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(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFYING PEPTIDE APTAMERS CAPABLE OF ALTERING A CELL PHENOTYPE

(57) Abstract: The invention provides methods and compositions for screening and identifying of peptide aptamers that can modulate a cell phenotype and further, can be used for the treatment of a disease involving a misregulated cell phenotype, such as, for example, a cancer. The invention encompasses methods and compositions for producing cyclic peptide aptamers, including peptide aptamers comprising a conotoxin sequence, having improved stability and bioactivity. The invention also provides methods and compositions for improved gene delivery and expression of a peptide aptamer in cell.

METHODS AND COMPOSITIONS FOR IDENTIFYING PEPTIDE APTAMERS CAPABLE OF ALTERING A CELL PHENOTYPE

Related Information

The contents of all patents, patent applications, and publications cited throughout this specification are hereby incorporated by reference in their entireties.

Background of the Invention

Peptides are often effective starting points for the design of therapeutic molecules. Peptides can themselves be highly effective therapeutic agents, despite the often attendant issues of delivery, bioavailability, or stability, when they serve as ligands for, *e.g.*, membrane-associated cellular receptors (*i.e.* agonists or antagonists). In the case of intracellular targets for peptide ligands, *in vitro* assay methods (*e.g.* biochemical assays) are most often used to identify effective peptides that manifest desirable activities or effects. Strategies for designing peptides in this latter class include the use of a peptide sequence composing the interaction domain within one partner of a known and important protein-protein interaction to compete and inhibit this interaction in assays where both protein interaction partners are provided and their interaction can be measured quantitatively.

An approach for the discovery of novel peptide ligands that modulate the activity of a known protein target involves the survey of peptide libraries to identify tightly binding peptides. Such libraries typically exist either within a phage display format or are chemically synthesized as combinatorial libraries of random peptides. This approach may be used to identify peptides that bind an enzyme in a manner that affects (increases or decreases) its catalytic properties, or may be used to identify high- affinity peptide ligands that affect interaction of the target protein with other proteins.

In each case, the identification of a peptide with desirable biological activities is only the beginning of a long, and sometimes, impossible process of converting that peptide into a molecule with therapeutic utility. Difficulties in this regard may arise from a number of issues: (*i*) mammalian cells are not permeable to most peptides, rendering them largely ineffective against intracellular targets; (*ii*) peptides (especially linear peptides) are most often unstable; (*iii*) linear peptides are structurally unconstrained, allowing essentially free rotation at each intramolecular bond along the peptide backbone; and, (*iv*) binding affinities of peptide ligands for their target proteins may be relatively low, often no better than μM or even mM. Moreover, in consideration of these significant shortcomings associated with many peptides, much effort has been devoted to the use of linear peptides as chemical entities to serve as effective conceptual starting

points for the design of non-peptidic small molecules. This approach, often referred to as "peptidomimetics" has met with very limited success.

Another type of issue that impacts the ability to identify therapeutic peptides, is the difficulty of specifying *a priori* a protein target within the cell that would result in a specific desired effect. This dilemma is exemplified by the fact that signal transduction pathways within eukaryotic cells are highly complex, convoluted, interconnected, and redundant. Thus, although it is common to formulate hypotheses regarding the utility of targeting a specific protein for the identification of peptides or other small molecules that might modulate its activity, it is quite common to discover either that it is difficult to direct the activity of such a molecule to that specific target, or that the hypothesized target is irrelevant, superfluous, or redundant with respect to the desired effect or function. In this regard, binding- mediated approaches such as phage display or screening of chemically synthesized peptide libraries for interactors may lead to peptides or peptide leads that do not carry out the desired activity within the cell, or that do so with insufficient potency or specificity.

Accordingly, improved methods and compositions for identifying therapeutic peptides capable, *e.g.*, of altering a cell phenotype, are needed.

Summary of the Invention

The present invention provides improved methods and compositions for screening and identifying peptides (aptamers) from random libraries that manifest identifiable phenotypes in cells. The methods and compositions of the invention have several important advantages over prior peptide screening technologies which allow for more rapid and accurate identification and characterization of therapeutic peptides from random libraries. As described herein, these advantages include improvements in the manner in which the peptide libraries are generated, such that they can be more easily and accurately screened, as well as improved strategies for screening the libraries.

Accordingly, in one aspect, the present invention provides methods for generating random peptide aptamer libraries which are modified to have a desired property, such as improved stability, intrinsic structural organization, affinity, bioavailability, or detectability. In one embodiment, this is achieved by introducing a sequence within the peptide that contributes to the desired property by, for example, cyclizing the peptide. Preferred cyclizable sequences include, for example, those derived from naturally occurring cyclic proteins, such as conotoxins. In another embodiment, the peptide is covalently linked to a cholesterol or sterol, which can direct it to a specific intracellular location or to a metabolic or signaling pathway within the cell.

The advantages of the present invention further include improved methods for delivering and expressing the peptide libraries in cells using, for example, improved receptor-mediated gene transfer systems and/or genetic regulatory elements to enhance expression of the peptide libraries, such as chromatin insulator elements. Other advantages of the present invention include more efficient methods for screening the peptide libraries by identifying populations of cells that have undergone a desired phenotypic change due to the activity of a given member of the peptide aptamer library. This allows for screening of a much larger number of random peptide sequences (*e.g.*, $>10^8$), and does not require that the identity of the target be known or specified as a pre-requisite for carrying out the screen.

The following is a summary of several particular embodiments of the present invention as described above.

In one embodiment, the present invention provides a method for identifying a peptide aptamer capable of modifying a cell phenotype by a) contacting cells with a library of expressible nucleic acid sequences encoding random peptide aptamers linked to a fusion moiety; b) selecting at least one cell having an altered phenotype compared to the phenotype of the cell prior to the contacting step (a); and c) identifying one or more peptide aptamers expressed in the selected cell. The method can further include the step of amplifying the nucleic acid sequences identified in step c) and repeating one or more times steps a)-c) using the amplified nucleic acid sequences as the library of expressible nucleic acid sequences. Preferably the nucleic acid sequences are amplified using the polymerase chain reaction (PCR) and a thermostable nucleic acid polymerase.

In one embodiment, the fusion moiety is a green fluorescent protein (GFP), thioredoxin, a regulatory polypeptide involved in apoptosis, bcl-2, p53, an NFκB-related polypeptide, a caspase, PTEN, myc, a BH3 domain, a death domain (DD), a BIR3 domain, a BIR domain, a nuclear localization signal sequence, a membrane localization signal sequence, a farnesylation signal sequence, a transcriptional activation domain, a transcriptional repression domain, or a functional fragment thereof.

In another embodiment, the fusion moiety is a moiety that undergoes processing (processing-fusion moiety) such as an intein or sterol conjugation domain (*e.g.*, hedgehog derived polypeptide), in which processing of the full length precursor results, respectively, in a cyclic or sterol-conjugated peptide product.

The method can further include the step of expressing the one or more peptide aptamers identified in step c) in a second sample of cells (which can be the same or a different cell type as the first sample), and identifying a change in the phenotype of the second sample of cells.

In another embodiment of the invention, the cells of the first sample discussed above are genetically modified to express a receptor, such as ecotropic receptor, not naturally expressed by the cells (or not abundantly expressed endogenously), to facilitate cellular uptake of the nucleic acid sequences or vectors encoding the random peptide aptamers.

In yet another embodiment of the invention, the methods discussed above further include contacting a sample of cells with a pathogen, for example, a virus or toxin, before or after expression of the one or more peptide aptamers. In another embodiment, a sample of cells is contacted with an agent which stimulates signal transduction before or after expressing the one or more random peptide aptamers.

In yet another embodiment of the invention, cells expressing the random peptide libraries are screened by identifying a change in the expression levels of an endogenous gene or gene products. In a related embodiment, the cells contain a reporter gene such that a change in the expression levels of the reporter gene are measured.

The screening methods of the present invention summarized above are used to identify peptide aptamers capable of altering a particular cell phenotype. Such phenotypes include, for example, a change in levels of apoptosis, signal transduction (*e.g.*, cellular responses mediated by a tyrosine kinase or a G protein coupled receptor), protein trafficking, cell adhesion, membrane transport, cell motility, metabolic state, survival or susceptibility to an infectious agent or toxin, or differentiation, as compared, for example, to a control cell. The methods can further be used to identify peptide aptamers capable of altering cellular lifespan or differentiation, hormone production, or metabolic processes such as cholesterol, sterol, or carbohydrate metabolism. Such peptides or aptamers include those relating to, cellular processes associated with hair growth or hair loss (*e.g.*, propecia). Such peptides include those involved in modulation of hair growth, modulation of hair follicle differentiation, modification of hair follicle development, melanogenesis, modulation of hair shaft elongation, modulation of skin differentiation, or a modulation of the hair growth cycle from telogen (resting stage) to anagen (growing stage).

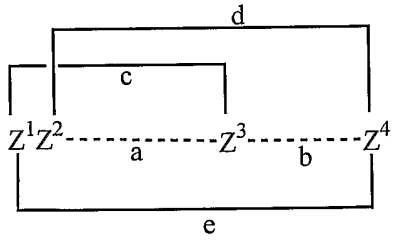
Suitable cells for use the above-described screening methods of the present invention include, for example, fungal cells (*e.g.*, yeast cells), insect cells, and mammalian cells, preferably human cells, and more preferably, clonal human cancer cells, or cells modified to exogenously express receptors or effectors of signal transduction, or cells that exhibit a hallmark of cellular differentiation, for example skin cells suitable for the study of hair growth or loss. The cells are transduced with a library of expressible nucleic acid sequences encoding random peptide aptamers that can be encoded, for example, in a eukaryotic expression vector, such as a retroviral vector. In a

particular embodiment, the vector (*e.g.*, the retroviral vector) includes an element to enhance or sustain gene expression, such as a chromatin insulator element.

The present invention further provides peptide aptamers identified using the improved methods described above. Peptide aptamers of the invention generally comprise between 5-9 (*e.g.*, 5, 6, 7, 8, or 9) amino acid residues or more. In one embodiment, the peptide aptamers are fused to an additional amino acid sequence, such as an intein, green fluorescent protein (GFP), a hedgehog polypeptide or functional derivative thereof, thioredoxin, a regulatory polypeptide involved in apoptosis, bcl-2, p53, an NFκB-related polypeptide, a caspase, PTEN, myc, a BH3 domain, a death domain (DD), a BIR3 domain, a BIR domain, a nuclear localization signal sequence, a membrane localization signal sequence, a farnesylation signal sequence, a transcriptional activation domain, a transcriptional repression domain, or a functional fragment thereof.

In a related embodiment, where the fusion moiety is an intein, suitable functional intein moieties are linked to each end of the peptide aptamer so that the peptide aptamer is flanked by intein splicing elements capable of catalyzing the formation of a cyclic peptide aptamer. The inteins flanking the peptide aptamer can further comprise a sequence which can be induced to join the inteins, such that the intervening peptide aptamer is cyclized in the presence of an inducing agent.

Peptide aptamers of the present invention can also include sequences derived from conotoxins which allow for cyclization of the peptide and enhance its ability to be efficiently and accurately screened. Such peptides are referred to herein as "conotides." In a particular embodiment, the conotide can be characterized by one of the following structural formulae:



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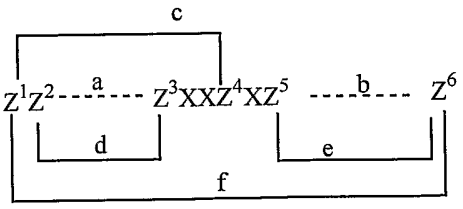

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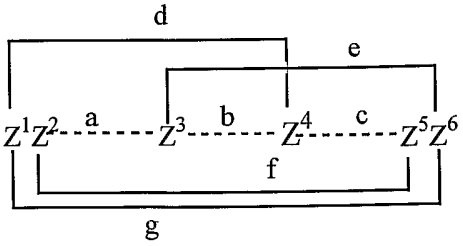

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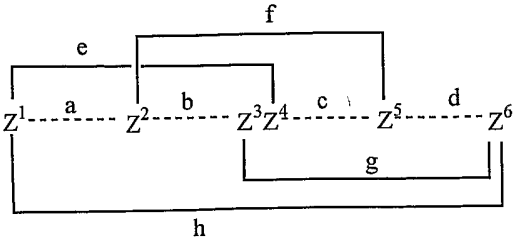

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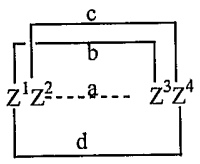

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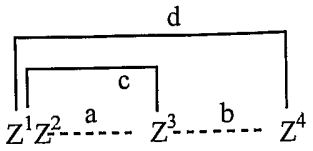

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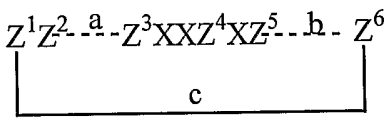

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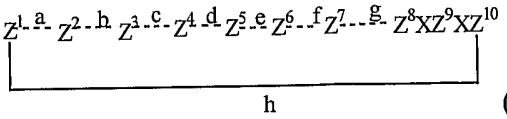

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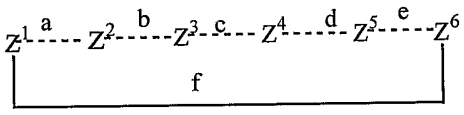


(IX)

wherein Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, Z^9, and Z^{10} are each independently selected linking residues as described below; and a, b, c, d, e, f, g, and h are each polypeptide linkers as described below. In a particular embodiment, the linkage (Z) is produced by intein-mediated splicing.

In still another embodiment, the invention provides a kit for screening a library of expressible nucleic acid sequences encoding peptide aptamers, conotides, or a panel of peptide aptamers or conotides, optionally with instructions for use.

Peptide aptamers and conotides of the present invention can be used according to art recognized techniques for the molecular modeling of an agent having similar structural and/or functional characteristics as the identified peptide aptamer. The peptides can also be used to treat a disease or condition associated with an aberrant (*e.g.*, misregulated) cell phenotype in a subject by administering to the subject, a therapeutically effective amount of the peptide, or a corresponding expressible nucleic acid (*e.g.*, by gene therapy). The misregulated cell phenotype can be associated, for example, with altered apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, differentiation, metabolic state, or a disease or condition such as cancer, neurodegenerative disease, viral infection or pathology (for example, due to an toxin), or a disorder associated with cellular differentiation, such as hair growth or loss.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 is an outline of the iterative phenotypic screens encompassed by the invention.

Figure 2 is an outline of the cyclic peptide libraries, and their structural and functional features, suitable for phenotypic screening and analysis, encompassed by the invention.

Figure 3 is a schematic illustrating the generation and screening of cyclic peptide libraries in cells using intein fusion moieties that allow for protein splicing and cyclization of the peptide aptamer in cells.

Figure 4 is an outline of the conotide libraries, and their structural and functional features, suitable for phenotypic screening and analysis, encompassed by the invention.

Figure 5 is a schematic illustrating the generation and screening of cholesterol or sterol-conjugated protein or peptide libraries through the use of hedgehog catalytic domains.

Figure 6 is a schematic illustrating the construction and use of a retroviral library to generate random peptide libraries for expression and phenotypic screening in mammalian cells. Nucleotide sequences encoding random amino acid residues are cloned into a retroviral vector for expression either as independent entities, or as fusions to other functional domains such as a functional self-processing domain derived from an intein or sterol-conjugating protein.

Figure 7 is a schematic illustrating intein-mediated protein splicing (panel A) and the functional residues found within the I_N and I_C elements of inteins that are directly involved in the splicing reaction and required for activity (panel B).

Figure 8 shows an alignment of the functional domains within naturally occurring inteins and a representative consensus sequence thereof.

Figure 9 shows a schematic of the chemistry of intein-mediated protein splicing whereby a cyclized peptide aptamer is produced.

Figure 10 shows a schematic of inducible intein-mediated protein splicing whereby the intein domains are inactive when fused to interaction domains Prot1 and Prot2 however, in the presence of a ligand, reassociate through Prot1 and Prot2 which restores protein splicing activity such that a cyclic peptide aptamer is produced.

Figure 11 shows the sequence of an exemplary sterol conjugating protein (hedgehog catalytic domain), peptide aptamer cloning site, and the Gly-Cys-Phe-Pro-Gly-Ser residues necessary for cleavage and sterol conjugation reactions to occur.

Figure 12 shows a schematic (panel A) and corresponding ribbon structure (panel B) of a constrained epitope (Myc) fused to GFP which is generated upon intein-mediated protein splicing.

Figure 13 shows the structure of a representative conotoxin (α-conotoxin) with free amino- and carboxy- termini and cysteine-mediated intramolecular disulfide bonds (panel A) and as fusion imbedded between self-splicing intein domains (I_N, I_C) before and after protein splicing (panel B).

Figure 14 shows a schematic of the screening method of the invention for identifying a peptide aptamer that alters a cell phenotype, in particular, an aptamer capable of causing apoptosis (cell death) in human leukemic cells (HL60) but not in normal cells.

Figure 15 shows a schematic of how the screening methods and peptide aptamers of the invention can be used to identify aptamers capable of altering a cell phenotype specific for a particular cancer cell which can then be tested in diverse panel of other cancer cell types to assess its range of action, such that an "aptamer profile" for each type of cancer can be assembled (see text).

Figure 16 shows a schematic of a screening method for identifying aptamers having antiviral activity such as the ability to block viral infection, viral replication, viral proliferation, or viral induced lysis or apoptosis.

Figure 17 shows a map of the pIMPS retroviral expression vector which allows for the convenient cloning of a nucleic acid or library of nucleic acids encoding peptide aptamer sequence(s) in frame with adjacent intein domains (I_C and I_N) for expression and intein-mediated peptide cyclization in cells.

Figure 18 shows a map of the an altered form of the pIMPS vector, in which a histidine leader epitope tag (6xHIS) is expressed at the carboxy-terminus of the intein flanked peptide for detection and/or quantitation, of either the unspliced or cyclic form of the peptide aptamer, by immunoblot or immunofluorescence.

Figure 19 shows a digital image of an immunoblot showing intein-mediated splicing of a test cyclic peptide (TCP) or green fluorescent protein (GFP), as compared to controls (*i.e.*, non-splicing (ns) forms), when expressed in mammalian cells.

Figure 20 shows a digital image of an immunoblot showing that the substitution of alanine residues (AA) for Gly-Pro ß-turn structures within a test cyclic peptide (TCP) decreases the efficiency of intein-mediated protein splicing.

Figure 21 shows a strategy for demonstrating constraint of epitopes presented by linking the amino and carboxy- termini of GFP over the "Top" of the cyclized GFP barrel structure.

Figure 22 shows a digital image of an immunoblot showing aptamers constrained over the "Top" of a cyclic GFP protein when expressed in mammalian cell extracts.

Figure 23 shows a digital image of an immunoblot showing the expression and intein-mediated splicing of a test cyclic peptide (TCP) and three different conotides when inducibly expressed in bacteria (*i.e.*, in presence (+) or absence (-) of IPTG). The lower panel shows the amino acid sequences of the test cyclic peptide (TCP) and representative conotides (CGRH, SCGS, and CCNP) expressed.

Figure 24 shows a schematic of how a sterol conjugation protein (catalytic domain of hedgehog) can be fused to a peptide aptamer (panel A) and, when expressed in cells and in the presence of a sterol(s), yield a sterol conjugated peptide sequence clipped free of the sterol conjugation protein (panel B).

Figure 25 shows a digital image of an immunoblot of cell extracts showing that a sterol conjugation protein (catalytic domain of hedgehog) can be fused to a test protein (GFP) and, when expressed in cells in the presence of a sterol, yield a sterol conjugated test protein clipped free of the sterol conjugation protein (hedgehog). "-" represents constructs containing alanine substitutions preventing cleavage; "SG" represents constructs with an additional Ser-Gly spacer to improve cleavage; and "*" indicates native test protein (GFP) which migrates faster than the conjugated GFP.

Figure 26 shows a diagram of the pcDNA4-ATRC-1 expression vector containing a cDNA encoding the mouse ATRC-1 receptor and a selectable marker for zeomycin resistance.

Figure 27 shows FACS results (panel A), and a graphical representation thereof (panel B), demonstrating that overexpression of the mouse ATRC-1 receptor in murine cells dramatically increases their susceptibility to transduction using a recombinant ecotropic retroviruses, as compared to a control.

Figure 28 shows a schematic of a strategy for selection and identification of activators of the erythropoietin receptor signal transduction pathway.

Figure 29 shows digital images of cells expressing two different test proteins (i.e., GFP and activated Ras) fused to a sterol conjugation domain (catalytic domain of hedgehog). GFP fused to a sterol conjugation domain remains functional (fluoresces) when expressed in cells and sterol conjugated in the presence of a sterol (top panels). Activated Ras (V12Ras) transforms cells in which it is expressed (middle left panel) but not if missing a membrane targeting domain (CAAX domain) (middle right panel). If activated V12Ras without a CAAX membrane targeting domain is fused to a sterol conjugation domain, the membrane targeting and transforming function of the mutant Ras protein is restored (lower left panel). This is dependent on a functional sterol conjugation domain (lower right panel).

Detailed Description of the Invention

The present invention provides improved methods and compositions for screening and identifying peptide aptamers (*e.g.*, from random libraries) that manifest identifiable phenotypes in cells. This includes not only the generation of peptide aptamer libraries, but also the delivery of the libraries into cells and the screening of the libraries by identifying alterations in a cell phenotype. Accordingly, the invention shall be described below under the following four (4) subheadings: 1) the making of peptide aptamers (libraries), 2) delivery and expression within cells of nucleic acids encoding peptide aptamers, 3) screening and identifying peptide aptamers capable of altering a cell phenotype, and 4) use of peptide aptamers for the prognosis, diagnosis, and/or treatment of a disease or disorder.

In describing the invention, the following definitions shall be applied:

Definitions

As used herein, the term "aptamer" or "peptide aptamer" refers to a polypeptide, generally between 2-40, preferably between 5-20 (*i.e.*, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20), most preferably between 5-10 (*e.g.*, 7) amino acid residues in length, capable of modifying a cell phenotype, *e.g.*, an observable characteristic of a cell, when introduced into or expressed in the cell (or organism). The polypeptide may be linear or cyclic. In a particular embodiment, the peptide aptamer exists intracellularly in a cyclic form generated through intein-mediated processing, or covalently conjugated to cholesterol or derivatized sterols generated through hedgehog-mediated processing (or functional derivative thereof). In another embodiment, the peptide aptamer comprises a sequence derived, in whole or in part, from a conotoxin peptide. Such peptide apatamers are referred to herein as "conotides." Cyclic peptides aptamers of the invention may also comprise one or more linking moieties. The peptide aptamers of the invention can be expressed intracellularly from a corresponding nucleic acid or produced (*e.g.*, synthesized) and used extracellularly (*e.g.*, for binding to a cell surface receptor) or intracellularly by introducing the peptide into a cell using art recognized techniques.

The term "conotide" refers to a class of peptide aptamers which include sequences and/or structural motifs derived from, or characteristic of, conotoxins. Conotoxins are naturally occurring, stable, and typically cyclic polypeptides having a range of biological activities, including, for example, modulating neuronal signals. The biological activity of conotoxins depends upon a looped structure that occurs through two or more cysteine-mediated intramolecular disulfide bonds. Accordingly, conotides of the invention are engineered to include sufficient conotoxin sequences such that, for example, the conotide exhibits improved stability, a cyclic structure (*i.e.* a contiguous,

cyclic polypeptide backbone), or improved biologic activity. The conotoxin-derived portion of the conotide is typically selected to provide for the cyclization of the peptide aptamer through one or more linkages, and may include, *e.g.*, the modification of one or more conotoxin derived residues, *e.g.*, cysteines, with a different amino acid or modified amino acid. The conotoxin-derived sequence of the conotide also may be substantially modified such that no original conotoxin-derived amino acid sequence remains but the spacing, or a similar spacing, of one or more cyclic linkages from the conotoxin does remain. Conotoxin-derived sequences may be derived from any known conotoxin protein, for example, thos included within the conotoxin superfamilies A, M, O, S, T, P, or non-disulfide rich conotoxins such as, *e.g.*, conantokin, contulakin, or contryphan (see, *e.g.*, Table 1).

The term "conotide library" refers to a group of two or more conotides or conotide-encoding nucleic acids.

The term "random" refers to differing sequences within the peptide aptamer which are not predetermined. Accordingly, a "random peptide aptamer" contains in whole, or in part, random sequences. In certain embodiments, the peptide aptamer further includes certain predetermined sequences, *e.g.*, to impart stability or secondary structure to the polypeptide. For example, certain peptide aptamers, such as conotides, typically contain a discontinuous random sequence that is interrupted by one or more predetermined or semi-random residues, such as linking residues. When referring to a codon, residue, or peptide, the term "random" means that, the codon, residue, or peptide may consist of any series of nucleotides or amino acids, except in the case of a stop codon which does not encode a residue and would result in an incomplete peptide aptamer.

The term "fusion moiety" refers to a polypeptide sequence which is linked (e.g., fused) to a peptide aptamer to improve its stability, affinity, bioavailability, visualization, detection and/or cause localization of the aptamer. Suitable fusion moieties include, but are not limited to, intein sequences (which allow for the cyclization of the peptide aptamer through intein-mediated protein splicing), hedgehog polypeptides and functional derivatives thereof (which can become linked to a sterol for membrane targeting of the peptide aptamer), localization sequences (*e.g.*, membrane localization sequence or nuclear targeting sequence which allow for targeting of the peptide aptamer to a particular cellular location), detectable marker proteins (*e.g.*, GFP (green fluorescent protein) or luciferase, which allow for visual detection of the peptide aptamer).

The term "processing-fusion moiety" refers to a subset of fusion moieties capable of being processed, *e.g.*, undergoing a catalytic cleavage reaction, and includes, *e.g.*, intein splicing moieties and sterol-conjunction moieties (*e.g.*, hedgehog derived proteins).

The term "hedgehog polypeptide" refers to any member of the class of hedgehog polypeptides (*e.g.*, mammalian, murine, or *Drosophila*), or derivative or homolog thereof, which has autoprocessing activity and the ability to mediate conjugation to a sterol, *e.g.*, cholesterol. The hedgehog polypeptide (or derivative thereof) may be produced by purifying from a native source, recombinantly produced, or synthesized. The hedgehog polypeptide can be modified, even substantially so, as long as its autoprocessing activity and the ability to become conjugated to a sterol remains intact. The catalytic domains for a number of hedgehog polypeptides, *i.e.*, the autoprocessing and/or sterol conjugation domains, have been described.

The term "peptide" or "polypeptide" and "protein" are used interchangeably throughout the specification and refers to two or more amino acid residues linked by a polypeptide bond.

The term "library of expressible nucleic acid sequences encoding random peptide aptamers" refers to a collection or plurality of nucleic acid sequences that encode different peptide aptamers (either alone or linked to a fusion moiety). Peptide aptamers differ randomly by one or more amino acids. Typically, the nucleic acid sequences are contained within a vector, for example, a plasmid, that can be propagated in a host cell, *e.g.*, a prokaryotic host, and can also be used to transfect or infect a eukaryotic cell. It is understood that the invention also encompasses peptide aptamer libraries, such as a collection of random peptide aptamers or conotides (or both), which can be produced recombinantly or synthetically and may exist in solution or in solid phase, for example, bound to a matrix, chip, or array.

The terms "vector", "vector construct", "expression vector", and "plasmid" are used interchangeably. The term "vector" also includes viral vectors, such as retroviral vectors derived from retroviruses, adenoviruses, SV40, adeno-associated viruses, or other suitable viruses recognized in the art.

The term "phenotype" includes any observable characteristic of a cell or organism. Thus, phenotypic changes selected for in the present invention include both histological changes which can be visually determined, as well as functional changes (including, *e.g.*, measurable changes in gene expression). Phenotypic changes also include a change in the ability of the cell or organism to undergo, *e.g.*, undesired cell growth, or survive a viral infection or a change of the ability of an infecting virus to replicate within the cell. The term phenotype also includes, for example, a change in levels of apoptosis, signal transduction (*e.g.*, cellular responses mediated by a receptor or a kinase, *e.g.*, a cell surface receptor, nuclear receptor, or G protein coupled receptor, or a tyrosine kinase or serine and/or threonine kinase), protein trafficking, cell adhesion, membrane transport, cell motility, metabolic state, survival or susceptibility to an infectious agent or toxin, or differentiation, as compared, for example, to a control cell.

In particular, the term phenotype includes a change in cellular lifespan or differentiation, hormone production, or metabolic processes such as cholesterol, sterol, or carbohydrate metabolism, or cellular processes associated with hair growth or hair loss (*e.g.*, propecia).

The term "intein" or "intein domain" includes any amino acid sequence which can undergo a protein/protein splicing reaction such that excision and/or cyclization of the amino acid or a linked amino acid sequence occurs. Accordingly, the term intein includes naturally-occurring or artificially engineered polypeptide sequence that can catalyze a protein / protein splicing reaction. A list of known inteins is published at http:www.neb.com/inteins.html and a partial list showing relevant consensus sequences is shown in Fig. 8 (see also Perler *et al.*, NAR, 25: 1087-1093 (1997)).

The term "split intein" includes an intein that has two or more separate regions which can catalyze a protein / protein splicing reaction but which are separated by an intervening sequence, *e.g.*, a protein of interest, *e.g.*, a random peptide aptamer.

The term "intein-mediated" includes a polypeptide (*e.g.*, aptamer) splicing or cyclization event that is catalyzed, at least in part, by an intein sequence, *e.g.*, an intein domain.

The term "protein splicing" includes the post translational cleaving and rejoining of a polypeptide.

The term "cyclization" refers to any chemical or enzymatic reaction which results in the cyclization of an amino acid sequence, preferably, *e.g.*, *via* a covalent bond.

The term "cyclic peptide", "cyclic polypeptide", or "cyclic protein" refers to a peptide or polypeptide sequence where at least a portion of its primary sequence is cyclized.

The term "retroviral vector" includes a vector containing, at least in part, retroviral sequences, which is capable of delivering a nucleic acid sequence (*e.g.*, a peptide aptamer encoding sequence) to a cell (*e.g.*, eukaryotic cell). Retroviral vectors (encoding a library of peptide aptamers) can be generated, for example, by transfecting a pre-constructed plasmid library into an appropriate retroviral packaging cell line.

The term "screen" includes an assay which comprises one or more parameters (or measurement thereof) to identify the presence of a particular aspect (*e.g.*, genotype or phenotype), whereas a "counterscreen" holds constant the foregoing aspect in the presence or measurement of other parameters to validate the predetermined aspect.

The term "phenotypic selection" refers to the assaying of any observable characteristic (including any measurable gene expression levels using, *e.g.*, gene array chips) of a cell or organism, *e.g.*, a cell or organism expresses or is contacted with one or more aptamers (*e.g.*, conotides) of the invention.

The term "linked", when referring to nucleic acids, includes the joining of two or more nucleotide sequences where each encodes an open reading frame and the open reading frame of each is preserved such that a genetic fusion polypeptide results. Accordingly, the term "linked", when referring to protein sequences, means conjugated as a single fusion protein, or embodied forms of intramolecular chemical conjugation.

The term "linking moiety" includes moieties which link non-adjacent amino acids (*e.g.*, linking residues) of the conotides of the invention. The linking moieties link the amino acid linking residues through covalent linkages (*e.g.*, carbon-carbon bonds, polypeptide linkages, disulfide bonds, thioether bonds, ether bonds, etc.), hydrophobic linkages (*e.g.*, aromatic stacking, *e.g.*, tryptophan stacking), or ionic linkages (*e.g.*, salt bridges, hydrogen bonds, etc.).

The term "linking residues" ("Z") includes one or more attaching moieties covalently bonded to a backbone (*e.g.*, an amino acid residue) of the conotides of the invention. The linking residue may be a natural amino acid (*e.g.*, cysteine or tryptophan) or it may be chemically modified (*e.g.*, to incorporate specific attaching moieties in the side chain) such that it is capable of performing its intended function. Methods for modifying side chains of amino acids are known in the art.

The term "attaching moiety" includes moieties which are capable of being linked (*e.g.*, independently or through the use of a linking reagent) to another attaching moiety (which may be the same or different) such that a linking moiety is formed. For certain linkages (*e.g.*, polypeptide linkages), the attaching moiety may be the C- or N- terminus of an amino acid.

The term "ionic linkages" includes ionic interactions between attaching moieties which enhance the stability, structure, or activity of the peptide aptamer, *e.g.*, conotide and, preferably, do not inhibit the peptide aptamer or conotide from performing its intended function. Attaching moieties suitable for forming ionic linkages ("ionic attaching moieties") include, but are not limited to, charged species such as ammonium, and species capable of forming hydrogen bonds (*e.g.*, sulfate, sulfite, phosphate, phosphite, nitrate, nitrite, SH, OH, NH_2, etc.). Generally, the ionic attaching moieties are attached *via* covalent bonds to a linking residue.

The term "hydrophobic linkages" includes interactions between attaching moieties which are mediated by, for example, van der Waals or other hydrophobic attractions, such as aromatic or tryptophan stacking, which may confer structural features, or such that the stability or activity of the conotide is not inhibited, and is preferably enhanced. Preferably, peptide aptamers or conotides comprising hydrophobic linkages have increased structural and chemical stability *in vivo*, as compared to such peptides without these linkages. Examples of attaching moieties which interact through

hydrophobic interactions include, but are not limited to, aromatic groups capable of aryl stacking, such as the side chains of tryptophan, phenylalanine or tyrosine.

The term "covalent linkages" includes covalently bonded chains of one or more atoms, which covalently link linking residues. The atoms of the chain may be, but are not limited to, carbon, oxygen, nitrogen, boron, phosphorous, sulfur, silicon, etc. In certain embodiments, covalent linkages may require one or more linking reagents for formation. The covalent linkage is generally formed through the interaction and chemical reaction of two or more attaching moieties and any necessary linking reagent, such that the covalent linking moiety is formed. Examples of attaching moieties for covalent linkages include, but are not limited to, alkenes, alkynes, amines, carboxylic acids, halogenated aromatics, aldehydes, and vinyl halides. Covalent linkages may be synthesized using, for example, transition metal catalyzed cross couplings, pinacol couplings, hydrozirconation, olefin metathesis, nucleophilic addition, aldol/Curtius rearrangements, and NBK coupling (Nozaki-Hlyama-Kishii,'Fiirstrier *et al.*, J Am. Chem. Soc. 1996, 118, 12349)). In a further embodiment, the covalently bonded chains of atoms in a particular covalent linkage has zero, one, two, three, four, five, six, seven, eight, nine, ten or more atoms in the chain, independent of any substitutions or cyclic moieties. In certain embodiments, such as when the linking residues of the covalent linkage are each cysteine, such as conotide derived from a naturally occurring conotoxins having such residues, the covalent linkage is not a disulfide linkage.

The term "polypeptide linkages" includes a covalent polypeptide linkage having at least one peptide bond. For example, the polypeptide linkages include moieties which comprise at least one peptide bond. Furthermore, the polypeptide linkages may comprise one or more amino acid residues connected through peptide bonds. The number of amino acid residues present in a particular polypeptide linkage may be selected such that the peptide aptamer or conotide manifests a specific function. The number may be selected for each occurrence separately. In certain embodiments, the polypeptide linkages may comprise zero, one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more amino acid residues. In another embodiment, the polypeptide linkage is produced through intein-mediated protein splicing.

The term "membrane transport" includes facilitated movement of a determined ion, protein, nucleic acid, or class of molecules across the plasma, mitochondrial or nuclear membrane of a cell.

The term "sterol" or "sterol derivative" includes any compound which contains a sterol functional group and can be conjugated to a polypeptide by a hedgehog polypeptide as defined above (*e.g.*, l, 7-dehydrocholesterol (5,7-cholestadien-3β-ol), 5-androsten-3β-ol, desmosterol; 24-dehydrocholesterol (5,24-cholestadion-3β-ol), β-

silosterol (5-stigmasten-3β-ol), 7β-hydroxycholestrol (5-cholestene-3β, 7β-dial), and cholesterol (5-cholestene-3β-ol).

The term "secondary library" includes a library that is derived from, or is a subset of, a first library such that the secondary library has a higher probability or bias for containing or encoding a molecule having a desired characteristic. Typically, the secondary library is the product of a selection or screening method using, *e.g.*, a biochemical assay (*e.g.*, a binding assay), cell-based assay, or in vivo assay (*e.g.*, where an animal is administered a library of aptamer peptides, *e.g.*, expressible aptamer peptides in the form of a gene therapy vector which after, *e.g.*, a period of time or challenge, the remaining expressible aptamers are isolated from the animal, *e.g.*, re-cloned or identified from a cell or tissue source).

The term "chromatin insulator" includes a cis-acting sequence within an expression vector that enhances expression of coding sequences within the vector, and which can perpetuate expression throughout passage or differentiation of the transduced cell.

The term "effector" includes a naturally-occurring cell-associated (*e.g.*, endogenous) polypeptide that is, directly or indirectly, responsible for a cellular phenotype.

