G)	A375	0	0	0	0	0	0	0
	Ea	(G)	HT29	0	0	0	0	0	0	0
	Fa	(G)	B054	0	0	0	0	0	0	0
15 min	Aa	05(G)	A375	3	1	0	0	0	0	0
	Bb	05(G)	HT29	0	0	0	0	0	0	0
	Ca	05(G)	B054	2	1	0	0	0	0	0
	Da	(G)	A375	0	0	0	0	0	0	0
	Ea	(G)	HT29	0	0	0	0	0	0	0
	Fa	(G)	B054	0	0	0	0	0	0	0
30 min	A	05(G)	A375	4	3	0	2	0	0	0
	B	05(G)	HT29	0	0	0	0	0	0	0
	C	05(G)	B054	3	2	0	1	0	0	0
	D	(G)	A375	0	0	0	0	0	0	0
	E	(G)	HT29	0	1	0	0	0	0	0
	F	(G)	B054	1	1	0	0	0	0	0
60	Aa	05(G)	A375	4	3	2	3	2	0	1
	Ba	05(G)	HT29	0	0	0	0	0	0	0
	Cc	05(G)	B054	3	2	0	2	0	0	1
	Da	(G)	A375	0	1	0	0	0	0	1
	Ec	(G)	HT29	1	1	0	1	0	0	0
	Fa	(G)	B054	1	2	0	1	0	0	0
24 hrs	Ab	05(G)	A375	2	1	1	2	4	0	2
	Ba	05(G)	HT29	0	1	1	2	3	0	0
	Cc	05(G)	B054	3	3	1	3	4	1	1

Table 3 Continued

Time	Grid	Product	Cell Line	Cell Surface	Primary vessicle micro/macro phagostasis pinocytosis	coated pit	secondary vessicle	lysosome	golgi	endoplasmic reticulum
	Da	(G)	A375	0	3	0	2	3	0	0
	Eb	(G)	HT29	0	3	0	3	1	0	0
	Fb	(G)	B054	0	2	0	2	3	0	0
					Cell Pellets					
1 min	1A	05(G)	A375	2	0	0	0	0	0	0
	7A	05(G)	HT29	0	0	0	0	0	0	0
	13A	05(G)	B054	3	0	1	0	0	0	0
	1B	(G)	A375	0	0	0	0	0	0	0
	7B	(G)	HT29	0	0	0	0	0	0	0
	13B	(G)	B054	0	0	0	0	0	0	0
5 min	2A	05(G)	A375	3	1	0	0	0	0	0
	8A	05(G)	HT29	0	0	0	0	0	0	0
	14A	05(G)	B054	2	1	0	0	0	0	0
	2B	(G)	A375	0	0	0	0	0	0	0
	8B	(G)	HT29	0	0	0	0	0	0	0
	15B	(G)	B054	0	0	0	0	0	0	0
15 min	3A	05(G)	A375	4	1	0	1	0	0	0
	9A	05(G)	HT29	0	0	0	0	0	0	0
	15A	05(G)	B054	1	1	0	0	0	0	0
	3B	(G)	A375	0	0	0	0	0	0	0
	9B	(G)	HT29	0	0	0	0	0	0	0
	15B	(G)	B054	0	0	0	0	0	0	0
30 min	4A	05(G)	A375	4	2	0	0	0	0	0
	10A	05(G)	HT29	0	0	0	0	0	0	0
	16A	05(G)	B054	2	1	0	0	0	0	0
	4B	(G)	A375	0	0	0	0	0	0	0
	10B	(G)	HT29	0	0	0	0	0	0	0
	16G	(G)	B054	0	0	0	0	0	0	0
60 min	5A	05(G)	A375	3	3	0	2	1	0	0
	11A	05(G)	HT29	0	0	0	0	0	0	0
	17A	05(G)	B054	2	2	0	2	0	0	0
	5B	(G)	A375	0	0	0	0	0	0	0
	11B	(G)	HT29	0	0	0	0	0	0	0
	17B	(G)	B054	0	0	0	0	0	0	0
24 hrs	6A	05(G)	A375	3	1	0	3	3	0	0

Table 3 Continued

Time	Grid	Product	Cell Line	Cell Surface	Primary vessicle micro/macro phagostasis pinocytosis	coated pit	secondary vessicle	lysosome	golgi	endoplasmic reticulum
	12A	05(G)	HT29	0	0	0	0	0	0	0
	18A	05(G)	B054	2	1	0	1	3	0	0
	6B	(G)	A375	0	0	0	0	0	0	0
	12B	(G)	HT29	1	2	0	2	2	0	0
	18B	(G)	B054	0	0	0	0	0	0	0

FIGURE 11 shows a tangential section parallel to the inner surface of a smooth muscle cell characterized by numerous endocytic vesicles, several of which contain antibody coated gold beads in the process of being internalized by the cell. These endocytic vesicles with particles attached to cell surface antigens were stimulated to fuse with lysosomes at a higher than expected rate for normal cell surface membrane recycling. The resultant marked accumulation of internalized particles was observed at the 24 hour time point and is shown in FIGURE 12.

The targeted gold bead vascular smooth muscle cell surface binding, internalization and lysosome concentration was observed in vivo as well. NR-AN-01 coated gold beads were infused via intravascular catheter, open ended with treated area occluded proximally and distally with slip ligatures, at 3 atm pressure applied for 3 minutes into the wall of a pig femoral artery immediately following balloon trauma. The bead internalization rate varied with the degree of damage sustained by the vascular smooth muscle cell during the balloon trauma. Cells with minimal or no damage rapidly internalized the particles by endocytosis and phagocytosis, concentrating the internalized particles in lysosomes. Cells that were killed by the trauma exhibited surface bead binding. Cells that were damaged by the trauma but survived were characterized by bead surface binding with delayed internalization and lysosome concentration. FIGURE 13 shows particulate concentration in the lysosomes in vivo at one week following bead administration.

EXAMPLE 10

Vascular Smooth Muscle In Vitro DNA and Protein Synthesis Inhibition By Staurosporin and Cytochalasin

The ability of staurosporin and cytochalasin to inhibit in vitro DNA and protein synthesis in vascular smooth muscle cells was tested. ^{3}H-leucine and ^{3}H-thymidine uptake and

cytotoxicity assays were conducted in accordance with the following protocols.

Cultured Cells:

BO54 cells (baboon smooth muscle cells) were derived from explants of aortic baboon smooth muscle cells. Cells were expanded in DMEM (Dulbecco's Modified Eagle's Medium):F-12 medium (Whittaker Bioproducts, Walkersville, Maryland) with 5% fetal bovine serum (FBS, Gibco) and 5% Serum Plus® (JRH Biologicals) ("complete medium"), and a seed lot of cells was frozen in liquid nitrogen for future use at passage seven.

5 Minute Exposure; Protein Synthesis Assay:

Vascular smooth muscle cells at 40,000-50,000 cells/ml were seeded and processed as described in Example 8, "5 minute exposure; ^{3}H-leucine uptake." Log dilutions of staurosporin (200 ng/ml, 20 ng/ml, 2 ng/ml, 0.2 ng/ml and 0.02 ng/ml) were dispersed in complete medium. For cytochalasin B, log dilutions at 20 μg/ml, 2.0 μg/ml, 0.2 μg/ml, 0.02 μg/ml and 0.002 μg/ml were dispersed in complete medium. Complete medium was then added to the control wells. One ml/well of each therapeutic agent dilution was added in quadruplicate wells, and the agent of interest was incubated with the vascular smooth muscle cells for 5 min at room temperature in a sterile ventilated hood. Following therapeutic agent incubation, the wells were subsequently treated as described in Example 8, "5 minute exposure; ^{3}H-leucine uptake."

5 Minute Exposure; DNA Synthesis Assay: Vascular smooth muscle (BO54) cells were seeded and processed in 24 well plates, as described above under "5 Minute Exposure: Protein Synthesis Assay." After 5 min incubation with the test therapeutic agent, the medium was aspirated and 1 ml/well of 1.0 μCi/ml ^{3}H-thymidine (rather than ^{3}H-leucine) dispersed in complete medium was added. The cells were then incubated overnight at 37°C in a humidified, 5% CO_2 environment. The

toxic effect of the therapeutic agent was then determined, as described in the Protein Synthesis Assay, above.

24 and 120 Hour Exposure; Protein Synthesis Assay: Vascular smooth muscle (BO54) cells at 20,000 cells/ml were seeded in sterile 24 well plates and incubated in complete medium (1 ml/well) overnight at 37°C, 5% CO_2, 95% air in a humidified atmosphere (saturation). Log dilutions of staurosporin (100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml and 0.01 ng/ml) were dispersed sequentially in the two media, as described below. For cytochalasin B, log dilutions at 10 μg/ml, 1.0 μg/ml, 0.1 μg/ml, 0.01 μg/ml and 0.001 μg/ml were dispersed sequentially in the two media, as described below:

Medium (1) = Complete medium; and

Medium (2) = DMEM (leucine-free) with 0.5 μCi/ml ^{3}H-leucine. Medium (2) is used for the final 24 hour incubation period of the experiment.

More specifically, in the 24 hour assay, each therapeutic agent was diluted in Medium (2), as noted above. Medium (1) was aspirated from the wells, and aliquots of therapeutic agent dilutions in Medium (2) were added in quadruplicate to the appropriate wells. Medium (2) was then added to the control wells.

In the 120 hour assay, each therapeutic agent was diluted in Medium (1), as noted above. Medium (1) was aspirated from the wells, and aliquots of therapeutic agent dilutions in Medium (1) were added in quadruplicate to the appropriate wells. Medium (1) was then added to the control wells. The medium was changed every 24 hours, and fresh therapeutic agent was added to the test wells. At 96 hr, (i.e., the fourth day), each therapeutic agent was diluted in Medium (2), as noted above. Medium (1) was aspirated from the wells, and aliquots of therapeutic agent dilutions in Medium (2) were added in quadruplicate to the appropriate wells. Medium (2) was then added to the control wells.

The test agents in ^{3}H-leucine (and controls) were incubated overnight at 37°C, 5% CO_2 in a humidified atmosphere. The toxic effect of the therapeutic agents was then determined, as described in the 5 Minute Exposure: Protein Synthesis Assay, described above. In addition, the changes in cells at each dilution were photographed using a Zeiss microscope (Zeiss, West Germany) at 320X. The medium was then aspirated, and the cells were processed with TCA, as described above.

24 and 120 Hour Exposure; DNA Synthesis Assay: This assay was performed according to the procedure described for "24 and 120 Hour Exposure; Protein Synthesis Assay", except Medium (2) in this 24 & 120 hr DNA Synthesis Assay is:

Medium (2) = Complete Medium with 1.0 μCi/ml ^{3}H-thymidine.

Medium (2) is used in the final 24 hour incubation of the experiment.

These protein and DNA synthesis assays are amenable for use with other target cell populations, especially adherent monolayer cell types.

Results: The minimum effective dose (MED) of each agent was determined as a percentage of the control that was treated with medium only; 50% of control values was chosen as the cytotoxicity benchmark. At a 5 min exposure, staurosporin demonstrated an MED of 100 ng/ml in the protein synthesis assay and 1 ng/ml in the DNA assay. The 24 hour MED for staurosporin was 10 ng/ml in the protein synthesis assay and 1 ng/ml in the DNA synthesis assay. Both assays gave an MED of 1 ng/ml for a 120 hour exposure of staurosporin.