The term "cancer" includes any neoplasm, such as a carcinoma (derived from epithelial cells) or sarcoma (derived from connective tissue cells) or a cancer of the blood, such as a leukemia.

The term "viral resistance" includes the level of infection or cytopathic effect measured after a cell is infected, transduced, or otherwise invaded by a virus.

The term "apoptosis" includes any non-necrotic, cell-regulated form of cell death, as defined by criteria well established in the art.

The term "cell" includes any prokaryotic or eukaryotic cell. A eukaryotic cell can be a fungal cell (*i.e.*, yeast cell), insect cell (*e.g.*, Schneider and sF9 cells), or somatic or germ line mammalian cell, or cell line *e.g.*, HeLa cell (human), NIH3T3 (murine), RK13 (rabbit) cells, an embryonic stem cell (*e.g.*, D3 and J1), and cell types such as a hematopoietic stem cell, myoblast, hepatocyte, lymphocyte, and epithelial cell and, *e.g.*, one of the following cancer cell lines. HL-60, HeLa, HepG2, Molt-4, NCI-H23, DMS114, HCC-2998, U251, SK-MEL-28, SK-OV-3, A498, PC-3, and SK-BR-3.

The term "surrogate signaling molecule" refers to a molecule that brings about a cellular response that phenotypically mimics the response of a cell to ligand engagement of a specific signal transducing receptor, but which is brought about in the absence of a ligand that would normally manifest that response. In addition, a surrogate signaling molecule may increase the sensitivity of a cell to receptor-ligand engagement, thereby allowing response to contacting the receptor and ligand, but at ligand concentrations

lower than those necessary for the activity of the ligand in the absence of a surrogate signaling molecule.

The term "predetermined" refers to aptamer sequences that are designed to have particular non-random amino acids or motifs.

The term "selecting" refers to the identifying of cells phenotypically or functionally, *e.g.*, as compared to control cells, as well as cells that are physically selected (*e.g.*, cell sorting, survival).

The term "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. Methods for generating such transgenic animals (*e.g.*, *via* embryo manipulation and microinjection), particularly animals such as mice, are well known in the art as described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo, Second Edition* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1994).

I. GENERATION OF PEPTIDE APTAMERS

1.1 Generation of Peptide Aptamers and Libraries Thereof

The peptide aptamers of the invention contain random combinations of a small number of amino acid residues, *e.g.*, 5, 6, 7 or more, but preferably less than 100, more preferably less than 50, and most preferably less than 20. The peptide aptamers of the invention can be produced recombinantly, from a corresponding nucleic acid sequence, or synthetically using art recognized techniques in peptide chemistry. A library of nucleic acid sequences encoding random peptide aptamers can be generated by combinatorial mutagenesis at the nucleic acid level. Alternatively, a variegated library of nucleic acid sequences encoding random peptide aptamers can be produced using chemical synthesis of a degenerate aptamer gene sequence using an automatic DNA synthesizer, where the synthetic gene is then ligated into an appropriate expression vector, *e.g.*, a retroviral vector. Use of a degenerate set of genes allows for, in one mixture, all of the sequences encoding the desired set of potential random peptide aptamer sequences to be represented. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

1.2 Generation of Peptide Aptamers Linked to Fusion Moieties

The peptide aptamers of the invention can be produced as free entities, or depending on the target of a given screen, as fusions to a heterologous protein, such as a protein, interchangeably referred to as a "protein scaffold" or "fusion moiety", *e.g.*, "processing-fusion moiety", that can promote, *e.g.*, expressibility, intracellular cyclization, or modification, localization, stability, secretability, isolatablitiy, or detectability of the peptide aptamer (see, *e.g.*, Figs. 1-4).

Fusion moieties can be "neutral" moieties that allow monitoring of expression (*e.g.* a catalytic or detectable moiety such as chloramphenicol acetyltransferase, ß-galactosidase, or green fluorescent protein). Alternatively, fusion moieties that encode targeting domains such as a nuclear or membrane localization signals, transcriptional activation or repression domains, a CAAX farnesylation signal sequence that directs membrane localization, MHC proteins, SH2, or SH3 domains, BH3, DD, BIR3, or BIR domains, can be used to direct peptide aptamers to other functional or regulatory cellular circuits. Still further, the fusion moieties can constrain the peptide aptamer, by for example, reducing flexibility, *e.g.,* rotational degrees of freedom (*e.g.*, using a proline), allowing flexibility (*e.g.*, using a Gly-Pro or Pro-Gly to increase the β-turn character of the peptide and facilitate cyclization), and/or providing certain residues, residue positions, and/or linkages that allow for intrapeptide bonds or cyclic backbone structures to be formed (*e.g.*, as contained within a conotide as described below).

The fusion moieties that constrain or loop a aptamer peptide thereby making it cyclic are desired in such phenotypic screens, because of the intrinsic structural organization they impose upon the peptide (*e.g.*, the backbone of the peptide is not free to swivel and allow for many transient conformations) and because cyclic peptide aptamers are much more stable than linear peptides (*e.g.*, have a longer intracellular half-life). Naturally-occurring peptides with looped structures often contain cysteine-mediated intramolecular disulfide bonds, which are not stable in the reducing environment of the mammalian cell cytoplasm. Thus, the peptide aptamers of the invention overcome the limitations of other strategies that use linear or looping sequences without cyclization by forming stable covalently closed peptide aptamer loops within the cell. Typically, the resulting peptide aptamer spontaneously cyclizes within the cellular milieu and contain no other sequence except random aptamer sequence or, optionally, a fusion moiety such as, *e.g.*, thioredoxin or GFP. The cells expressing such modified peptide aptamers are then monitored for any phenotypic change as described herein.

Other fusion moieties include processing-fusion moieties (and products thereof) which are capable of catalyzing a protein modification, *e.g.*, splicing of the protein (including inducible splicing) or conjugating the protein to a sterol, and these aspects of the invention are introduced in following three subsections (1.3, 1.4, and 1.5).

1.3 Generation of Cyclic Peptide Aptamers Using Protein Splicing

In a particular embodiment, the invention provides for the generation of cyclic peptide aptamers with improved stability using protein splicing. The cyclization of the peptide aptamer is achieved by linking to the peptide aptamer a fusion moiety that contains an intein sequence (or functional derivative thereof) (see Figs. 3 and 7). Intein sequences are naturally-occurring protein sequences which have been identified in a number of proteins and can catalyze self-splicing of the protein in which they are found (see Fig. 8).

Inteins are the protein equivalent of introns, in the sense that they are internal sequences that are removed from a full-length immature host protein in order to form a functional product. This catalytic reaction results in the excision of the intein and in the formation of a mature host protein. This naturally occurring, spontaneous process is depicted in Fig. 7. Inteins exist in a large number of organisms and so far, all known inteins have been compiled in a database (http://www.neb.com/neb/inteins.html; see also Fig. 8.). Exemplary intein sequences which can be fused to a random peptide sequence include, *e.g.*, those comprising or derived from *DnaE* from *Synechocysti* (see *e.g.* Example 1).

Importantly, inteins can be split to generate cyclic peptides (see, *e.g.*, WO 00/36093). In that case, intein moieties capable of carrying out the intein splicing reaction are split in two parts surrounding the peptide to be cyclized. The chimeric immature product is composed of the carboxyl-terminus half-part of the intein (Int-C), the peptide of interest, followed by the amino-terminus half-part of the intein (Int-N). The association of Int-C and Int-N reconstitutes a functional intein and results in the auto-catalytic excision of the central peptide in a cyclic form (cyc-Pep). The generation of a cyclic peptide as opposed to a linear peptide (lin-Pep) presents several advantages: an increased stability of cyc-Pep compare to lin-Pep due to its potential resistance to peptidases and a conformational stability due to the structural constraints imposed by the cyclization (see Fig. 9).

Accordingly, using genetic engineering techniques, these elements can be used to modify a target sequence such that, when expressed as a polypeptide, the added intein motifs will catalyze a protein splicing reaction (as described, for example in U.S. Pat. No. 5,496,714 and WO 00/36093). Spontaneous self-splicing of the intein yields a cyclized form of the intervening peptide: the product of this reaction is a cyclized

peptide in which the boundary features have been extruded during the intein slicing reaction. Accordingly, in one embodiment, the nucleic acids encoding the peptide aptamers are artificially engineered to further encode self-splicing intein elements flanking the intervening random peptide aptamers.

The self-splicing peptide aptamer libraries can be expressed in any eukaryotic cell, *e.g.*, mammalian cells, by means well known in the art, including standard expression plasmids, *e.g.*, retroviral expression vectors. Typically, such libraries contain flanking sequences capable of self splicing the intervening peptide, which is composed of random amino acid sequences of specified length (*e.g.*, at least three, preferably four, five, six, seven or more amino acids).

The vectors encoding the self-cyclizing peptide libraries are then introduced into the test cell, *e.g.*, a mammalian cell, and the cells are subsequently analyzed for acquisition of a specified peptide aptamer-dependent phenotypic change.

1.4 Generation of Cyclic Peptide Aptamers Using Inducible Protein Splicing

The invention can also be performed using aptamers that are first expressed as inteins which then are processed under inducible control. Normally, the split inteins, as discussed above, undergo cyclic catalysis without regulation. By contrast, the invention provides a system where the formation of the cyc-Pep can be controlled at any time by engineering an intein where the critical residues are retained that participate directly in the chemical reactions leading to peptide backbone cleavage and rejoining, but where the 2 independent components of the split-intein that act to bring together these reactive sites are replaced, in whole or in part, by proteins or domains capable of interacting only under controlled conditions (see Fig. 10). This confers the advantage of inducing the production of cyclic peptides at very defined times and under very defined conditions. Accordingly, an important advantage of this inducible system is the ability to identify cyclic peptides during a biological screen that may have secondary effects or toxicity that otherwise would not normally allow their evaluation or detection.

Inteins have been well studied and the critical residues necessary for the self-splicing catalysis and for the endonuclease activity have been identified (Liu 2000, Paulas 2000, and Perler 1997). Accordingly, the invention provides two polypeptide domains, Prot1 and Prot2, which replace respectively, Int-C and Int-N, for producing cyclic peptides. As opposed to the constitutive splicing activity catalyzed by split-inteins, the catalytic reaction of the engineered intein Prot1/Prot2 is based on the inducible propensity of Prot1 and Prot2 to interact under precise conditions. These conditions can occur by the addition of a chemical compound that promotes the interaction of Prot1 and Prot2 or the induction of a signaling pathway in the cell that

results in the covalent modification of either Prot1 or Prot2 which will then induce their binding to each other.

Preferably, Prot1 and Prot2 are selected for their small size; that they do not interact in the absence of the defined conditions or compound; that they do interact with a strong affinity in the presence of the defined conditions or compound; that the compound allowing the interaction of Prot1/Prot2 is preferentially cell permeable and with no additional or strong interfering effect than its binding to Prot1 and Prot2; and that once associated, the C-terminal domain of Prot1 is within a relatively small distance of the N-terminal domain of Prot2.

Accordingly, in one embodiment, the Prot1 and Prot2 is the immunophilin FKBP12 which forms a dimer in presence of the chemical compound FK1012. The dimerization of FKBP12 can be reverted by the monomeric molecule FK506.

In another embodiment, the Prot1 and Prot2, are, respectively, calmodulin and the regulatory domain of the protein kinase CaMPKII, which forms a dimer in the presence of calcium. Release of intracellular calcium can be achieved by , *e.g.,* various art recognized cellular stimuli or by the addition of a calcium ionophore, such as A23187. Calcium levels can also be modulated through calcium chelators.

In another embodiment, *Escherichia coli* dihydrofolate reductase enzyme (DHFR) binds the ligand methotrexate (MTX) and can form a dimer in presence of bisMTX, a homobifunctional version of MTX. Thus, this domain can be used for the Prot1 and Prot2 domains.

In yet another embodiment, the vitamin D receptor (VDR) binds to NCoR or to Alien and these proteins are used for the Prot1 and the Prot2 domains. The 1,25-dihydroxyvitamin D3 dissociates these complexes and stimulates the transcriptional activity of the receptor.

In still another embodiment, the human growth hormone (hGH) and the extracellular portion of the growth hormone receptor (hGHR) are engineered so that they can no longer interact. Mutations introduced into human growth hormone (hGH) (Thr175 Gly-hGH) and the extracellular domain of the hGH receptor (Trp104 Gly-hGHbp) created a cavity at the protein-protein interface that resulted in binding affinity being reduced by a factor of 10^6. A small library of indole analogs was screened for small molecules that bind the cavity created by the mutations and restore binding affinity. The ligand 5-chloro-2-trichloromethylimidazole was found to increase the affinity of the mutant hormone for its receptor more than 1000-fold (Guo *et al.*, 2000). These hGH domains can be fused to catalytic intein sequences, such that the Ic and In catalytic residues are brought in to proximity by addition of 5-chloro-2-trichloromethylimidazole, thereby inducing intein mediated splicing and peptide

cyclization. Accordingly, these domains can be used for Prot1 and Prot2, as depicted in Fig. 10.

Finally, the SH2 domains are structural protein domains that recognize phosphorylated-tyrosine (P-Tyr) in a specific peptide context. The phosphorylation of tyrosine is a common event that follows the activation of tyrosine kinase in signal transduction pathways in response to a specific treatment of the cells. Thus, SH2 can be used, *e.g.,* for Prot1 and the domain containing a particular Tyr, as Prot2.

1.5 Generation of Sterol-Conjugated Peptide Aptamers

In another preferred embodiment, the invention also includes fusion moieties that can be attached to a peptide aptamer and are capable of conjugating the peptide aptamer to a ligand, *e.g.*, a sterol. In a particular embodiment, the aptamer sequence is linked to a sufficient portion of the hedgehog polypeptide such that the aptamer is subsequently linked to a sterol, *e.g.*, cholesterol, and targeted to a membrane (see, *e.g.*, Figs. 5, 11, and 24)

As described in detail herein, the peptide aptamer libraries can be generic (*i.e.*, encode only minimal, random peptide aptamers), incorporate structural features for stability (*e.g.*, the peptides aptamers are fused to scaffold proteins or produced as cyclic peptides) and/or detection (*e.g.*, fused to GFP), or incorporate features that suit them for the study of particular phenotypes associated with certain processes or specific intracellular locations (*e.g.*, the aptamer is fused to a heterologous localization domain).

1.6 Generation of Peptide Aptamers "Conotides" Derived from Conotoxins

Peptide aptamers (and libraries thereof) can also be generated using sequence motifs derived from conotoxins to produce peptide aptamers (referred to herein as "conotides") which have desired structural and biologic properties (Jones *et al.*, Composition and Therapeutic Utility of Conotoxins from Genus *Conus*. Patent Status 1996-2000. Exp. Opin. Ther. Patents 11: 603-623 (2001)). Conotoxins were originally isolated from predatory cone snails (genus *Conus*) which can immobilize and kill their prey using a cocktail of neurotoxic peptides referred to as conotoxins. These conotoxins are small, 10-30 amino acids in length, and they are constrained, usually by two or more sets of cysteine-mediated disulfide bonds. Naturally occurring conotoxins function by inhibiting the function of various ion channels present in neuronal or muscular tissues.

The present invention uses *in vivo* and synthetic combinatorial peptide chemistry to exploit the use of conotoxin backbones, to identify novel conotoxin or conotoxin-like peptides (conotides) with improved or altered target affinity or specificity. In addition, the invention also provides a variety of novel strategies that can be used alone or in combination to generate and screen peptide libraries in which certain conotoxin- like

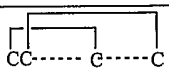
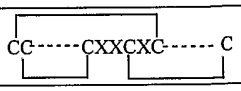
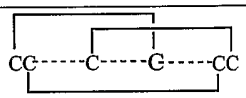
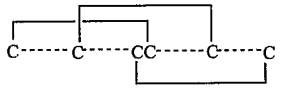
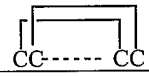
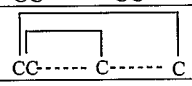
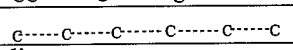
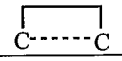
structural elements are retained, but which improve various characteristics of the conotoxin peptides, including but not limited to, increasing peptide stability, or removing the need for one or more of the disulfide bonds present in the naturally occurring prototypic backbone structures.

In accordance with the present invention, one or more cysteine residues is removed and the conotides generated are preferably highly stable and constrained. Cysteine independent constraints, *e.g.*, linking moieties, featured in this type of molecule are designed to be less susceptible to the redox environment within the cell, thereby maintaining their structural integrity in various cellular microenvironments (*e.g.* endoplasmic reticulum, Golgi apparatus, lysosomes), or to degradation or redox effects in the extracellular environment or bloodstream of an animal or human. Moreover, synthesis of such random peptide libraries within the cell allows examination of their effects upon intracellular targets. This is in contrast to naturally occurring conotoxins, which evolved to act upon extracellular targets or ligands.

The constrained structural features and characteristics of the conotoxin peptides represent an ideal scaffolding strategy for the design and synthesis of peptide aptamer libraries. Indeed, the processes of natural selection that have led to their existence and prevalence in nature suggest that these are optimized formats for the generation of biologically active peptides, particularly peptides that act upon ionic or voltage gated channels. Due to their specificity, the effectiveness of small helical shapes, and the availability of a cysteine framework, conotoxin-based conotides are suitable for use as the basis for drugs with importance in a number of therapeutic areas.

The known, naturally occurring conotoxins have been divided into families based on conserved cysteine residues and function. A summary of the various conotoxins, their classification, structure, and mode of action is shown in Table 1, below. The several major superfamilies include the A, M, S, and O superfamilies; at least 4 other superfamilies may exist. A given conotoxin is usually highly specific for its target. Some examples include the α-conotoxins, which inhibit nicotinic acetylcholine receptors; ω-conotoxins, which block voltage-gated calcium channels; and δ-conotoxins, which alter activation of voltage-gated sodium channels

Table 1. Summary of the various conotoxins, their classification, structure, and mode of action.

Super Family	Family	Mode of Action	Linkage Pattern in Native Conotoxin
A	α	Competitive antagonist of nAchRs	
	β	Antagonist of α_1-adenoreceptors	
	αA	Competitive antagonist of nAchRs	
	κA	Inhibits conductance of voltage-gated K^+ channels	CC-----CXXCXC------ C
M	μ	Blocks Na^+ channels by binding to Site I	
	ψ	Non-competitive inhibitor of nAchRs	
O	δ	Delays Na^+ channel inactivation	
	ω	Blocks Ca^{+2} channels	
	γ	Modulates channels	
	μO	Blocks Na^+ channels but does not bind to Site I	
	κ	Blocks K^+ channels	
	Bromosleeper	Unknown	
	Conotoxin GS	Blocks Na^+ channels	
S	σ	Competitive antagonist of the 5-HT_3 Receptor	C--C--C--C--C-C-C-C---CXCXC
T	τ	Unknown	
	χ	Noradrenaline transporter blocker	
P	N/A	Unknown	
Non-disulfide rich	Conantokin	Antagonist at NMDA receptor	linear
	Contulakin	Agonist at neurotensin receptor	linear
	Contryphan	Unknown	

In Table 1, C represents cysteine residues, X represents a single amino acid residue, the dotted line represent a polypeptide linkage and the solid line represents a disulfide covalent linkage.

The A superfamily includes the α and αA families, which target nicotinic acetylcholine receptors, the ρ family, which is an antagonist of α1-adreno receptors, and the κA family, which inhibits conductance of voltage-gated K^+ channels. The M superfamily includes the μ family of conotoxins, which block Na^+ channels, as well as the ψ family, which noncompetitively inhibit nicotinic acetylcholine receptors.

The O superfamily consists of a number of families with an identical cysteine pattern. Members of the O superfamily include the δ, ψ, γ, μO, and κ families. These families can be used to block or deactivate ionic channels, including Na^+, Ca^{2+}, and K^+

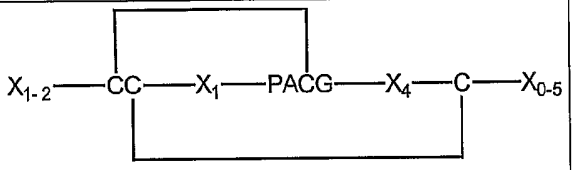
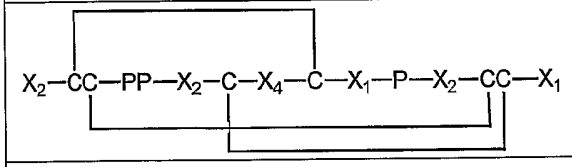
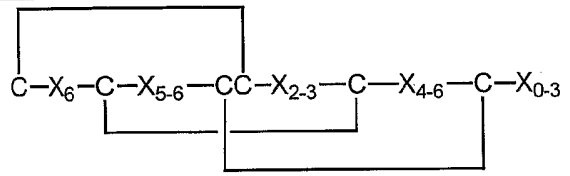
channels. The O superfamily also includes the conotoxin-GS family, which can be used to block Na^+ channels, and this family also includes Bromosleeper.

There are several other families of conotoxins. Three other currently known superfamilies are S, T, and P. These superfamilies include the σ, τ, and χ. There are also several conotoxin-like peptides; these include conantokins, contulakin, and contryphan. Members of the conotoxin-related conantokin peptides function similarly to conotoxins, but do not contain disulfide bonds. Instead, the conantokin structure is dependent on the presence of calcium binding by numerous gamma-carboxy-glutamic acid residues within these peptides (Prorok *et al.*, Biochemistry, 35(51):16528-34 (1996)). Contryphan contains only one disulfide bond. The activity of these conotoxin-like peptides requires the constrained helical shape present in the conotoxin/conantokin family and the disulfide bonds that sponsor and stabilize this helical configuration.

One exemplary conotoxin isolated and characterized was α–conotoxin GI. The 13 amino acid α–conotoxin GI peptide (ECCNPACGRHYSC-NH_2) has a pair of disulfide bonds. The intramolecular disulfide bond between Cys3 and Cys13 serves as a "clamp", to keep the N- and C- terminal ends of the peptide in proximity. The N-terminal glutamic acid residue of GI can be deleted without affecting biological activity. The disulfide bond between Cys2 and Cys7 establishes the conserved small-loop structure characteristic of all α–conotoxins. The importance of this structure is evidenced by the observations that mutations in this small-loop region lead to a loss of biological activity, and substitution of Pro5 eliminates the biological activity of α–conotoxins MI and GI.

It is understood that any of the conotides described herein can, in lieu of or in addition to any actual or potentially linkages, can be cyclized using intein-mediated protein splicing (see, *e.g.*, Fig. 13), for example, as indicated below in Table 2. Here, the left panel shows the cysteine positioning and spacing associated with the various subtypes of naturally occurring conotoxins, along with the configuration of structurally essential cysteine-mediated intramolecular disulfide bonds that are known to occur in these peptides. The right panel exemplifies the manner in which these respective subtypes can be equivalently integrated into an intein- splicing format and expressed in cells for the generation of conotides.

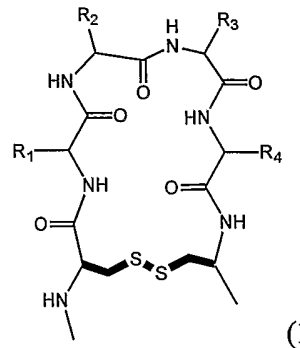
Table 2. Exemplary Conotides

Canonical Conotoxin Sequences	I_C-Conotide-I_N
	I_C X_{1-2}—CC—X_1—PACG—X_4—C—X_{0-5} I_N
	I_C X_2—CC—PP—X_2—C—X_4—C—X_1—P—X_2—CC—X_1 I_N
	I_C C—X_6—C—X_{5-6}—CC—X_{2-3}—C—X_{4-6}—C—X_{0-3} I_N

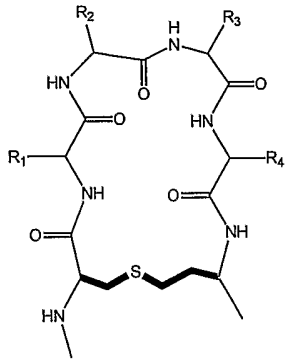
1.7 Generation of Synthetic Conotides

This invention also describes synthetic chemical approaches to the improvement of conotoxins or conotoxin derived peptides. The linking moieties of the conotides of the invention generally replace one or more of the disulfide bridges of the native conotoxins, respectively. Linking moieties can also be introduced at positions where a linkage would not inhibit the conotide from performing its intended function. Advantageously, the linking moieties enhance the stability of the aptamer in *in vivo* environments.

Conotides linked through polypeptide linking moieties may be synthesized *in vivo* through intein mediated cyclization chemistry described above. Alternatively, a variety of chemical ligation or linking methods, such as those described below, may also be used. Examples of linking moieties, include, for example, moieties which comprise disulfide bonds (**1**) and moieties which comprise thioether linkages (**2**). Disulfide bonds and thioether bonds are found in some naturally occurring peptides such as conotoxins and antimicrobial tachyplesins, protegrins and defensins are shown in the formulae below.

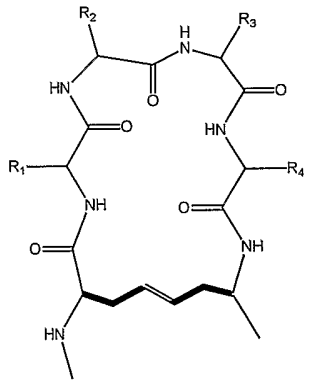


(1)

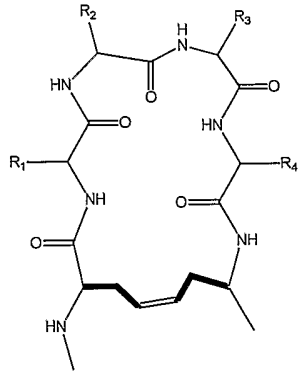


(2)

The linking moiety may also comprises carbon atoms in the atom chain. For example, the linking moiety may be alkyl, alkenyl, or alkynyl. Alkenyl linking moieties may be synthesized using ring-closing-metathesis (RCM) reactions. This reaction has been exploited for the synthesis of cyclic aptamers with alkenyl linking moieties (**3, 4**). The RCM reaction can be performed either in solution or on a solid support giving the cyclic peptide in a yield of 50-65%.

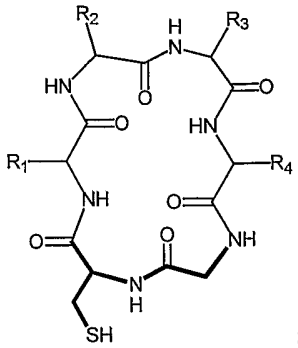


(3)



(4)

Other linking moieties also include those which link the N- and C- termini of the polypeptide aptamers of the invention together. End-to-end cyclic peptides are comparatively more stable to enzymatic digestion and have a better bioavailability due to the lack of N- and C- termini. Examples of linking moieties which can be used to link the ends of an aptamer include those shown in formulae **5**, **6**, **7**, and **8**. Other linking moieties which may be used include linking moieties which comprise one or more peptide bonds.



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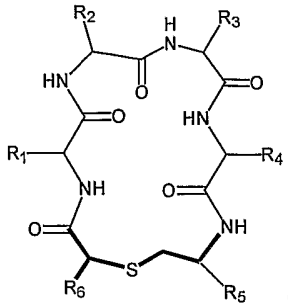

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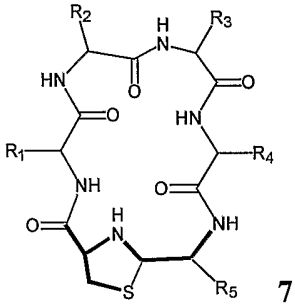

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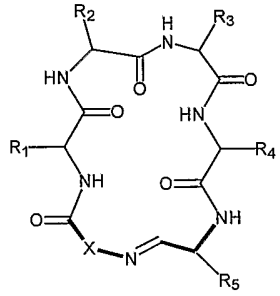
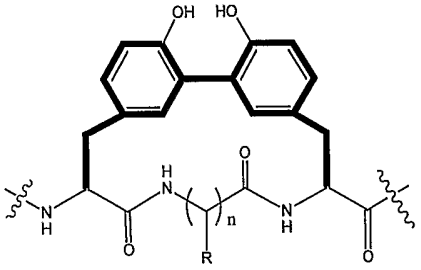


8

Conotides with peptidic linking moieties (such as those shown in **5**) can be synthesized using an intramolecular thioester ligation reaction using art recognized techniques. For example, the unprotected N-Cys peptide thioester precursor undergoes a two-step reaction in 6 M Gua·HCl at pH 7.6 to afford the conotide in a quantitative yield [24]. Cyclic aptamers of type **6** can be obtained by a cascade thioester ligation.

Other linking moieties also include moieties such as the thiozolidine linking moiety shown in **7**. The key intermediate for the synthesis of this compound is the unprotected peptide aldehyde. The unprotected peptide aldehyde can also be used for the synthesis of the cyclic aptamer **8** through an oxime ligation.

Conotides also can be formed by reacting at least two attaching moieties of linking residues with each other under appropriate conditions, such that the linking moiety is synthesized such as shown in structures 9-17 below. For example, in the formation of certain linking moieties the appropriate conditions comprise one or more linking reagents.



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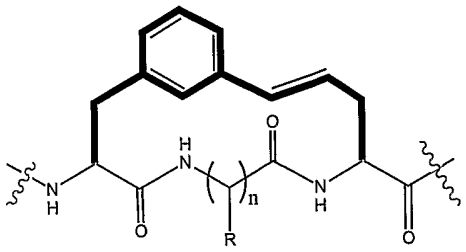

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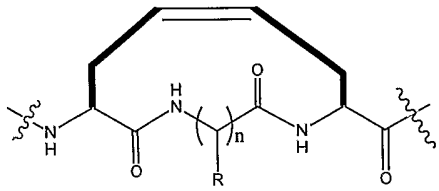

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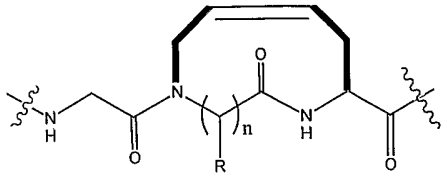

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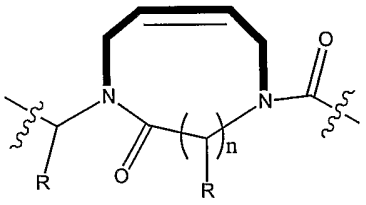

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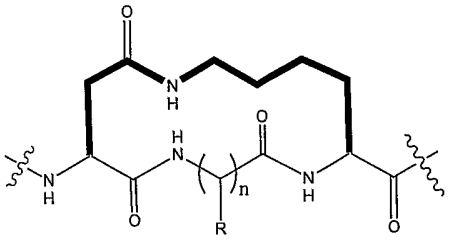

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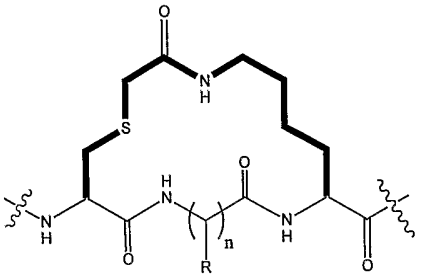

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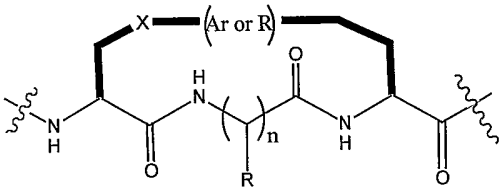

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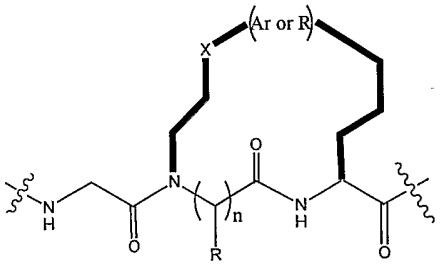


17

Linking reagents, such as sulfonyl chloride, dimethyl dichlorosilane, diisopropylamino, chlorophosphoramidite and metals (such as boron and titanium) are capable of temporary binding as a linking moiety. One of ordinary skill in the art will realize that other linking reagents can be employed to generate mixed carbonates, carbamates, disulfides, ureas, acetals, ortho esters, phosphates and oxides as linking moieties.

The linking moiety may be synthesized at any amino acid residue site which includes a covalent attaching moiety, *e.g.*, functionality capable of reacting with another covalent attaching moiety, optionally in the presence of a linking reagent, to form a

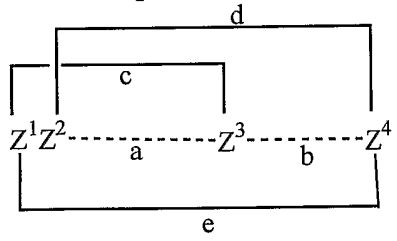
linking moiety. Preferably, the attaching moiety comprises a hydroxyl, amino, or thiol group. Examples of attaching moieties include hydrogen, protecting groups, alkenes, alkynes, amines, carboxylicacids, halogenated aromatics, aldehydes and vinyl halide.

In one embodiment, the linking moiety comprises a silicon atom. In this embodiment, the linking residues ("Z") are linked by a linking reagent. In a further embodiment, the linking residues each have a heteroatom-containing attaching moieties and are linked together by means of a silicon linking reagent such as dimethy1dichlorosilane. In another embodiment of the invention, the linking residues each contain a hydroxyl or amine attaching moiety and diisopropylamino chlorophosphoramidite is used as the linking reagent.

In one embodiment, the linking residues each comprise alkene attaching moieties. These linking residues are then subjected to conditions appropriate for an intramolecular metathesis reaction, such that an alkene linking moiety is generated. Examples of suitable linking reagents include, but are not limited to transition metal catalysts (*e.g.*, Grubbs' catalyst and others cited in *Tet. Lett.*, 1999,40., 2247).

Transition metal mediated crosscoupling reactions may also be used to synthesize linking moieties. Examples of transition metal mediated cross coupling reactions which can be used include but are not limited to, palladium catalyzed Heck Reactions (*e.g.*, in which a haloaromatic linking residue is reacted with a second linking residue under palladium (0) catalysis to yield aromatic and alkenyl linking moieties).

In an embodiment, the invention pertains to conotides of the formula (I):



(I)

wherein

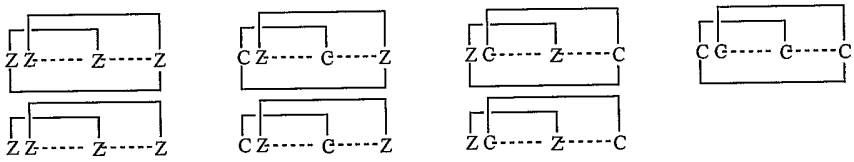
Z^1, Z^2, Z^3, and Z^4 are each independently selected linking residues;

a and b are each polypeptide linkers comprising one or more amino acid residues; and

c, d and e are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of c, d, and e is not a disulfide linking moiety; and provided that at least one of c, d, and e is not absent.

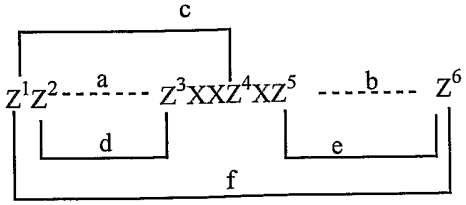
In a further embodiment, conotides of formula (I) comprise one or more linking moieties which are covalent linkages (*e.g.*, c, d, or e), as described above. In a further embodiment, at least one of c, d, or e is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, and Z^4 are cysteine. In another embodiment, at least one of c, d, and e is not a disulfide linkage. In certain embodiments, at least one of c, d, or e is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3 and Z^4 are tryptophans. Examples of e include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of c, d, or e, include ionic linkages. Preferably, the conotide of formula (I) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at c and d and with e absent. In a further embodiment, a and b comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a and b each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a and b polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (I), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

The invention also pertains to conotides of the formula (II):



(II)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

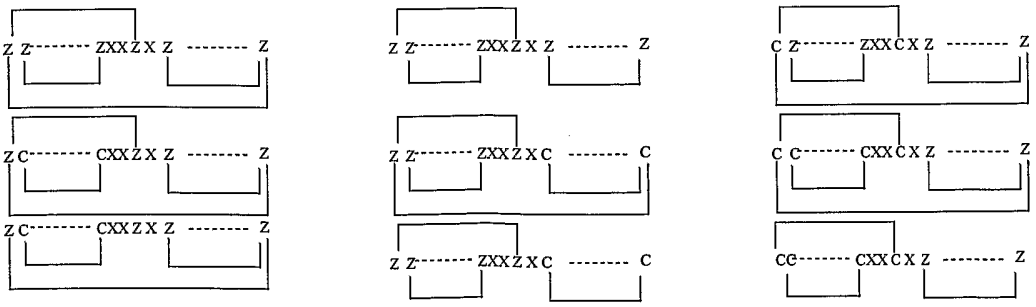
X is an independently selected amino acid residue for each occurrence;

a and b are each independently selected polypeptide linker moieties comprising one or more amino acid residues; and

c, d, e, and f are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are cysteine, then at least one of c, d, e, and f is not a disulfide liking moiety; and provided that at least one of c, d, e, or f is not absent.