At a 5 minute exposure, cytochalasin B demonstrated an MED of 10 μg/ml in the protein synthesis assay as well as in the DNA assay. The 24 hour MED for cytochalasin B was 1.0 μg/ml in the protein synthesis assay and 0.1 μg/ml in the DNA

synthesis assay. Both assays gave an MED of approximately 0.1 μg/ml for a 120 hour exposure of staurosporin.

Cytochalasin C and cytochalasin D therapeutic agents were tested at 24 and 48 hour exposures using the same dilutions as described for cytochalasin B, above. At 24 hours, cytochalasin C demonstrated an MED of 1.0 μg/ml in the protein synthesis assay and an MED of 0.01 μg/ml in the DNA synthesis assay. At 48 hours, cytochalasin C demonstrated an MED of 0.1 μg/ml in the protein synthesis assay and 0.01 μg/ml in the DNA synthesis assay. Cytochalasin D demonstrated an MED of 1.0 μg/ml in the 24 hour protein synthesis assay and an MED of 0.1 μg/ml in the 24 hr DNA synthesis assay. A 48 hour exposure to cytochalasin D gave an MED ranging between 0.1 and 0.01 μg/ml in both the protein synthesis and DNA synthesis assays.

EXAMPLE 11

Vascular Smooth Muscle Cell Migration Inhibition

Scratch assays to determine the extent of smooth muscle cell migration inhibition by cytochalasin B were performed in accordance with the following protocol:

Vascular smooth muscle cells (BO54) were derived from explants of baboon aortic smooth muscle, as described in Example 10. The cells were grown in flat bottom, six well tissue culture plates, which hold about 5 ml of medium. The vascular smooth muscle cells were plated at 200,000 cells/well and placed at 37°C in a humidified 5% CO_2 incubator for 18 hours. The wells were then scratched with a sterile portion of a single edge razor blade that was held by clamp or pliers and was brought aseptically into contact with the bottom of the well at a 90° angle. The cells from a small area along the scratch were removed by a sterile cotton tipped applicator while the blade was in contact with the bottom of the well. After incubation, the presence of cells in the "scratched" area is indicative of cell migration across the scratch line. A control incubation showed significant cellular migration,

and serves as the standard against which the migration of cells exposed to the therapeutic agent is compared.

Briefly, a stock solution of cytochalasin B (Sigma Chemical Co.) in dimethyl sulfoxide (DMSO) at 1 mg/ml was prepared. Test dilutions of cytochalasin B or control medium were added. Each experiment included two sets of plates:

A set: Test agent exposure for 1, 3, 6, 8 and 10 days only; and

B set: Test agent exposure for 1, 3, 6, 8 and 10 days, followed by a seven day recovery time with control medium.

Both sets of plates were fixed (10% formalin in PBS) and stained (0.02% crystal violet) at the end of the timed exposures. Test concentrations for cytochalasin B were 1, 0.1 and 0.01 μg/ml, and a negative medium control was included. Fresh medium and drug were supplied 3 times per week.

Table 4 shows the results of these experiments. In this Table, "M" indicates Migration Grade, wherein - = no migration; +1 = minimal; +2 = mild; +3 = moderate; and +4 = marked (maximum density; limit of cell contact inhibition) migration of vascular smooth muscle cells into the cleared area adjacent to the scratch. In this Table, "T" denotes a morphological Toxicity Grade, wherein - = no toxicity; +1 = minimal; +2 = mild; +3 = moderate; and +4 = marked toxicity. The migration results are expressed as "Grade in the Cleared Area of the Well / Grade in an Undisturbed Region of the Well." The toxicity values represent a grade for all cells in each well.

The data indicate that cytochalasin B inhibits the migration (+1 to +2) of vascular smooth muscle cells into the cleared area adjacent to the scratch at a dose of 0.1 μg/ml with only minimal (- to +1) morphological toxicity. The data also show that the treated cells (0.1 μg/ml) regain the ability to migrate (+3 to +4) following removal of the therapeutic agent, even after 10 days of continuous exposure to the therapeutic agent.

Table 4

SCRATCH-MIGRATION ASSAY: INHIBITION OF VASCULAR SMOOTH MUSCLE CELL MIGRATION BY CYTOCHALASIN B

	Continuous Exposure				7-day Recovery Post Exposure			
	Dosage µg/mL				Dosage µg/mL			
Day	Control 0.0	0.01	0.1	1.0	Control 0.0	0.01	0.1	1.0
1 M	+1/+3	+1/+3	+1/+3	-/+2	+3/+4	+3/+4	+3/+4	+2/+3
T	-	-	-	+3	-	-	-	+2
3 M	+3/+4	+3/+4	+1/+4	-/+2	+3/+4	+3/+4	+3/+4	+2/+3
T	-	-	+1	+3	-	-	-	+1
6 M	+3/+4	+3/+4	+2/+4	-/+2	+4/+4	+4/+4	+3/+4	+2/+3
T	-	-	+1	+4	-	-	-	+3
8 M	+3/+4	+3/+4	+2/+4	-/+2	+4/+4	+4/+4	+3/+4	+2/+3
T	-	-	+1	+4	-	-	-	+3
10 M	+3/+4	+3/+4	+2/+4	-/+2	+4/+4	+4/+4	+4/+4	+2/+3
T	-	-	+1	+4	-	-	-	+3

EXAMPLE 12

Therapeutic Agent Cytotoxic Effects on Vascular Smooth Muscle Cells - Pulse and Continuous Exposure

Vascular smooth muscle cells were exposed to a therapeutic agent in one of two exposure formats:

Pulse exposure: The pulse exposure protocol is described in Example 8 above (see "Morphological Cytotoxicity Evaluation - Pulsed Exposure").

Continuous exposure: The same methodology is used for continuous exposure morphological cytotoxicity evaluation as for the pulse exposure, except that the test wells were continuously exposed to therapeutic agent in medium during the exposure period. The medium and therapeutic agent were aspirated from each well daily, including from the untreated control well, and were replaced with 1 ml of fresh medium and therapeutic agent (or medium alone for control wells). Re-incubation followed, until each of the incremental evaluation points of the long term continuous exposure protocol was achieved. These incremental evaluation time points were at 6, 24, 48, 72, 96, 120, 168, 216 and 264 hours. At the designated time period, the appropriate cells were fixed, stained and evaluated as in the pulse exposure protocol. The results of a continuous exposure experiment are shown in Table 5 for suramin, staurosporin and cytochalasin B. The 5 min and 24 hr data presented in Table 5 are correlates of the data contained in Figures 10A, 10B and 10C.

Table 5

MORPHOLOGICAL CYTOTOXICITY ASSAY
Drug & Dose

Exposure Protocol	Cytochalasin B				Suramin				Staurosporine			
	10 ug	1 ug	0.1 ug	0.01ug	10 mg	1 mg	0.1 mg	0.01mg	100 ng	10 ng	1 ng	0.1 ng
5 min + 2 hrs	0.5	0	0	-	0	0	0	-	0	0	0	-
5 min + 6 hrs	4	1	0	-	1	0	0	-	0	0	0	-
5 min + 24 hrs	4	0.5	0	-	1	0	0	-	0	0	0	-
5 min + 48 hrs	4	1	0	-	2	0	0	-	2	1	0	-
5 min + 72 hrs	4.5	1	0	-	3	1	0	-	3	1.5	0	-
5 min + 96 hrs	5	1	0	-	3	1	0	-	3.5	1.5	0	-
5 min + 120 hrs	5	1	0	-	3	1	0	-	4	1.5	0	-
Continuous 6 hrs	-	3	0	0	3	1	0	-	0	0	0	0
Continuous 24 hrs	-	3	1	0	3	2	0	-	-	0	0	0
24 hrs + 24 hrs	-	3	0.5	0	4	3	0	-	-	0.5	0	0
24 hrs + 48 hrs	-	4	1	0	4	3	0	-	-	2	0	0
24 hrs + 72 hrs	-	4	0.5	0	4	3	0.5	-	-	1	0	0
24 hrs + 96 hrs	-	4	0	0	4	3.5	1	-	-	1.5	0	0
24 hrs + 120 hrs	-	4	0	0	-	-	-	-	-	1.5	0	0
Continuous 24 hrs	-	3	0	0	-	1	1	0	-	3	1	0
Continuous 48 hrs	-	3	1	0	-	3	2	0	-	3	2	0
Continuous 72 hrs	-	3	1	0	-	4	3	0	-	3	2	0
Continuous 96 hrs	-	3	2	0	-	4	3	0	-	3	2	1
Continuous 120 hrs	-	3	1	0	-	5	4	0	-	3	2	1
Continuous 168 hrs	-	4	1	0	-	5	4	0	-	3	2	1
Continuous 216 hrs	-	4	1	0	-	5	4	0	-	3	2	1
Continuous 264 hrs	-	4	1	0	-	5	4	0	-	4	2	1

At an in vitro effective dosage, cytochalasin B (1 μg/ml; an anti-migration/contraction effective dose) and staurosporin (1 ng/ml; an anti-proliferative effective dose) exhibited a cytotoxicity grade of 1 (minimal) and 2 (mild), respectively. Independent studies have indicated that a grade of 3 (moderate) or less is preferred for a cytostatic, anti-proliferative agent of the present invention.

EXAMPLE 13

In Vivo BRDU Assay: Inhibition of Vascular Smooth Muscle Cell Proliferation

BRDU assay: In vivo vascular smooth muscle proliferation was quantitated by measuring incorporation of the base analog 5-bromo-2'-deoxyuridine (BRDU, available from Sigma Chemical Co.) into DNA during cellular DNA synthesis and proliferation. BRDU incorporation was demonstrated histochemically using commercially available anti-BRDU monoclonal antibodies. The 1 hour pulse labeling permits assessment of the number of cells undergoing division during the pulse period.

The BRDU pulse labeling protocol described above is used as a standard evaluation technique with in vivo pig vascular studies. Following surgical and treatment procedures (discussed, for example, in Examples 7 and 11 herein) and a post-surgical recovery period, pigs were sedated and pulsed with BRDU 1 hour prior to tissue collection.

Briefly, the pigs were sedated with tiletamine hydrochloride and xylazine (as in Example 7, "Gross Pathology and Histological Evaluation") by intramuscular injection. BRDU was then administered intravenously via the lateral ear vein. Two ml of BRDU at a concentration of 50 mg/ml was administered to each 30-40 lb pig. One hour later, the pigs were sacrificed by intravenously administered pentobarbital. Test artery segments were then removed (a segment included normal vessel located proximally and, if possible, distally with respect to the treated artery segment). The artery segments were transected at 2 mm intervals; arranged in order

in cryomolds with O.C.T. (optimum cutting temperature) compound (Tissue Tek®, Miles Laboratories, Inc., Elkhart, IN); and frozen in liquid nitrogen. The blocks were sectioned at 5 microns and immunohistologically stained to detect BRDU using the following procedure.