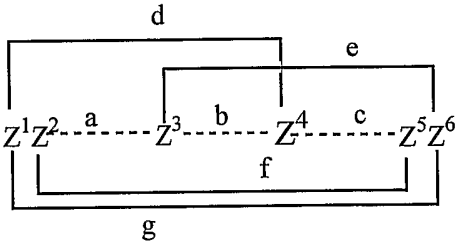
In a further embodiment, conotides of formula (II) comprise one or more linking moieties which are covalent linkages (*e.g.*, c, d, e, or f), as described above. In a further embodiment, at least one of c, d, e, or f is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are cysteine. In another embodiment, at least one of c, d, e, and f is not a disulfide linkage. In certain embodiments, at least one of c, d, e, and f is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are tryptophans. Examples of f include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of c, d, e, or f, include ionic linkages. Preferably, the conotide of formula (II) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at c, d, and e and with f absent. In a further embodiment, a and b comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a and b each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a and b polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (II), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

In another embodiment, the invention pertains to conotides of formula (III):



(III)

wherein

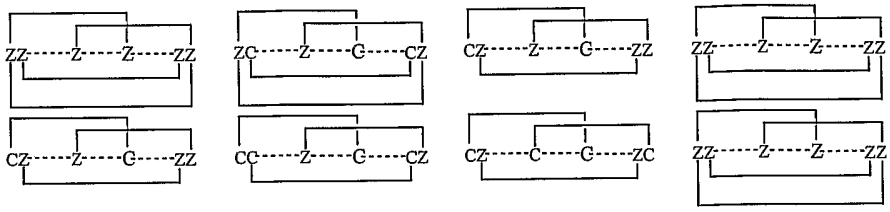
Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

a, b, and c are each independently selected polypeptide linkers comprising one or more amino acid residues; and

d, e, f, and g are each independently selected linking moieties or absent; provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are cysteine, then at least one of e, d, f, and g is not a disulfide linking moiety; and provided that at least one of d, e, f, or g is not absent.

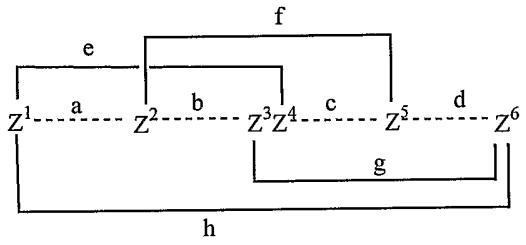
In a further embodiment, conotides of formula (III) comprise one or more linking moieties which are covalent linkages (*e.g.*, d, e, f, or g), as described above. In a further embodiment, at least one of d, e, f, or g is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are cysteine. In another embodiment, at least one of d, e, f, and g is not a disulfide linkage. In certain embodiments, at least one of d, e, f, and g is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are tryptophans. Examples of g include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of d, e, f, or g include ionic linkages. Preferably, the conotide of formula (III) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at d, e, and f and with g absent. In a further embodiment, a, b, and c comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a, b, and c each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a, b and c polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (III), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

The invention also pertains to conotides of the formula (IV):



(IV)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

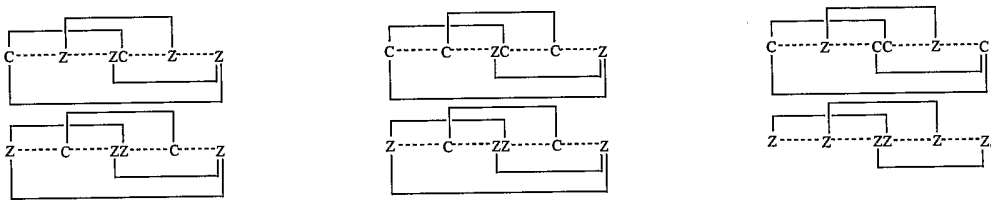
a, b, c, and d are each independently selected polypeptide linkers comprising one or more amino acid residues; and

e, f, g, and h are each independently selected linking moieties or absent; and, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are each cysteine, then at least one of e, f, g, and h is not a disulfide linking moiety; and provided that at least one of e, f, g, or h is not absent.

In a further embodiment, conotides of formula (IV) comprise one or more linking moieties which are covalent linkages (*e.g.*, e, f, g, or h), as described above. In a further embodiment, at least one of e, f, g, or h is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are cysteine. In another embodiment, at least one of e, f, g and h is not a disulfide linkage. In certain embodiments, at least one of e, f, g, and h is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are tryptophans. Examples of h include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of e, f, g, and h include ionic linkages. Preferably,

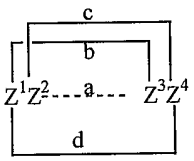
the conotide of formula (IV) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at e, f and g and with h absent. In a further embodiment, a, b, c, and d comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a, b, c, and d each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a, b, c, and d polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (IV), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

In yet another embodiment, the invention pertains to conotides of the formula (V):



(V)

wherein

Z^1, Z^2, Z^3, and Z^4 are each independently selected linking residues;

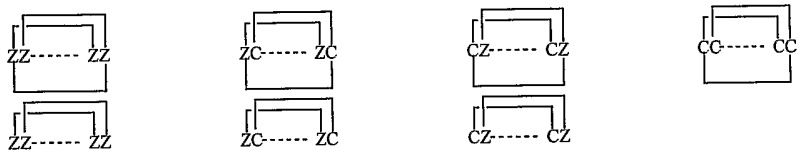
a is a polypeptide linker comprising one or more amino acid residues; and

b, c and d are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of b, c, and d is not a disulfide linking moiety; and provided that at least one of b, c, and d is not absent.

In a further embodiment, conotides of formula (V) comprise one or more linking moieties which are covalent linkages (*e.g.*, b, c, or d), as described above. In a further embodiment, at least one of b, c, or d, is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, and Z^4 are cysteine. In another embodiment, at least one of b, c, and d is not a disulfide linkage. In certain embodiments, at least one of b, c, or d is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3 and Z^4 are

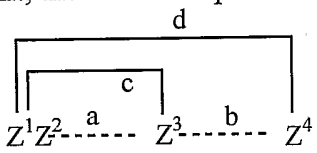
tryptophans. Examples of d include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of b, c, or d , include ionic linkages. Preferably, the conotide of formula (V) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at b and c and d absent. In a further embodiment, a comprises between zero and twenty independently selected amino acid residues. In particular embodiments, a comprises from about one to about ten amino acid residues. The number of amino acid residues present in a polypeptide linkers may vary, however, generally the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (V), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

In a further embodiment, the invention pertains to conotides of the formula (VI):



(VI)

wherein

Z^1, Z^2, Z^3, and Z^4 are each independently linking residues;

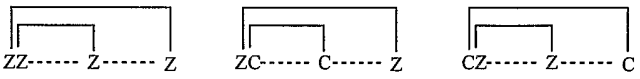
a and b are polypeptide linkers comprising one or more amino acid residues; and

c and d are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of c and d is not a disulfide linking moiety; and provided that at least one of c and d is not absent.

In a further embodiment, conotides of formula (VI) comprise one or more linking moieties which are covalent linkages (*e.g.*, c or d) as described above. In a further embodiment, at least one of c or d is a disulfide covalent linkage and at least two of Z^1, Z^2, Z^3, and Z^4 are cysteine. In another embodiment, at least one of c, and d is not

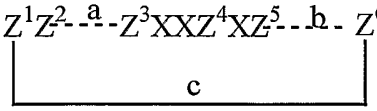
a disulfide linkage. In certain embodiments, at least one of c or d is a linking moiety with a hydrophobic linkage. For this embodiment, at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl group. In a further embodiment, at least one of Z^1, Z^2, Z^3 and Z^4 are tryptophans. Examples of d include a polypeptide linkage (*e.g.*, comprises at least a peptide bond). Other examples of linking moieties which may be used for one or more of c or d include ionic linkages. Preferably, the conotide of formula (I) is more stable to the redox environment *in vivo*, than a conotide with disulfide bonds at c and d. In a further embodiment, a and b comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a and b each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a and b polypeptide linkers may vary, however, generally the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

Examples of conotides of the invention of formula (VI), include, but are not limited to, conotides of the formulae:



wherein each occurrence of Z is an independently selected linking residue other than cysteine, C is a cysteine residue, the solid lines are covalent linkages and the dotted lines represent polypeptide linkages.

In one embodiment, the invention pertains to conotides of the formula (VII):



(VII)

wherein:

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

X is an independently selected amino acid residue for each occurrence;

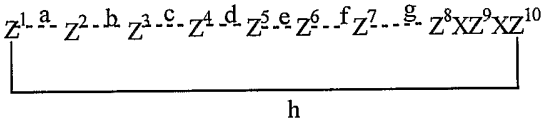
a and b are each independently selected polypeptide linkers comprising one or more amino acid residues; and

c is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are cysteine, then c is not absent.

In a further embodiment, conotides of formula (VII) comprise a covalent linkage (*e.g.*, c), as described above. In a further embodiment, c is a disulfide covalent linkage and Z^1 and Z^6 are each cysteine. In another embodiment, c is not a disulfide linkage. In certain embodiments, c is a linking moiety with a hydrophobic linkage. For this

embodiment, Z^1 and Z^6 may comprise an aryl group. In a further embodiment, Z^1 and Z^6 are tryptophans. The linkage c also may be a polypeptide linkage (*e.g.*, comprises at least a peptide bond). The linkage c also may be an ionic linkage. Preferably, the conotide of formula (VII) is more stable to the redox environment *in vivo*, than a conotide without the linkage at c. In a further embodiment, a and b comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a and b each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a and b polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

The invention also pertains, at least in part, to conotides of the formula (VIII):



(VIII)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, Z^9, and Z^{10} are each independently selected linking residues;

X is an independently selected amino acid residue for each occurrence;

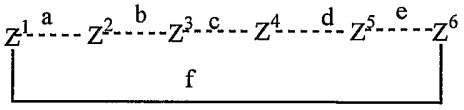
a, b, c, d, e, f, and g are each independently selected polypeptide linkers comprising one or more amino acid residues; and

h is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, Z^9, and Z^{10} are cysteine, then h is not absent.

In a further embodiment, conotides of formula (VIII) comprise a covalent linkage (*e.g.*, h), as described above. In a further embodiment, h is a disulfide covalent linkage and Z^1 and Z^{10} are each cysteine. In another embodiment, h is not a disulfide linkage. In certain embodiments, h is a linking moiety with a hydrophobic linkage. For this embodiment, Z^1 and Z^{10} may comprise an aryl group. In a further embodiment, Z^1 and Z^{10} are tryptophans. The linkage h also may be a polypeptide linkage (*e.g.*, comprises at least a peptide bond). The linkage h also may be an ionic linkage. Preferably, the conotide of formula (VIII) is more stable to the redox environment *in vivo*, than a conotide without the linkage at h. In a further embodiment, a, b, c, d, e, f, and g each comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a, b, c, d, e, f, and g each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a, b, c,

d, e, f, and g polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

In yet another embodiment, the invention pertains to conotides of the formula (IX):



(IX)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

a, b, c, d, and e are each independently selected polypeptide linkers comprising one or more amino acid residues; and

f is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6, are cysteine, then f is not absent.

In a further embodiment, conotides of formula (IX) comprise a covalent linkage (*e.g.*, f), as described above. In a further embodiment, f is a disulfide covalent linkage and Z^1 and Z^6 are each cysteine. In another embodiment, f is not a disulfide linkage. In certain embodiments, f is a linking moiety with a hydrophobic linkage. For this embodiment, Z^1 and Z^6 may comprise an aryl group. In a further embodiment, Z^1 and Z^6 are tryptophans. The linkage f also may be a polypeptide linkage (*e.g.*, comprises at least a peptide bond). The linkage f also may be an ionic linkage. Preferably, the conotide of formula (IX) is more stable to the redox environment *in vivo*, than a conotide without the linkage at f. In a further embodiment, a, b, c, d, and e, each comprise between zero and twenty independently selected amino acid residues. In particular embodiments, a, b, c, d, and e each comprise from about one to about ten amino acid residues. The number of amino acid residues present in a, b, c, d, and e polypeptide linkers may vary, although the number of amino acid residues is selected such that the conotide is capable of performing its intended function.

1.8 Additional Chemical Modifications of Peptide Aptamers

In addition to the above described modifications of peptide aptamers using fusion moieties, cysteine replacement, and intramolecular linkages, it is also understood that the peptide aptamer of the invention can be further modified to alter a specific property of the peptide aptamer while retaining the ability of the aptamer to, *e.g.*, bind to a ligand. For example, in one embodiment, the peptide aptamer is further modified to alter a pharmacokinetic property of the peptide aptamer, such as *in vivo* stability or half-

life. In another embodiment, the peptide aptamer is further modified to label the peptide aptamer with a detectable substance. In yet another embodiment, the peptide aptamer is further modified to couple the peptide aptamer to an additional therapeutic moiety.

To further chemically modify the peptide aptamer, such as to alter the pharmacokinetic properties (*e.g.*, stability or half-life) of the peptide aptamer, reactive groups can be derivatized. For example, linear peptide aptamers can be modified at the carboxy-terminal end to reduce the ability of the peptide aptamer to act as a substrate for carboxypeptidases. Examples of preferred C-terminal modifiers include an amide group (*i.e.*, a peptide amide), an alkyl or aryl amide group (*e.g.*, an ethylamide group or a phenethylamide group) a hydroxy group (*i.e.*, a peptide alcohol) and various non-natural amino acids, such as D-amino acids and β-alanine. Alternatively, the amino-terminal end of the peptide aptamer can be further modified, for example, to reduce the ability of the peptide aptamer to act as a substrate for aminopeptidases.

A peptide aptamer of the invention can also be reacted and labeled with a detectable substance. Suitable detectable substances include various prosthetic groups, fluorescent materials, luminescent materials, and radioactive materials. Examples of suitable prosthetic groups include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{14}C, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{99}Tc, ^{35}S or ^{3}H. In a preferred embodiment, a peptide aptamer is radioactively labeled with ^{14}C, either by incorporation of ^{14}C into the modifying group or one or more amino acid structures in the peptide aptamer. Labeled aptamer compounds can be used to assess the *in vivo* pharmacokinetics of the peptide aptamer, as well as to detect a desired activity of the peptide aptamer, *e.g.*, affinity, stability, bioavailability, for example, for diagnostic purposes. Such activities can be detected using a labeled peptide aptamer either *in vivo* or in an *in vitro* sample derived from a subject or art recognized animal model.

In an alternative chemical modification, a peptide aptamer of the invention is prepared in a "prodrug" form. For example, in this type of peptide aptamer, the modulating group can be present in a prodrug form that is capable of being converted upon metabolism into the form of an active modulating group. Such a prodrug form of a modifying group is referred to herein as a "secondary modifying group." A variety of strategies are known in the art for preparing peptide prodrugs that limit metabolism in order to optimize delivery of the active form of the peptide-based drug (see *e.g.*, Moss, J. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 18. Additionally strategies have been specifically tailored to achieving CNS delivery based on "sequential metabolism" (see

e.g., Bodor, N., *et al.* (1992) *Science* 257:1698-1700; Prokai, L., *et al.* (1994) *J. Am. Chem. Soc.* 116:2643-2644; Bodor, N. and Prokai, L. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 14. In one embodiment of a prodrug form of a peptide aptamer of the invention, the modifying group comprises an alkyl ester to facilitate blood-brain barrier permeability. In another embodiment, the prodrug aptamer is in the form of an unspliced intein/aptamer fusion.

II. GENE DELIVERY AND GENE EXPRESSION

2.1 Improved Gene Delivery Using Receptors to Increase Vector Uptake

The above described peptide aptamers (and libraries thereof) can be generated using art recognized nucleic acid synthesis and vector systems suitable for expressing nucleic acid sequences in cells, *e.g.*, prokaryotic or eukaryotic cells. Various vectors, as well as amphotropic and ecotropic packaging cell lines, can be used for production of high titers of retroviruses that infect eukaryotic cells, *e.g.*, mouse or human cells. In a preferred embodiment, the invention employs high-titer retroviral packaging systems to produce peptide aptamer libraries (Burns *et al.*, 1993; Pear *et al.*, 1993) (see Fig. 6). These delivery and expression systems can be adapted for the efficient infection of a variety of cell types.

However, the efficient screening of retroviral peptide aptamer libraries requires that the percentage of cells in a population of cells undergoing transduction (gene uptake) be optimized. Like most other viruses, retroviruses use endogenous proteins associated with the cell membrane to mediate attachment and entry into the host mammalian cell. Proteins associated with the retroviral envelope interact specifically with host cell proteins, therefore the pattern of expression of a receptor associated with a given receptor is a major determinant of the animal species (or even specific tissues within a given species) that are susceptible to infection by a given retrovirus. Thus, the proteins associated with the envelope of a specific infectious retrovirus, combined with the identity of mammalian cells expressing the cognate receptor for such viral envelope proteins, determines the range of infectivity of a given retroviral particle.

Accordingly, the invention includes the efficient gene delivery of peptide aptamer libraries using libraries incorporated into a retrovirus where, either the retrovirus, recipient cell type, or both, are optimized for virus uptake. For example, retroviral particles can be packaged to present amphotropic receptors, which mediate viral transduction and entry into all types of mammalian cells, and even a variety of non-vertebrate cell types. In the case of ecotropic retroviruses, packaging is carried out in cell lines that produce virus containing the env protein of Moloney murine leukemia virus (MoMuLV). The MuLV receptor has been identified as a membrane spanning

amino acid transporter protein known as ATRC-1 (Albritton *et al.*, 1989). Only cells expressing the mouse form of ATRC-1 are susceptible to infection or transduction by ecotropic retroviruses or retroviral vectors. Thus, cells that are not normally susceptible to ecotropic infection by MuLV infection (*e.g.* Chinese hamster ovary cells, human cells) become infectable upon expression of the ATRC-1 receptor (Albritton *et al.*, 1993). Moreover, as disclosed in this invention, mouse cells that are not naturally susceptible to efficient retroviral gene transduction using recombinant ecotropic retroviral vectors, are rendered highly susceptible upon stable over-expression of mouse ATRC-1.

The invention provides an additional use of the ATRC-1 receptor in the screening of retrovirus-encoded peptide aptamer libraries which addresses the safety of use and handling of retroviral libraries encoding random peptides. By definition, libraries encoding highly diverse random peptide libraries have the potential to produce peptides with a wide variety of biological functions. Moreover, in cells in which stable integration and expression of the retroviral genome occurs. the effects manifest by the encoded peptide aptamer are stable and irreversible. As such, a randomly derived retrovirus within a library could potentially encode a peptide with biological activity that by definition renders it potentially hazardous. For example, a peptide aptamer capable of immortalization or even transformation of mammalian cells would pose a limited but real biological threat to individuals handling it, were it able to infect human cells.

Accordingly, the invention provides for the use of retroviruses containing ecotropic receptors in the production and screening of peptide aptamer libraries. The invention further provides for the screening of such libraries in human (or other cell lines not normally susceptible to infection by ecotropic retroviruses) using cell lines that have been genetically altered to stably express the ATRC-1 ecotropic receptor. This approach offers the opportunity to screen and test retroviral aptamer libraries in mammalian cells, but using methods that minimize or eliminate potential risk to the experimenter.

2.2 Improved Gene Expression Using Chromatin Insulator Elements

Even with efficient gene delivery, optimal gene expression must be achieved in order to carry out an effective phenotypic screen of peptide aptamer libraries. It is known that, even though gene delivery using retroviral transduction generally results in stable integration and maintenance of the gene, expression *per se* occurs in only a subset of the recipient cells, and that expression frequently declines even in those cells that initially expressed the gene at high levels. For phenotypically screening peptide libraries that are transduced (delivered) into cells, it important to optimize the percentage of cells in which expression of the insert encoding the library aptamer

occurs, and furthermore, under most conditions, to perpetuate that expression to the greatest extent possible.

This invention provides methods and compositions for using chromatin insulator elements for the improvement of retroviral vectors and their use in generating and screening retroviral libraries in mammalian cells.

The invention also encompasses the use of chromatin insulator elements to optimize the establishment of populations of cells expressing retroviral libraries early after transduction and proviral integration, as well as their utility in perpetuating expression throughout the growth of these cells, and the use of these cells in phenotypic selection assays either in tissue culture or in animals.

The chromatin insulator is a genetic element that is engineered into the retroviral library vector itself, and serves to augment and perpetuate expression of the retroviral library inserts at each site of integration within a plurality of cells, and throughout the continued growth or differentiation of the cells. This is of particular use in perpetuated expression in transgenic mouse models, or in experiential systems where expression in myeloid or lymphoid cell systems is desired.

III. SCREENING METHODS

3.1 Screening Methods for Identifying Peptide Aptamers Capable of Altering a Cell Phenotype

The above peptide libraries and improved methods for gene delivery and expression can be used to carry out high efficient screens to identify peptide aptamers capable of altering a cell phenotype.

To carrying out a typical screening method of the invention, a population of cells, preferably a clonal population of eukaryotic cells is transfected or infected and cultured under appropriate conditions. Then, a cell or cells exhibiting a desired phenotype or a phenotype which differs from other cells in the cell population, is selected or isolated, for example, using FACS. Coding sequences of aptamers selected in the first round of screening can be amplified by PCR, re-cloned, and re-introduced into naïve cells. Phenotypic selection can then be repeated in order to validate individual aptamers within the original pool (*e.g.*, as shown in Fig. 14). Aptamer coding sequences within cells identified in subsequent rounds of selection can be iteratively amplified and subcloned and the sequences of active aptamers can then be determined by DNA sequencing using standard techniques. This strategy can be applied to the identification of aptamers associated with a wide variety of cellular processes including, *e.g.*, cell proliferation, regulation of apoptosis, protein trafficking or transport, cell motility or differentiation, metabolic state, and modulation of various signal transduction.

Peptide aptamer libraries of the present invention can have a complexity of up, *e.g.*, up to ~10^8 or more allowing for the screening of a large numbers of aptamers (*e.g.*,) in a single experiment using any appropriate cell type (*e.g.*, to test 10^7 or preferably, 10^9 combinations, 100 tissue culture vessels each containing 10 mls of media with 10^6 cells/ml can be used). Thus, the present invention surpasses existing strategies that rely on target identification and selection, including those based on elucidation of specific protein-protein interactions, phenotypic gene expression profiling, or genotypic analysis. This is especially advantageous in the study of complex and highly diverse diseases, such as cancer and other diseases involving signal transduction pathways, as well as infectious diseases, such as viral diseases, where the virus has co-opted one or more cellular pathways (*e.g.*, in order to replicate or avoid detection by the host immune system).

In a particular embodiment, the invention is used for the identification of aptamers that modulate measurable cellular processes (*i.e.*, phenotypes). These include, but are not limited to: apoptosis, susceptibility to pathology associated with infection by a virus, bacterium, toxin, or prion, induction or repression of expression of one or more genes or gene products, differentiation, senescence, transport of intracellular molecules, including ions, nucleic acids, proteins, lipids, hormones, or metabolites, stability of any specified intracellular molecules, including ions, nucleic acids, proteins, lipids, or metabolites, or modulation of signalling by cellular receptors or their respective downstream signaling effectors. Other cellular processes may also be probed using the above approach, such as hormone or lipid metabolism, metabolism or homeostasis, and pathological conditions thereof.

In a specific embodiment, the cellular process of hair growth or hair loss, hair follicle differentiation, modification of hair follicle development, melanogenesis, modulation of hair shaft elongation, modulation of skin differentiation, or modulation of the hair growth cycle from telogen (resting stage) to anagen (growing stage) can be carried out using the peptides of the invention and any art recognized assay for measuring a change in one of the foregoing characteristics associated with hair growth or loss (see *e.g.*, U.S. Patent Nos. 5,767,152; 5,840,690; 5,527,772; and 5,407,944).

3.2 In Vitro Screening Methods for Identifying a Peptide Aptamer Capable of Killing a Cancer Cell

As indicated above, the screening methods of the invention can be adapted to identify aptamers that can alter any number of cellular phenotypes, including, *e.g.*, the cancer phenotype. Accordingly, in one embodiment, a peptide aptamer library of the invention is tested for its ability to inhibit the growth, induce apoptosis, or kill a cancer cell, as compared to a normal cell. For example, the peptide aptamer can be tested in a

clonal cancer cell line, and preferably, a panel of clonal cancer cell lines, such as those in the DTP Human Tumor Cell Line Screen which represents a diverse set of clonal cancer cells derived from various types of human tumors (Monks *et al.*, 1991; see also Fig. 15). The susceptibility of each cancer cell type to exhibit a phenotypic change, for example inhibited growth or apoptosis when expressing the peptide aptamer, is then measured. This approach allows for determining the specificity of the bioactivity of the peptide aptamer across a range of different cancer types.

The library screening process can then be repeated in a second cell line in which expression of the first aptamer did not induce apoptosis. Aptamers identified in screening of this second cell line can also be tested against an entire cell line panel representative, *e.g.*, of different cell types or cancers. This iterative process of identifying an aptamer with activity in one cell line, determining its activity against other cell lines, followed by identification of additional aptamers active against other cell lines, eventually leads to coverage of the entire cell line panel: a set of aptamers that induces apoptosis in at least one type of cancer cell line in the panel. An idealized compilation of the outcome of this process is shown in Fig. 15. It is important to reiterate that the pattern of susceptibility of a cancer cell to the identified set of aptamers can serve as a phenotype in and of itself, since this susceptibility can indicate the manifestation of the aggregate of changes that the cell underwent in its pathogenesis. Thus, this functional categorization of cancer cells can be extremely valuable, even in the absence of a full understanding of the molecular basis of the action of a given aptamer.

3.3 Screening Method for Identifying Aptamers Having Antiviral Activity

The screening methods of the invention can also be used to identify peptide aptamers that have antiviral activity. In particular, the screening methods of the invention are suitable for identifying aptamers that fall into three classes: *i)* aptamers that modulate cellular processes that render the cell resistant to lysis or other pathological effects resulting from viral infection, but in which virus production by the cell can still occur; (*ii*) aptamers that inhibit or alter viral functions required for viral replication; and/or (*iii*) aptamers that inhibit a cellular function required for viral replication.

A typical screen for identifying an aptamer having antiviral activity in one of the above-mentioned classes is carried out by introducing the peptide aptamers into an appropriate cells as described herein that have either been previously infected, coinfected, or are later infected with a virus. Aptamers that alter the viral-mediated cell phenotypes and/or viral activity (*e.g.*, viral replication, production) are determined using

art recognized techniques (*e.g.*, PCR, histological criteria, infectivity assays, or cell viability).

The screening methods of the invention are uniquely well suited for identifying aptamers that influence a phenotypic property associated with an infected cell that is not observed in an equivalent or comparable non-infected cell. For example, such a phenotypic property can be the expression of a specific cellular or viral antigen by the infected cell, or other functions that influence the pathogenesis or disease-causing functions mediated by infection of an animal by a given virus.

Accordingly, the first class of aptamers identified includes aptamers that alters any cellular function that, as a result of a virus-induced response, leads to cell death or pathogenesis. For example, induction of cellular PKR activity or transcriptional activation by NF-kappaB is associated with apoptosis of virally infected cells; thus attenuation of signals that manifest this response will render the infected host cell refractory to virus-induced death. Identifying peptide aptamers capable of modulating interferon response or other events associated with the intracellular signaling cascade response to viral infection is also within the scope of this invention.

The second class of aptamers identified includes aptamers that inhibit functions of virus encoded enzymes, as well as cell entry, aberrant localization of viral proteins, inhibition of viral packaging, or inhibition of virus release.

The third class of aptamers identified includes aptamers that effect cellular events necessary for the virus life cycle.

It is also understood that the above screening methods of the invention can also be applied to identifying aptamers that alter the activity of pathogens that have certain viral-like properties but are not a virus *per se*, for example, a prion.

3.4 In Vivo Screening Methods for Identifying Peptide Aptamers Capable of Altering a Cell Phenotype Using Transgenic Animals

The screening methods of the invention can also be used to identify peptide aptamers capable of altering a cell phenotype in a non-human transgenic or gene-knockout animal. For example, in one embodiment, a library of peptide aptamers encoded in an eukaryotic expression vector, *e.g.*, a retroviral vector can be introduced into a transgenic animal having a detectable phenotype. The detectable phenotype may be a visually or molecularly recognizable occurrence and includes, for example, an alteration in the growth, maintenance, migration, or function of a cell type or tissue of the animal.

Transgenic animals suitable for introducing an aptamer library include animals engineered to have, *e.g.*, a cancer (*e.g.*, an animal having a constitutive promoter driving the expression of an oncogene or, alternatively, an animal engineered to lack a tumor

suppressor) thereby allowing for the screening of aptamers which can abrogate a cancer phenotype. Alternatively, animals engineered to have a gene disruption, *i.e.*, a transgenic "knock-out" animal, can be used to screen the aptamer library for peptide aptamers that can rescue the function normally provided by the disrupted gene. Using either of the foregoing strategies, peptide aptamers that can affect a "gain of function" or "loss of function" can be screened or selected for in vivo. Then, the cells or tissue from an animal exhibiting the desired phenotype are then used as a source of biological material for the isolation and identification of the nucleic acid encoding the peptide aptamer associated with the phenotype using art recognized techniques.

The invention also encompasses the *ex vivo* treatment of cells, *e.g.*, cells derived from one of the transgenic animal described above, with a peptide aptamer library. The treated cells can then be studied *in vitro* or introduced into a host animal and monitored using art recognized techniques. For example, desired cell types or tissues that can be treated *ex vivo* and then reintroduced into a host animal following exposure to an aptamer library include, but are not limited to cells of the nervous system, muscle cells, and hematopoietic cells. In a preferred embodiment, hematopoietic cells lacking a gene needed for normal blood cell development or function, for example, a growth factor or a receptor, *e.g.*, a T cell receptor, are contacted with an aptamer library and then introduced into a host animal, for example a host animal that has been treated so as to lack its normal blood cell repertoire (using, *e.g.*, radiation). The animals treated with the cells exposed to the aptamer library are then monitored for the appearance of a desired phenotype (*e.g.*, the repopulation of a particular blood compartment or outgrowth of a certain cell type), and such cells can then be isolated and used as a source of material for identifying an aptamer associated with the phenotype. Using the foregoing approach, the invention is suitable for screening peptide aptamers that are capable of affecting, *e.g.*, cancers of the blood (*e.g.*, mechanisms of leukemogenesis), immune cell function (*e.g.*, T cell receptor function and/or other immune cell interactions), and various other diseases of the blood (*e.g.*, hemochromatosis, or viral infections, *e.g.*, an HIV infection).

3.5 Other Screening Advantages of the Invention

Another advantage of the present invention is integration of the primary screen with effective counter-screens that demonstrate the specificity of the phenotype. For example, cancer cells frequently undergo epigenetic changes that allow them to ignore normal growth regulatory signals, including apoptosis. In searching for aptamers that induce apoptosis in a given type of tumor cell, it is important to make sure that these aptamers do not induce apoptosis in normal cells.

In other examples, where an aptamer might be identified that modulates trafficking or transport of a particular protein, the invention provides the ability to incorporate counterscreens to determine that aptamer induced changes in localization are relatively specific to the phenotype of interest.

IV. USES

4.1 Use of Peptide Aptamers as a Prognostic Tool

The peptide aptamer of the invention can also be used as a prognostic tool for, for example, determining the likelihood of a cell to respond to a certain therapy based on the ability of an aptamer or panel of aptamers to alter the cell phenotype.

In a particular embodiment, the invention provides the ability to generate an apoptotic aptamer phenotype, *i.e.,* a profile of aptamers that induces apoptosis and therefor represents important information about a given tumor cell. The aptamers allows for the categorization of any clonal population of tumor cells with respect to the most pertinent and important types of information, for example, how to destroy the cancer and with what selectivity and specificity.

In a clinical setting, for example, profiling of aptamer-associated apoptosis, conducted using, for example, a kit containing a panels of aptamers that can be used by investigators to draw correlations between the aptamer apoptosis phenotype and clinical prognosis, or serve as a predictive tool for the effectiveness of a given therapeutic strategy.

4.2 Use of Peptide Aptamers as a Diagnostic Tool

The methods and compositions of the invention can also be used for diagnostic purposes.

Accordingly, the retroviral aptamer libraries disclosed herein, or aptamers encoded by these libraries, can be packaged into kits with instructions for use. These kits can be used to screen for desirable aptamers using a format described herein for phenotyping, *e.g.*, a cancer cell or tissue derived from, *e.g.*, a biopsy sample. For example, a panel of vectors expressing peptide aptamers that induce apoptosis in cancer cells can be used for the "functional phenotyping" of tumor biopsy tissue, in which susceptibility to a particular aptamer can have predictive value for determining the efficacy of a certain treatment regimen. Alternatively, the kit may be used in conjunction with the cancer cell lines disclosed herein, other art recognized cell lines, or a combination thereof. In addition, the aptamer libraries may be used in conjunction with other screening technology involving, *e.g.*, phage display and/or yeast two-hybrid systems for testing or validating a given aptamer. Still further, the methods and compositions described herein may also be used in conjunction with various art

recognized gene chip technologies to, *e.g.*. phenotype or diagnose a cancer. For example, the aptamer approach can be combined with gene chip technologies in order to enable the high-throughput quantitation of the expression of thousands of genes in a sample. This combined approach can be applied to the study of, *e.g.*, diffuse large B-cell lymphoma (DLBCL), the most common subtype of non-Hodgkin's lymphoma, in order to discover identifiable differences in aptamer susceptibility and also gene expression patterns that correlate with and distinguish tumor proliferation rate, host response, and differentiation state of the tumor (Alizadeh *et al.*, *Nature* 403:503-511 (2000)). Any of the forgoing composite diagnostic approaches are understood to be within the scope of the invention.

4.3 Use of Peptide Aptamers for Altering a Cell Phenotype

Aptamers can be identified that induce apoptosis in cells associated with other hyperproliferative disorders. These include, for example, prostatic hyperplasia in aging men and psoriasis. In addition, apoptosis is associated with diseases like osteoporosis, in which induction of osteoclast apoptosis (thus, decreasing cells that resorb bone), is of potential therapeutic benefit (Rezka *et al.*, 1999). Conversely, aptamer screens can also be performed to identify inhibitors of osteoblast apoptosis. The therapeutic benefit of inhibition of apoptosis in the bone for generating needed cells is a desirable result (Plotkin *et al.*, 1999).

4.4 Use of Peptide Aptamers in Gene Therapy

The therapeutic peptide aptamers capable of modulating a cell phenotype can be delivered to cells by methods of gene therapy. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Viral vectors include, for example, recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1. Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. Alternatively they can be used for introducing exogenous genes *ex vivo* into cells in culture.

A major prerequisite for the use of viruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271).

Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag, pol, env*) is replaced by a gene of interest rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:9079-9083; Julan *et al.* (1992) *J. Gen. Virol.* 73:3251-3255; and Goud *et al.* (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda *et al.* (1991) *J. Biol. Chem.* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (*e.g.* lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (*e.g.* single-chain antibody/*env* fusion proteins). Thus, in a specific embodiment of the invention, viral particles containing a nucleic acid molecule containing a gene of interest, *e.g.*, encoding a suitable peptide aptamer operably linked to appropriate regulatory elements, are modified for example according to the methods described above, such that they can specifically target desired cells.

Other methods relating to the use of viral vectors in gene therapy can be found in, *e.g.*, Kay, M.A. (1997) *Chest* 111(6 Supp.):138S-142S; Ferry, N. and Heard, J. M. (1998) *Hum. Gene Ther.* 9:1975-81; Shiratory, Y. *et al.* (1999) *Liver* 19:265-74; Oka, K. *et al.* (2000) *Curr. Opin. Lipidol.* 11:179-86; Thule, P.M. and Liu, J.M. (2000) *Gene Ther.* 7:1744-52; Yang, N.S. (1992) *Crit. Rev. Biotechnol.* 12:335-56; Alt, M. (1995) *J.*

Hepatol. 23:746-58; Brody, S. L. and Crystal, R. G. (1994) *Ann. N.Y. Acad. Sci.* 716:90-101; Strayer, D. S. (1999) *Expert Opin. Investig. Drugs* 8:2159-2172; Smith-Arica, J. R. and Bartlett, J. S. (2001) *Curr. Cardiol. Rep.* 3:43-49; and Lee, H. C. *et al.* (2000) *Nature* 408:483-8.

For the particular treatment of a cancer, for example, following optional testing of the cancer biopsy sample, a vector encoding a predetermined aptamer can be injected directly into the tumor, or delivered in any other art-recognized manner of gene therapy. An advantage of the invention is that treatment of a given cancer in a subject with a vector encoding a therapeutic aptamer is an acute undertaking, which does not require perpetual expression of the introduced gene, which has been a difficulty in most other gene therapy approaches (Verma and Somia, 1997). In one approach, aptamer expressing viruses are used either as stand-alone therapeutics, or as adjuncts to other therapeutic regimens. For example, the viruses (*e.g.*, retroviruses) can be injected directly into solid tumor sites to minimize the possibility of side-effects. In addition, aptamers can be identified that act in concert with other cancer therapeutic drugs or radiation therapy in a manner that lowers their effective doses, thereby decreasing toxicity or side effects of these treatments. Indeed, library screens can be undertaken in which aptamer expression results in apoptosis of a cell line in the presence of a sub-apoptosis inducing concentration of a conventional cancer therapeutic agent (*e.g.* tamoxifen or camptothecin).

4.5 Use of Peptide Aptamers as Therapeutics

Still further, the aptamer peptides of the invention, *e.g.*, the conotoxin-derived peptides of the invention, *i.e.*, conotides (and libraries thereof), are particularly well suited for use or for identifying an agent for any of the indications that the naturally-occurring conotoxins can be used for with the advantage being that the conotide of the invention has been improved in one or more aspects such as, *e.g.*, affinity, *e.g.*, to a cognate receptor, stability, or bioavailability.

Accordingly, conotides based on a backbone sequence from the A superfamily, *i.e.*, conotoxins from the family, α, β, αA, and κA, can be used to modulate nAchRs, α_1-adenoreceptors, or voltage-gated K^+ channels (see Table 1). Conotides based on a backbone sequence from the M superfamily, *i.e.*, conotoxins from the family, μ and ψ, can be used to modulate Na^+ channels or nAchRs . Similarly, conotides for the O superfamily, *i.e.*, conotoxins from the families such as δ, ω, γ, μO, κ, Bromosleeper, and Conotoxin GS, can be used to modulate a variety of different ion channels (see Table 1). Other conotides based on a backbone sequence from a conotoxin from the S, T, P, and non-disulfide rich superfamilies (*i.e.*, superfamilies that include σ, τ, χ, Conantokin,

Contulakin, and Contryphan) can be used to, *e.g.*, modulate the 5-HT_3 receptor, noradrenaline transporter, NMDA receptor or neurotensin receptor.

Disease or disorders which can be modulated (*i.e.*, prevented or treated) using the conotides of the invention include, but are not limited to, pain, epilepsy stroke/ischemia, Parkinson's disease, dystonias, demyelinating disorders, congestive heart failure, cardiovascular disorders, neuromuscular disorders, chemotherapy-induced nausea and vomiting, psychiatric indications, cerebrovascular ischemia, cardiovascular disease, neurodegenerative disease, head injury, spasticity, neurotoxic injury associated with conditions of hypoxia, anoxia or ischemia which typically follows stroke, cerebrovascular accident, brain or spinal cord trauma, Alzheimer's disease, senile dementia, Amyotrophic Lateral Sclerosis, Multiple Sclerosis, Parkinson's disease, Huntington's disease, Down's Syndrome, Korsakoff's disease, schizophrenia, AIDS dementia, multi-infarct dementia, Binswanger dementia, psychiatric disorders, anxiety, major depression, manic-depressive illness, obsessive-compulsive disorder, schizophrenia and mood disorders, ophthalmic disorders, urinary incontinence, memory/cognition enhancement, *i.e.*, treating memory, learning or cognitive deficits, analgesic agents, treatment of migraine, acute pain or persistent, non-addictive (*e.g.*, non opioid based) pain management, convulsions, HIV infection and ophthalmic indications, agents and anti-pain agents for the treatment of acute and persistent pain, decrease bladder/sphincter dyssynergia (see, *e.g.*, Jones *et al.*, Exp Opin. Ther. Patents 11:603-623 (2001).

4.6 Use of Peptide Aptamers for the Research and Development of Other Therapeutics

Elucidation of aptamer targets can also serve as a powerful tool for the discovery of novel cellular targets that advance our understanding of the impacted cellular pathway/s. As such, the invention also encompasses retroviral aptamer libraries (*e.g.*, in the form of kits), for use by basic researchers for genetic exploration of complex pathways in mammalian cells.

Accordingly, the invention can be used for the molecular classification of tumors and identification of previously undetected and clinically significant subtypes of cancer. In addition, the invention can be used as therapies that regulate or manage tumor growth (Balis, 1998).

Another embodiment of the invention includes the use of the aptamers as lead molecules for drug development. For example, using any art recognized molecular modeling techniques, an aptamer can be used for designing and synthesizing other molecules having the desirable function of the aptamer but also having other desirable traits such as cell solubility, potency, time-release properties, *etc.*

4.7 Use of Peptide Aptamers as Pharmaceutical Compositions

Another aspect of the invention pertains to pharmaceutical compositions of the peptide aptamers of the invention. In one embodiment, the composition includes an peptide aptamer in a therapeutically or prophylactically effective amount sufficient to alter, a desired activity, *e.g.*, a cellular activity or a disorder or disease indication as described herein, and a pharmaceutically acceptable carrier. In another embodiment, the composition includes a peptide aptamer in a therapeutically or prophylactically effective amount sufficient to modify one or more of the modes of a action listed in Table 1 and a pharmaceutically acceptable carrier. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as improved modulation of a receptor. A therapeutically effective amount of a peptide aptamer may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the aptamer compound to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the aptamer compound are outweighed by the therapeutically beneficial effects. The potential toxicity of the aptamer compounds of the invention can be assayed using the cell-based assays or art recognized animal models and a therapeutically effective modulator can be selected which does not exhibit significant toxicity. In a preferred embodiment, a therapeutically effective amount of a peptide aptamer is sufficient to alter, and preferably inhibit, undesired cell growth (*e.g.*, a cancer or a viral pathology or mechanism). A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting undesired cell growth (*e.g.*, a cancer or a viral pathology or mechanism). A prophylactically effective amount can be determined as described above for the therapeutically effective amount. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

It is to be noted that dosage values may vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens can be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The amount of active aptamer compound in the composition may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Preferably, the carrier is suitable for administration into the central nervous system (*e.g.*, intraspinally or intracerebrally). Alternatively, the carrier can be suitable for intravenous, intraperitoneal or intramuscular administration. In another embodiment, the carrier is suitable for oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, or

sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the aptamers can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active aptamer compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active aptamer compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

A peptide aptamer of the invention can be formulated with one or more additional compounds that enhance the solubility of the aptamer compound. Preferred compounds to be added to formulations to enhance the solubility of the aptamer compounds are cyclodextrin derivatives, preferably hydroxypropyl-γ-cyclodextrin. Drug delivery vehicles containing a cyclodextrin derivative for delivery of peptides to the central nervous system are described in Bodor, N., *et al.* (1992) *Science* 257:1698-1700.

Another preferred formulation for the aptamer compounds to enhance brain uptake comprises the detergent Tween-80, polyethylene glycol (PEG) and ethanol in a saline solution. A non-limiting example of such a preferred formulation is 0.16% Tween-80, 1.3% PEG-3000 and 2% ethanol in saline.

In another embodiment, a pharmaceutical composition comprising a peptide aptamer of the invention is formulated such that the modulator is transported across the blood-brain barrier (BBB). Various strategies known in the art for increasing transport across the BBB can be adapted to the aptamer compounds of the invention to thereby enhance transport of the aptamers across the BBB (for reviews of such strategies, see *e.g.*, Pardridge, W.M. (1994) *Trends in Biotechnol.* 12:239-245; Van Bree, J.B. *et al.* (1993) *Pharm. World Sci.* 15:2-9; and Pardridge, W.M. *et al.* (1992) *Pharmacol.*

Toxicol. 71:3-10). In one approach, the aptamer compound is chemically modified to form a prodrug with enhanced transmembrane transport. Suitable chemical modifications include covalent linking of a fatty acid to the modulator through an amide or ester linkage (see *e.g.*, U.S. Patent 4,933,324 and PCT Publication WO 89/07938, both by Shashoua; U.S. Patent 5,284,876 by Hesse *et al.*; Toth, I. *et al.* (1994) *J. Drug Target.* 2:217-239; and Shashoua, V.E. *et al.* (1984) *J. Med. Chem.* 27:659-664) and glycating the aptamer compound (see *e.g.*, U.S. Patent 5,260,308 by Poduslo *et al.*). Also, N-acylamino acid derivatives may be used in an aptamer to form a "lipidic" prodrug (see *e.g.*, 5,112,863 by Hashimoto *et al.*).

In another approach for enhancing transport across the BBB, the aptamer is conjugated to a second peptide or protein, thereby forming a chimeric protein, wherein the second peptide or protein undergoes absorptive-mediated or receptor-mediated transcytosis through the BBB. Accordingly, by coupling the modulator to this second peptide or protein, the chimeric protein is transported across the BBB. The second peptide or protein can be a ligand for a brain capillary endothelial cell receptor ligand. For example, a preferred ligand is a monoclonal antibody that specifically binds to the transferring receptor on brain capillary endothelial cells (see *e.g.*, U.S. Patents 5,182,107 and 5,154,924 and PCT Publications WO 93/10819 and WO 95/02421, all by Friden *et al.*). Other suitable peptides or proteins that can mediate transport across the BBB include histones (see *e.g.*, U.S. Patent 4,902,505 by Pardridge and Schimmel) and ligands such as biotin, folate, niacin, pantothenic acid, riboflavin, thiamin, pryridoxal and ascorbic acid (see *e.g.*, U.S. Patents 5,416,016 and 5,108,921, both by Heinstein). Additionally, the glucose transporter GLUT-1 has been reported to transport glycopeptides (L-serinyl-β-D-glucoside analogues of [Met5]enkephalin) across the BBB (Polt, R. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7114-1778).

A peptide aptamer can be coupled to such a glycopeptide to target the modulator to the GLUT-1 glucose transporter. For example, a modulator compound which is modified at its amino terminus with the modifying group Aic (3-(*O*-aminoethyl-*iso*)-cholyl, a derivative of cholic acid having a free amino group) can be coupled to a glycopeptide through the amino group of Aic by standard methods. Chimeric proteins can be formed by recombinant DNA methods (*e.g.*, by formation of a chimeric gene encoding a fusion protein) or by chemical crosslinking of the aptamer to the second peptide or protein to form a chimeric protein. Numerous chemical crosslinking agents are known in the art. A crosslinking agent can be chosen which allows for high yield coupling of the modulator to the second peptide or protein and for subsequent cleavage of the linker to release bioactive aptamer. For example, a biotin-avidin-based linker system may be used.

In yet another approach for enhancing transport across the BBB, the aptamer is encapsulated in a carrier vector which mediates transport across the BBB. For example, the aptamer can be encapsulated in a liposome, such as a positively charged unilamellar liposome (see *e.g.*, PCT Publications WO 88/07851 and WO 88/07852, both by Faden) or in polymeric microspheres (see *e.g.*, U.S. Patent 5,413,797 by Khan *et al.*, U.S. Patent 5,271,961 by Mathiowitz *et al.* and 5,019,400 by Gombotz *et al.*). Moreover, the carrier vector can be modified to target it for transport across the BBB. For example, the carrier vector (*e.g.*, liposome) can be covalently modified with a molecule which is actively transported across the BBB or with a ligand for brain endothelial cell receptors, such as a monoclonal antibody that specifically binds to transferrin receptors (see *e.g.*, PCT Publications WO 91/04014 by Collins *et al.* and WO 94/02178 by Greig *et al.*).

In still another approach to enhancing transport of the modulator across the BBB, the modulator is coadministered with another agent which functions to permeabilize the BBB. Examples of such BBB "permeabilizers" include bradykinin and bradykinin agonists (see *e.g.*, U.S. Patent 5,112,596 by Malfroy-Camine) and peptidic compounds disclosed in U.S. Patent 5,268,164 by Kozarich *et al.*

A peptide aptamer of the invention can be formulated into a pharmaceutical composition wherein the aptamer is the only active compound or, alternatively, the pharmaceutical composition can contain additional active compounds. For example, two or more aptamer compounds may be used in combination.

In another embodiment, a pharmaceutical composition of the invention is provided as a packaged formulation. The packaged formulation may include a pharmaceutical composition of the invention in a container and printed instructions for administration of the composition for treating a subject having a disorder, *e.g.*, undesired cell growth (*e.g.*, a cancer) or a viral pathology.

Other features of the invention will be apparent from the following examples which should not be construed as limiting.

EXEMPLIFICATION

Throughout the examples, the following materials and methods are used unless otherwise stated.

Materials and Methods

Cyclic Peptide for Drug Discovery

Unless otherwise noted, cyclic peptides for drug discovery using the methods and compositions of the invention can made according to Al-Obeidi *et al.*, J. Med. Chem. 32:2555 (1989); Carpentier *et al.*, J. Med. Chem. 32:1184 (1989); Rizo *et al.*, Annu. Rev. Biochem. 61: 387 (1992); Hruby, Life Sci. 31:189 (1981); Hruby *et al.*, Biochem J. 268: 249 (1990); Shan *et al.*, *J. Pharm. Sci.* 86: 765-768 (1997); Takasaki *et*

al., *Nature Biotechnology,*15, 1266-1270 (1997); and Clarke *et al.*, *Biochemistry,* 32:4322 (1993).

Cyclic Peptide Synthesis by Conventional Methods - Unless otherwise noted, cyclic peptides for use in the methods and compositions of the invention can made according to Kopple *et al., J. Pharm. Sci.* 61, 1345 (1972); Perlow *et al., J. Org. Chem.,* 57, 4394 (1992)l; McGuiness *et al.,* in *" Peptides: Chemistry and Biology, Proceedings of the Fourteenth American Peptide Symposium"* (P. T. P. Kuaumaya and R. S. Hodges, eds.), p.125. Mayflower Worldwide, Birmingham, UK, (1996); and Arttamangkul *et al.*, Lett. Pept. Sci.m, 3:357 (1996).

Peptide Ligation Methods - Unless otherwise noted, peptide ligation methods for use in the methods and compositions of the invention can be performed according to Tam *et al., Biopolymers* (Peptide Science) 51:311-322 (1999); Dawson *et al., Science,* 266:776 (1994); and Tam *et al., Proc. Natl. Acad. Sci. U.S.A., 92:12485 (1995).*

Cyclic Peptide Synthesis by Thioester Ligation - Unless otherwise noted, cyclic peptide synthesis by thioester ligation for use in the methods and compositions of the invention can made according to Zhang *et al., J. Am. Chem. Soc.*, 119:2363-2370 (1997); Tam *et al.*, Tetrahedron Lett. 38:5599 (1997); Camarero *et al., Chem. Inlt Ed.*, 37:347-349 (1998); and Tam *et al., Protein Sci.* 7:1583-1592(1998); Zhang *et al., J. Am. Chem. Soc.*, 121: 3311-3320 (1999); Sun *et al., Org. Lett.*, 3 (11):1681-1684 (2001); Tam *et al., J. Am. Chem. Soc.*, 121:4316-4324 (1999); and Yan *et al., J. Am. Chem. Soc.*, 123:526-533 (2001).

Cyclic Peptide Library Production in Retroviruses - Oligonucleotides were synthesized using standard nucleic acid synthesis to typically encode 9 amino acid residues structured as a cyclic-Ser- X-X-X -Gly-Pro-X-X-X (SEQ ID NO: 1). The Serine residue is an acceptor of protein splicing, each X is represents any amino acid residue (except for a stop codon) and the Glycine and Proline (Gly-Pro) motif is designed to facilitate the cyclization of the peptide. The synthetic oligonucleotides were cloned in between the *Mfe* I and the *Hind* III sites between the N-terminal (In) and C-terminal (Ic) intein fragments of the DnaE gene engineered into a murine retroviral vector (*i.e.*, the pIMPS vector, see also Fig. 17, SEQ ID NO: 2). Recombinant products were electroporated into Top10 bacteria to generate a library having a complexity of approximately 10^8 independent colonies (clones).

Cell transfection and Virus Production - To generate infectious virions, 293T cells were transfected with 15 μg of retroviral peptide library DNA and 5 μg of helper virus DNA (EcoPac vector) using standard calcium phosphate precipitation. Briefly, 62.5μl of calcium chloride (2M) was added to the DNA, diluted in 425μl of distilled water, and a precipitate was obtained by adding 500μl of Hepes buffer 2X dropwise while vortexing slowly. The precipitate was then added to 293T cells in 5ml of DMEM

+ 10% FCS and allowed to incubate at 37° C for 7 hours. The cells were then washed and incubated with fresh media and supernatants containing infectious virions were collected at 24 and 48 hours post-transfection. Typically, packaging of the retroviruses was accomplished by transfecting a proviral vector (*e.g.*, pIMPS) into a commercially available packaging cell line such as, *e.g.*, EcoPack-293 (HEK293 cells; Cat. No. C3200-1; available from Clontech). For producing pantropic virus, co-transfections were performed using the VSV-G plasmid and GP-293 packaging cell line (available from Clontech; Cat. No. K1063-1)

Transduction of Retroviral Libraries- Cells were plated at $5x10^5$ cells per 10 cm diameter plate 24 hours prior to retroviral transduction infected using supernatant from 293T cells transfected with the library. Infections were carried out for 6 hours in the presence of 8 mg/ml of polybrene and continued overnight after the addition of additional growth media. The following day, cells were rinsed once with PBS and allowed to recover one day in growth media before phenotypic selection.

Titering of Retroviral Libraries – The titer of retroviral libraries was carried out either by packaging retroviral vectors encoding green fluorescent protein (GFP), transduction of these retroviruses, and quantitation of the number of cells demonstrating fluorescence by microscopy. Preferably, retroviral titer is also more directly established by Southern blot analysis of transduced cells. Serial dilutions of packaged retroviral stock are used to transduce host cells, and copy number is assessed by quantitative Southern blot; titer is extrapolated by determining the viral dilution at which transduction of host cells occurs at an average of one copy per cell.

Titering of EMCV Virus – Virions were produced by infection of L929 cells in a large 150 cm^2 flask with 0.5 ml aliquot of viral supernatant which were then allowed to incubated overnight. The next day, supernatants bearing infectious virions were harvesting and titered using a plaque forming unit (PFU) assay as described below. Briefly, murine L929 cells were plated as an ~80% confluent monolayer and inoculated with a 100 µl aliquot of various virus dilutions, incubated and then covered in media and agar and allowed incubate for 2 days. Cells were then fixed with formalin, the agar was removed, and cells attached to the plate were stained with 2% crystal violet/ 20% ethanol to allow for enumeration of the clear zones or plaque forming units (PFU) formed by as a result of successful infection by virus.

Titering of EMCV Virus by Cell Viability Assay: An Alternative High-Throughout Method – Cells (L929) were plated at a density of 10^4 cells/well (in 96 well plates), incubated overnight, and then infected with cell supernatants or serial dilutions of EMCV preparations in 100 µl of RPMI + 10% FBS. Infections were performed in a 37° C humidified CO2 incubator for about 20 hours. The viability of cells after the lytic infection of EMCV was measured using a colorimetric assay, according to the

manufacturer's instructions (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega).

Immunoblot Analysis – Cellular protein extracts were prepared by harvesting the cells, after transfection or transduction, washing the cells once with PBS and resuspending the cells in 1 ml of cold lysis buffer (50 mM Tris. HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, and 0.5% Triton X-100). The cells were then incubated on ice for 15 min, vortexed, centrifuged at low speed to eliminate nuclei, and the resultant supernatant was recovered for analysis. Protein samples were analyzed by SDS-PAGE gel (4-20% polyacrylamide pre- cast gel, Invitrogen) followed by immunoblotting onto nitrocellulose membranes using standard techniques. Proteins of interest were then resolved using immunodetection with an appropriate primary antibody (*e.g.,* murine anti-HIS or murine anti-GFP), blotting with a secondary antibody coupled to horseradish peroxidase (anti-mouse-HRP diluted at 1/2000), and visualized using ECL (Enhanced ChemoLuminescence; Amersham).

Throughout the examples, unless otherwise indicated, the practice of the present invention will employ conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA technology, cell culture, and animal husbandry, which are within the skill of the art and are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: Cold Spring Harbor Laboratory Press* (1989); *DNA Cloning*, Vols. 1 and 2, (D.N. Glover, Ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait, Ed. 1984); *PCR Handbook Current Protocols in Nucleic Acid Chemistry*, Beaucage, Ed. John Wiley & Sons (1999) (Editor); *Oxford Handbook of Nucleic Acid Structure*, Neidle, Ed., Oxford Univ Press (1999); *PCR Protocols: A Guide to Methods and Applications*, Innis *et al.*, Academic Press (1990); *PCR Essential Techniques: Essential Techniques*, Burke, Ed., John Wiley & Son Ltd (1996); *Nucleic Acid Hybridization* (B.D. Hames and S.J. Higgins, Eds. 1984); the series *Methods In Enzymology* (Academic Press, Inc.), particularly Vol. 154 and Vol. 155 (Wu and Grossman, Eds.; *Antibody Engineering Protocols (Methods in Molecular Biology),* 510, Paul, S., Humana Pr (1996); *Antibody Engineering: A Practical Approach (Practical Approach Series, 169)*, McCafferty, Ed., Irl Pr (1996); *Antibodies: A Laboratory Manual,* Harlow *et al.*, C.S.H.L. Press, Pub. (1999); Coffin, *et al., Retroviruses*, Cold Spring Harbor Laboratory Press (1997); Weiner, *et al., Chemical and Structural Approaches to Rational Drug Design (Pharmacology and Toxicology),* CRC Press (1995); Bodanszky, *et al., Peptide Chemistry: A Practical Textbook,* Springer Verlag Press (1993); McClelland, *et al., Expression Genetics: Accelerated and High-Throughout Methods (Biotechniques Update Series),* Eaton Pub Co., (1999); Devlin, *High Throughout Screening*, Marcel Dekker Press, (1997); Sucholeike, *High Throughput Synthesis*, Marcel Dekker Press (2001); Murray, *Principles and Practice of*

High Throughput Screening, Blackwell Science, Inc. (2001); Weiner, *et al.*, Biological Approaches to Rational Drug Design (Pharmacology and Toxicology), CRC Press (1994); Roth, *Methods in Cell Biology: Protein Expression in Animal Cells*, Academic Press, (1994), *Large-Scale Mammalian Cell Culture Technology*, Lubiniecki, A., Ed., Marcel Dekker, Pub., (1990); *Molecular and Cell Biology of Yeasts*, Yarranton *et al.*, Ed., Van Nostrand Reinhold, Pub., (1989); *Yeast Physiology and Biotechnology*, Walker, G., John Wiley & Sons, Pub., (1998); *Baculovirus Expression Protocols*, Richardson, C., Ed., Humana Press, Pub., (1998); *Methods in Plant Molecular Biology: A Laboratory Course Manual*, Maliga, P., Ed., C.S.H.L. Press, Pub., (1995); *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992) and *Manipulating the Mouse Embryo*, Hogan *et al.*, C.S.H.L. Press, Pub (1994)).

EXAMPLE 1

METHODS AND COMPOSITIONS FOR PRODUCING CYCLIC PEPTIDE APTAMER LIBRARIES USING PROTEIN SPLICING

In this example, methods and compositions are described for the production and phenotypic screening of peptide aptamers that undergo spontaneous cyclization through intein-mediated protein splicing.

An intein derived from *Synechocysti* (see also WO/36093) is used as proof of principle that intein sequences can be used to bring about targeted protein splicing in mammalian cells for the purpose of creating a cyclic polypeptide, *e.g.*, a cyclic peptide aptamer (*e.g.*, cPEP).

In brief, the pMFG vector, which is based on the Moloney Murine Leukemia Virus (MMLV), was modified to generate the pIMPS vector (SEQ ID NO: 2). Using methods well known in the art, the pIMPS vector, and derivatives thereof, can be transfected into appropriate cell lines for packaging and production of recombinant retrovirus. The pIMPS vector (see Fig. 17) was designed to encode and express an I_C intein splicing element, followed by a multiple cloning site and an -I_N intein splicing element. (The endogenous *Hind* III site in pMFG was removed by *Hind* III cleavage, Klenow fill-in, and religation.) A split intein, derived from the two *DnaE* genes of *Synechocysti* PCC6803 (Accession Nos: D90917 AB001339 and D90904 AB001339), was incorporated into the resulting vector by standard cloning techniques. A schematic drawing of the pIMPS vector is shown in Fig. 17. (see also SEQ ID NO: 2).

A general strategy for the insertion of nucleotide inserts encoding library peptides or proteins that undergo intein-mediated splicing upon intracellular expression using the pIMPS vector is described below and in Table 3. The pIMPS vector is digested with *Mfe* I and *Hind* III. The I_C-I_N junction at the Hind III site was engineered to create a frameshift in the absence of an insert between I_C and I_N. In addition, the

pIMPS vector is designed not to encode key catalytic residues at the Ic/In insertion site. Instead, the 5' end of the aptamer peptide or polypeptide insert is designed to be compatible with the *Mfe* I overhang and digesting with *Mfe* I, *EcoR* I, or *Apo* I enzymes will generate a suitable 5' 'AATT' extension. The 'AAT' of the extension is the codon for the catalytically-required asparagine of I_C. The remaining 'T' of the overhang is the first nucleotide of the I_C nucleophile. Depending on the restriction enzyme used to digest the insert, and therefore the nucleotide immediately following the restriction site, the resultant I_C nucleophile will vary as provided below in Table 3.

Table 3

Insert restriction site (5')	Codon/I_C nucleophile	splicing competent
CAATTGy (Mfe I)	TGY / C	yes
CAATTGa (Mfe I)	TGA / STOP	no
CAATTGg (Mfe I)	TGG / W	yes
GAATTCn (EcoR I)	TCN / S	yes
RAATTYn (Apo I)	TTN / F or L	yes
RAATTYn (Apo I)	TCN / S	yes

The 3' end of the insertion site of pIMPS is designed to contain a *Hind* III site, but lacks coding sequences for cysteine and leucine residues that are required for optimal splicing. Accordingly, the optimal aptamer insert ends with the following sequence: TGY YTA AGC TT (SEQ ID NO: 5), has a *Hind* III site and sticky end, and its insertion reconstitutes the correct number of nucleotides and codons necessary for I_N to function in the intein splicing reaction. If desired, the cysteine can be converted to other residues to modulate activity by changing the 'TGY' sequence to a codon encoding another amino acid. Combining the guidelines from above, the "top" or "coding" strand of a "functional" pIMPS insert (following digestion or other modifications necessary to prepare it for insertion into the pIMPS vector) has the following sequence: AAT TSY $(NNN)_X$ TGY YTA (SEQ ID NO: 6), and therefore has a total length of 3X+12 where X is the number of random amino acid residues desired in the translated and circularized final peptide product.

Using the methods described above, a library of self-cyclizing random peptides was constructed in the following manner. Sequences encoding and flanking the peptide aptamer sequence were designed to be cleavable by *EcoR* I and *Hind* III sites, which is then digested and inserted in frame with the upstream I_C coding sequences and the downstream I_N coding sequences:

G AAT TCT $(NNN)_x$ TGC TTA AGC TT (SEQ ID NO: 7)

N S X C L S (SEQ ID NO: 8)

Accordingly, the complete nucleotide sequence will have a total length of 3X+18 basepairs (counting from the 'G' in *EcoR* I to the second '*T*' in *Hind* III) plus any additional residues used to flank the *EcoR* I and *Hind* III sites. The resulting plasmids are then transformed and propagated in *E. coli* using standard techniques. The plasmids are then harvested, transfected into packaging cells and the resultant virions produced are used to transduce the random peptide library into cells in which expression and subsequent splicing of the library inserts is allowed to occurs in cells. The cells containing peptides that bring about a phenotypic change are then identified as described herein.

Upon completion of requisite screening and counter-screening steps or procedures, DNA from the population of cells possessing the desired phenotype is harvested by standard methods. For example, the PUREGENE DNA Isolation kit from Gentra Systems yields genomic DNA in sufficient amounts and purity to generate PCR products from as few as 100 cells. The primer pair for retrieving the sequence encoding the identified candidate aptamer having functional activity is:
(forward, for I_C, termed ENOL 351:

GCCGTCTAGAGCCG**CCATGG**TTAAAGTTATCGGTCGTCGTTCCCTC (SEQ ID NO: 9)
(*Nco* I site in bold) (reverse, for I_N, termed ENOL 354)

GCTA**GTCGAC**TTATTTAATTGTCCCAGCGTCAAGTAATGGAAAGGG (SEQ ID NO: 10)
(*Sal I* site in bold).

The resultant PCR product using this primer pair encodes the entire ORF (open reading frame) of a retrovirally transduced I_C-CP-I_N, flanked by *Nco* I and *Sal* I cloning sites. In the example shown, the expected PCR product is 533 bp with a 507 bp ORF (aptamer library insert). This insert can be re-cloned into pIMPS (or any other vector appropriate for expression in mammalian cells or repackaging into a secondary library. Alternatively, or in parallel, the amplified insert can be sequenced using standard techniques.

In addition to the pIMPS vector, a modified derivative was produced to allow facile monitoring of intein splicing reaction. This vector, referred to as the pIMPS-cHIS and shown in Fig. 18, contains Ic encoding sequences and a site for insertion of peptide aptamer coding sequences identical to those of pIMPS. In addition, a sequence encoding a 6XHIS tag (*i.e.* six consecutive histidine codons) was added to the I_N moiety by insertion of a short synthetic fragment.

This modification of pIMPS-cHIS provides two useful features. First, cell lysates from cells transfected with a pIMPS-cHIS plasmid into which peptide or protein coding sequences have been cloned into the insertion site, can be analyzed by standard methods (*i.e.*, immunoblot) to confirm expression of an I_C-Insert-I_N protein. The presence of a 6XHIS tagged protein confirms that an insert (or family of inserts in the case of a peptide library) is present in pIMPS-cHIS, since, as described above, an appropriate insertion event is necessary to adjust the reading frame downstream of the insertion site to encode an In moiety that contains a polyhistidine tag, in the case of pIMPS-cHIS.

Results of intein mediated splicing of proteins or peptides expressed by the pIMPS-cHIS vector in mammalian cells are shown in Fig. 19. pIMPS-cHIS vectors into which coding sequences for the green fluorescent protein (GFP) or a short test cyclic peptide (TCP) were cloned and expressed in frame between the I_C and I_N intein elements. Cell lysate samples in which splicing- competent forms of these respective precursors were transfected are denoted as sGFP and sTCP, and non-splicing controls are denoted as nsGFP and nsTCP. Unprocessed and processed forms of these proteins are detected in the left panel by probing by immunoblot with an antibody that recognizes the 6XHIS tag at the carboxy- terminus of the I_C-INSERT-I_N-HIS precursor and the smaller In-HIS splicing product, respectively (Fig. 22).

Analogous experiments can be performed analyzing lysates of cells that have been transduced by recombinant pIMPS-cHIS- derived retroviruses or retroviral libraries.

EXAMPLE 2
INCORPORATION OF ß-TURN MOTIFS TO IMPROVE SPLICING EFFICIENCY AND INTRACELLULAR PEPTIDE CYCLIZATION THROUGH INTEIN- MEDIATED PROTEIN SPLICING

In this example, methods and compositions for producing intein peptide aptamers that comprise β-turn motif for improved intein-mediated protein splicing, are described.

Libraries encoding random peptides in which all residues in the cyclized product are random, with the exception of residues that are required for splicing itself (*i.e.*, the serine or cysteine nucleophiles that are necessary for intein-mediated splicing, and that remain in the cyclic peptide product after the reaction), can also be engineered to contain one or more ß-turn motifs. For example, Gly-Pro or Pro-Gly residues can be fixed within the random peptide.

```
AAT NNN NNN NNN NNN GGN CCN NNN NNN NNN NNN NNN        SEQ ID NO: 12
    NN NNN NNN NNN CCN GGN NNN NNN NNN NNN NNN TCGA    SEQ ID NO: 13
      X   X   X   X   G   P   X   X   X   X            SEQ ID NO: 14
```

Peptide libraries containing this sequence have the following advantages which are: (i) the cyclic peptide product contains a structural motif that serves as context for the remaining random residues, and (ii) the flexibility within the cyclic peptide coding sequence improves the protein splicing efficiency of the modified peptide flanked by intein domains (I_C-cPEP-I_N) (Cochran *et al.*, *Proc. Natl. Acad. Sci. USA* 98: 5578-5583 (2001)). This was demonstrated with the following experiment.

The following *Hind* III compatible / *Mfe* I compatible double-stranded oligonucleotide sequences were cloned into the *Mfe* I-*Hind* III sites of the pIMPS-cHIS vector:

```
AAT TTG TTC CTG TAC GGG CCC TGG AAA GAC TTG TTA        SEQ ID NO: 15
     AC AAG GAC ATG CCC GGG ACC TTT CTG AAC AAT TCGA   SEQ ID NO: 16
      L   F   L   Y   G   P   W   K   D   L           SEQ ID NO: 17

AAT TTG TTC CTG TAC GCC GCC TGG AAA GAC TTG TTA        SEQ ID NO: 18
 AC AAG GAC ATG CGG CGG ACC TTT CTG AAC AAT TCG AAA    SEQ ID NO: 19
     L   F   L   Y   A   A   W   K   D   L   L        SEQ ID NO: 20
                   (G) (P)

AAT TCG TTC CTG TAC GCC GCC TGG AAA GAC TGC TTA        SEQ ID NO: 21
 GC AAG GAC ATG CGG CGG ACC TTT CTG ACG AAT TCG A      SEQ ID NO: 22
     S   F   L   Y   A   A   W   K   D   C   L        SEQ ID NO: 23
                   (G) (P)
```

The resulting plasmids were transfected into 293T cells, and splicing efficiency was determined by immunoblot detection of the I_N-HIS reaction product in cell lysates of the transfected cells (see Fig. 20). Comparison of the ratio of unspliced products in extracts from cells expressing a test cyclic peptide (TCP) containing a Gly-Pro ß-turn structure compared to an analogous peptide in which the Gly-Pro ß-turn structure is substituted by Ala-Ala, were measured and indicated a splicing efficiency of the peptide containing a Gly-Pro ß-turn structure to be approximately 30% more efficient.

EXAMPLE 3
METHODS AND COMPOSITIONS FOR PRODUCING CYCLIC PEPTIDE APTAMER LIBRARIES FUSED TO GREEN FLUORESCENT PROTEIN (GFP)

In this example, methods and compositions for producing peptide aptamers fused to an intein that can undergo protein splicing and are fused to green fluorescent protein (GFP), are described.

While several contexts for insertion of random amino acid domains into GFP have been described, these strategies have focused on identification of looped or structurally constrained domains within GFP that are presented and accessible on the outer surface of the GFP protein, which can tolerate insertion of exogenous amino acids without decreasing fluorescence (see U.S. Patent Nos. 6,025,485 and 6,180,343).

Several such domains within GFP have been identified and used in this manner. However, in each case, the fluorescent properties of GFP are significantly lower than the native protein: even in the best cases, fluorescence is decreased by at least an order of magnitude upon insertion of peptide coding regions within looped domains of GFP.

The present invention provides specific regions of the green fluorescent protein which can be altered to accommodate an aptamer sequence without any deterioration of the fluorescent activity of the GFP. The GFP protein is composed of a series of ß-strands arranged as a cylinder, with the free N- and C- termini of the protein projecting upward, away from the "top" of the barrel structure (see Fig. 21). The projection of these free N- and C- termini facilitate the fusion of heterologous proteins to either end of GFP without adversely affecting the function of the fused protein or the fluorescent properties of GFP. This invention uses I_C and I_N intein splicing domains that are fused to the amino and carboxy termini of native GFP. These intein domains then carry out peptide ligation, resulting in a covalently closed, cyclized GFP protein with no free N- or C- terminus, and producing liberated I_N and I_C products.

This reaction is used to generate libraries in which random peptides are presented in a constrained loop that extends across the space that normally exists between the N- and C- termini of native GFP. This is carried out as follows: nucleotides encoding random amino acids are inserted into a retroviral vector downstream of an I_C coding sequence, and upstream of the beginning of GFP. Sequences encoding a second set of random amino acids are positioned in frame following the GFP coding sequence, followed by coding sequences for the I_N intein domain. These respective random amino acid sequences, which flank the GFP coding region, and which are themselves flanked by I_C and I_N, are referred to respectively as "X" and "Y". Accordingly, these random elements are expressed in the cell as part of a contiguous precursor protein (I_C-X-GFP-Y-I_N). The I_C and I_N intein elements of this precursor protein undergo splicing to produce a cyclic GFP product that joins the X and Y peptide domains, generating a contiguous (XY) peptide that extends across the top of the GFP barrel. The resulting GFP product has no free N- or C- termini, but instead presents the peptide aptamer as a constrained peptide fused to GFP.

By expressing a chimeric GFP, intein-mediated splicing results in a covalent cyclization of a GFP protein in which the aptamer is presented in a way that does not intervene in any GFP sequence such that its fluorescence is diminished. The expression of cyclized GFP proteins that present random peptide aptamers can then be accurately monitored by the presence of green fluorescent protein activity using standard techniques, *e.g.*, fluorescent activated cell sorting (FACS).