BRDU-labeled cell detection: After BRDU (1 g BRDU diluted in 17 ml sterile water and 3 ml 1 N NaOH) pulse labeling and test artery segment removal and sectioning (as above), immunohistochemical staining with anti-BRDU monoclonal antibody provides a visual means of determining a mitotic index over a specified time period. The immunohistochemical staining method was performed as follows:

1) 5 μm sections of test artery were dehydrated in cold acetone (-20°C) for 10 minutes;
2) Sections were mounted on glass microscope slides, and the slides were dried in a 37°C oven for 10 minutes;
3) Slides were rehydrated in PBS for 10 minutes;
4) Slides were subjected to Feulgen's acid hydrolysis using 1 N HCl, wherein two aliquots of 1 N HCl are preheated to 37°C and 60°C prior to proceeding;
5) Slides were rinsed with 1 ml of 1 N HCl at 37°C for 1 min;
6) Slides were transferred to 60°C 1 N HCL for 15 min;
7) Slides were rinsed with 1 ml of 1 N HCl at 37°C for 1 min;
8) Slides were washed with room temperature PBS, using 3 changes of PBS at 5 min intervals;
9) Endogenous, cross-reactive sites on the sections were blocked with normal goat serum (1:25 in PBS) for 20 min;
10) Slides were washed with PBS, as in step 8;
11) Sections were incubated with mouse anti-BRDU antibody (DAKO Corporation, Carpinteria, CA) at 10 μg/ml for 30 min;
12) Slides were washed with PBS, as in step 8;

13) Sections were incubated with horseradish peroxidase-labeled (HRPO) goat anti-mouse IgG_1 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA; diluted 1:20 in PBS) and 4% human AB serum for 30 min;

14) Slides were washed with PBS, as in step 8;

15) Sections were incubated with chromogen (3,3'-diaminobenzidine (DAB; Sigma) at 5 mg/ml in 200 ml PBS) and 200 μl of 30% H_2O_2 for 10 min;

16) Slides were washed with PBS, as in step 8;

17) Samples were counterstained with Gill I hematoxylin (Gill I Lerner Laboratories, Pittsburgh, PA; 30 dips);

18) Slides were washed with PBS, as in step 8; rinsed with a bluing solution (1 gm lithium carbonate in 500 ml dH_2O); washed with deionized water; and

19) Test samples were then dehydrated, cleared and coverslipped.

At the conclusion of this procedure, a positive immunohistological stain exhibits a brown color at the site(s) of reactivity.

Cytocidal agents inhibited BRDU uptake relative to a PBS control; however, cytochalasin B and staurosporin inhibited BRDU uptake (i.e., cell proliferation) without killing the vascular smooth muscle cells. The number of vascular smooth muscle cells labeled with BRDU was assigned a grade at 400X magnification as follows:

1 = $\leq$ 1/high power field (HPF);

2 = 2 to 5/HPF;

3 = > 5 to $\leq$ 10/HPF; and

4 = > 10/HPF.

Both cytochalasin B and staurosporin inhibited proliferation for 24 hours following balloon trauma (grade 1), yielding a BRDU labeling grade equivalent to that of a pre-trauma baseline (grade 1). PBS and monoclonal antibody controls exhibited grade 2.5 to 4 BRDU labeling during the

same time period. At 4 days post-trauma, arteries treated with cytochalasin B or staurosporin, as well as PBS and monoclonal antibody controls, exhibited a BRDU labeling grade of 4. The anti-proliferative, non-cytocidal properties of cytochalasin B and staurosporin suggest that these agents are amenable to sustained release dosage formulations for reduction of vascular stenosis.

EXAMPLE 14

Biological Stenting of Balloon Traumatized Pig Arteries Using Cytochalasin B

Balloon traumatized pig arteries that had been treated with cytochalasin B displayed a larger luminal area at the 4 day and 3 week post-treatment time points, as compared to arteries treated with other test agents or controls. Ten femoral arteries (two arteries obtained from each of the 5 pigs that were treated according to the single dose protocol described in Example 7) were evaluated histologically. The maximal luminal area of each artery was measured and calculated from digitized microscopic images by a BQ System IV computerized morphometric analysis system (R & M Biometrics, Inc., Nashville, TN). This experiment was repeated with 5 additional pigs (two arteries per pig; cytochalasin B dose = 0.1 μg/ml, applied for 3 min at 1 atm pressure; same time points). The data obtained from the two experiments were combined. An increase in lumen area at the 3 week post-cytochalasin B treatment time point was observed.

The luminal area of the traumatized and cytochalasin B-treated segments of the arteries were also compared to the luminal area of the normal, untreated region of the femoral artery proximal to the test area. The results showed that the lumen area in the test region was approximately two times as large as the area of the normal control segment of the same artery. The negative control agents, PBS and monoclonal antibody NR-AN-01, showed no increase or a slight decrease in

lumen area as compared to the normal control segment of the same artery.

A cytochalasin B dose response study was then conducted on 10 pigs, following the experimental protocol described in Example 7. Briefly, both arteries in each of 2 pigs were treated with one of the following doses of cytochalasin B: 0.0 μg/ml (i.e., PBS negative control); 0.01 μg/ml; 0.10 μg/ml; 1.0 μg/ml; and 10.0 μg/ml. The agent was delivered by intraluminal catheter at 1 atm pressure for 3 min, and the arteries were evaluated 3 weeks later by the morphometric analysis system described above. The ratio of treated artery luminal area to proximal normal artery luminal area was determined as a percent change in treated vs. normal area. A significant threshold effect was observed at doses from 0.1 μg/ml (≈140% increase) to 1.0 μg/ml (FIGURE 14). The 10 μg/ml dose appeared to be toxic to the vascular smooth muscle cells (data not shown). The subthreshold dose (0.01 μg/ml) and negative control (PBS) exhibited a ± ≈20% change in luminal area. These data suggest that cytochalasin B acts as a "biological stent" when delivered to traumatized arteries.

EXAMPLE 15

Direct Conjugation of NR-AN-01 Antibody to Carboxylic Functional Groups of a Latex Particle

Antibody-coated latex particles (a model of an antibody-coated, sustained release dosage form) may be obtained using the following aseptic technique:

Conjugation:

To 4 ml 0.05 M sodium borate, pH 8.5, containing 0.01% Tween-20® (polyoxyethylene sorbitan monolaurate, Sigma) is added 0.5 ml PBS containing 5 mg NR-AN-01 monoclonal antibody. To this solution at room temperature is added, with vortexing, 2.5 ml of an aqueous suspension containing 50 mg of 1 μm diameter carboxylated latex particles. Immediately thereafter, 0.50 ml of water containing 100 mg of freshly dissolved 1(3-dimethyl-aminopropyl)3-ethyl carbodiimide HCl

is added with vortexing. The solution is then incubated with shaking for 1-2 hr at room temperature. The reaction mixture is then diluted with 50 ml of 50 mM phosphate buffer, pH 6.6, containing 0.2% gelatin stabilizer (phosphate/gelatin buffer). The mixture is centrifuged at 40,000 x g for 2 hr at 4-10°C. The supernatant is decanted, and the pellet is resuspended in 50 ml phosphate/gelatin buffer using low level sonication for 10 sec. Centrifugation is repeated, and the pellet is resuspended two times, followed by resuspension in the phosphate/gelatin buffer. The conjugated particles are then lyophilized using standard protocols and sorbitol excipients.

Characterization:

(a) Sizing: Particle size homogeneity is assessed by laser anisotropy or, for particles larger than 1 μm, by microscopic examination.

(b) Specific Binding Assessment: Specific binding to smooth muscle cells is determined by histological examination of tissue or cell pellet microtome slices after incubation of protein/peptide conjugates with conjugated particles, with or without blocker protein/peptide included in the incubation mixture. Preferred detection techniques include second antibody assays (i.e., anti-mouse Ig) or competition assays (i.e., radioscintigraphic detection in conjunction with radioisotopically labeled protein/peptide conjugates).

(c) Assessment of the extent of protein/peptide derivitization: This determination is performed by coating the latex particles with radioisotopically labeled antibody, followed by detection of radioactivity associated with the coated particles.

The characterization of antibody-coated particles is described in Table 6.

Table 6

Characterization of NR-AN-01-Coated Latex Particles

Particle Diameter	Offering of Ab/Particle	µg Ab Bound/ 5 mg Latex	Ab Molecules Per Particle
1.2 µm	40,000	42	3520
1.2 µm	84,000	66	5470
0.4 µm	32,000	99	3160
0.4 µm	64,000	140	4550
0.1 µm	932	140	65

The particle aggregation effect of pH during antibody conjugation is presented in Table 7.

Table 7

Effect of pH During Antibody Conjugation - Particle Aggregation

Particle Diameter	pH* During Conjugation	Particle Aggregation** +Tween 20®	Particle Aggregation** -Tween 20®
1.2 µm	8.5	< 5%	< 2.5%
1.2 µm	7.0	≈ 20%	≈ 10%
1.2 µm	5.5	10%	100%
0.4 µm	8.5	< 10%	< 5%
0.4 µm	7.0	≈ 30%	≈ 20%
0.4 µm	5.5	100%	100%
0.1 µm	8.5	< 20%	< 10%
0.1 µm	7.0	≈ 50%	≈ 40%
0.1 µm	5.5	100%	100%

* Using 50 mM MES (pH 5.5); phosphate (pH 7.0); or borate (pH 8.5) buffer, as described.

** As assessed by microscopic examination, on a scale of 0-100%.

These data suggest that proteins or peptides may be directly conjugated with sustained release dosage forms of the present invention. More specifically, poly-lactic/glycolic acid particulates having terminal carboxylic acid groups will be conjugated according to the procedure described herein or the alternative procedures described in the specification hereof.

Citations

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11. Sclingemann et al. 1990. Am. J. Pathol. 136: 1393-1405.

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While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for inhibiting one or more pathological activities of normal mammalian vascular smooth muscle cells for a period of time sufficient to maintain an expanded vessel luminal area, which method comprises:

administering to a mammal an effective amount of a cytostatic therapeutic agent capable of inhibiting one or more pathological activities of vascular smooth muscle cells, wherein the therapeutic agent is administered directly or indirectly to a traumatized vessel.

2. The method of Claim 1 wherein the administering step is accomplished with a catheter.

3. The method of Claim 1 wherein the therapeutic agent is a cytoskeletal inhibitor or an analog thereof.

4. The method of Claim 3 wherein the cytoskeletal inhibitor is cytochalasin B, cytochalasin C, cytochalasin D or an analog thereof.

5. The method of Claim 1 further comprising the step of subsequently administrating a sustained release dosage form having dispersed therein an effective amount of a cytostatic therapeutic agent capable of inhibiting one or more pathological activities of vascular smooth muscle cells.

6. The method of Claim 5 wherein the sustained release dosage form is coated with a covalently attached binding peptide or protein capable of specifically localizing to vascular smooth muscle cells, stromal cells or interstitial matrix surrounding vascular smooth muscle cells.

7. A method for inhibiting one or more pathological activities of normal mammalian vascular smooth muscle cells for a

period of time sufficient to maintain an expanded vessel luminal area, which method comprises administering to a mammal the following:

a cytocidal conjugate comprising a cytocidal agent and a binding partner capable of specifically localizing to vascular smooth muscle cells, stromal cells or interstitial matrix surrounding vascular smooth muscle cells; and

a sustained release dosage form having dispersed therein an effective amount of a cytostatic therapeutic agent capable of inhibiting one or more pathological activities of vascular smooth muscle cells, wherein the dosage form is coated with a covalently attached binding peptide or protein capable of specifically localizing to vascular smooth muscle cells, stromal cells or interstitial matrix surrounding vascular smooth muscle cells.

8. The method of Claim 7 wherein the cytocidal agent comprises a toxin or toxin subunit and the therapeutic agent is a protein kinase inhibitor, a cytoskeletal inhibitor, TGF-beta, an analog thereof, a TGF-beta activator, or a TGF-beta production stimulator.