As a proof of principle, the myc epitope consisting of the amino acid sequence EQKLISEEDL (SEQ ID NO: 11) that is recognized by the 9E10 monoclonal antibody was genetically fused to GFP using standard genetic engineering. Specifically, oligonucleotide linkers were designed to place half of the myc epitope in front of the GFP reporter protein and the other half behind the GFP protein, *i.e.*, as SEEDL [green fluorescent protein (GFP)] EQKLI and as shown in Fig. 12 (SEQ ID NOS: 24-25). The subsequent intein-mediated splicing reaction generates a contiguous EQKLISEEDL polypeptide sequence, which is presented as a novel loop "across the top" of the GFP, resulting in a restoration of the myc epitope sequence which is not present in the absence of cyclization. This can be confirmed using the 9E10 monoclonal antibody that only detects the restored, *i.e.*, correctly spliced sequence of the myc epitope.

To determine that the above myc GFP fusion encoded the expected protein, cells were transfected with a vector also containing a histidine epitope (for purification and immunodetection; SEQ ID NOS: 3-4) and transduced using packaged retrovirus and the resultant HIS tagged proteins were harvested and purified using standard NiNTA chromatography followed by elution with imidazole. The purified HIS tagged proteins were then cleaved with enterokinase protease and analyzed by SDS-PAGE and determined by immunoblot (Fig. 22).

EXAMPLE 4
METHODS FOR PRODUCING CONOTIDES

In this example, methods and compositions for making cyclized conotoxins or conotides, by intein-mediated protein splicing and/or disulfide bond replacement, are described.

Briefly, conotoxins or conotides are generated either through chemical cyclization of a linear synthesized peptide, or through the intein-mediated splicing as described herein. In either approach, the naturally occurring proximity of the amino- and carboxy- termini of the conotoxin peptide are maintained through a covalent (amide) bond, thereby rendering these molecules structurally constrained but protease resistant. In the case of cyclic peptides (conotides) derived from naturally occurring conotoxins, the cyclic peptides produced in this manner are tested for improved activity corresponding to that of the naturally occurring conotoxin peptide. For example, the cyclized form of the α–conotoxin GI peptide sequence (ECCNPACGRHYSC; SEQ ID NO: 26) and the improved activity of this cyclized form compared to that of the naturally occurring α–conotoxin GI peptide can be measured by standard binding assays.

In one embodiment, forms of these cyclic peptides are produced in which one or more cysteine pairs are eliminated. In these peptides, the stable structural constraints that are normally provided by cysteine-mediated disulfide bonds are instead provided by the amide closure. For example, in the cyclized α–conotoxin GI peptide, the cysteine residues at positions 3 and 13, which normally form a structurally important disulfide linkage, are replaced in the cyclic peptide with other non disulfide forming amino acids (*i.e.* alanine, methionine, serine, threonine, glycine, leucine, isoleucine, valine), and the resulting cyclized peptides are tested for α–conotoxin GI activity. The same process is carried out for replacement of the cysteines that mediate the disulfide bridge between residues 2 and 7 of the α–conotoxin GI peptide. In addition to these strategies that examine replacement of these disulfide bond functions separately, peptides can also be generated and tested that replace all four structurally important cysteines, thereby eliminating both endogenous disulfide bonds, for improved activity.

Within the process described above, in which replacement of one or more disulfide bonds is examined systematically, additional replacement strategies are contemplated. In the case of the α–conotoxin GI peptide, replacement of disulfide bond forming cysteine pairs with aromatic amino acid residues (*e.g.* tryptophan, tyrosine, or phenylalanine) allows ring stacking, thereby stabilizing critical secondary structures within the cyclic peptide, but alleviating the need for one or both disulfide bridges. Such ring stacking by aromatic residues has been shown to facilitate the formation of stable monomeric ß- hairpins in linear peptides (Cochran *et al.*, 2001) which sponsors the formation of functional conotoxin structures in conotoxin-derived peptides where one or more cysteine pairs have been replaced. This added structural organization associated with cyclization of the conotides augments the efficacy of aromatic ring stacking in forming secondary structures associated with conotoxin activity.

An alternative to this approach is the use of salt bridges to fulfill the role of cysteine mediated disulfide bonds. For example, in the case of the α–conotoxin GI peptide, a positively charged amino acids such as lysine or arginine is used to replace the cysteine in position 3, in conjunction with glutamic acid or aspartic acid substitution of the partnering cysteine residue at position 13 to create a conotide with improved activity. The opposite configuration, in which an acidic residue is substituted for cysteine at position 3 and a basic residue replaces the cysteine at residue 13 can also be used. This substitution strategy is applied to either disulfide- forming cysteine pair (*e.g.* 3-13 and 2-7 pairs in the α–conotoxin GI peptide example) either singly (one at a time) or in combination (one or more disulfide bond forming cysteine pairs at a time).

The conotides described above are then tested by comparing the conotide against the conotoxin it was derived from (*e.g.*, synthesizing α–conotoxin GI in cyclic and non-cyclic form) for binding to, *e.g.*, nAChRs, *e.g.*, using the *T. californica* electric organ assay, as recognized in the art.

EXAMPLE 5
METHODS AND COMPOSITIONS FOR PRODUCING CONOTIDES USING INTEIN-MEDIATED PROTEIN SPLICING

In this example, methods and compositions for using intein-mediated protein splicing to generate cyclic conotoxin-derived peptides ("conotides"), are described.

Briefly, conotoxin coding sequences are expressed as a fusion protein between the I_C and I_N intein splicing domains. Upon translation of the I_C-conotoxin-I_N fusion protein or conotide, followed by spontaneous intein-mediated protein splicing, a cyclic peptide product composed of conotoxin residues is produced, along with the I_C and I_N side products. This can be carried out in a variety of contexts, including *in vitro* translation of the I_C-conotoxin-I_N fusion protein, as well as expression of the I_C-conotoxin-I_N fusion protein in bacteria, yeast, insect cells (*e.g.* recombinant baculoviral expression), or in mammalian cells (*e.g.*, CHO cells).

For expression of conotoxin-derived peptides (conotides) in mammalian cells, conotoxin derived coding sequences are inserted between the I_C and I_N elements of the pIMPS vector. Expression of the I_C-conotoxin-I_N peptide results in intein splicing and production of the cyclized conotide. A preferred class of conotides are derived from a class of conotoxins that do not depend on extensive post-translational modification. The α and αA–conotoxins meet this requirement as well as other members of conotoxin families that have an amidated C-terminus such that there are no post-translational modifications in the "small loop" structure believed to be the binding site.

In generation of cyclized conotoxin- derived peptides (conotides) such as the α-conotoxin GI peptide with the naturally occurring sequence ECCNPACGRHYSC, gene cassettes express the following cyclic permutations of this sequence. Scanning of cyclic permutations allows the examination of several configurations of the splicing reaction and insert, and allows selection of the most efficient intein splicing form for conotide production. (However, it is important to maintain a cysteine or serine nucleophile at the first position of the conotide coding sequence, in frame and immediately downstream of the I_C intein splicing element, in order for intein splicing to occur.) Exemplary amino acid and oligonucleotide sequences for the α- conotoxin GI peptide are listed below:

```
tgc tgc aac ccg gcg tgc ggt cgt cat tat agc tgc ggt agc gaa
C   C   N   P   A   C   G   R   H   Y   S   C   G   S   E
(SEQ ID NOS: 27, 28)

tgc ggt cgt cat tat agc tgc ggt agc gaa tgc tgc aac ccg gcg
C   G   R   H   Y   S   C   G   S   E   C   C   N   P   A
```

(SEQ ID NOS: 29, 30)

tcg tgc ggt agc gaa tgc tgc aac ccg gcg tgc ggt cgt cat tat
S C G S E C C N P A C G R H Y

(SEQ ID NOS: 31, 32)

An analogous approach, using an appropriate bacterial expression vector, such as the pET derived vector, can be used to generate such conotoxin-derived peptides in bacteria. Results from the expression and processing of these respective intein flanked conotides (*i.e.*, I_C-conotoxin-I_N-HIS) in *E. coli* are shown in Fig. 23. In particular, ratios of spliced products and unspliced precursors were determined by immunoblot analysis of bacterial lysates using antibodies against a engineered epitope (6XHIS epitope tag).

In addition, replacement strategies analogous to those described above can be performed, using recombinant DNA techniques to replace designated cysteine residues within the cyclizing peptides with codons encoding other amino acids, and the resulting peptides are then tested for activity. One advantage associated with the use of intein splicing to generate cyclic conotoxin-derived peptides is the economy and high throughput of this approach, which permits the systematic replacement of one or both sets of cysteines in the α–conotoxin GI peptide with amino acids that feature non-disulfide forming side chains described above. Therefore, all possible permutations can be readily generated and tested. The use of recombinant DNA techniques to generate all possible permutations facilitates this.

One class of conotides of the invention is derived from the α and αA–conotoxins which are competitive antagonists of nicotinic acetylcholine receptors (nAChRs). There are two distinct families of nAChRs in vertebrates; muscle-type nAChRs are located at skeletal neuromuscular junctions, while neuronal nAChRs are located in the central and peripheral nervous system. Muscle and neuronal nAChRs consist of five homologous subunits arranged around a central pore. α–conotoxins with subtype specificity for muscular and neuronal nAChRs have been characterized. α–conotoxins EI, GI, MI and SI target the muscular nAChRs, which are composed of two α units and one each β, γ, and δ unit. Other α and αA–conotoxins, which are composed of several α and β units, target neuronal nAChRs

Conotides based on the foregoing preferably have an insertion of charged amino acids that result in changes in the affinity of the conotide for a particular target, as for example, compared to a conotoxin control. For instance, in α–conotoxin SI (ICCNPACGPLYSC-NH2; SEQ ID NO: 33), Pro9 replaces the Arg9 found in α–conotoxin GI. This change results in α–conotoxin GI that are lethal to mammals at low concentrations. α–conotoxin SI, however, while active in *T. californica*, produces essentially no effect in mammals. The sequence differences between the conotoxins also results in different affinity for different sites on the same receptor. α–conotoxin SI

does not distinguish between the α/δ and α/γ sites in *T. californica* electric organ, but α–conotoxin GI finds the α/δ site preferable to the α/γ site. The exemplary α-conotoxin GI and its derivatives described above can be screened for receptor binding and specificity.

EXAMPLE 6

METHODS AND COMPOSITIONS FOR PRODUCING AND SCREENING CONOTIDE LIBRARIES

As described above, the conotoxin peptides, or more particularly, structural motifs found in naturally occurring conotoxin peptides, are privileged structures that have been evolutionarily selected to be ideally suited for binding to receptors, including nAch receptors, as well as various ion and voltage gated channels. This invention contemplates the use of recombinant DNA techniques to generate and phenotypically screen random peptide libraries in which residues of a given conotoxin that do not have important structure-determining roles are replaced by random amino acids to produce a conotide library. Conotide libraries are generated by insertion of the coding sequences below into a vector suitable for expression of the encoded peptide. Although this expression can be carried out in a variety of cells (*e.g.* bacteria, yeast, insect cells or mammalian cells), in preferred embodiments, retroviral vector systems are used to generate and express the random peptide libraries in mammalian cells, where phenotypic screening can be carried out.

Conotoxin peptides, as they exist in nature, are secreted and act extracellularly upon target membrane proteins of cells within the prey organism. Thus, although the targets of individual conotoxins may vary, the tasks for which they were evolutionarily selected did not require that they act upon intracellular targets. However, in consideration of the observation that conotoxin-like molecules are uniquely suited for highly specific interaction with various membrane receptors and channels, intracellular expression of conotoxin-like peptides, accompanied by screening of cell populations expressing random peptides that incorporate structural elements of conotoxin-derived backbones, allows for the ability to screen random, structurally constrained conotide libraries for unique functions. By expressing and screening these libraries inside the cell, a broad range of conotides capable of altering a cell phenotype can be identified.

Several types of libraries are contemplated. For example, libraries based on naturally occurring conotoxin families can be generated by inserting DNA fragments shown below into a vector suitable for their expression and translation.

α-Conotoxin- Based Random Peptide Libraries:

ATG $(NNN)_{1-2}$ TGT TGT NNN CCC GCC TGT GGG $(NNN)_4$ TGT $(NNN)_{0-4}$ TGA

M X C C X P A C G X C X *

(SEQ ID NOS: 34, 35)

μ-Conotoxin- Based Random Peptide Libraries:

ATG $(NNN)_2$ TGT TGT $(NNN)_2$ CCC CCC TGT $(NNN)_4$ TGT NNN CCC $(NNN)_2$ TGT TGT NNN TGA

M X C C X P P C X C X P X C C X *

(SEQ ID NOS: 36, 37)

ω-Conotoxin- Based Random Peptide Libraries:

ATG TGT $(NNN)_6$ CTT $(NNN)_{5-6}$ TGT TGT $(NNN)_{2-3}$ TGT $(NNN)_{4-6}$ TGT $(NNN)_{0-6}$ TGA

M C X P X C C X C X C X *

(SEQ ID NOS: 38, 39)

In the libraries described above, the cysteine residues are retained. However, additional libraries can be made that incorporate the substitution strategies described above. Namely, codons encoding charged amino acids capable of forming salt bridges can be used to replace cysteine partners. For example, such a library based on an α–conotoxin can be composed using the following oligonucleotide inserts:

α-Conotoxin- Based Random Non-Cysteine/Salt Bridged Peptide Libraries:

ATG $(NNN)_{1-2}$ GAN AAA NNN CCC GCC AAA GGG $(NNN)_4$ GAN $(NNN)_{0-4}$ TGA

AGA AGA

M X E/D R/K X P A R/K G X E/D X *

(SEQ ID NOS: 40, 41)

Analogous libraries based on the μ- and ω-conotoxins incorporate complementarily charged residues at respective cysteine partners, in such a way that the salt bridges formed can fulfill the structural roles of the endogenous disulfide bonds in these conotoxin backbone families.

Stacking of aromatic rings is another strategy for the replacement of structural cysteines in this format. Again, using the α-conotoxin backbone as an example:

α-Conotoxin- Based Random Peptide Libraries Constraining Stacked Aromatics:

ATG $(NNN)_{1-2}$ TGG TGT NNN CCC GCC TGG GGG $(NNN)_4$ TGT $(NNN)_{0-4}$ TGA

M X W C X P A W G X C X *

(SEQ ID NOS: 42, 43)

ATG $(NNN)_{1-2}$ TGT TGG NNN CCC GCC TGT GGG $(NNN)_4$ TGG $(NNN)_{0-4}$ TGA

M X C W X P A C G X W X *

(SEQ ID NOS: 44, 45)

ATG $(NNN)_{1-2}$ TGG TGG NNN CCC GCC TGG GGG $(NNN)_4$ TGG $(NNN)_{0-4}$ TGA

M X W W X P A W G X W X *

(SEQ ID NOS: 46, 47)

In these libraries, codons encoding one or more pairs of aromatic amino acids replace one or more cysteine pairs in the backbone. Although tryptophan (W) is shown in this example, all possible combinations of aromatic amino acids (*i.e.* tryptophan, tyrosine, or phenylalanine) are also contemplated. In addition, the same strategy is applicable to libraries based on the μ- and ω-conotoxin family backbones.

In one embodiment, the receptor binding activity and specificity of the identified conotide(s) is compared to a naturally occurring conotoxin. In another embodiment, the conotide is assayed for its ability to alter a cell phenotype, *e.g.*, when expressed in a cell. This process identifies conotides with desired biochemical or biological activities, along with improvement of other chemical or physiological properties. In addition, this process allows the systematic derivation of a structure/activity relationship profile that informs the transformation of other conotoxins into conotides, or the design of random peptide library schemes with improved properties, as described below.

EXAMPLE 7

USE OF INTEIN- MEDIATED SPLICING TO GENERATE AND PHENOTYPICALLY SCREEN CONOTIDE LIBRARIES

The substitution strategies used for the libraries described above describes the generation of linear peptide libraries that can be expressed inside cells for phenotypic screening. This invention also encompasses the generation and screening of libraries derived from the conotoxin backbones, but that are spontaneously cyclized intracellularly through intein mediated splicing.

In all examples below, the nucleotide sequences shown are inserted in frame with both upstream Ic sequences and downstream In sequences, resulting in the production of a self-processing fusion protein composed of Ic-Conotoxin Derived Library Peptide-In. In using the pIMPS vector, oligonucleotide primers are designed such that an *Mfe* I compatible cloning site is present upstream of the library insert, and a *Hind* III compatible cloning site downstream of the library insert (see Fig. 17).

In this approach, intein-mediated cyclization facilitates the ability to eliminate structurally important cysteines, and, using the substitution strategies described above (*i.e.*, amino acid substitutions that lead to salt bridge formation, stacking of aromatic amino acid residues, etc.), leads to stable, structurally constrained and organized cyclic amino acids that feature privileged conotoxin-like backbone structures, but that do not rely upon cysteines for their structural integrity and function, and that are present intracellularly, where they have access to a milieu of novel targets. Although only libraries based on themes of the α–conotoxin backbone are shown herein, the invention also encompasses these strategies applied to the generation and screening of libraries

based on the μ- and ω-conotoxin family backbone, using, for example, the basic formula I_C-conotide insert (*e.g.*, SEQ ID NOS: 34-47)-I_N.

In summary, libraries generated in this manner use conotoxin peptide backbone structures, further informed and improved through identification and systematic replacement of critical but potentially labile or unstable secondary structures (*i.e.* cysteine replacement and formation of a cyclically contiguous amide backbone) to screen and identify conotides that modulate categories of targets known to be susceptible to the activities of naturally occurring conotoxin peptides, as well as novel classes of targets, including intracellular or other novel targets for which a phenotypic or functional activity in mammalian cells is known. This includes all varieties of signal transduction cascades, metabolic regulatory systems, susceptibility to infection or pathogenesis of viruses, bacteria, or toxins thereof.

EXAMPLE 8
METHODS AND COMPOSITIONS FOR PRODUCING APTAMER LIBRARIES FUSED TO HEDGEHOG FOR STEROL CONJUGATION AND MEMBRANE LOCALIZATION

In this example, methods and compositions for producing peptide aptamers that undergo hedgehog polypeptide-mediated sterol conjugation are described.

The aptamer libraries of the invention can be engineered to take advantage of the sterol conjugation domain of the hedgehog polypeptide. Previous experiments examining the mechanism of hedgehog autocleavage have demonstrated that although the integrity of the C-terminal region of the hedgehog protein (*i.e.*, Hh-C domain) is critical for function, most of the N-terminal domain (*i.e.*, Hh-N domain) is dispensable (Fig. 24A).

Accordingly, aptamer peptide libraries can be constructed in which the coding sequences for random peptides are fused in-frame with the essential C-terminal region of hedgehog polypeptides such that hedgehog splicing results in the covalent modification of cholesterol with random peptides (see Fig. 24B). Moreover, this esterification reaction can occur with cholesterol derivatives and other sterols, *e.g.*, 7-dehydrocholesterol, 5-androsten-3ß-ol (Mann and Beachy, 2000)). Accordingly, this same library of fusion polypeptides can be used to produce a library of peptides conjugated to any cholesterol or sterol molecule in which such splicing can occur.

The utility of such a library is that cholesterol-conjugated peptides can be localized to the cell membrane. Accordingly, directed localization of these peptide/sterol conjugates can facilitate the identification of molecules that act through targets localized at the cell surface (*e.g.* ion channels or membrane transporters, signal transduction receptors, or effectors of receptor mediated signal transduction). Alternatively, cholesterol or sterol- conjugated peptides can be discovered that affect

various aspects of cholesterol metabolism, both with respect to lipid metabolism and homeostasis (*i.e.* LDL, HDL, etc.) and synthesis or activities of various cholesterol derived sterol hormones. In other specific aspects of the invention, libraries affecting phenotypes associated with the functions of cellular proteins with cholesterol sensing activity (*e.g.*, SCAP, HMG CoA reductase, patched, dispatched) or cholesterol or sterol metabolism (*e.g.* SREBP, ACAT1, ACAT2, CETP), ABC1 mediated cholesterol transport, or other intracellular cholesterol trafficking or localization, can be used.

In addition, multiple functions have been ascribed to lipid rafts and caveolae, cholesterol-rich sub-compartments of the plasma membrane (reviewed in Galbiati *et al.*, 2001). These include, but are not limited to, modulation of the activities of signaling proteins, including oncogenes (*e.g.* ras, raf, rac, rho) and G- protein coupled receptors or their effectors, ion channels, receptor kinases or effectors of their activity, protein import or export, intracellular lipid or protein compartmentalization, as well as viral entry or egress.

An exemplary aptamer/hedgehog expression vector was constructed as follows. Briefly, a cDNA fragment encoding the carboxy terminus of human sonic hedgehog (SEQ ID NO: 48) was cloned into the *BamH* I and *Xho* I sites of the pMFG expression vector (other species of hedgehog polypeptide can also be used). The resultant hedgehog cleavage and corresponding cloning sites for the aptamer sequence is illustrated in Fig. 11 and the sequence of the essential hedgehog polypeptide region (human sonic hedgehog) is provided in SEQ ID NO: 49. Since splicing activity of this minimal C-terminal hedgehog domain coupled to heterologous upstream peptide or protein sequences has not been previously examined or demonstrated, a proof-of-concept experiment was performed in which a gene cassette encoding the GFP protein was cloned upstream of and in frame with the C-terminal hedgehog domain. This plasmid, encoding the fusion protein in the pMFG vector, was transfected into mammalian cells. Cleavage and processing was assessed by immunoblot analysis of transfected cells (Fig. 25). These results demonstrate the presence of an intracellular cleaved GFP in cells expressing a splicing-competent GFP-Shh fusion protein. Importantly, spliced GFP protein in the hedgehog- processing samples had slightly slower mobility in SDS-PAGE gel analysis than an unmodified intracellularly expressed GFP protein, consistent with the covalent attachment of cholesterol to the cleaved GFP product of the intracellularly processed GFP-Shh fusion protein.

In a manner analogous to the introduction of GFP coding sequences upstream of the C-Shh coding sequences in the experiment described above, oligonucleotides encoding random amino acid sequences can be inserted, and expressed intracellularly to yield spontaneously processed cholesterol-ligated peptide libraries. The length of this random coding region is pre-determined in the design of the oligonucleotides. In

addition, specific features of peptides or proteins may be incorporated into the design of such libraries and their coding sequences. These include such elements as SH2 or SH3 domains, or libraries that fix certain elements of these domains (*i.e.* one or more amino acid residues identified as consensus sequences), while randomizing the remaining positions. Such an approach allows for the exploration of additional sequences that improve the binding, activity, or specificity against a given target, while in other cases, such a strategy expands the range of potential targets that can be addressed using a given library. The foregoing steps result in a retroviral library in which sequences encoding a random peptide of determined length is expressed in frame with the C-terminal domain of a selected hedgehog polypeptide. The species of origin or the exact identity of the hedgehog polypeptide is not critical, as long as the polypeptide selected retains the ability to spontaneously auto-process and conjugate a sterol (*e.g.*, to a peptide).

The oligonucleotide library can then be packaged into retroviruses by co-transfection into EcoPac cells with the pVSV-G vector as described herein. The resulting retroviral library can be used to transduce a plurality of test cells and phenotypic selection is carried out. Cells expressing a peptide-hedgehog fusion that is processed to form a cholesterol-peptide conjugate that manifested a desired phenotypic can then be identified.

As a proof of principle for this approach, the green fluorescent protein (GFP) was fused with the catalytic domain of hedgehog (human) having either a functional or non-functional cleavage site (e.g., the functional cleavage site has the sequence Ser-Gly-Gly-Cys-Phe whereas the nonfunctional cleavage site has the sequence Ser-Gly-Gly-Ala-Phe where the absence of a Cys destroys the cleavage site) and cloned into a retroviral expression vector. Constructs were packaged into ecotropic retroviruses and correct protein expression and effective cleavage and sterol conjugation, based on altered mobility, was confirmed by immunoblot. Supernatants of the transfected cells bearing retroviral particles containing the constructs were also used to infect NIH 3T3 cells using standard techniques. Cells were then washed and examined for phenotypic changes. Cells infected by virus containing a GFP hedgehog fusion fluoresced showing that the GFP sterol conjugated by hedgehog was functionally active. (Fig. 29).

To confirm that biologically functional sterol conjugations can be made, cells having an activated Ras, Ras lacking a membrane targeting domain (CAAX), and Ras lacking a membrane targeting domain but fused to the catalytic domain of hedgehog, were examined for their ability to transform cells using the above approach. the activated form of Ras (V12Ras) is strongly transforming whereas Ras lacking a membrane targeting domain (CAAX) is not. However, Ras lacking the membrane targeting domain but fused to hedgehog was sterol conjugated, targeted to a membrane, and rescued for its transforming activity (see Fig. 29). Controls indicated that this

restoration of a biologically functional sterol conjugation, depended on a functional hedgehog protein. Further, immunoblotting of cholesterol rich membrane fractions confirmed that activated Ras and Ras sterol conjugated by hedgehog where correctly targeted to the membrane whereas Ras lacking a membrane targeting domain (CAAX) and Ras lacking a membrane targeting domain (CAAX) fused to a nonfunctional hedgehog protein where not.

An additional use of the library is as an inducible system, in which although the peptide-hedgehog fusion protein is expressed intracellularly, expression is initially carried out in "cholesterol starved" cells. Such starvation is sufficient to halt self-processing of the native hedgehog precursor protein (Guy *et al.*, PNAS, 97:7307-12 (2000)). In the absence of cholesterol, the cells retain viability, but autoprocessing stops. Thus, at a desired time or circumstance, cholesterol, a specified sterol, or a mixture of sterols is then introduced to the media and the cell population expressing the peptide-hedgehog library initiates processing, sterol conjugation, and membrane targeting of the fused polypeptide, *e.g.*, peptide aptamer.

In another application of the inducible system, prokaryotic cells may be used because they have the advantage of lacking any potential substrate sterols. Accordingly, the sterol conjugation of a given polypeptide by hedgehog can be precisely controlled and will only occur upon addition of an exogenous sterol (e.g., cholesterol). Other advantages for sterol conjugating peptides in prokaryotic cells include the fast generation time of prokaryotic cells, the ease of their manipulation, their ability to produced large amounts of heterologous proteins or peptides, and their ability to be arrayed (e.g. 96 well or other multiwell plate formats) to form vast libraries in either the conjugated or unconjugated form for various screening applications as described herein.

A typical application using prokaryotic cells can be performed as follows. Briefly, prokaryotic expression vectors (e.g., comprising a pET vector backbone) encoding proteins or peptides fused to a sterol conjugation domain (e.g., hedgehog) are introduced into a suitable strain of prokaryotic cells (e.g., BL21 cells, see e.g., Studier *et al.*, *Methods Enzymol.* 185:60-89 (1990) and Ausabel *et al.*, *Current Protocols in Molecular Biology*, John Wiley, Pub., (2002) for an extended list of suitable prokaryotic expression hosts). Then, upon exposure to a sterol, the sterol conjugation domain carries out an autoprocessing reaction in the cells that results in the covalent conjugation of a sterol molecule (e.g., cholesterol) to the protein or peptide (e.g. a random peptide aptamer). In one approach, a single sterol is added to a culture of prokaryotic cells expressing the random peptide coding sequences expressed in frame with the sterol conjugation domain. This results in a library of peptides all conjugated to an identical sterol. In another approach, two or more sterols are added to a mixed culture (or separate cultures) such that a library of peptides fused to different sterols is obtained.

Typically, the resultant sterol conjugated peptide libraries are isolated by either arranging for their secretion into the media or by lysing the cells expressing the peptides. The resultant sterol conjugated peptides are then purified to a desired homogeneity for further applications using standard techniques (e.g., by engineering an immunotag in the peptide, e.g., HIS tag for capture using metal chelating resins or cysteine capture using thiol reactive capture).

EXAMPLE 9
METHODS AND COMPOSITIONS FOR INCREASING EXPRESSION OF RETROVIRAL LIBRARIES AND EFFICIENCY OF PHENOTYPIC SCREENING IN TRANSDUCED MAMMALIAN CELLS

In this example, methods and compositions for producing high expression retroviral vectors for aptamer peptide expression and screening, are described.

Typically, upon entry of a retrovirus into a host mammalian cell, a DNA copy of the RNA genome is generated by reverse transcription, and this proviral form is stably integrated into the host cell genome. Frequently, however, an integrated retrovirus may fail to express (be transcriptionally inert). Moreover, it is well established that within a given clonal cell population of transduced cells, although a given proviral integrant may be transcriptionally active early after integration, gene expression may decrease significantly as the cell passes through subsequent cell divisions or differentiation.

The invention provides for the use of chromatin insulator elements to optimize the establishment of populations of cells expressing retroviral libraries early after transduction and proviral integration, as well as their utility in perpetuating expression throughout the growth of these cells, and the use of these cells in phenotypic selection assays either in tissue culture or in animals. The chromatin insulator is a genetic element that is engineered into the retroviral library vector itself, and serves to augment and perpetuate expression of the retroviral library inserts at each site of integration within a plurality of cells, and throughout the continued growth or differentiation of the cells. This is of particular use in perpetuated expression in transgenic mouse models, or in experimental systems where expression in myeloid or lymphoid cell systems is desired.

In brief, a fragment such as that described in Reitman, *et al.* (Mol. Cell. Biol., 10:2774-2786 (1990)), which comprises the element provided in SEQ ID NO: 50, is introduced at or near the LTR region of the retroviral vector as described by, *e.g.*, Emery *et al.*, (Proc. Natl. Acad. Sci. U S A 97: 9150-9155 (2000)) and in U.S. Pat. No. 5,610,053.

The modified retroviral vector encoding the insulator element(s) is then used to express one or more aptamers in the test cell line without the disadvantage of poor expression (*e.g.*, transcriptional silencing). Accordingly, the inherent complexity and

sensitivity of the aptamer library screen is improved such that every recipient test cell receives an actively expressed aptamer whose potential alteration of the cell phenotype can be detected.

EXAMPLE 10

METHODS AND COMPOSITIONS FOR PRODUCING CELLS WITH HIGH TRANSDUCTION EFFICIENCY FOR HIGH THROUGHPUT SCREENING OF RETROVIRAL LIBRARIES

In this example, the genetic engineering of a cell to express an ecotropic receptor for improved infectivity by a retroviral vector, is described.

The receptor for ecotropic murine retroviruses has been identified and cloned. The receptor protein, known as ATRC-1, is a membrane spanning cationic amino acid transporter. Although expressed ubiquitously in mammalian cells, differences exist between the human and mouse proteins that render the murine version of the gene specific for murine retroviruses; ecotropic murine retroviruses do not infect human cells.

The present invention provides for the use of the ATRC-1 receptor in two general ways: (*i*) to improve infectivity of mouse or rat cells by ecotropic murine retroviruses by establishing mouse cell lines or transgenic animals overexpressing the receptor; and (*ii*) expansion of the host range of retroviral libraries in ecotropic murine retrovirus vectors by expression of the mouse receptor in human cells or cells of other species not normally infected by ecotropic murine retroviruses.

Accordingly, efficiency is improved in terms of the number of transduced and integrated viral genomes per infected cell, and/or by increasing the number of individual cells in which retroviral transduction occurs. Moreover, the phenotypic screening of random peptides or RNA aptamers in mammalian cells requires very high efficiency in transduction of the retroviral library containing the sequences to be screened. This frequently a limiting step in the ability to carry out such screens in a manner that allows expression and examination of large libraries in a way that ensures as thorough a survey as possible of the complexity of the library in each phenotypic screen. Still further, another advantage is that multiple retroviruses can be used to transduce and integrate into a given cell, effectively allowing for the screening of multiple candidates aptamers per cell.

In brief, the selected cell line to be used for phenotypic screening is transfected with a vector encoding the ATRC-1 receptor (Accession No. M26687; Fig. 26; SEQ ID NO: 51-52; see also, Sommerfelt, *J. Gen. Virol.* 80: 3049-3064 (1999); Albritton *et al. Cell* 57: 659-666 (1989); Albritton *et al., J. Virol.* 67: 2091-2096 (1993)). Preferably, the receptor is stably integrated into the genome of the test cell, is stably expressed after many cell passages, and has no phenotypic consequences for the cell. The receptor is

introduced into the cell using standard nucleic acid transfer techniques and the vector used to express the receptor can be, *e.g.*, a retroviral vector or recombinant expression vector.

Use of ATRC-1 to improve the transduction efficiency of ecotropic retrovirus in mouse cell lines is demonstrated in Fig. 27. In this experiment, mouse L929 cells, which undergo low transduction efficiency using ecotropic retrovirus, were transfected with a plasmid expressing the ATRC-1 protein using the CMV enhancer/promoter. The expression vector also contains a zeomycin resistance selectable marker. Following transfection of the ATRC-1 expression plasmid and selection for zeomycin resistant cells, resistant cells were collected, and are referred to as the "pools" in this figure. Selection of clonal cells derived from this mixture were also generated. These clonal lines, along with the cell pool and mock transfected cells were tested for transduction efficiency using a recombinant ecotropic virus encoding GFP. Following transduction (and selection using zeomycin), efficiency was assessed both with respect to the percentage of cells expressing GFP, and the relative brightness of GFP expressing cells as compared top a control transduced with vector alone using FACS (Fig. 27A-B). The latter parameter is an approximate indication of the average number of transduction events per cell genome (*i.e.* copy number per cell). L929-ATRC-5 and L929-ATRC-8 are clonal cell lines that were highly transducible subclones selected from the original transfection pool of zeomycin resistant transfectants. L929-ATRC-Pool cells represent a mixed population of cells that were transfected and with pcDNA4-ATRC-1 and selected for zeomycin resistance.

Control experiments in which human 293T cells that are not transducible using ecotropic retrovirus, demonstrate that stable expression of mouse ATRC-1 in these cells results in a population of cells that are transducible with almost 100% efficiency using a recombinant ecotropic retrovirus expressing GFP.

EXAMPLE 11

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING APOPTOSIS IN A HUMAN MYELOID LEUKEMIA

In this example, methods for screening peptide aptamers capable of modulating apoptosis in human myeloid leukemia HL-60 cells are described.

HL-60 cells are a well characterized human myeloid leukemia cell line in which apoptosis is inducible. These cells also grow in suspension, and their apoptotic response to multiple stimuli has been characterized (reviewed in Darzynkiewicz *et al.*, 1992). Accordingly, in one approach, cells pre-cleared of spontaneously apoptotic cells are contacted with an aptamer library encoded in an expressible form on a plasmid, preferably a retrovirally derived vector, that can efficiently enter the cell and express a

particular aptamer. Then, HL-60 cells in which apoptosis has been induced by an expressed aptamer, are identified. Any art recognized FACS or panning strategies can be used for detecting the approximate 100-1000 apoptotic cells per 10 million cells that represent a desirable level of sensitivity and selectivity required for the apoptotic aptamer screen.

After selection of apoptotic cells is achieved, the aptamers that induce apoptosis in HL-60 cells, are then tested for their ability cause apoptosis in non-cancerous human cells, such as primary fibroblasts, with a preferably result being that the selected aptamer works preferentially in only cancerous cells.

Each aptamer identified in the HL-60 screen is then tested in a diverse panel of human cancer cell lines. This aspect of the invention allows for the identification of a set of aptamers sufficient to induce apoptosis in as many different types of cancer cells as possible.

In the initial screening steps, apoptotic cells are identified using APOPTEST ™ or an analogous method, both before and after induction of aptamer expression. Briefly, in contrast to TUNEL staining methods for identifying apoptotic cells, which identify apoptosis by end-labeling DNA fragments that arise late, APOPTEST ™ stains cells early in apoptosis, and does not require fixation of the cell sample (Martin *et al.*, 1994). This method uses an annexin V antibody to detect cell membrane re-configuration that is characteristic of cells undergoing apoptosis. Apoptotic cells stained in this manner can then sorted either by fluorescence activated cell sorting (FACS), or by adhesion and panning using immobilized annexin V antibodies.

Retroviral sequences in cells identified and segregated in this manner can be amplified by PCR, and the aptamers can be recloned and validated. In later rounds of aptamer re-screening and validation, at the point where aptamers are being re-tested individually, other methods of apoptosis can be employed as a counterscreen. These include such methods as TUNEL staining or propidium iodide staining. This is necessary to ensure that the selected phenotype is in fact apoptosis, and not an aptamer-induced alteration in membrane metabolism. Validated apoptotic aptamers are then expressed in a variety of non-cancerous cells and other cancer cell lines to determine their specificity and range of action. Aptamers can be identified that do not induce apoptosis in non-tumor cells, although they can also be evaluated for their ability to induce apoptosis in other cell lines, as described herein.

The invention also encompasses screens that can be conducted for identification of aptamers that augment the sensitivity of cancer cells to radiation or cancer chemotherapeutic agents. In addition, aptamers can act synergistically with the apoptotic response to these agents, either by impacting the same pathways, or by targeting novel but parallel cellular responses to these agents. In each case, aptamer

library expression can be induced in a population of transduced cells, followed by treatment with an agent known to induce apoptosis, but at a dose below the threshold for this response.

EXAMPLE 12

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING A CELLULAR PHENOTYPE USING A PEPTIDE LIBRARY THAT CAN BE INDUCIBLY EXPRESSED OR WHICH CAN BE FUNCTIONALLY ACTIVATED

In this example, methods for screening peptide aptamers capable of modulating apoptosis in human myeloid leukemia HL-60 cells, whereby the peptide aptamers can be inducibly expressed, are described.