FIG. 1

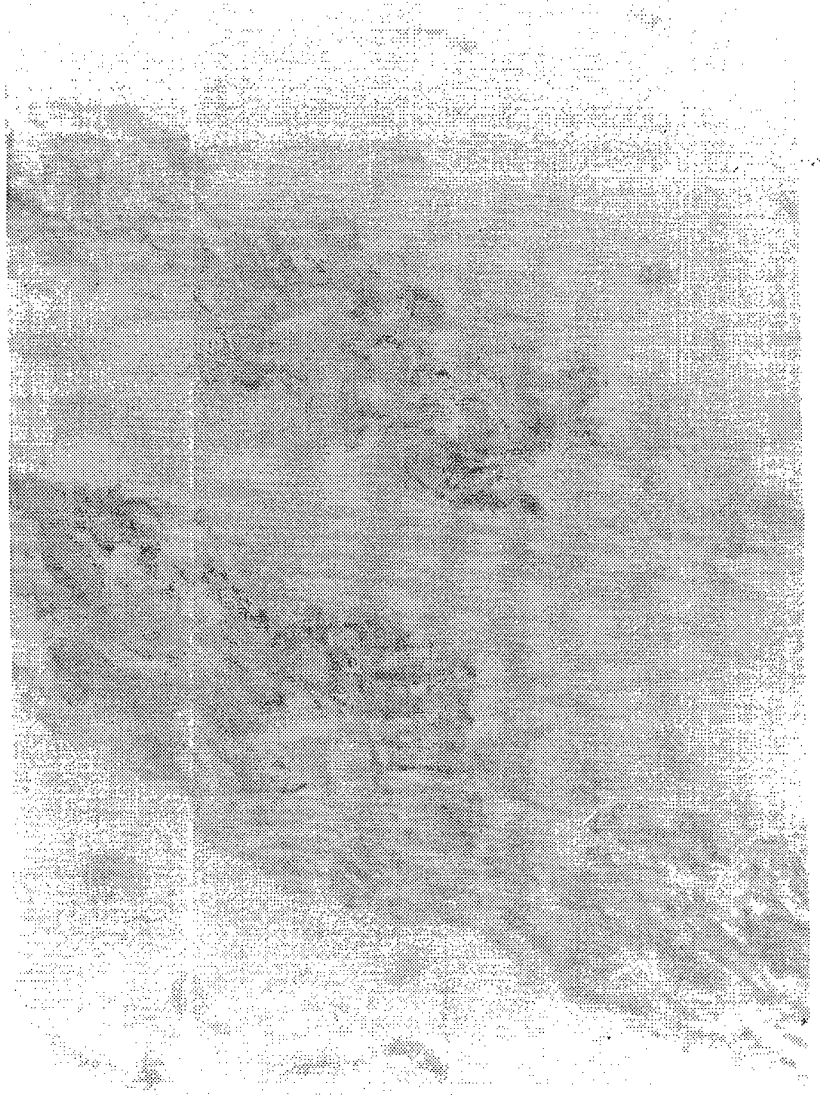


FIG. 1B

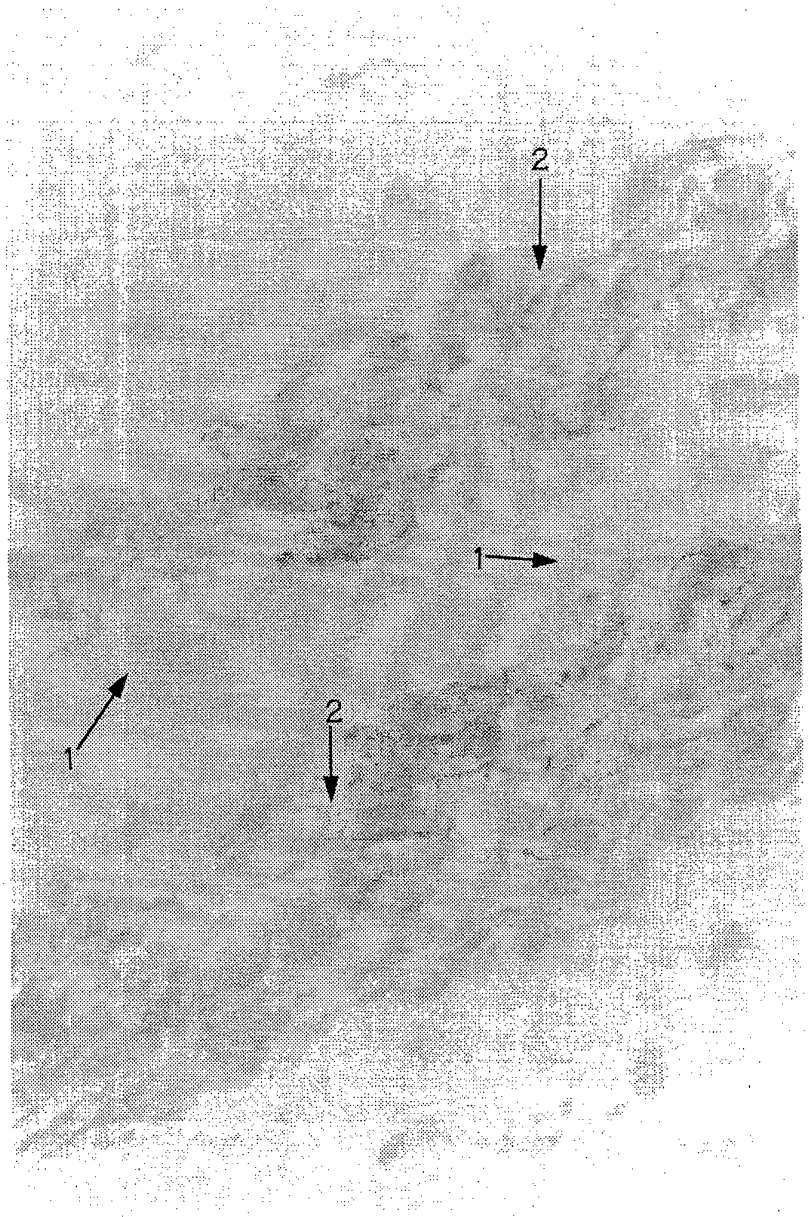
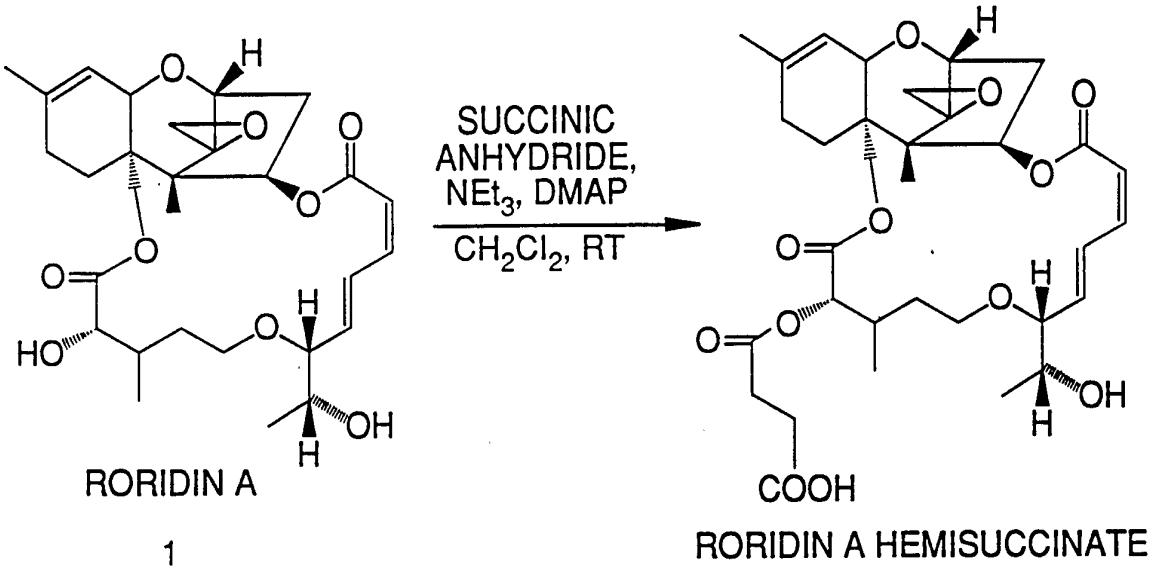


FIG. 2

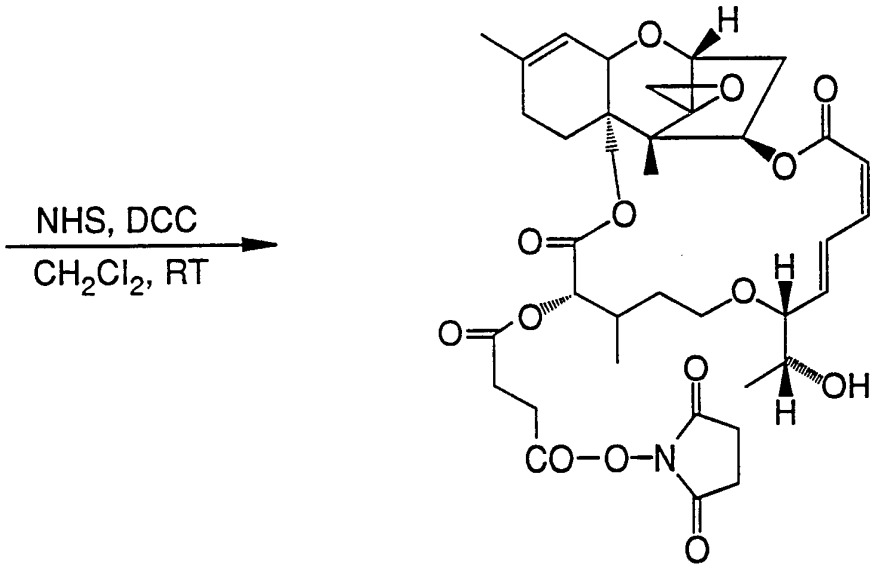


RORIDIN A

1

RORIDIN A HEMISUCCINATE

2



RORIDIN A HEMISUCCINYL SUCCINIMIDATE
(RA-HS-NHS)

3

FIG. 3

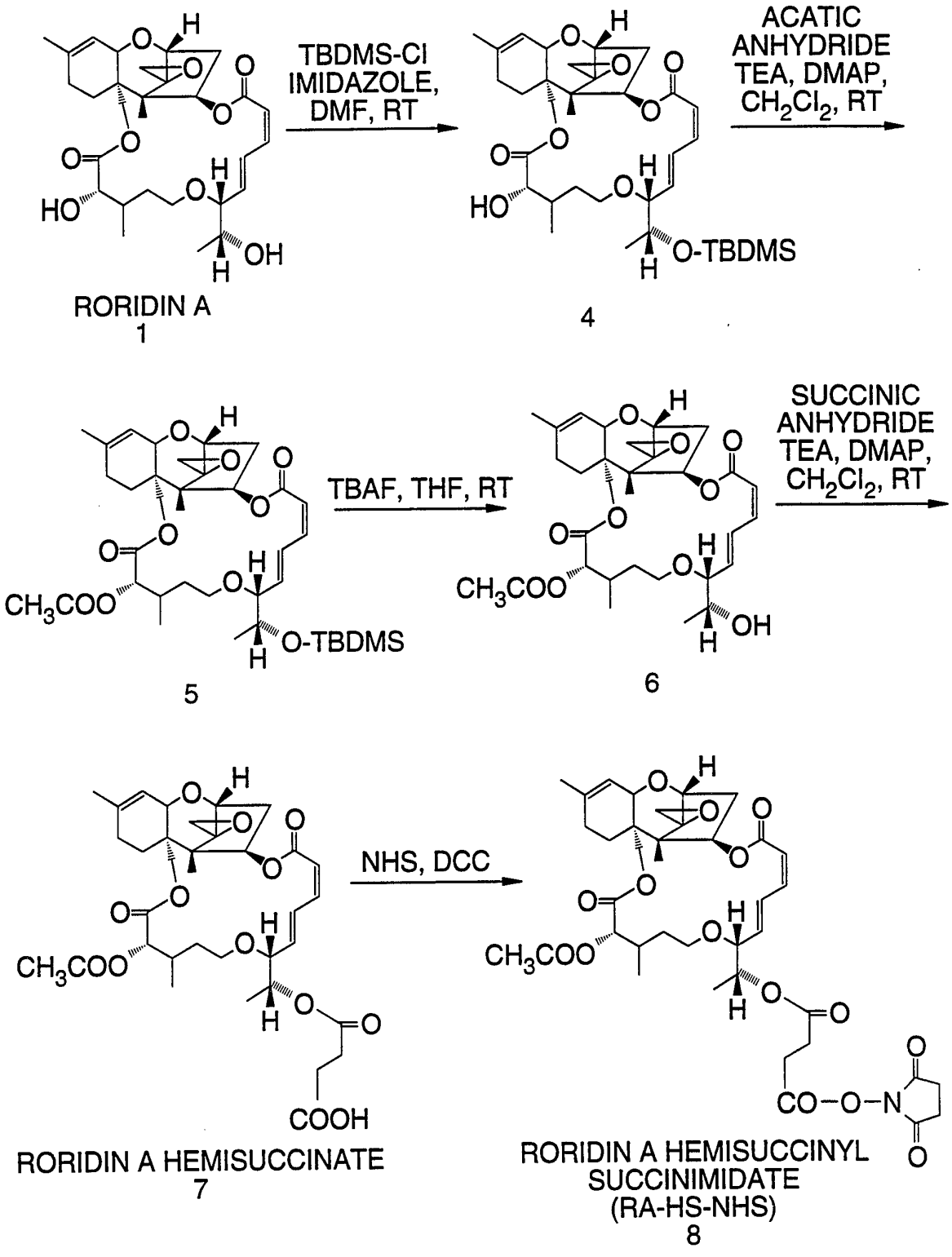

TBDMS-Cl
IMIDAZOLE,
DMF, RT
ACATIC
ANHYDRIDE
TEA, DMAP,
CH_2Cl_2, RT
TBAF, THF, RT
SUCCINIC
ANHYDRIDE
TEA, DMAP,
CH_2Cl_2, RT
NHS, DCC
RORIDIN A
1
4
5
6
RORIDIN A HEMISUCCINATE
7
RORIDIN A HEMISUCCINYL
SUCCINIMIDATE
(RA-HS-NHS)
8

FIG. 4A

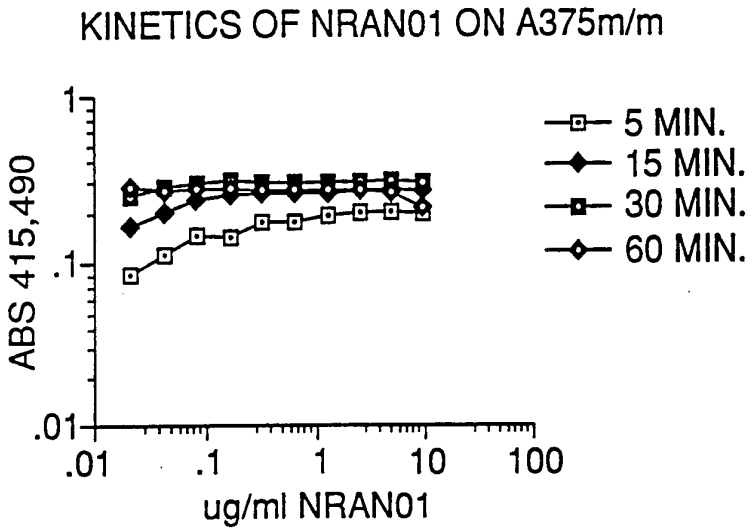

KINETICS OF NRAN01 ON A375m/m
ABS 415,490
1
.1
.01
.01
.1
1
10
100
ug/ml NRAN01
5 MIN.
15 MIN.
30 MIN.
60 MIN.

FIG. 4B

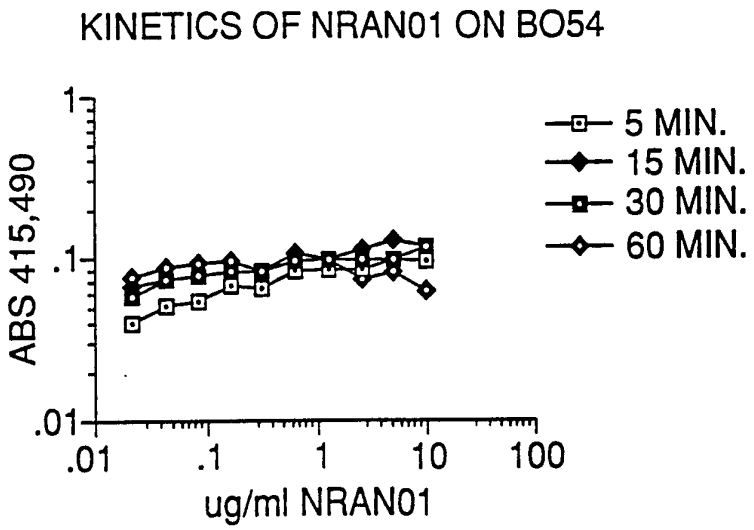

KINETICS OF NRAN01 ON BO54
ABS 415,490
1
.1
.01
.01
.1
1
10
100
ug/ml NRAN01
5 MIN.
15 MIN.
30 MIN.
60 MIN.

FIG. 5A

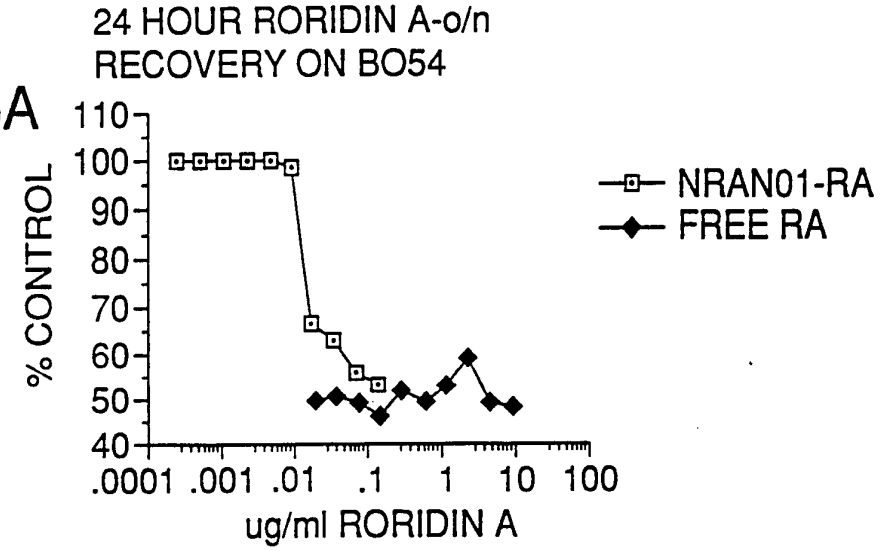

24 HOUR RORIDIN A-o/n
RECOVERY ON BO54
% CONTROL
110
100
90
80
70
60
50
40
.0001 .001 .01 .1 1 10 100
ug/ml RORIDIN A
NRAN01-RA
FREE RA

FIG. 5B

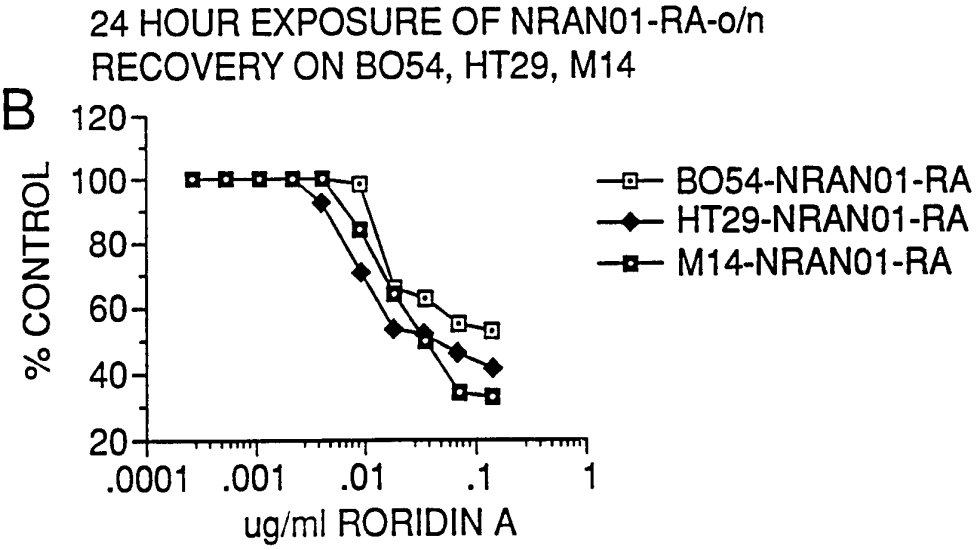

24 HOUR EXPOSURE OF NRAN01-RA-o/n
RECOVERY ON BO54, HT29, M14
% CONTROL
120
100
80
60
40
20
.0001 .001 .01 .1 1
ug/ml RORIDIN A
BO54-NRAN01-RA
HT29-NRAN01-RA
M14-NRAN01-RA

FIG. 6A

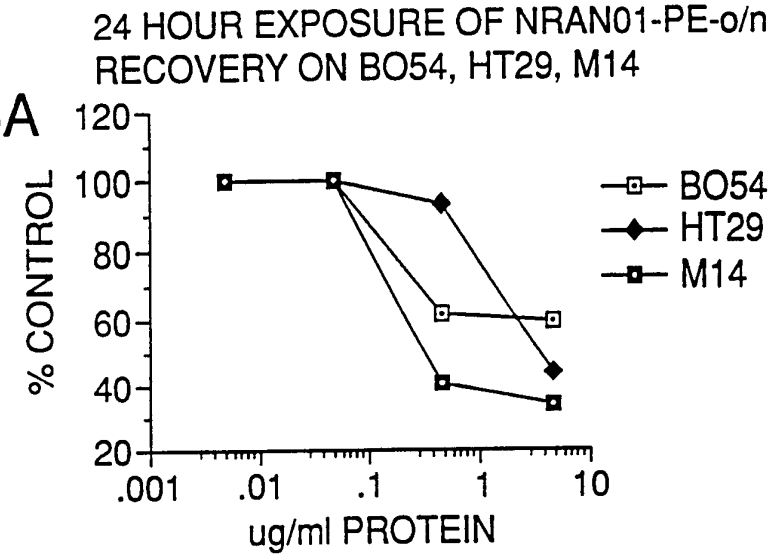

24 HOUR EXPOSURE OF NRAN01-PE-o/n
RECOVERY ON BO54, HT29, M14
% CONTROL
120
100
80
60
40
20
.001
.01
.1
1
10
ug/ml PROTEIN
BO54
HT29
M14