This assay is performed in a manner similar to the assay described above, with the advantage that the expression of the peptide aptamer is inducible. In this variation of the screen, it is important to discriminate between spontaneous apoptosis and aptamer induced apoptosis by inducing aptamer expression at a given time, and identifying the cells that have undergone apoptosis subsequent to this induction. A number of commercially available transcriptional regulation systems exist that regulate transcription upon exposure or removal of specific compounds (*e.g.* ecdysone or tetracycline). However, these systems require multiple time consuming modifications of the host cell, including integration and stable expression of several plasmids harboring the numerous components of the system. Expression of these various required components can be unstable and unreliable. Moreover, such reagents are not amenable to use in high- throughput systems (*i.e.* generation and screening of large libraries): populations of cells in which appropriate regulation is achieved cannot be derived *en masse*. Instead, for expression of any given protein using these systems, it is often necessary to screen numerous individual clones to identify a given clonal cell population in which desired regulation of expression (*i.e.* acceptable shut-off and induction) has been achieved.

One approach to achieving wholesale and reliable regulation of the peptide within each individual transduced cell is through the use of regulatory elements that are integrated into the retroviral vector itself, and do not require the co-expression and activity of secondary activator or repressor proteins. One such approach is exemplified in a manner described by Werstuck and Green (1998), in which an RNA element that binds an exogenous ligand is introduced into the 5' UTR of a gene. When cells expressing RNA containing this regulatory element are exposed to this cell- permeable ligand (in this case a Hoechst dye), translation of a downstream reporter is drastically reduced. By incorporating such a feature in the 5'UTR of retroviral vectors encoding

random peptide libraries in mammalian cells, such a regulatory element allows for coordinated and controlled repression/induction of library expression within a population of transduced cells.

Alternatively, instead of regulating expression of the expressed library proteins *per se*, activity of the proteins or precursors proteins may be coordinately regulated. For example, in use of the intein mediated cyclization technology described above, "crippled" inteins that retain critical splicing residues, but do not undergo autonomous Ic- In association sufficient to sponsor efficient spontaneous splicing, may be fused to protein sequences known to carry out inducible protein- protein interaction through the activity of extraneous ligands. For example, the FK506 binding domain of FKBP12, when expressed within both the Ic and In elements of a crippled Ic-pep-In precursor, can undergo inducible splicing upon introduction of FK1012, a dimeric form of FK506 that has been shown to mediate such interactions in other systems (Rollins *et al.*, 2000). Other protein/ ligand systems that can be used include, but are not limited to, the growth hormone-inducible system as described (Guo *et al.*, Science, 288:2042-2045 (2000).

Accordingly, an inducible retroviral peptide aptamer library can be used to transduce ten to 100 million cells growing in suspension. For example, HL-60 cells can be used since they have robust growth in suspension, and have well characterized responses to various apoptotic stimuli, which can be useful in examining the apoptotic phenotypes. Apoptosis resulting from activity of peptides encoded by the transduced library can be distinguished from spontaneous apoptosis by presorting and removing cells undergoing apoptosis prior to induction of library expression or activity, either by washing cells to remove the translational- repressing RNA binding agent (allowing translation of the aptamer peptide) or by introduction of a dimerization- inducing ligand that prompts intein splicing and production of library peptide cyclization. (In the latter case, the system has the additional advantage of selecting for peptides that have activity only when they are present in a cyclic form.) Cells are then re-sorted to identify cells in which apoptosis occurred following aptamer expression.

EXAMPLE 13

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING APOPTOSIS USING GROWTH FACTOR DEPENDENT CELLS

In this example, methods for screening peptide aptamers capable of modulating apoptosis using the BaF3 cell line are described.

To identify and catalog aptamers that carry out signaling *via* various signal transduction pathways, a well established growth factor dependent (IL-3) BaF3 cell-based assay system can be employed (see, *e.g.*, Kitamura *et al.*, 1995). BaF/3 cells

require continuous stimulation of certain pathways in order to forgo apoptosis: removal or attenuation of some certain stimulatory signals or signaling cascades, results in BaF/3 apoptosis. For example, withdrawal of IL-3 induces apoptosis of BaF/3 cells. Erythropoietin signaling can substitute for that of IL-3 in BaF/3 cells engineered to express the erythropoietin receptor (EpoR). In these cells, the erythropoietin mediated signal cascade is sufficient to rescue cells from apoptotic death in the absence of IL-3 growth factor. In this screen, a retroviral library is used to infect mouse BaF/3 cells, which normally undergo apoptosis upon withdrawal of IL-3. BaF/3 cells stably expressing EpoR are used to screen and identify retrovirus encoded peptide aptamers that abrogate apoptosis of BaF3 cells in response to withdrawal of erythropoietin. (Growth and library selection are carried out in the absence of IL-2.) A schematic summary of this selection strategy is shown in Fig. 28. Aptamer coding sequences from the surviving cells are then amplified by PCR, recloned into a mammalian expression vector, and re-screened by reintroduction into naive BaF3 cells, and the process is repeated iteratively.

This assay system allows for the discovery of active aptamers, and phenotypes associated with aptamer expression can be easily deconvoluted. For example, this abrogation of the BaF/3 apoptotic phenotype is not observed in the presence of activated forms of the *abl* oncogene. Accordingly, expression of aptamers that stimulate Abl kinase activity, or the activity of appropriate downstream components that signal transduction pathway, can result in cell survival; aptamers that cause survival of BaF3 cells through stimulation of Abl can be identified by the susceptibility of these cells to Novartis STI571, a specific inhibitor of the Abl kinase that is in clinical use for the treatment of some leukemias (Carroll *et al.*, 1997). The survival phenotype of these cells can the be reversed by the STI571 kinase inhibitor, whereas aptamer mediators of Jak2 or STAT5 activity can be identified by examination of these proteins and their activities in aptamer expressing cells (Nosaka *et al.*, 1999; McCubrey *et al.*, 2000).

In another selection scheme, a library of random peptides is transduced into a population of myeloid or lymphoid precursor cells from mice that are defective for development or differentiation. Aptamers can then be expressed ectopically in erythropoietin receptor knockout mice. These mice are deficient for erythropoiesis, which is reconstituted by activation of the Abl kinase (Ghaffari *et al.*, 1999).

Aptamers that specifically substitute for erythropoietic signals can be used as lead compounds for the development of small molecule drugs for therapeutic use in the treatment of anemia in kidney dialysis and cytotoxic chemotherapeutic treatments.

Further, if desired, aptamers identified in the BaF3 anti-apoptotic screen can also be tested for their effects on hematopoietic stem cell development and differentiation.

Accordingly, this system allows for the identification of active aptamers capable of modulating apoptosis and a method for understanding their mode of action.

EXAMPLE 14

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING INTRACELLULAR SIGNALING CASCADES

In this example, methods for screening peptide aptamers capable of modulating intracellular signaling cascades are described.

In general, signaling cascades refer to networks of molecular interactions and activities through which an environmental or developmental stimulus is received and interpreted by a cell. This carefully orchestrated molecular response is a designated sequence of events that ultimately leads to an alteration in cellular metabolism or function. G protein coupled receptors (GPCRs) are a large and growing gene family of transmembrane proteins. To date, over 1000 GPCRs have been cloned. These receptors are classified both by the types of extracellular signals to which they respond (*e.g.* photons, odors, ions, monoamines, or peptides), and by the particular trimeric G protein effector complex that mediates intracellular transmission and amplification of receptor signaling. Ligand mediated signaling through these receptors results in a broad spectrum of responses.

The present invention provides aptamer libraries that can be screened for members that modulate a cellular response analogous to that resulting from ligand engagement by a given receptor, or that inhibit such a response, in the presence of ligand. The invention also provides methods for screening libraries to identify aptamers that abrogate, attenuate, or alter the specificity of receptor mediated signaling that occurs upon binding of the receptor by a cognate (endogenous or exogenous) ligand. Receptor tyrosine kinase signaling cascades, as well as receptor mediated signaling cascades that mediate signaling through src family kinases, are other pathways that can also be targeted using this system.

EXAMPLE 15

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING PROTEIN TRANSPORT AND TRAFFICKING

In this example, methods for screening peptide aptamers capable of modulating protein transport and trafficking are described.

The present invention provides methods for identifying aptamers that can affect trafficking of specific proteins to the cell surface. This has particular utility in cases where misdirection of proteins is associated with disease. Aptamer libraries can be introduced into clonal cell lines stably expressing the mislocalized protein. Cells

expressing aptamers that affect membrane localization of the desired protein can be identified by staining non-permeabilized cells with a specifically reactive antibody. Positively staining cells can then be physically separated by either fluorescence activated cell sorting (FACS) or other appropriate art recognized techniques. Aptamers can be identified that correct the mislocalization or induce the relocalization of any protein at the cell surface, including various receptors and channels, antigens, or proteins involved in the immune response. In the latter case, involving modulation of antigen presentation in an immune response, an aptamer can either augment an immune response to specific infections, especially in immunocompromised individuals, or attenuate certain aspects of immunity that can be beneficial in autoimmune syndromes or conditions.

An example of a particular application of the foregoing methods can be for the development of therapeutics for hemochromatosis, an autosomal recessive disorder in which approximately 95% of the non-functional protein encoded by mutant alleles is no longer directed to the cell surface (Waheed *et al.*, 1997). This leads to an alteration of iron transport in certain intestinal cells of individuals homozygous for this mutant allele, and chronic accumulation of iron in the serum to levels that lead to long-term organ toxicity. Hemochromatosis is, in fact, the most common hereditary disorder among Caucasians, affecting up to one in every two hundred Americans, and leading to liver, kidney, and other organ failure, the etiology of which, had not been previously appreciated. Analogous screens can be carried out to identify modulators of transport of disease- associated alleles of the cystic fibrosis (CF) chloride channel protein, which, unlike its non-disease associated counterparts, is not efficiently transported to the surface of the cell.

Still another application of the foregoing methods is the following. Many viruses, including HIV infected T cells in which the HIV *nef* gene product down-regulates MHC-mediated antigen presentation (reviewed in Collins and Baltimore, 1999), various herpesviruses, including cytomegalovirus (CMV) (del Val *et al.*, 1997; Kleijnen *et al.*, 1997) , and papillomavirus, actively suppress antigen presentation as a means of eluding or evading immune recognition and response (reviewed in McFadden and Kane, 1994). Clonal cell lines either chronically infected by these viruses, or constitutively expressing virus encoded proteins that affect these functions involving protein transport or trafficking, can be infected with a retroviral aptamer library, and cells in which antigen presentation was augmented or reconstituted as measured by, *e.g.*, FACS, can be scored using the subsequent steps described above, and a candidate aptamer that can modulate the pathway can be identified.

In other embodiments, cells can be identified for their ability to express or not express specific markers characteristic of certain types of immune cells (*e.g.* markers associated with T cell subtypes).

EXAMPLE 16
METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING CELL ADHESION

In this example, methods for screening peptide aptamers capable of modulating cell adhesion are described.

Cell adhesion is an important element of development and the immune response. Cell surface adhesion molecules function both as mediators of physical association between cells and as important sensors and transmitters of intracellular signals. For example, the integrin proteins of leukocytes and neutrophils serve as adhesive molecules that immobilize these cells to sites of localized immune or allergic response, and in turn, trigger intracellular responses upon adherence (*i.e.* degranulation). The cell sorting protocols described herein can be easily adapted for panning, in which aptamers that can modulate (*e.g.*, induce certain adhesive properties in a cell) are identified.

EXAMPLE 17
METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING MEMBRANE TRANSPORT

In this example, methods for screening peptide aptamers capable of modulating membrane transport are described.

Membrane transport of ions and other ligands plays an important role in many physiological processes and disease states. For example, ATP cassette transport proteins have a wide variety of functions, including mediating efflux of drugs. The human multiple drug receptor membrane protein (MDR) presents a significant clinical problem in patients undergoing cancer chemotherapy, by efficiently pumping cancer therapy drugs out of cancer cells, thereby limiting their efficacy. Other members of this family are associated with peroxisomes, mutant forms of which are associated with disease, including adenoleukodystrophy. Art recognized dyes exist that can be used to identify cells in which these transporters are unable to mediate efflux of certain types of compounds.

Accordingly, these techniques can be used to screen retroviral peptide aptamer libraries when used to infect clonal cell lines that endogenously overexpress an MDR or other ATP cassette protein, or in which this gene or a mutant form is stably expressed. Cells expressing aptamers capable of increasing dye retention in these cells can be sorted, and the sequence of the encoded aptamer can be determined.

EXAMPLE 18

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING CELL MOTILITY AND CHEMOTAXIS

In this example, methods for screening peptide aptamers capable of modulating cell motility and chemotaxis are described.

Neutrophils are among the first leukocytic cells to migrate into tissues in response to invading pathogens or other initiators of inflammatory injury. One of the first steps of neutrophil involvement in acute inflammation is chemotaxis, directed movement toward chemotactic agents, such as complement fragments (C5a), cytokines (IL-8), leukotrienes, and bacteria-derived peptides such as formyl-methionine-leucine-phenylalanine (fMLP). Inhibition of this chemoattractive response is an effective means of abrogating inflammation, especially in diseases like asthma and the chronic inflammation associated with cystic fibrosis.

Accordingly, cell lines stably expressing a chemotactic receptor can be infected with a retroviral peptide aptamer library, and migration toward a specific chemoattractant can be measured using art recognized transwell assays in which the cells are placed in an upper chamber, and the chemoattractant is placed in a lower chamber. After a time sufficient for transmigration of the chemotactic cells across the chamber barrier, cells remaining in the upper chambers can be pooled, grown out, and re-assayed serially until a population of truly non-responsive cells is identified.

Thus, using this approach, which can be readily adapted to a high throughput format, aptamers that can modulate cell motility and/or chemotaxis can be identified.

EXAMPLE 19

METHODS FOR SCREENING PEPTIDE APTAMERS CAPABLE OF MODULATING VIRAL RESISTANCE

In this example, methods for screening peptide aptamers capable of modulating viral resistance, are described.

In particular, using split inteins, a random cyclic peptide library was engineered by placing random oligonucleotides between the coding sequences of I_C and I_N intein domains and the library was then expressed in cells. The peptides expressed in cells are then determined if they perturb the course of a viral infection. As a model system, a virus was used that promotes certain cell death and belongs to a family that has been implicated as being pathogenic in humans. Specifically, the encephalomyocarditis virus (EMCV), a cardiovirus of the picornavirus family, was used. EMCV is a cardiovirus of the picornavirus family. Its single, positive-stranded RNA genome encodes a large open reading frame that, upon infection, is translated into a single polyprotein. This

polyprotein is subsequently processed *via* a series of proteolysis events into its component proteins. EMCV can infect a wild-range of cells with a preference for rodents. Infections by EMCV induce a massive programmed cell death, also named apoptosis. The screen is performed, to test whether cyclic peptides can interfere with any viral associated processes, *e.g.*, viral infection: membrane attachment, reverse translation of the genomic RNA, inhibition of antiviral responses of the cells, inhibition of the apoptotic machinery or production of new viral particles.

The assay was performed as described above and number of colonies resistant to viral mediated apoptosis were enumerated. Significantly more colonies are observed on plates containing cells transduced with the retroviral peptide library than in the control plates (GFP retrovirus) indicating that peptide aptamers that confer viral resistance are present in the library . The peptide aptamers are recovered from the cells as described, *e.g.*, in Example 1.

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method of identifying a peptide aptamer capable of modifying a cell phenotype comprising:

a) contacting a first sample of cells with a library of expressible nucleic acid sequences encoding random peptide aptamers linked to a fusion moiety;

b) selecting at least one cell having an altered phenotype compared to the phenotype of the cell prior to the contacting step (a); and

c) identifying one or more peptide aptamers expressed in the selected cell.

2. The method of claim 1 further comprising amplifying the nucleic acid sequences encoding the one or more peptide aptamers identified in step c) and repeating steps a)-c) using the amplified nucleic sequences as the library of expressible nucleic acid sequences specified in step a).

3. The method of claim 2, wherein steps a)-c) are repeated two or more times.

4. The method of claim 1 further comprising genetically modifying the first sample of cells to express a receptor not naturally expressed by the cells.

5. The method of claim 4, wherein the receptor is an ecotropic receptor.

6. The method of claim 1 wherein a sample of cells expresses an ecotropic receptor.

7. The method of claim 1, further comprising expressing the one or more peptide aptamers identified in step c) in a second sample of cells, and identifying a change in the phenotype of the second sample of cells.

8. The method of claim 7, wherein the cell type of the first and second sample of cells differs.

9. The method of claim 7, further comprising contacting the second sample of cells with a pathogen before or after expression of the one or more peptide aptamers in the second sample of cells.

10. The method of claim 7, further comprising contacting the second sample of cells with an agent which stimulates signal transduction before or after expressing the one or more peptide aptamers in the second sample of cells.

11. The method of claim 7, wherein the change in the phenotype of the cells comprises a change in the expression levels of an endogenous gene or gene products associated with said phenotype.

12. The method of claim 7, wherein the change in the phenotype of the cells is selected from the group consisting of a change in levels of apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, viral resistance, metabolic state, or cellular differentiation, as compared to a control cell.

13. The method of claim 7, wherein said cellular differentiation is selected from the group consisting of the induction of hair growth, promotion of hair growth, hair follicle differentiation, hair follicle differentiation development, melanogenesis, hair shaft elongation, skin cell differentiation, and a shift in the hair growth cycle from telogen (resting stage) to anagen (growing stage).

14. The method of claim 4, wherein the cells of the second sample contain a reporter gene.

15. The method of claim 14, wherein the change in phenotype of the cells comprises a change in the expression levels of the reporter gene.

16. The method of claim 1, wherein the altered phenotype is associated with a change in levels of apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, viral resistance metabolic state, or cellular differentiation as compared to a control cell.

17. The method of claim 16, wherein the change in levels of apoptosis is measured using immunohistochemistry.

18. The method of claim 1, wherein the altered phenotype is associated with a change in levels of signal transduction.

19. The method of claim 1, wherein the change in levels of signal transduction is primarily mediated by a receptor or downstream effectors of a receptor.

20. The method of claim 1, wherein the change in levels of signal transduction is primarily mediated by an exogenous receptor or downstream effectors of an exogenous receptor.

21. The method of claim 1, wherein the change in levels of signal transduction is primarily mediated by an erythropoietin receptor or downstream effectors of an erythropoietin receptor.

22. The method of claim 18, wherein the change in levels of signal transduction is primarily mediated by a receptor kinase or one or more downstream effectors of a receptor kinase.

23. The method of claim 18, wherein the change in levels of signal transduction is primarily mediated by a G protein coupled receptor or downstream effectors of a G protein coupled receptor.

24 The method of claim 1, wherein the cells are selected from the group consisting of fungal cells, insect cells, and mammalian cells.

25. The method of claim 24, wherein the fungal cells are yeast cells.

26. The method of claim 24, wherein the mammalian cells are human cells.

27. The method of claim 24, wherein the mammalian cells are cancer cells.

28. The method of claim 1, wherein the library of expressible nucleic acid sequences is encoded in a eukaryotic expression vector.

29. The method of claim 28, wherein the eukaryotic expression vector is a retroviral vector.

30. The method of claim 28, wherein the eukaryotic expression vector comprises a chromatin insulator element.

31. The method of claim 1, wherein the peptide aptamer comprises 5 to 9 amino acid residues.

32. The method of claim 1, wherein the peptide aptamer comprises a conotide amino acid sequence.

33. The method of claim 1, wherein the peptide aptamer comprises a predetermined sequence.

34. The method of claim 1, wherein the fusion moiety is selected from the group consisting of an intein, inducible-intein, green fluorescent protein (GFP), a hedgehog polypeptide or functional derivative thereof, thioredoxin, a regulatory polypeptide involved in apoptosis, bcl-2, p53, an NFκB-related polypeptide, a caspase, PTEN, myc, a BH3 domain, a death domain (DD), a BIR3 domain, a BIR domain, a nuclear localization signal sequence, a membrane localization signal sequence, a farnesylation signal sequence, a transcriptional activation domain, a transcriptional repression domain, Rac, Raf, and fragments thereof.

35. The method of claim 1, wherein the fusion moiety is a processing-fusion moiety selected from the group consisting of an intein, inducible-intein, a hedgehog polypeptide or functional derivative thereof, and fragments thereof.

36. The method of any one of claim 34 or 35, wherein the intein is selected from the group consisting of DnaE, RecA, DnaB, Psp, Pol-I, and Pfu.

37. The method of any one of claim 34 or 35, wherein the fusion moiety comprises an intein linked to either end of the peptide aptamer so that the peptide aptamer is flanked by intein splicing elements capable of catalyzing the formation of a cyclic peptide aptamer.

38. The method of claim 37, wherein the inteins flanking the peptide aptamer further comprise a sequence which can be induced to join the inteins, such that the intervening peptide aptamer is cyclized in the presence of an inducing agent.

39. The method of claim 38, wherein the aptamer is linked to green fluorescent protein (GFP).

40. The method of claim 37, wherein the peptide aptamer is a conotoxin.

41. The method of claim 37, wherein the peptide aptamer is a conotide.

42. The method of claim 2, wherein the amplification of the nucleic acid sequences is performed by polymerase chain reaction (PCR).

43. A peptide aptamer, derivative thereof, or corresponding nucleic acid, identified according to the method of claim 1.

44. Use of a peptide aptamer, derivative thereof, or corresponding nucleic acid, identified according to the method of claim 1 for the molecular modeling of an agent having similar binding characteristics as said peptide aptamer.

45. A pharmaceutical composition comprising a peptide aptamer, derivative thereof, or corresponding expressible nucleic acid, identified according to the method of claim 1, and a pharmaceutically acceptable carrier.

46. A method for treating a disease or condition associated with an aberrant cell phenotype in a subject comprising:

administering to the subject, a therapeutically effective amount of a peptide aptamer, derivative thereof, or corresponding expressible nucleic acid, identified according to the method of claim 1.

47. The method of claim 46, wherein the aberrant cell phenotype is associated with a change in levels of apoptosis, viral resistance, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, metabolic state, or differentiation, when compared to a control cell.

48. The method of claim 46, wherein the disease or condition is a cancer.

49. The method of claim 46, wherein the expressible nucleic acid is administered using a retrovirus.

50. The method of claim 49, wherein the retrovirus comprises a chromatin insulator element.

51. A peptide aptamer, derivative thereof, or corresponding expressible nucleic acid, identified according to the method of claim 1, in a form suitable for treating or inhibiting a disease or condition characterized by an aberrant cell phenotype.

52. The peptide aptamer of claim 51, wherein the aberrant cell phenotype is associated with altered apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, metabolic state, or differentiation.

53. The peptide aptamer of claim 51, wherein the disease or condition is a cancer.

54. A viral vector encoding a peptide aptamer identified according to the method of claim 1.

55. A viral vector encoding a peptide aptamer identified according to the method of claim 1, wherein the peptide aptamer is suitable for treating a disease characterized by an aberrant cell phenotype.

56. The viral vector of claim 54, wherein said aberrant cell phenotype is associated with a tumor cell.

57. A kit for identifying a peptide aptamer capable of modifying a cell phenotype comprising a library of expressible nucleic acid sequences encoding peptide aptamers linked to a fusion moiety.

58. A kit for identifying a cancer phenotype comprising a library of expressible nucleic acid sequences encoding peptide aptamers linked to a fusion moiety.

59. The method of claim 1, wherein the cells contacted with a library of expressible nucleic acid sequences are contacted before, after, or concurrently with a pathogenic agent or associated toxin.

60. The method of claim 59, wherein the altered phenotype selected for is associated with a change in levels of cell susceptibility, infectivity, or pathogenesis by the pathogenic agent or associated toxin.

61. The method of claims 59, wherein the pathogenic agent is selected from the group consisting of a prion, a virus, a bacterium, and a parasite.

62. The method of claims 59, wherein the pathogenic agent is a virus.

63. The method of claim 62, wherein the virus is selected form the group consisting of Picornaviridae, Togaviridae, Coronaviridae, Rhabdoviridae, Paramyxoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae, Rotaviridae, Retrroviridae, Polyomaviridae, Adenoviridae, Parvoviridae, Herpesviridae, Poxviridae, Hepadvaviridae, and Hepatitis delta virus.

64. The method of claim 62, wherein the virus is a lytic virus.

65. The method of claim 59, wherein the altered phenotype selected for is associated with increased resistance to cellular lysis, necrosis, or apoptosis.

66. The method of claim 62, wherein the virus comprises a reporter gene.

67. The method of claim 62, wherein the altered phenotype selected for is associated with a change in levels of expression of a viral RNA, viral polypeptide, or cellular localization of a viral polypeptide.

68. A method of producing a random peptide aptamer library comprising:

linking a library of expressible nucleic acid sequences encoding random peptide aptamers to a nucleic acid encoding a hedgehog polypeptide or a functional derivative thereof, and

introducing said library into cells in the presence of a sterol,

such that the hedgehog polypeptide or functional derivative thereof mediates the conjugation of the sterol to the random peptide aptamers.

69. A method of producing a random peptide aptamer library conjugated to a sterol comprising:

linking a library of expressible nucleic acid sequences encoding random peptide aptamers to a nucleic acid encoding a hedgehog polypeptide or a functional derivative thereof, and

introducing said library into cells in the absence of a sterol; and

adding to the cells one or more sterols to promote conjugation of the peptide aptamer library to one or more sterols.

70. A method of inducing sterol conjugation of a random peptide aptamer comprising:

linking an expressible nucleic acid sequence encoding a random peptide aptamer to a nucleic acid encoding a hedgehog polypeptide or a functional derivative thereof, and

introducing said peptide aptamer into cells in the absence of a sterol; and

adding to the cells one or more sterols to promote conjugation of the peptide aptamer to one or more sterols.

71. The method of any one of claims 68-70, wherein the sterol is selected from the group consisting of 7-Dehydrocholesterol (5,7-Cholestadien-3β-ol), 5-Androsten-3β-ol, Desmosterol; 24-Dehydrocholesterol (5,24-Cholestadion-3β-ol), β-Silosterol (5-Stigmasten-3β-ol), 7β-Hydroxycholestrol (5-Cholestene-3β, 7β-dial), and cholesterol (5-Cholestene-3β-ol).

72. The method of any one of claim 71, wherein the sterol is cholesterol.

73. The method of any one of claims 68-70, further comprising selecting one or more random peptide aptamers of the library which are expressed in a majority of the cells and processed to bring about a desired phenotypic change in the cell.

74. The method of any one of claims 68-70, wherein the phenotypic change is associated with a change in levels of apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, viral resistance, metabolic state, or cellular differentiation, activity of a oxysterol receptor, as compared to a control cell.

75. The method of claim 74, wherein the change in levels of signal transduction is primarily mediated by a receptor kinase or one or more downstream effectors of a receptor kinase.

76. The method of claim 74, wherein the change in levels of signal transduction is primarily mediated by a G protein coupled receptor or downstream effectors of a G protein coupled receptor.

77. The method of claim 73, wherein the phenotypic change is associated with a change in the activity of cellular proteins with cholesterol sensing activity (e.g. SCAP, HMG CoA reductase, patched, dispatched), cholesterol or sterol metabolism (e.g. SREBP, ACAT1, ACAT2, CETP), ABC1 mediated cholesterol transport, or other intracellular cholesterol trafficking or localization.

78. The method of claim 77, wherein the cellular protein is selected from the group consisting of SCAP, HMB CoA reductase, patched, ,dispatched, SREBP, ACAT1, ACAT2, CETP, ABC1, and oxysterol receptor.

79. The method of any one of claims 68-70, wherein the sterol comprises a mixture of one or more sterol derivatives thereby resulting in the generation of a mixture of random peptide aptamers conjugated to one or more different sterol derivatives.

80. The method of any one of claims 68-70, further comprising cleavage of the hedgehog polypeptide free of the peptide aptamer.

81. The method of any one of claims 68-70, wherein the cells are selected from the group consisting of fungal cells, insect cells, and mammalian cells.

82. The method of claims 68-70, wherein said library of expressible nucleic acids encoding random peptide aptamers are encoded in a eukaryotic expression vector.

83. The method of claim 82, wherein the eukaryotic expression vector is a retroviral vector.

84. This method of claim 82, wherein the eukaryotic expression vector comprising a chromatin insulator element.

85. The method of claims 68-70, wherein said peptide aptamer comprises about 5 to about 9 amino acid residues, fused upstream of a functionally active hedgehog polypeptide.

86. A nucleic acid encoding a peptide aptamer linked to an intein sequence and a GFP polypeptide sequence.

87. A nucleic acid sequence encoding a peptide aptamer linked to a hedgehog polypeptide sequence.

88. The nucleic acid sequence of any one of claims 86-87, wherein the peptide aptamer comprises a random sequence.

89. The nucleic acid sequence of any one of claims 86-87, wherein the peptide aptamer comprises a predetermined sequence.

90. The nucleic acid sequence of claims 86-87, wherein the peptide aptamer comprises about 5 to 9 amino acid residues.

91. A random peptide aptamer linked to a fusion moiety whereby the stability, affinity, bioavailability, or detectability of the peptide aptamer is altered or improved by the presence of the fusion moiety.

92. The peptide aptamer of claim 91, wherein the fusion moiety is selected from the group consisting of an intein, green fluorescent protein (GFP), a hedgehog polypeptide or functional derivative thereof, thioredoxin, a regulatory polypeptide involved in apoptosis, bcl-2, p53, an NFκB-related polypeptide, a caspase, PTEN, myc, a BH3 domain, a death domain (DD), a BIR3 domain, a BIR domain, a nuclear localization signal sequence, a membrane localization signal sequence, a farnesylation signal sequence, a transcriptional activation domain, a transcriptional repression domain, Rac, Raf, and fragments thereof.

93. A nucleic acid encoding the peptide aptamer of claim 91.

94. A method of identifying a conotide capable of modifying a cell phenotype comprising:

a) contacting a first sample of cells with one or more conotides or nucleic acid encoding one or more conotides; and

b) determining if at least one cell has an altered phenotype compared to the phenotype of the cell prior to the contacting step.

95. The method of claim 94, wherein one or more conotides is a library of conotides.

96. The method of claim 95, further comprising a subset of at least one or more conotides from the library with a second sample of cells and determining if at least one cell in the second sample has an altered phenotype compared to the phenotype of the cell in the first sample.

97. The method of claim 96 further comprising,

determining a difference in cell phenotypes in the first and second samples of cells thereby indicating the presence or absence of a conotide capable of altering a cell phenotype and

identifying one or more conotides capable of altering a cell phenotype from either the first or second sample of cells.

98. The method of claim 96, wherein the cell type of the first and second sample of cells differs.

99. The method of claim 96, further comprising contacting the second sample of cells with a pathogen before or after the contacting of one or more conotides.

100. The method of claim 96, further comprising contacting the second sample of cells with an agent which stimulates signal transduction before or after expression of the one or more peptide aptamers.

101. The method of claim 96, wherein the change in the phenotype of the cells is selected from the group consisting of a change in levels of apoptosis, signal transduction, protein trafficking, cell adhesion, membrane transport, cell motility, viral resistance metabolic state, or cellular differentiation, as compared to a control cell.

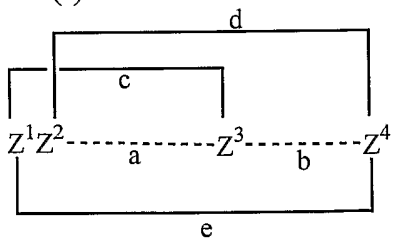
102. The method of claim 96, wherein the cells of the second sample contain a reporter gene.

103. The method of claim 102, wherein the change in phenotype of the cells comprises a change in the expression levels of the reporter gene.

104. Use of a conotide, derivative thereof, or corresponding nucleic acid, identified according to the method of claim 1 for the molecular modeling of an agent having similar binding characteristics as said conotide.

105. A pharmaceutical composition comprising a conotide, derivative thereof, or corresponding expressible nucleic acid, identified according to the method of claim 79, and a pharmaceutically acceptable carrier.

106. A conotide of the formula (I):



(I)

wherein

Z^1, Z^2, Z^3, and Z^4 are each independently selected linking residues;

a and b are each polypeptide linkers comprising one or more amino acid residues; and

c, d and e are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of c, d, and e is not a disulfide linking moiety; and provided that at least one of c, d, and e is not absent.

107. The conotide of claim 106, wherein at least one of said linking moieties is a covalent linkage.

108. The conotide of claim 107, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

109. The conotide of claim 106, wherein e is a covalent linkage.

110. The conotide of claim 109, wherein e is a polypeptide linkage.

111. The conotide of claim 106, wherein at least one of said linking moieties is a hydrophobic linking moiety.

112. The conotide of claim 111, wherein at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl moiety.

113. The conotide of claim 112, wherein at least one of said linking moiety is an ionic linkage.

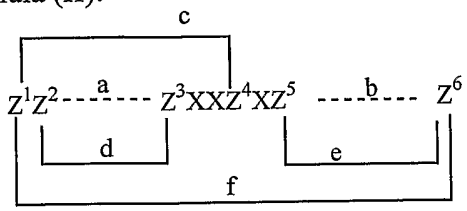
114. The conotide of claim 106, wherein Z^1 and Z^3 are each cysteine and c comprises a disulfide bond.

115. The conotide of claim 106, wherein Z^2 and Z^4 are each cysteine and d comprises a disulfide bond.

116. The conotide of claim 106, wherein said conotide is resistant to degradation *in vivo*.

117. The conotide of claim 106, wherein none of said linking moieties are disulfide linkages.

118. A conotide of the formula (II):



(II)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

X is an independently selected amino acid residue for each occurrence;

a and b are each independently selected polypeptide linkers moieties comprising one or more amino acid residues; and

c, d, e, and f are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are cysteine, then at least one of c, d, e, and f is not a disulfide linking moiety; and provided that at least one of c, d, e, or f is not absent.

119. The conotide of claim 118, wherein at least one of said linking moieties is a covalent linkage.

120. The conotide of claim 119, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

121. The conotide of claim 118, wherein f is a covalent linkage.

122. The conotide of claim 121, wherein f is a polypeptide linkage.

123. The conotide of claim 118, wherein at least one of said linking moieties is a hydrophobic linking moiety.

124. The conotide of claim 123, wherein at least two of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 comprise an aryl moiety.

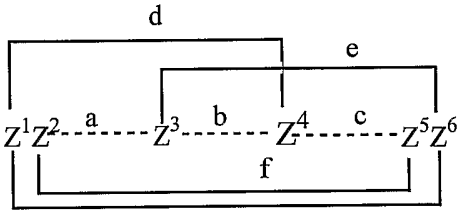
125. The conotide of claim 124, wherein at least one of said linking moiety is an ionic linkage.

126. The conotide of claim 118, wherein at least one of c, d, e, or f is a disulfide linkage.

127. The conotide of claim 118, wherein said conotide is resistant to degradation *in vivo*.

128. The conotide of claim 118, wherein none of said linking moieties are disulfide linkages.

129. A conotide of the formula (III):



(III)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

a, b, and c are each independently selected polypeptide linkers comprising one or more amino acid residues; and

d, e, f, and g are each independently selected linking moieties or absent; provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are cysteine, then at least one of e, d, f, and g is not a disulfide linking moiety; and provided that at least one of d, e, f, or g is not absent.

130. The conotide of claim 129, wherein at least one of said linking moieties is a covalent linkage.

131. The conotide of claim 130, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

132. The conotide of claim 131, wherein g is a covalent linkage.

133. The conotide of claim 132, wherein g is a polypeptide linkage.

134. The conotide of claim 129, wherein at least one of said linking moieties is a hydrophobic linking moiety.

135. The conotide of claim 134, wherein at least two of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 comprise an aryl moiety.

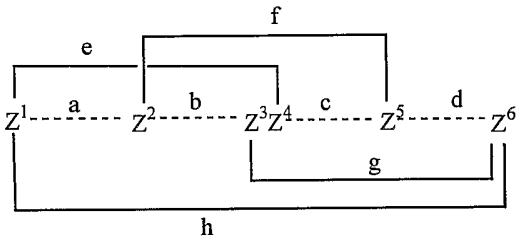
136. The conotide of claim 129, wherein at least one of said linking moiety is an ionic linkage.

137. The conotide of claim 129, wherein at least one of d, e, f, or g is a disulfide linkage.

138. The conotide of claim 129, wherein said conotide is resistant to degradation *in vivo*.

139. The conotide of claim 129, wherein none of said linking moieties are disulfide linkages.

140. A conotide of the formula (IV):



(IV)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

a, b, c, and d are each independently selected polypeptide linkers comprising one or more amino acid residues; and

e, f, g, and h are each independently selected linking moieties or absent; and, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 are each cysteine, then at least one of e, f, g, and h is not a disulfide linking moiety; and provided that at least one of e, f, g, or h is not absent.