FIG. 6B

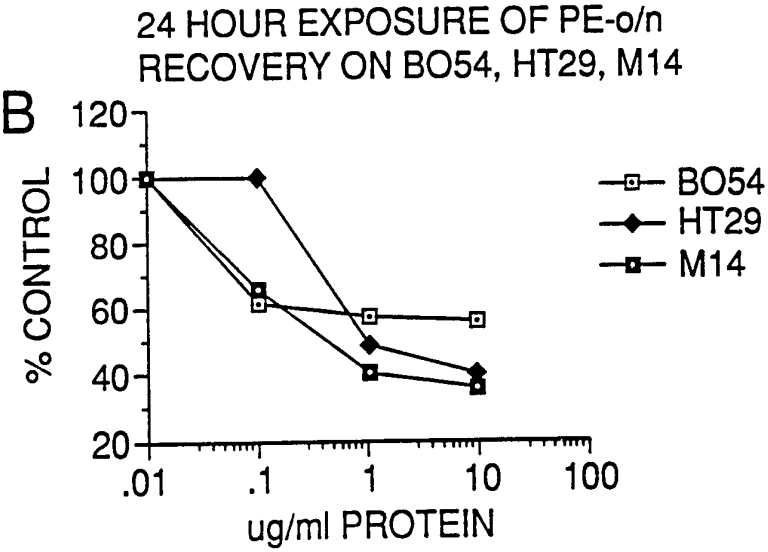

24 HOUR EXPOSURE OF PE-o/n
RECOVERY ON BO54, HT29, M14
% CONTROL
120
100
80
60
40
20
.01
.1
1
10
100
ug/ml PROTEIN
BO54
HT29
M14

FIG. 7A

5 MIN. RORIDIN A DIRECT [3H] LEUCINE-4 HOURS ON HT29

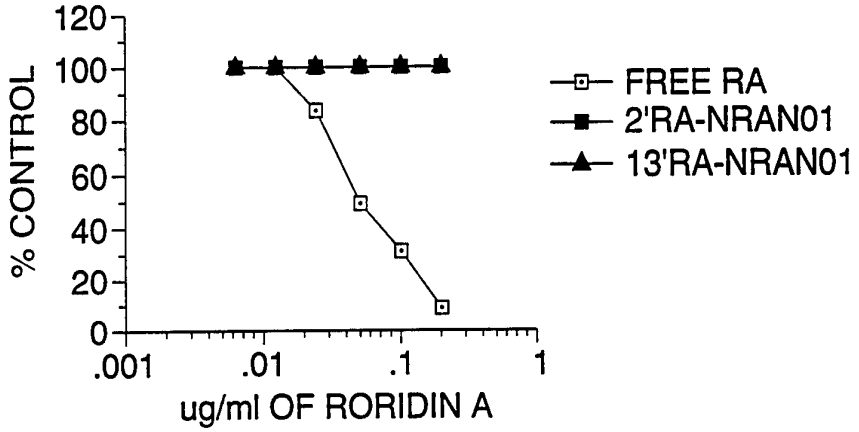


FIG. 7B

5 MIN. RORIDIN O/N RECOVERY, [3H] LEUCINE-4 HOURS ON HT29

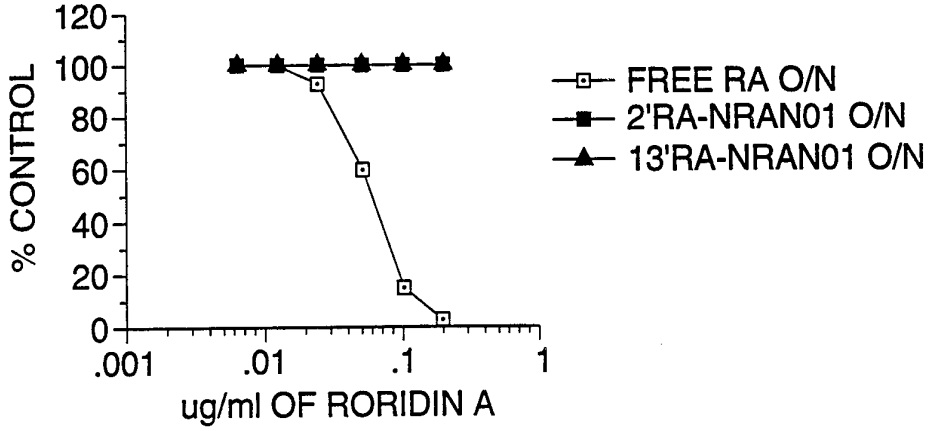


FIG. 7C

5 MIN. RORIDIN A DIRECT [3H] LEUCINE-4 HOURS ON A375m/m

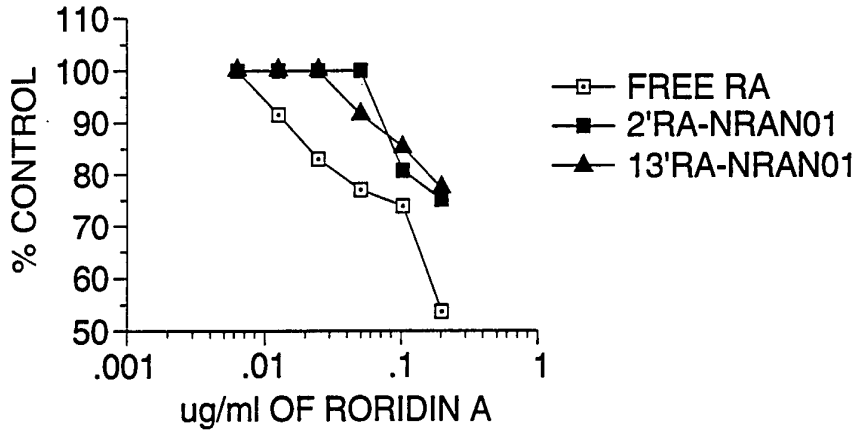


FIG. 7D

5 MIN. RORIDIN O/N RECOVERY, [3H] LEUCINE-4 HOURS ON A375m/m

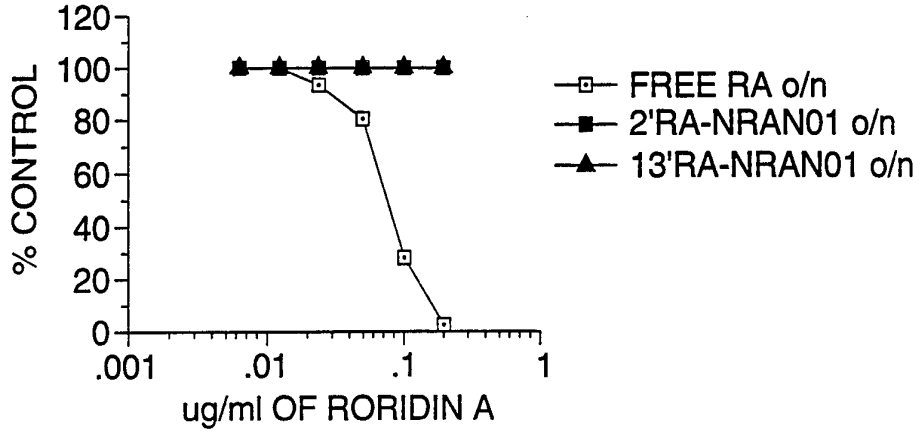


FIG. 8A

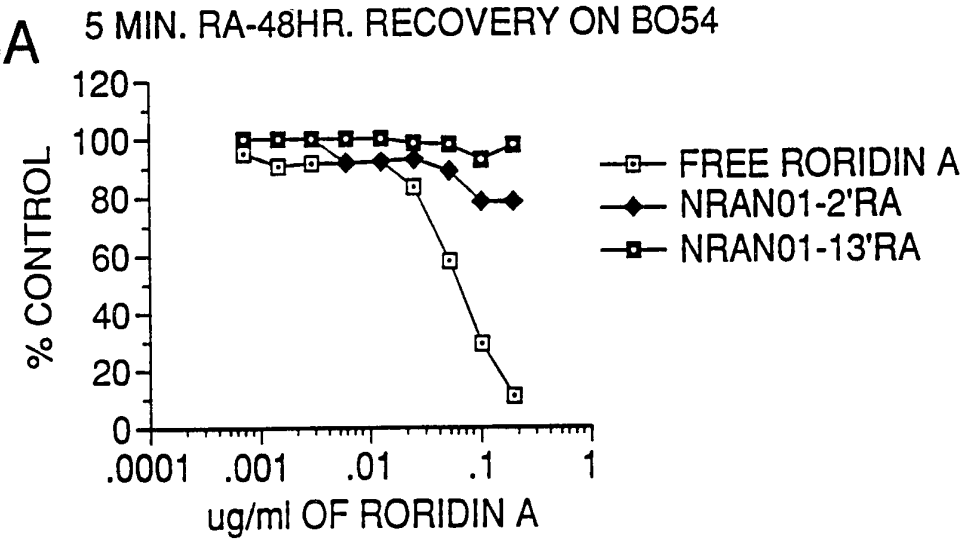


FIG. 8B

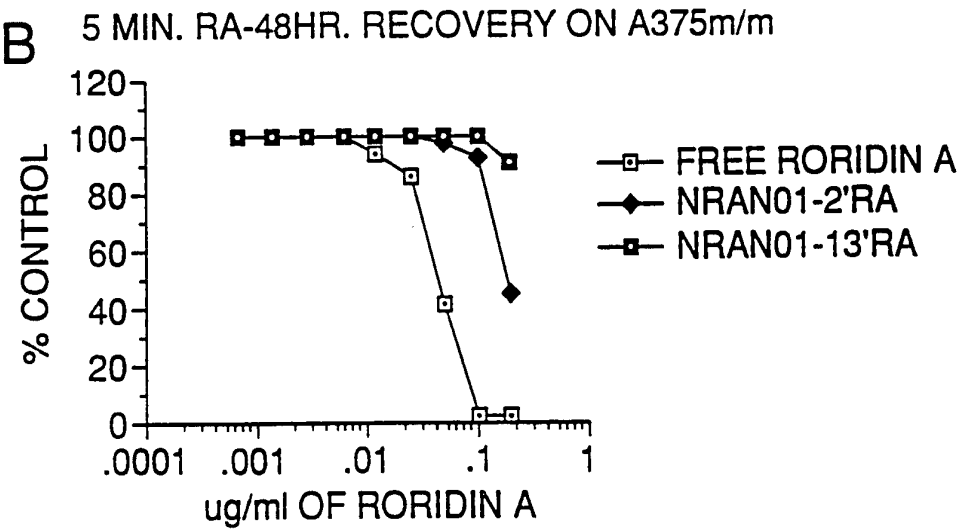


FIG. 8C

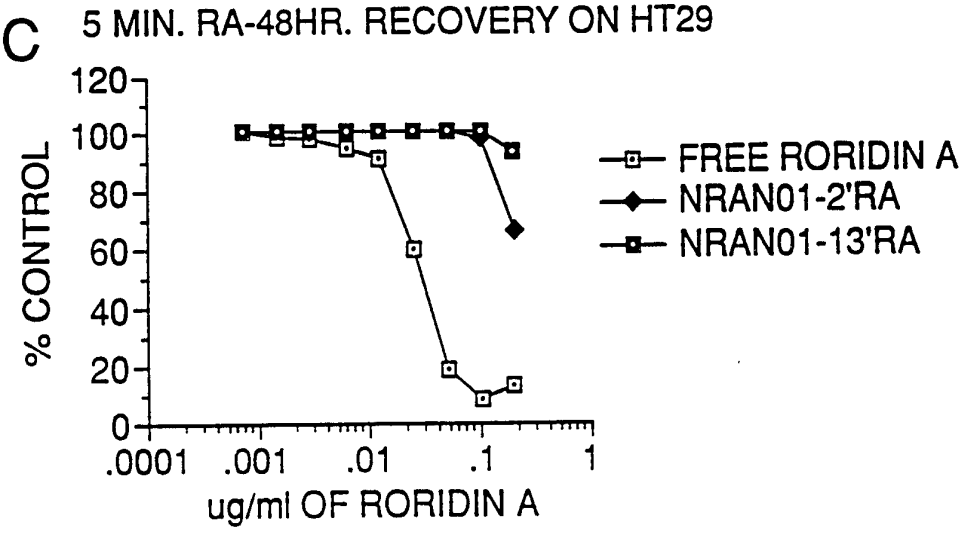


FIG. 9A

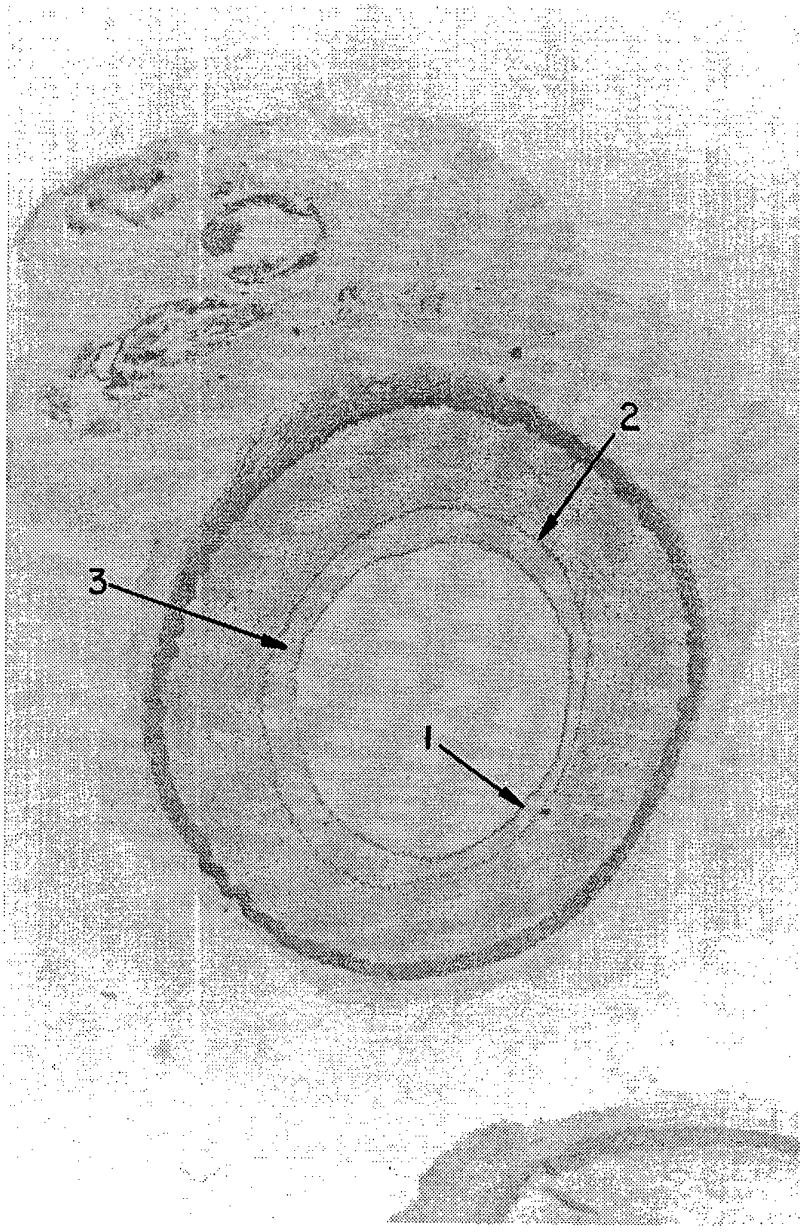


FIG. 9B

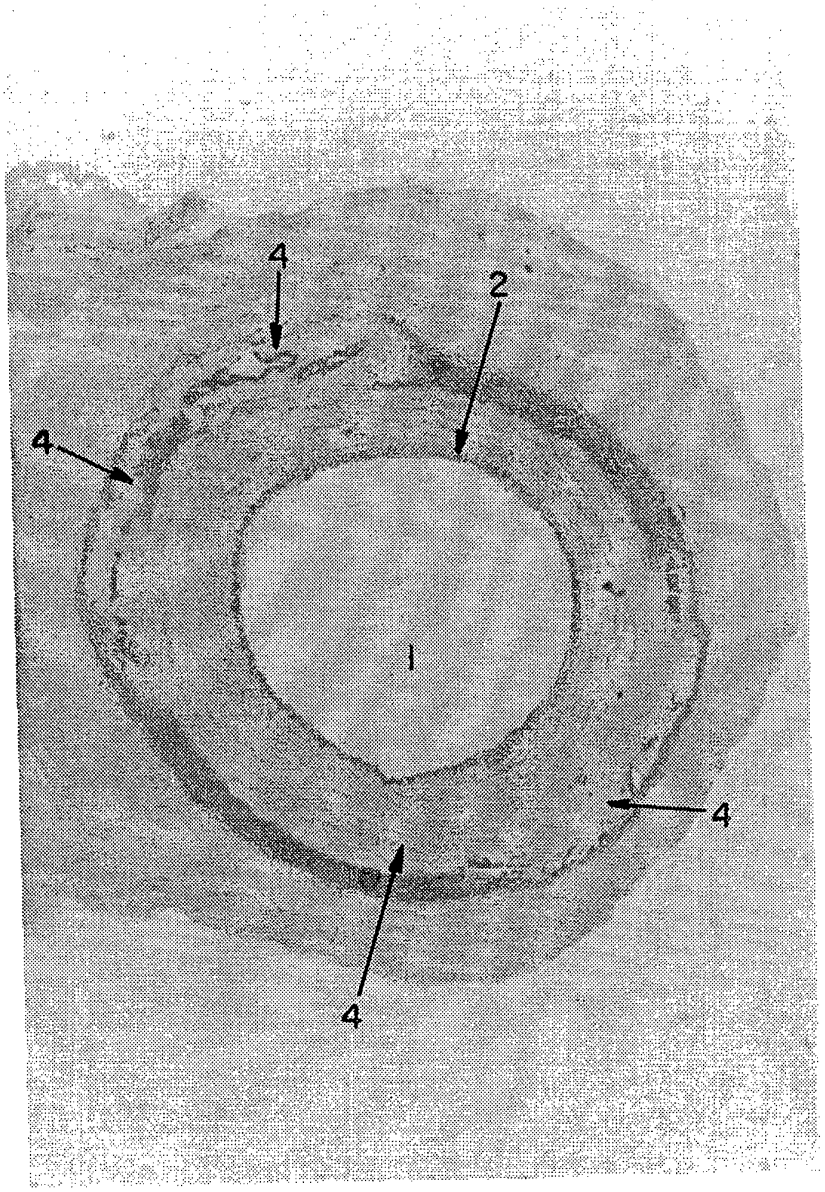


FIG. 10A

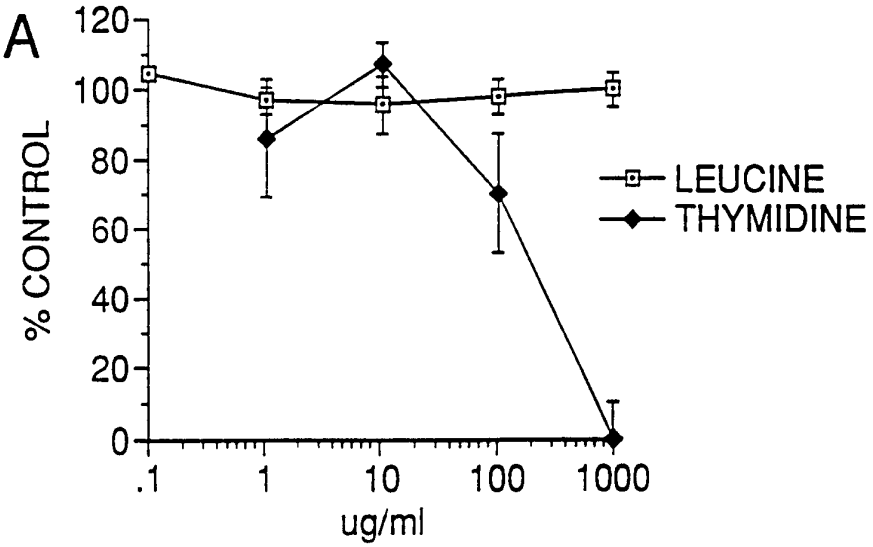


FIG. 10B

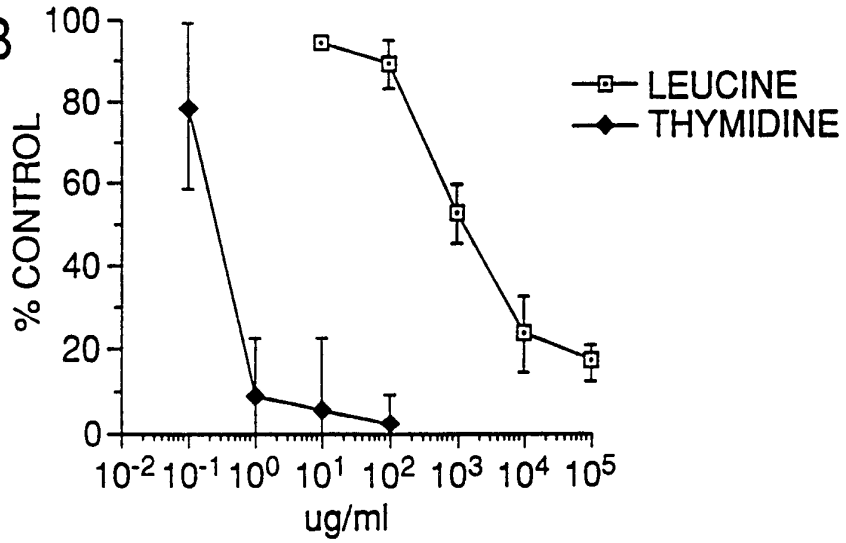


FIG. 10C

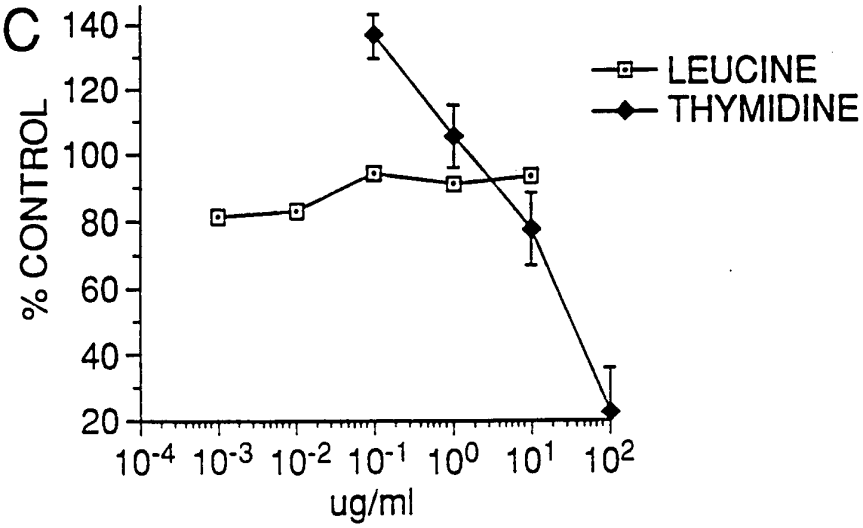


FIG. 11

FIG. 12

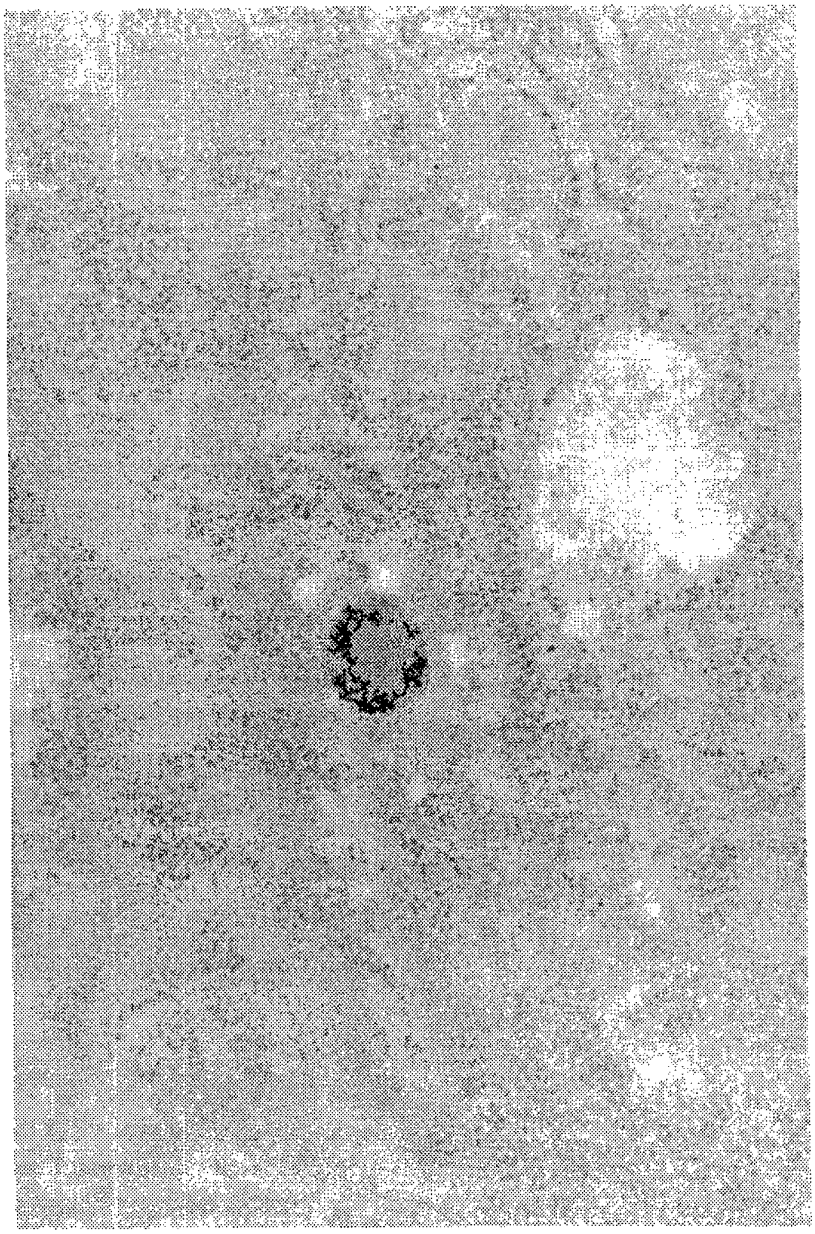


FIG. 13

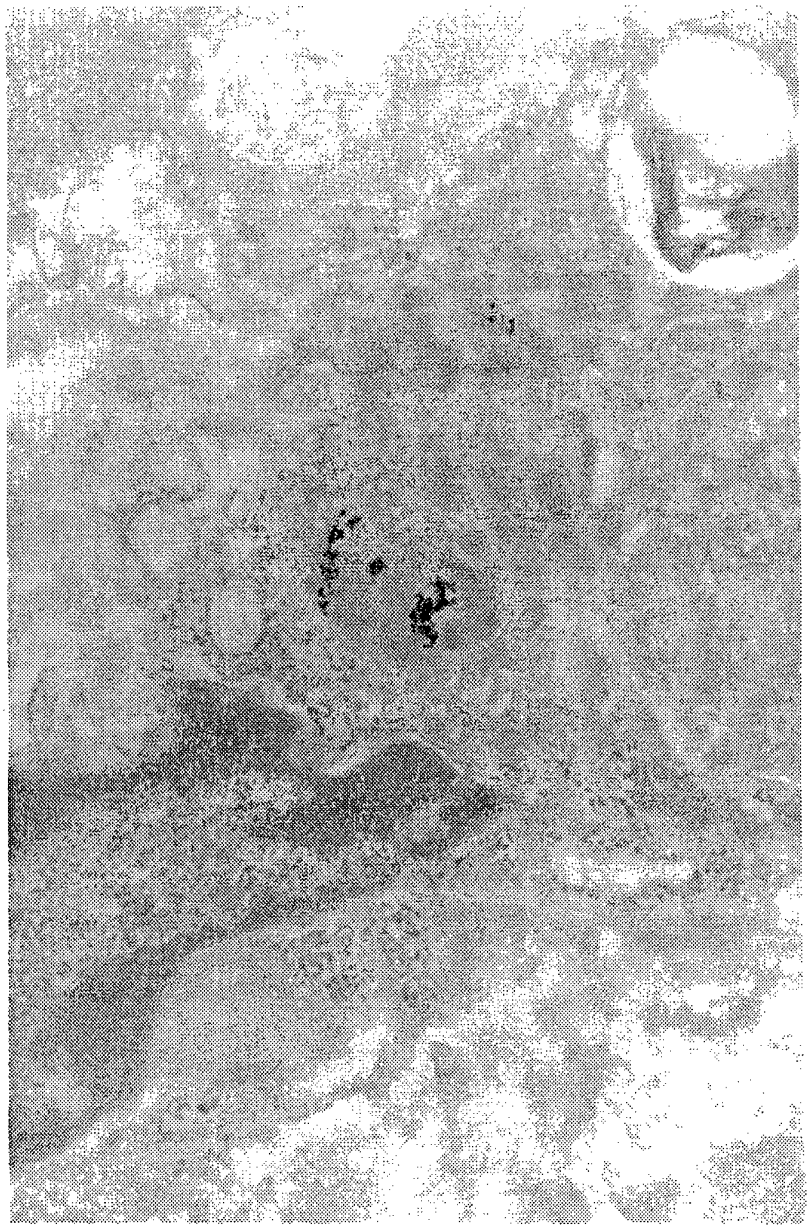
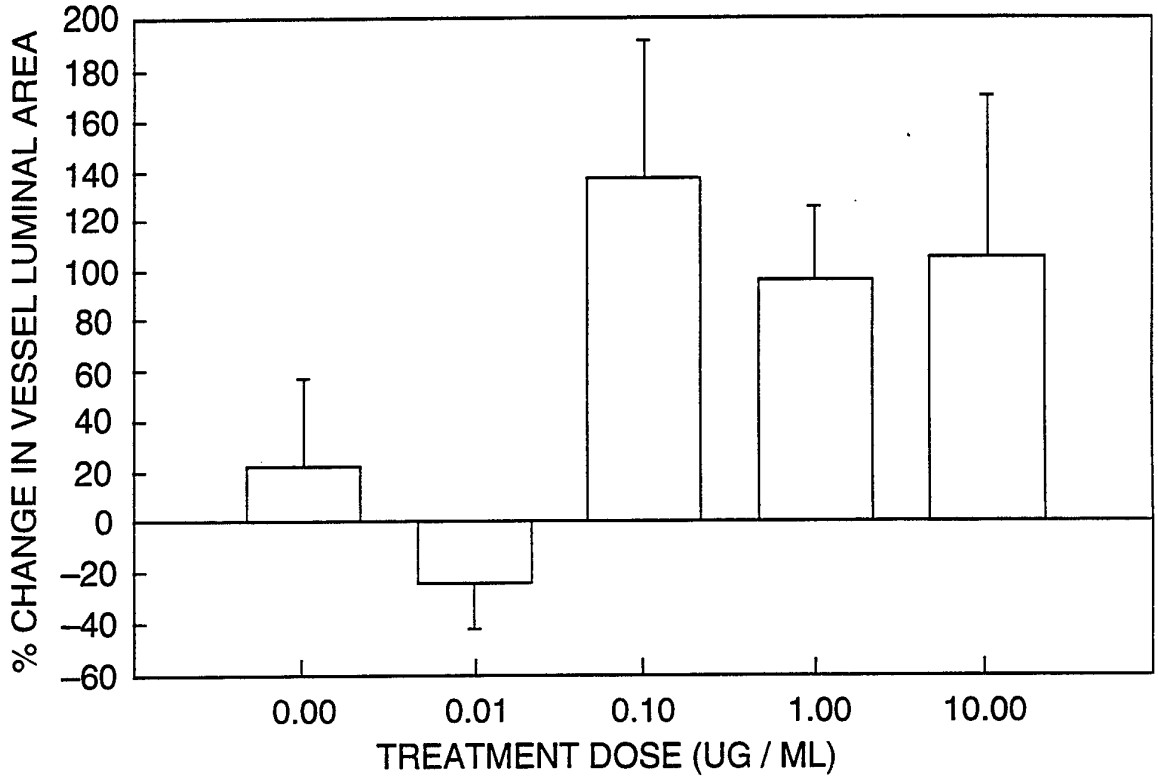


FIG. 14

PERCENT CHANGE IN LUMINAL AREA RATIO (TREATED / NORMAL)
IN PIG ARTERY (N=4) 3 WEEKS POST–CYTOCHALASIN B TREATMENT



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01034

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/535
US CL : 514/230

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/230

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,270,047 (Kauffman et al.) 14 December 1993. See entire document.	1-8
Y,P	US, A, 5,268,358 (Fretto) 07 December 1993. See entire document.	1-8
Y,P	US, A, 5,232,911 (Vidal) 03 August 1993 . See entire document.	1-8
Y	US, A, 5,171,217 (March et al.) 15 December 1992. See entire document.	1-8
Y	US, A, 5,166,143 (Ondetti et al.) 24 November 1992. See entire document.	1-8
Y	US, A, 5,140,012 (McGovern et al.) 18 August 1992. See entire document.	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 MARCH 1994	Date of mailing of the international search report APR 19 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SAMUEL BARTS Telephone No. (703)308-1235

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01034

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,280,016 (Conrad et al.) 18 January 1994. See entire document.	1-8
Y	Budavari et al, "THE MERCK INDEX", published 1989 by MERCK & CO., INC.(N.J.), see pages 438-439, especially compound 2796.	1-8