141. The conotide of claim 140, wherein at least one of said linking moieties is a covalent linkage.

142. The conotide of claim 141, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

143. The conotide of claim 140, wherein h is a covalent linkage.

144. The conotide of claim 143, wherein h is a polypeptide linkage.

145. The conotide of claim 140, wherein at least one of said linking moieties is a hydrophobic linking moiety.

146. The conotide of claim 145, wherein at least two of Z^1, Z^2, Z^3, Z^4, Z^5 and Z^6 comprise an aryl moiety.

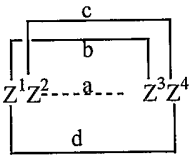
147. The conotide of claim 140, wherein at least one of said linking moiety is an ionic linkage.

148. The conotide of claim 140, wherein at least one of e, f, g, or h is a disulfide linkage.

149. The conotide of claim 140, wherein said conotide is resistant to degradation *in vivo*.

150. The conotide of claim 140, wherein none of said linking moieties are disulfide linkages.

151. A conotide of the formula (V):



(V)

wherein

Z^1, Z^2, Z^3, and Z^4 are each independently selected linking residues;

a is a polypeptide linker comprising one or more amino acid residues; and

b, c and d are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of b, c, and d is not a disulfide linking moiety; and provided that at least one of b, c, and d is not absent.

152. The conotide of claim 151, wherein at least one of said linking moieties is a covalent linkage.

153. The conotide of claim 152, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

154. The conotide of claim 151, wherein d is a covalent linkage.

155. The conotide of claim 154, wherein d is a polypeptide linkage.

156. The conotide of claim 151, wherein at least one of said linking moieties is a hydrophobic linking moiety.

157. The conotide of claim 156, wherein at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl moiety.

158. The conotide of claim 151, wherein at least one of said linking moiety is an ionic linkage.

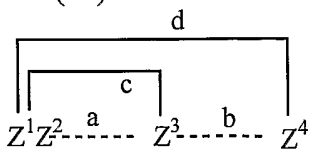
159. The conotide of claim 151, wherein Z^1 and Z^3 are each cysteine and b comprises a disulfide bond.

160. The conotide of claim 151, wherein Z^2 and Z^4 are each cysteine and c comprises a disulfide bond.

161. The conotide of claim 151, wherein said conotide is resistant to degradation *in vivo*.

162. The conotide of claim 151, wherein none of said linking moieties are disulfide linkages.

163. A conotide of the formula (VI):



(VI)

wherein

Z^1, Z^2, Z^3, and Z^4 are each independently linking residues;

a and b are polypeptide linkers comprising one or more amino acid residues; and

c and d are each independently selected linking moieties or absent, provided that if each of Z^1, Z^2, Z^3 and Z^4 are cysteine, then at least one of c and d is not a disulfide linking moiety; and provided that at least one of c and d is not absent.

164. The conotide of claim 143, wherein at least one of said linking moieties is a covalent linkage.

165. The conotide of claim 164, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

166. The conotide of claim 163, wherein d is a polypeptide linkage.

167. The conotide of claim 163, wherein at least one of said linking moieties is a hydrophobic linking moiety.

168. The conotide of claim 167, wherein at least two of Z^1, Z^2, Z^3, and Z^4 comprise an aryl moiety.

169. The conotide of claim 168, wherein at least one of said linking moiety is an ionic linkage.

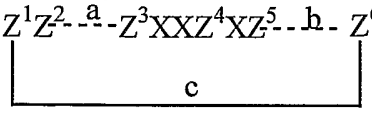
170. The conotide of claim 163, wherein Z^1 and Z^3 are each cysteine and c comprises a disulfide bond.

171. The conotide of claim 163, wherein Z^2 and Z^4 are each cysteine and d comprises a disulfide bond.

172. The conotide of claim 163, wherein said conotide is resistant to degradation *in vivo*.

173. The conotide of claim 163, wherein none of said linking moieties are disulfide linkages.

174. A conotide of the formula (VII):



(VII)

wherein:

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

X is an independently selected amino acid residue for each occurrence;

a and b are each independently selected polypeptide linkers comprising one or more amino acid residues; and

c is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are cysteine, then c is not absent.

175. The conotide of claim 174, wherein c is a covalent linkage.

176. The conotide of claim 175, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

177. The conotide of claim 175, wherein c is a polypeptide linkage.

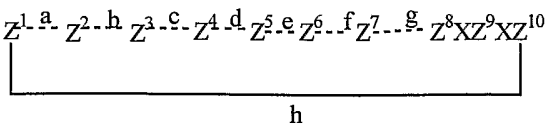
178. The conotide of claim 174, wherein c is a hydrophobic linking moiety.

179. The conotide of claim 178, wherein Z^1 and Z^6 comprise an aryl moiety.

180. The conotide of claim 174, wherein c is an ionic linkage.

181. The conotide of claim 175, wherein said conotide is resistant to degradation *in vivo*.

182. A conotide of the formula (VIII):



(VIII)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, Z^9, and Z^{10} are each independently selected linking residues;

X is an independently selected amino acid residue for each occurrence;

a, b, c, d, e, f, and g are each independently selected polypeptide linkers comprising one or more amino acid residues; and

h is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, Z^9, and Z^{10} are cysteine, then h is not absent.

183. The conotide of claim 182, wherein h is a covalent linkage.

184. The conotide of claim 183, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

185. The conotide of claim 182, wherein h is a polypeptide linkage.

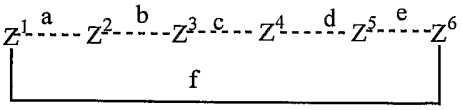
186. The conotide of claim 182, wherein h is a hydrophobic linking moiety.

187. The conotide of claim 186, wherein Z^1 and Z^{10} comprise an aryl moiety.

188. The conotide of claim 182, wherein h is an ionic linkage.

189. The conotide of claim 182, wherein said conotide is resistant to degradation *in vivo*.

190. A conotide of the formula (IX):



(IX)

wherein

Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6 are each independently selected linking residues;

a, b, c, d, and e are each independently selected polypeptide linkers comprising one or more amino acid residues; and

f is a linking moiety or absent, provided that if each of Z^1, Z^2, Z^3, Z^4, Z^5, and Z^6, are cysteine, then f is not absent.

191. The conotide of claim 190, wherein f is a covalent linkage.

192. The conotide of claim 191, wherein said covalent linkage is alkyl, alkenyl, alkynyl, aryl, amido, or thioether.

193. The conotide of claim 190, wherein f is a polypeptide linkage.

194. The conotide of claim 194, wherein f is a hydrophobic linking moiety.

195. The conotide of claim 194, wherein Z^1 and Z^6 comprise an aryl moiety.

196. The conotide of claim 190, wherein f is an ionic linkage.

197. The conotide of claim 190, wherein said conotide is resistant to degradation *in vivo*.

198. A library comprising a nucleic acid encoding a conotide having a formula selected from the group consisting of I, II, III, IV, V, and VI

199. A library comprising a conotide having a formula selected from the group consisting of I, II, III, IV, V, and VI .

200. The conotide of any one of claims 106, 118, 129, 140, 151, 163, 174, 182, and 190, whereby the conotide further comprises an intein linked to either end of the conotide so that the peptide aptamer is flanked by intein splicing elements capable of catalyzing the formation of a cyclic peptide aptamer.

201. The conotide of claim 200, wherein the inteins flanking the conotide further comprise a sequence which can be induced to join the inteins, such that the intervening peptide aptamer is cyclized in the presence of an inducing agent.

202. The conotide of claim 200, wherein one or more linkages (Z) is produced by intein-mediated splicing.

203. The method of any one of claims 68-72, wherein the cells are prokaryotic cells.

204. The method of any one of claims 68-72, wherein said library of expressible nucleic acids encoding random peptide aptamers is encoded in a prokaryotic expression vector.

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Fig. 1

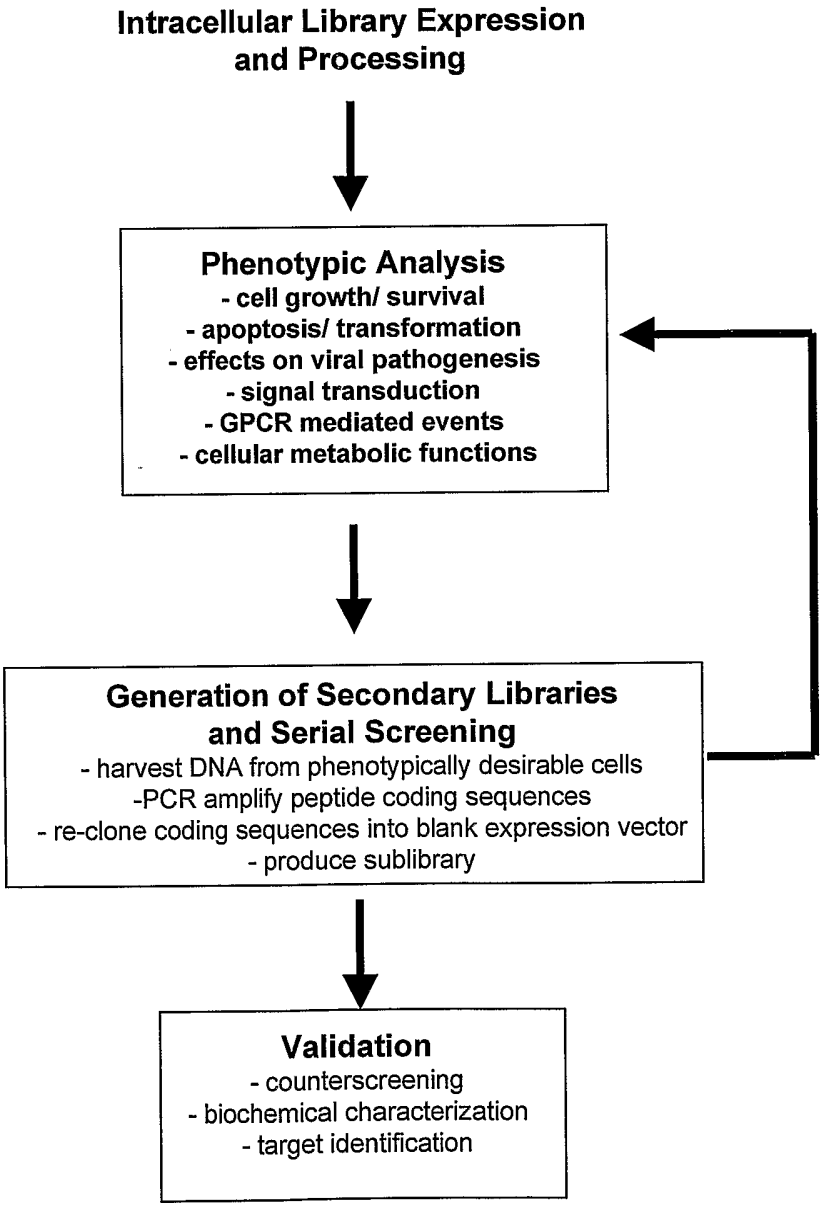
Intracellular Library Expression and Processing
Phenotypic Analysis
- cell growth/ survival
- apoptosis/ transformation
- effects on viral pathogenesis
- signal transduction
- GPCR mediated events
- cellular metabolic functions
Generation of Secondary Libraries and Serial Screening
- harvest DNA from phenotypically desirable cells
-PCR amplify peptide coding sequences
- re-clone coding sequences into blank expression vector
- produce sublibrary
Validation
- counterscreening
- biochemical characterization
- target identification

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Fig. 2

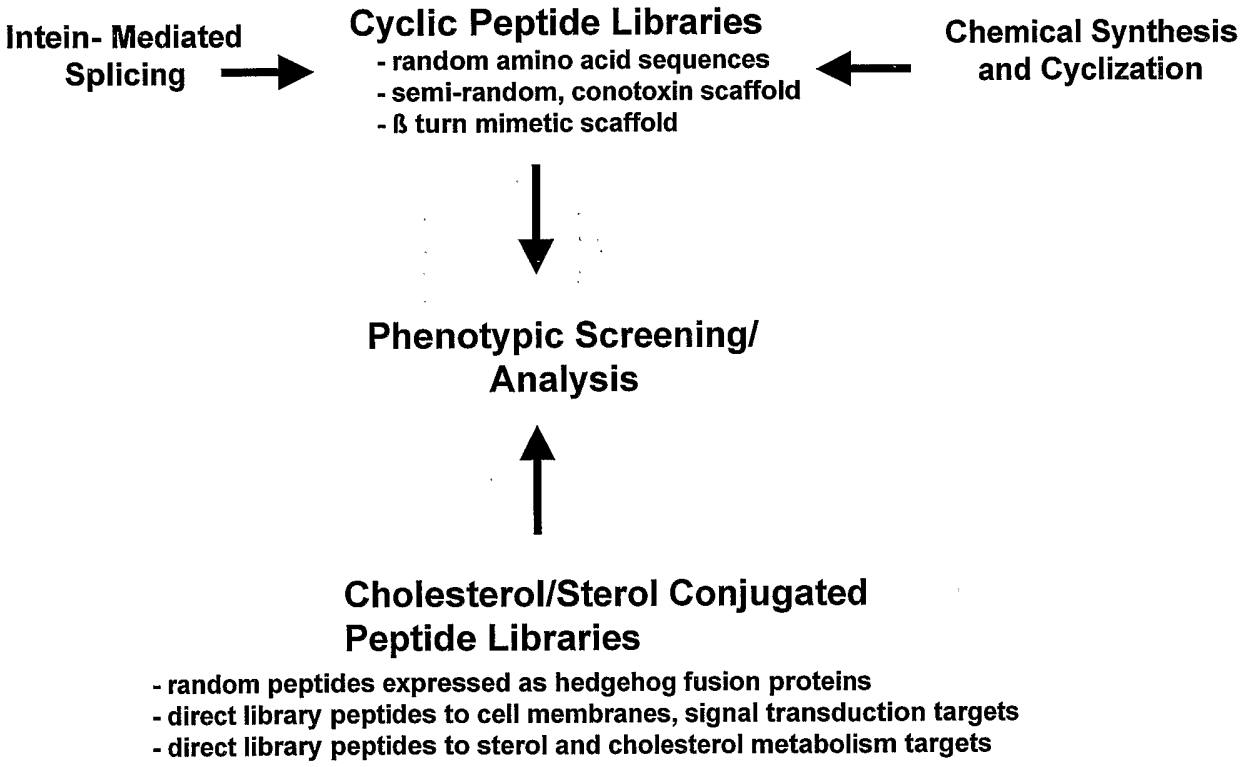
Intein- Mediated Splicing
Cyclic Peptide Libraries
- random amino acid sequences
- semi-random, conotoxin scaffold
- ß turn mimetic scaffold
Chemical Synthesis and Cyclization
Phenotypic Screening/ Analysis
Cholesterol/Sterol Conjugated Peptide Libraries
- random peptides expressed as hedgehog fusion proteins
- direct library peptides to cell membranes, signal transduction targets
- direct library peptides to sterol and cholesterol metabolism targets

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Fig. 3

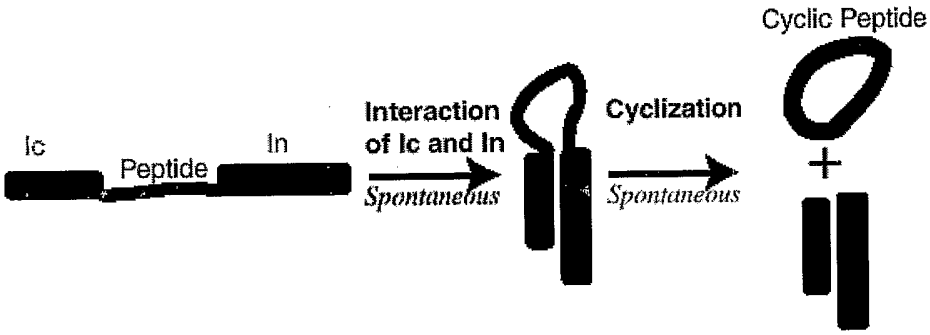
Cyclic Peptide
Ic
Peptide
In
Interaction
of Ic and In
Spontaneous
Cyclization
Spontaneous
+

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Fig. 4

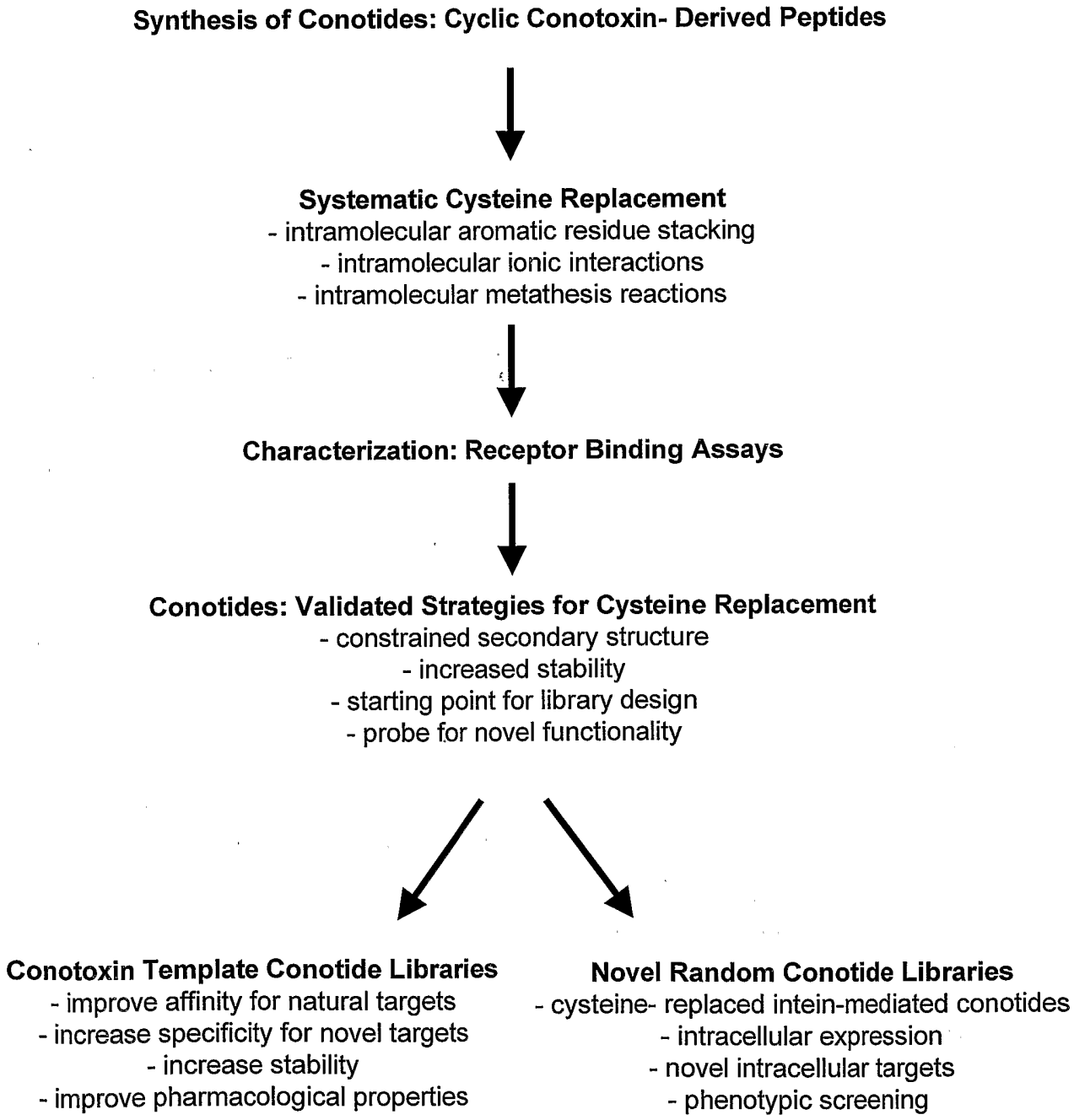
Synthesis of Conotides: Cyclic Conotoxin- Derived Peptides
Systematic Cysteine Replacement
- intramolecular aromatic residue stacking
- intramolecular ionic interactions
- intramolecular metathesis reactions
Characterization: Receptor Binding Assays
Conotides: Validated Strategies for Cysteine Replacement
- constrained secondary structure
- increased stability
- starting point for library design
- probe for novel functionality
Conotoxin Template Conotide Libraries
- improve affinity for natural targets
- increase specificity for novel targets
- increase stability
- improve pharmacological properties
Novel Random Conotide Libraries
- cysteine- replaced intein-mediated conotides
- intracellular expression
- novel intracellular targets
- phenotypic screening

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Fig. 5

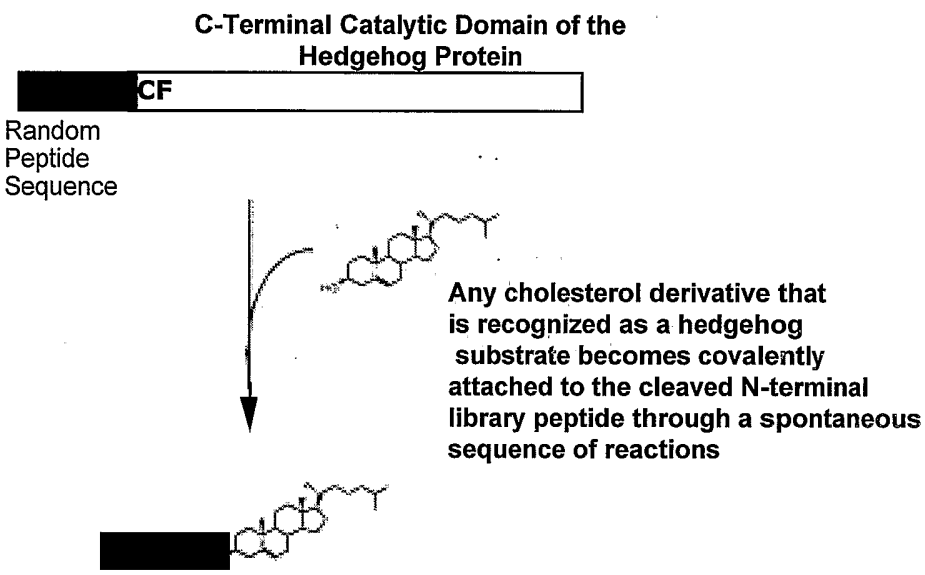
C-Terminal Catalytic Domain of the Hedgehog Protein
CF
Random Peptide Sequence
Any cholesterol derivative that is recognized as a hedgehog substrate becomes covalently attached to the cleaved N-terminal library peptide through a spontaneous sequence of reactions

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Fig. 6

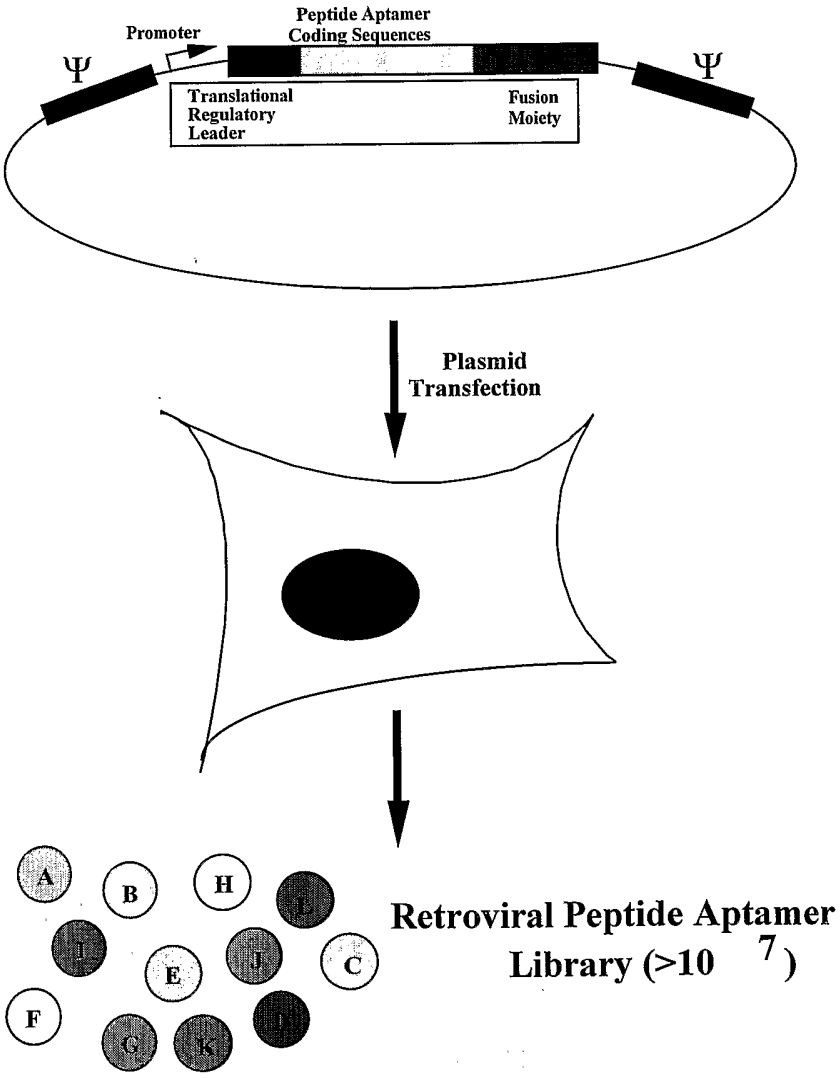
Peptide Aptamer
Coding Sequences
Promoter
Ψ
Ψ
Translational
Regulatory
Leader
Fusion
Moiety
Plasmid
Transfection
A
B
H
E
C
F
G
K
Retroviral Peptide Aptamer
Library (>10 7)

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Fig. 7

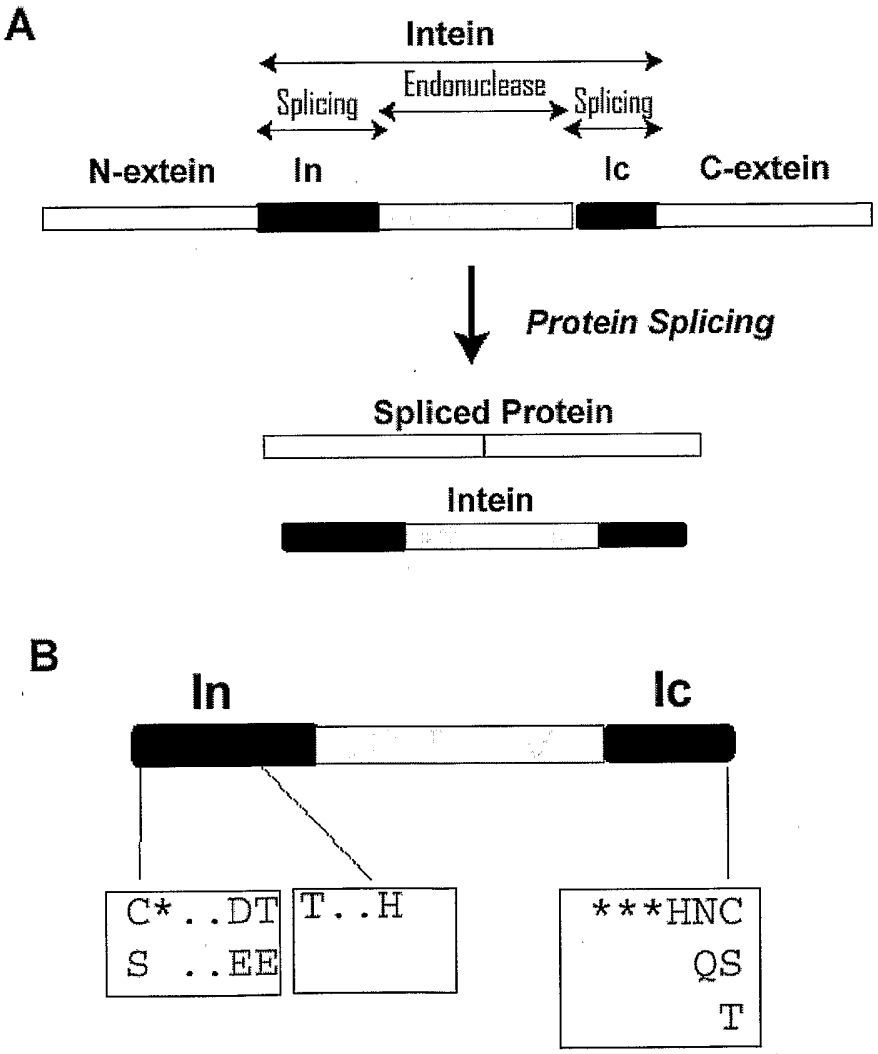
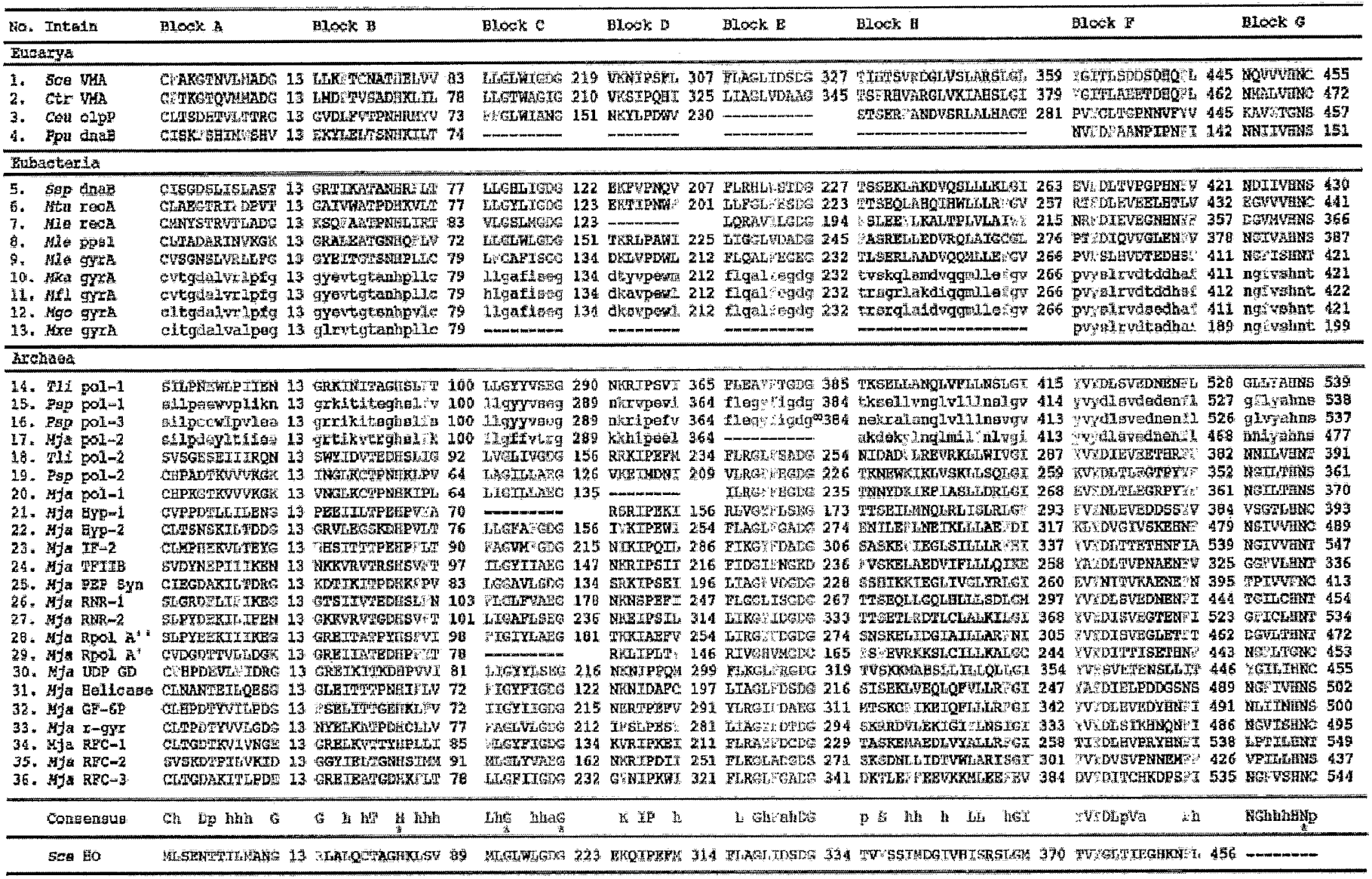
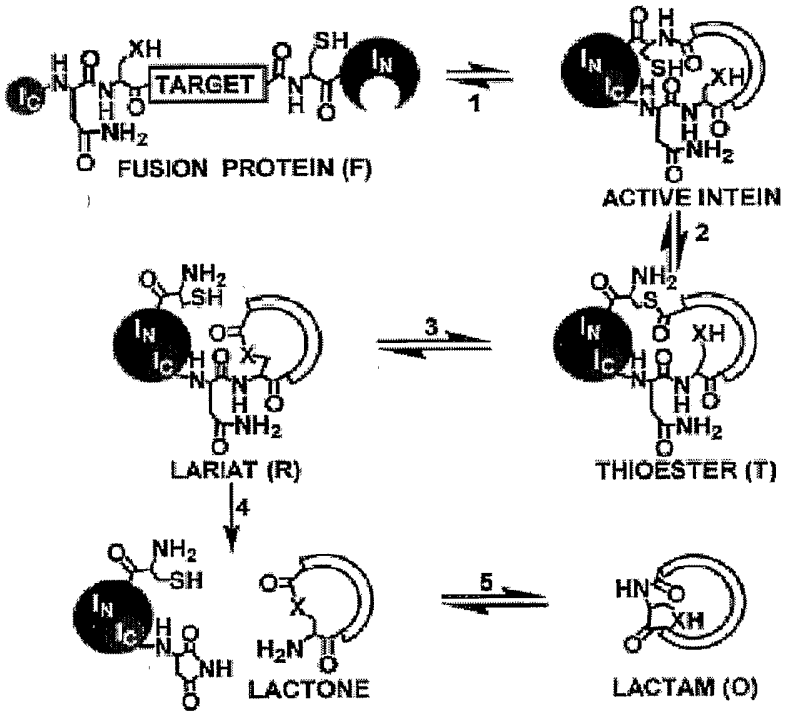

A
Intein
Splicing
Endonuclease
Splicing
N-extein
In
Ic
C-extein
Protein Splicing
Spliced Protein
Intein
B
In
Ic
C*..DT
S ..EE
T..H
***HNC
QS
T

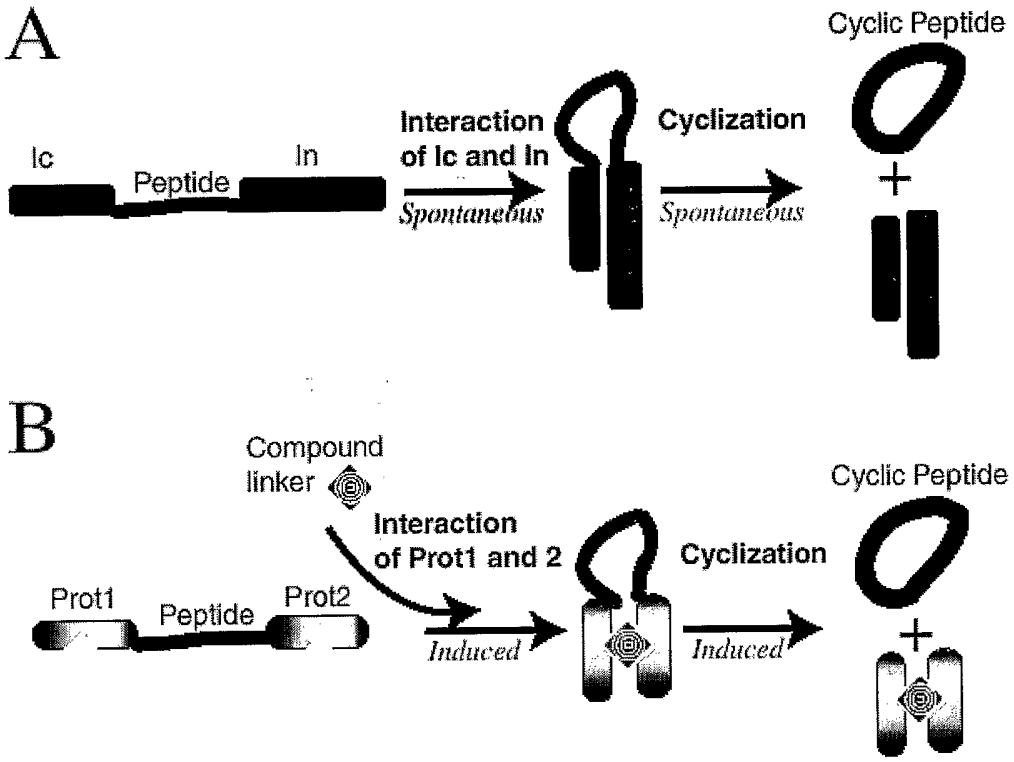
Fig. 8



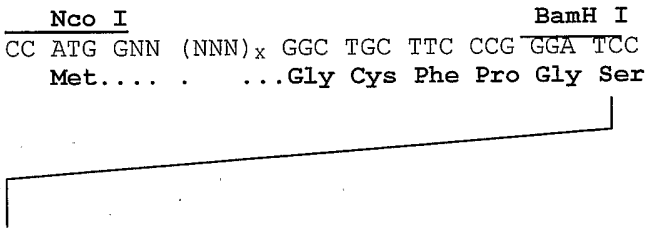
9/29
Fig. 9


TARGET
FUSION PROTEIN (F)
ACTIVE INTEIN
LARIAT (R)
THIOESTER (T)
LACTONE
LACTAM (O)
1
2
3
4
5

10/29
Fig. 10


A
Cyclic Peptide
Ic
Peptide
In
Interaction
of Ic and In
Spontaneous
Cyclization
Spontaneous
B
Compound
linker
Cyclic Peptide
Interaction
of Prot1 and 2
Cyclization
Prot1
Peptide
Prot2
Induced
Induced

11/29
Fig. 11



BamH I Human Sonic Hedgehog C-Terminal Sequence

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gacctgagccccggggaccgcgtgctggcggcggacgaccagggccggctgctctacagc
 D  L  S  P  G  D  R  V  L  A  A  D  D  Q  G  R  L  L  Y  S
gacttcctcactttcctggaccgcgacgacggcgccaagaaggtcttctacgtgatcgag
 D  F  L  T  F  L  D  R  D  D  G  A  K  K  V  F  Y  V  I  E
acgcgggagccgcgcgagcgcctgctgctcaccgccgcgcacctgctctttgtggcgccg
 T  R  E  P  R  E  R  L  L  L  T  A  A  H  L  L  F  V  A  P
cacaacgactcggccaccggggagcccgaggcgtcctcgggctcggggccgccttccggg
 H  N  D  S  A  T  G  E  P  E  A  S  S  G  S  G  P  P  S  G
ggcgcactggggcctcgggcgctgttcgccagccgcgtgcgcccgggccagcgcgtgtac
 G  A  L  G  P  R  A  L  F  A  S  R  V  R  P  G  Q  R  V  Y
gtggtggccgagcgtgacggggaccgccggctcctgcccgccgctgtgcacagcgtgacc
 V  V  A  E  R  D  G  D  R  R  L  L  P  A  A  V  H  S  V  T
ctaagcgaggaggccgcgggcgcctacgcgccgctcacggcccagggcaccattctcatc
 L  S  E  E  A  A  G  A  Y  A  P  L  T  A  Q  G  T  I  L  I
aaccgggtgctggcctcgtgctacgcggtcatcgaggagcacagctgggcgcaccgggcc
 N  R  V  L  A  S  C  Y  A  V  I  E  E  H  S  W  A  H  R  A
ttcgcgcccttccgcctggcgcacgcgctcctggctgcactggcgcccgcgcgcacggac
 F  A  P  F  R  L  A  H  A  L  L  A  A  L  A  P  A  R  T  D
cgcggcggggacagcggcggcggggaccgcgggggcggcggcggcagagtagccctaacc
 R  G  G  D  S  G  G  G  D  R  G  G  G  G  G  R  V  A  L  T
gctccaggtgctgccgacgctccgggtgcgggggccaccgcgggcatccactggtactcg
 A  P  G  A  A  D  A  P  G  A  G  A  T  A  G  I  H  W  Y  S
cagctgctctaccaaataggcacctggctggctcctggacagcgaggccctgcacccgct
 Q  L  L  Y  Q  I  G  T  W  L  A  P  G  Q  R  G  P  A  P  A
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 G  H  G  G  Q  V  Q  L  T  R
                              XhoI
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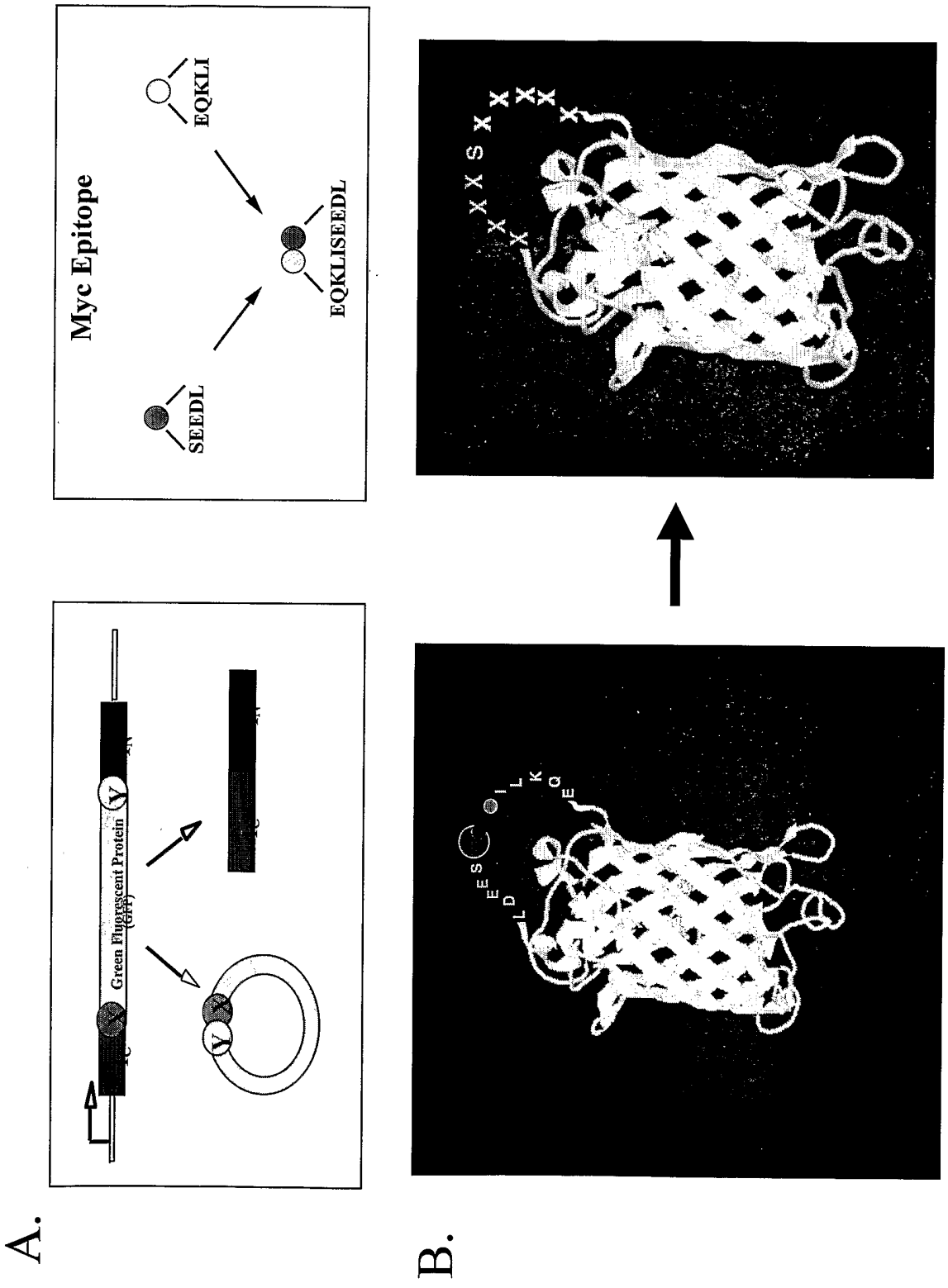
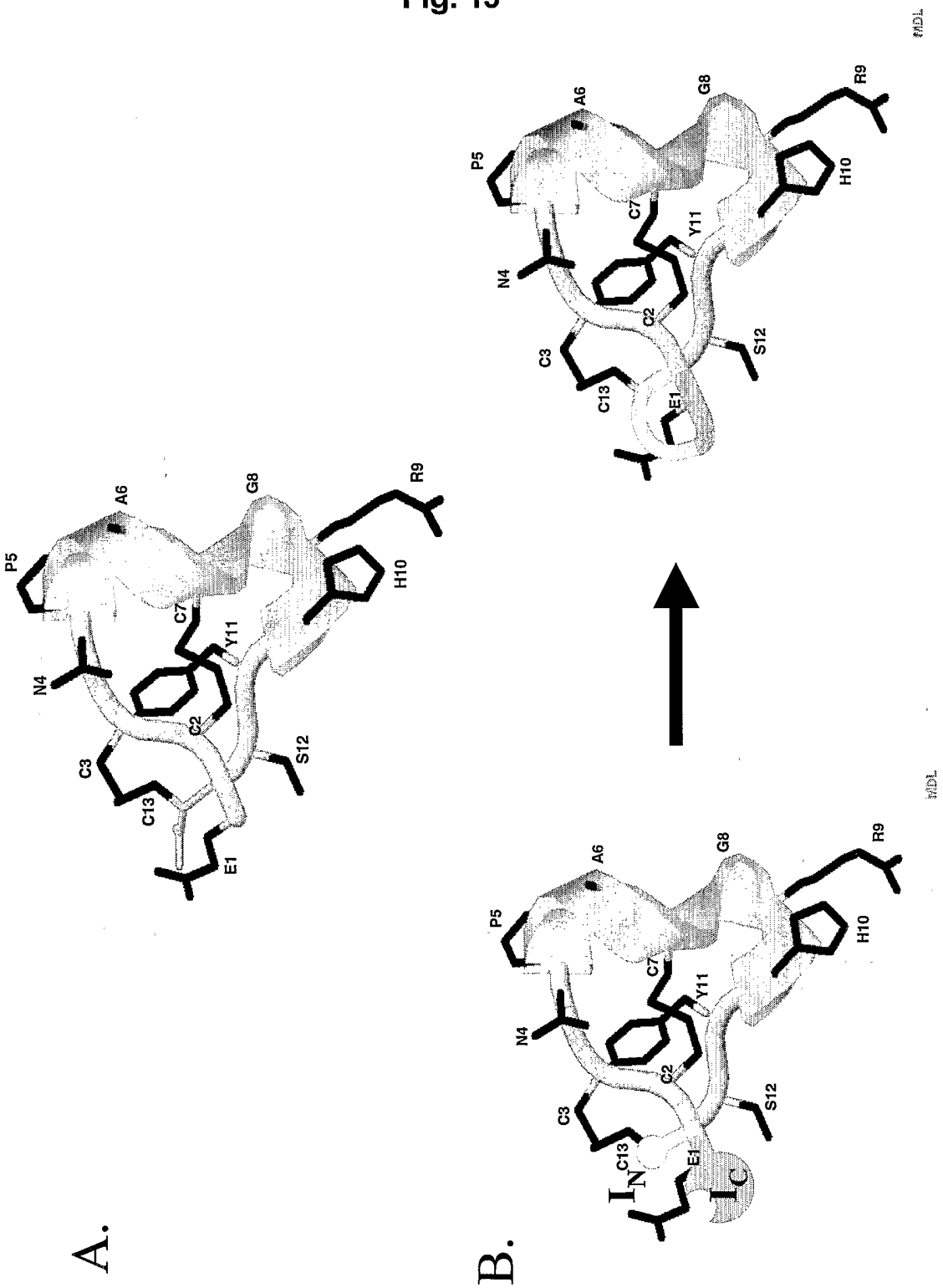
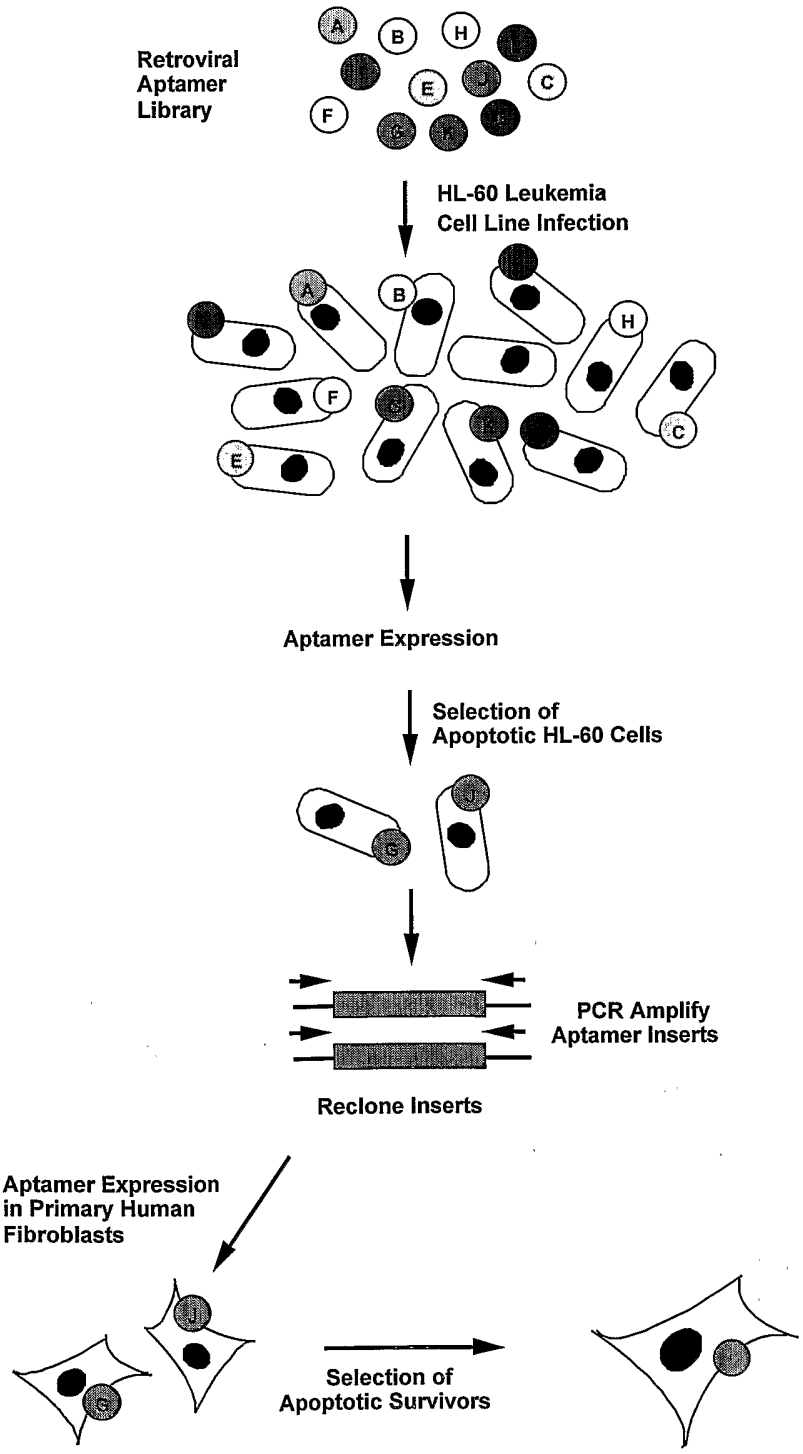
Fig. 12


Myc Epitope
EQKLI
SEEDL
EQKLISEEDL
Green Fluorescent Protein
(GFP)
A.
B.

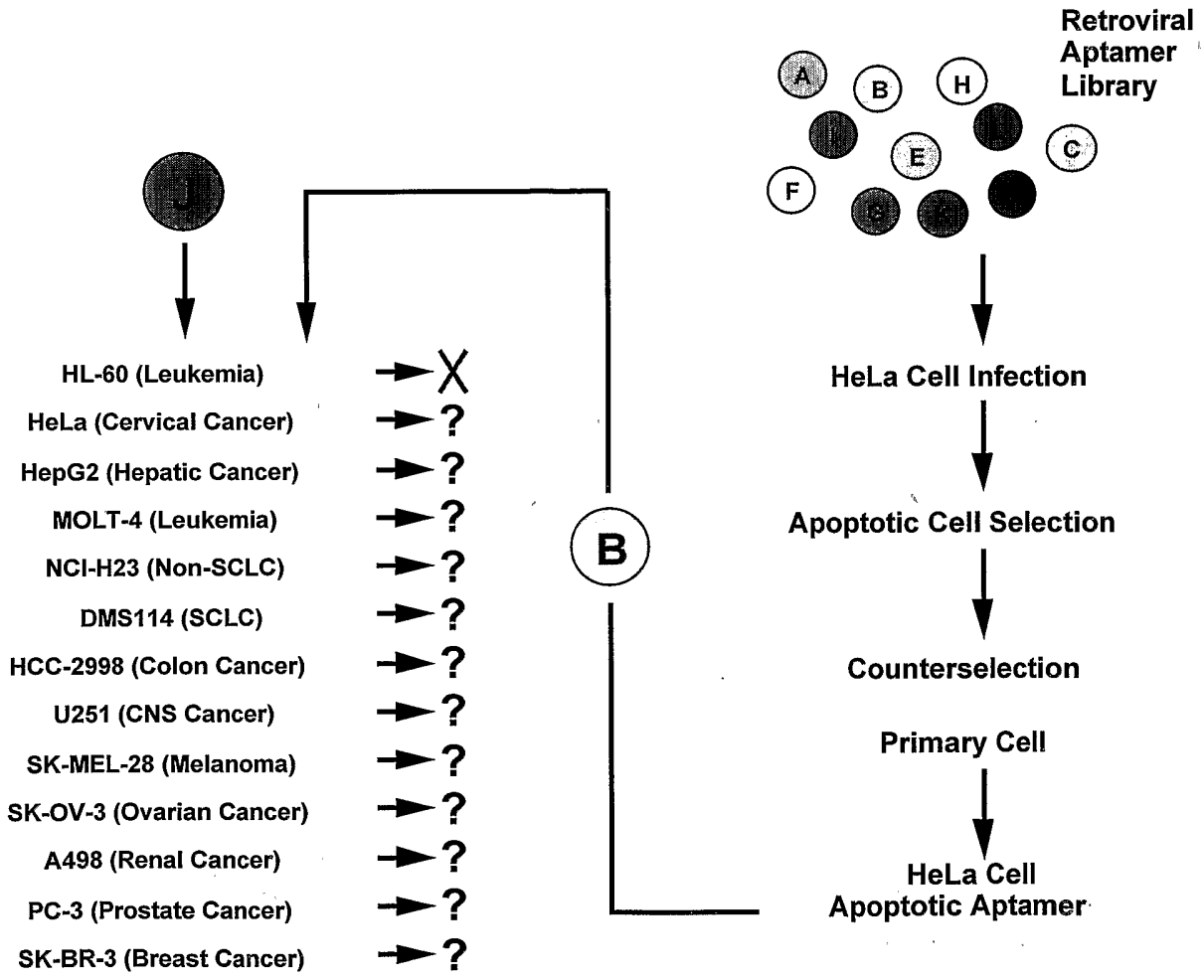
13/29
Fig. 13



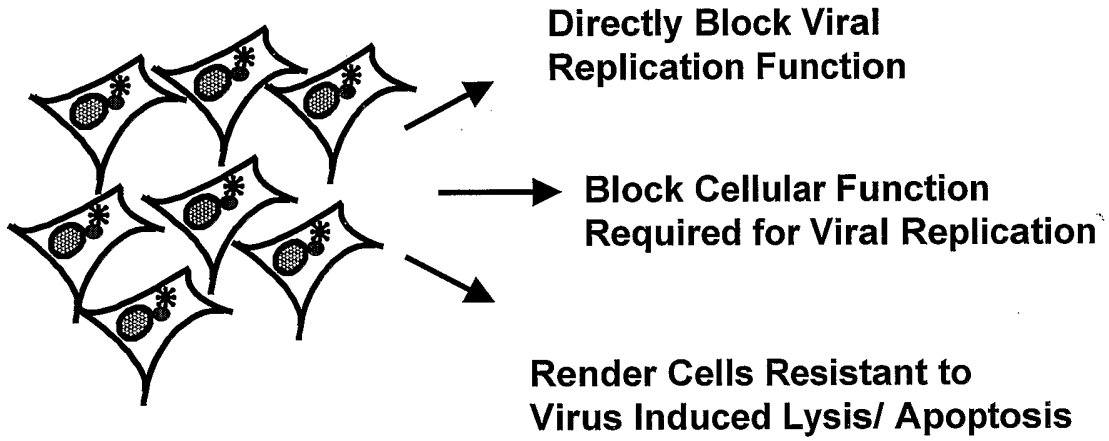
14/29
Fig. 14


Retroviral
Aptamer
Library
HL-60 Leukemia
Cell Line Infection
Aptamer Expression
Selection of
Apoptotic HL-60 Cells
PCR Amplify
Aptamer Inserts
Reclone Inserts
Aptamer Expression
in Primary Human
Fibroblasts
Selection of
Apoptotic Survivors

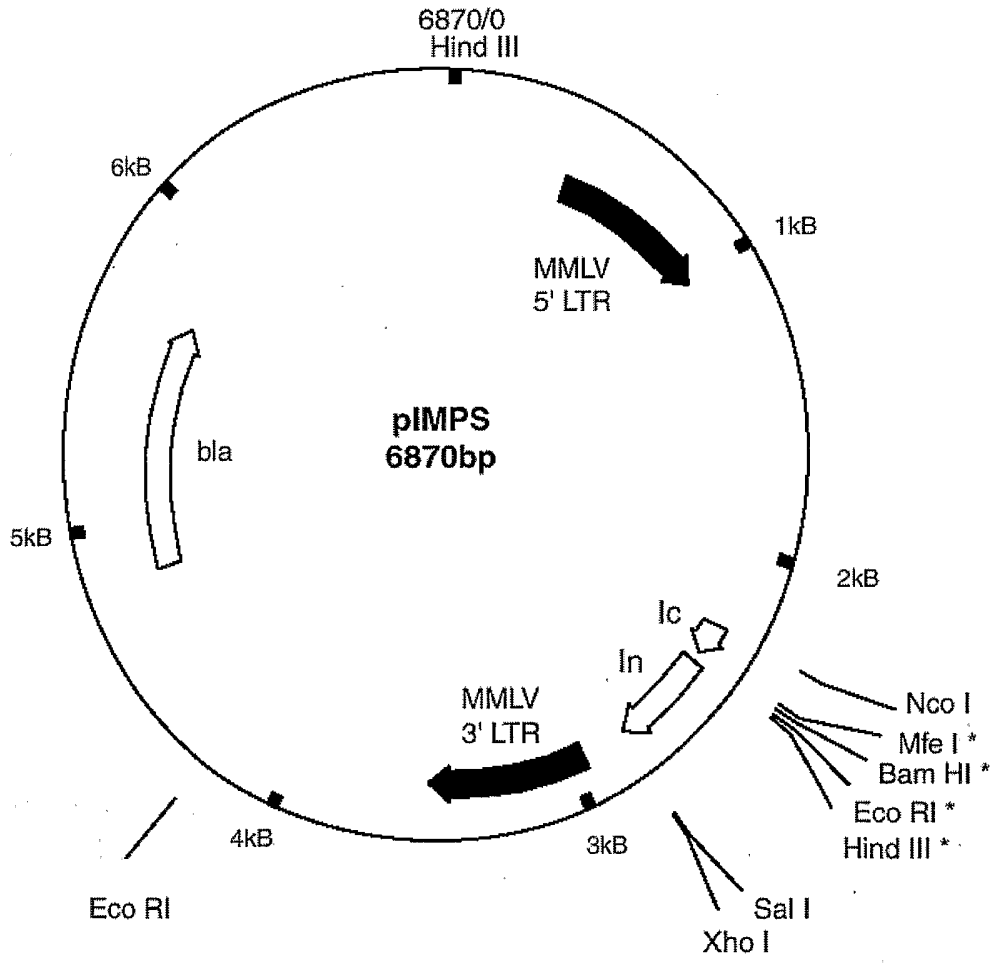
15/29
Fig. 15


Retroviral
Aptamer
Library
A
B
H
E
C
F
J
HL-60 (Leukemia)
HeLa (Cervical Cancer)
HepG2 (Hepatic Cancer)
MOLT-4 (Leukemia)
NCI-H23 (Non-SCLC)
DMS114 (SCLC)
HCC-2998 (Colon Cancer)
U251 (CNS Cancer)
SK-MEL-28 (Melanoma)
SK-OV-3 (Ovarian Cancer)
A498 (Renal Cancer)
PC-3 (Prostate Cancer)
SK-BR-3 (Breast Cancer)
B
HeLa Cell Infection
Apoptotic Cell Selection
Counterselection
Primary Cell
HeLa Cell
Apoptotic Aptamer

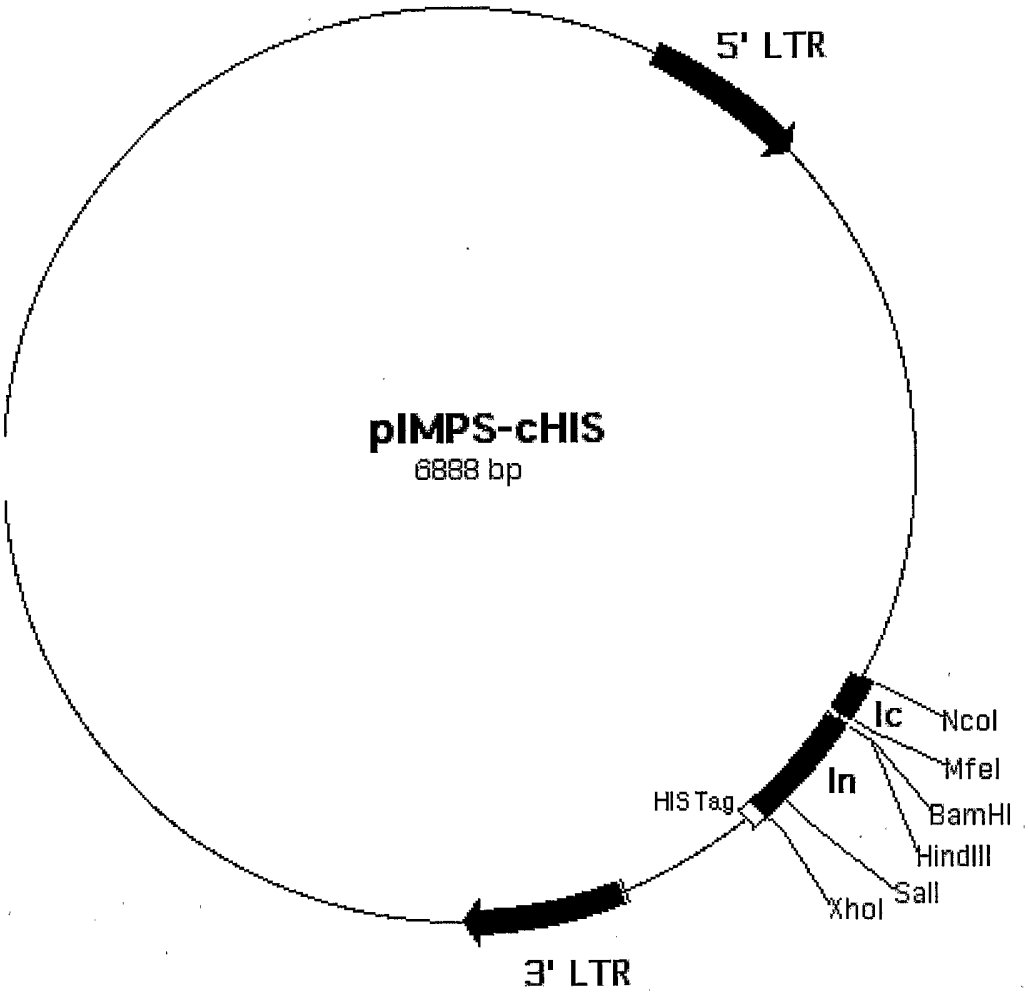
16/29
Fig. 16


Directly Block Viral Replication Function
Block Cellular Function Required for Viral Replication
Render Cells Resistant to Virus Induced Lysis/ Apoptosis

17/29
Fig. 17


6870/0
Hind III
1kB
2kB
3kB
4kB
5kB
6kB
MMLV
5' LTR
pIMPS
6870bp
bla
Ic
In
MMLV
3' LTR
Nco I
Mfe I *
Bam HI *
Eco RI *
Hind III *
Sal I
Xho I
Eco RI

18/29
Fig. 18


5' LTR
pIMPS-cHIS
6888 bp
Ic
In
NcoI
MfeI
BamHI
HindIII
SalI
XhoI
HIS Tag
3' LTR

19/29
Fig. 29

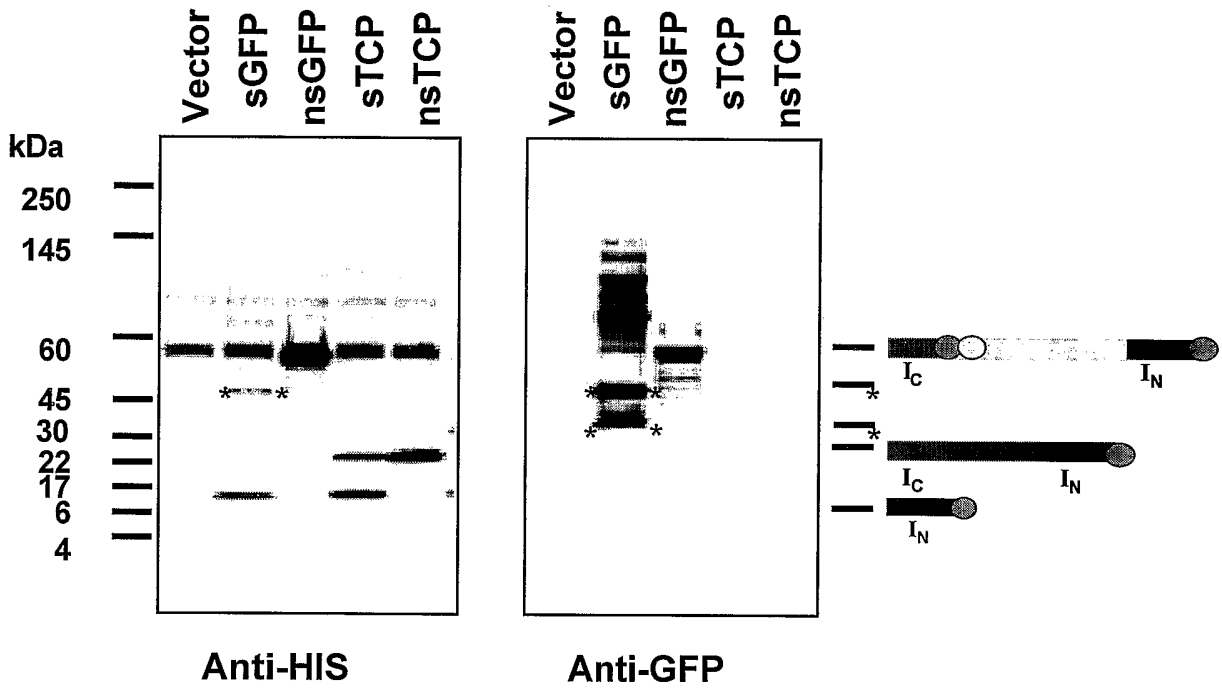
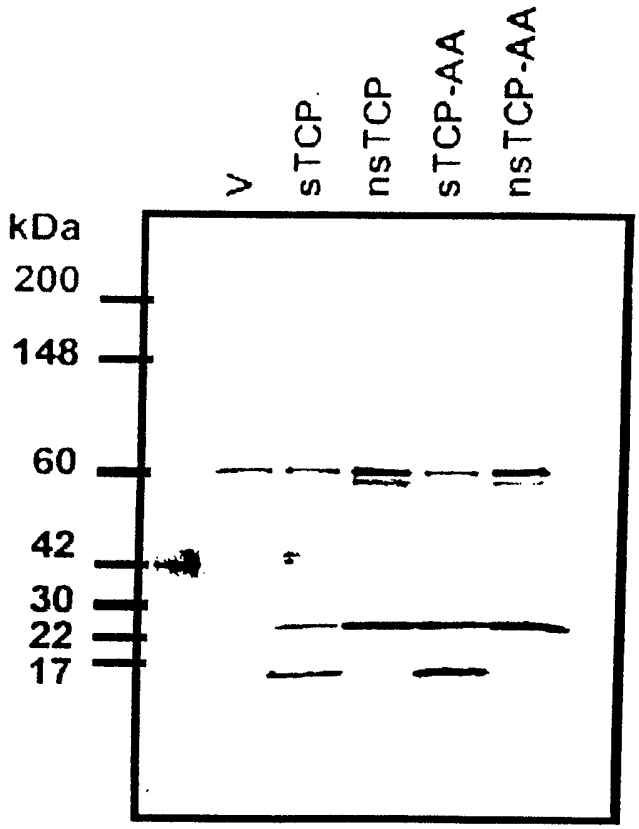
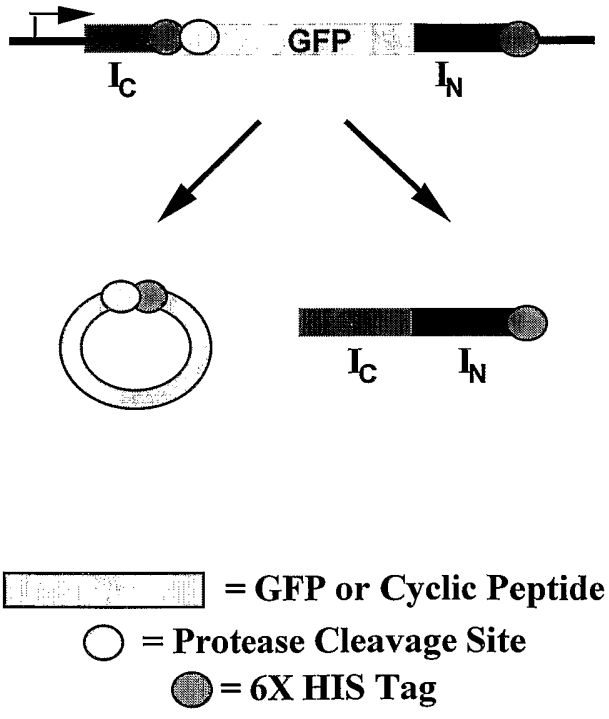

Vector
sGFP
nsGFP
sTCP
nsTCP
kDa
250
145
60
45
30
22
17
6
4
Anti-HIS
Vector
sGFP
nsGFP
sTCP
nsTCP
Anti-GFP
I_C
I_N
I_C
I_N
I_N

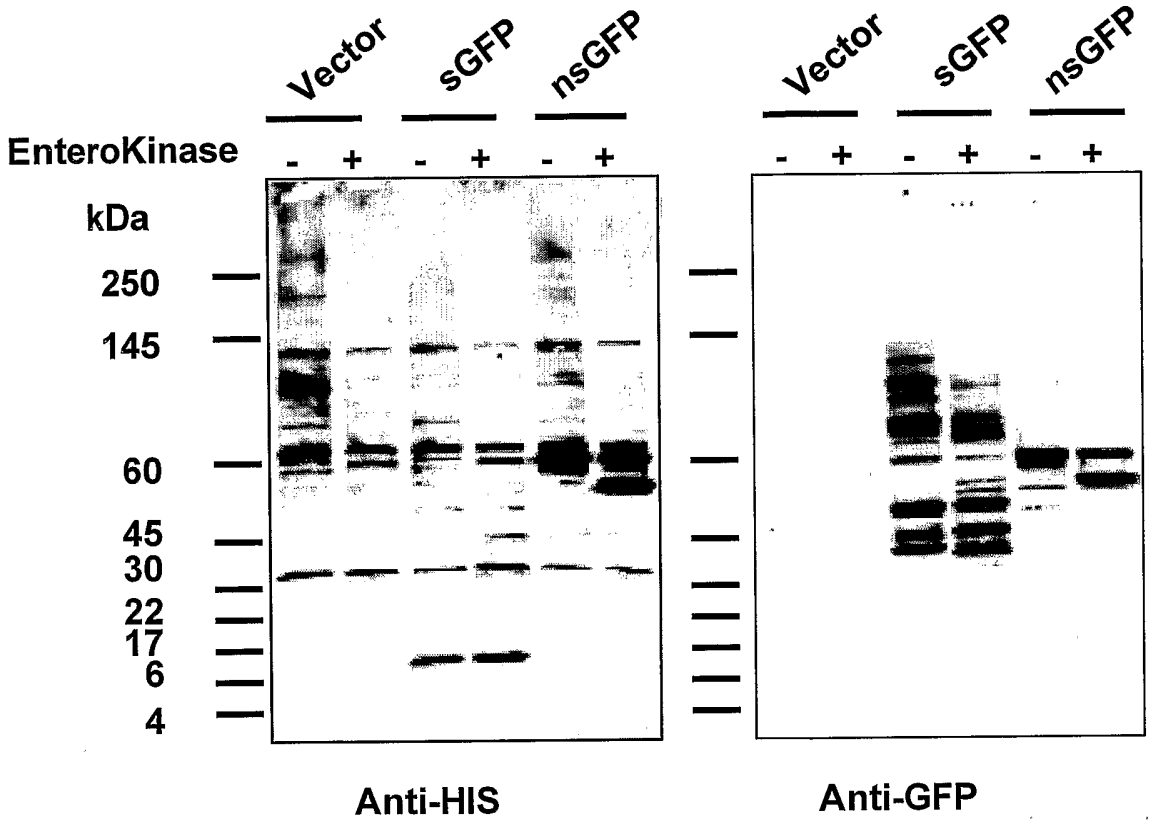
Figure 20



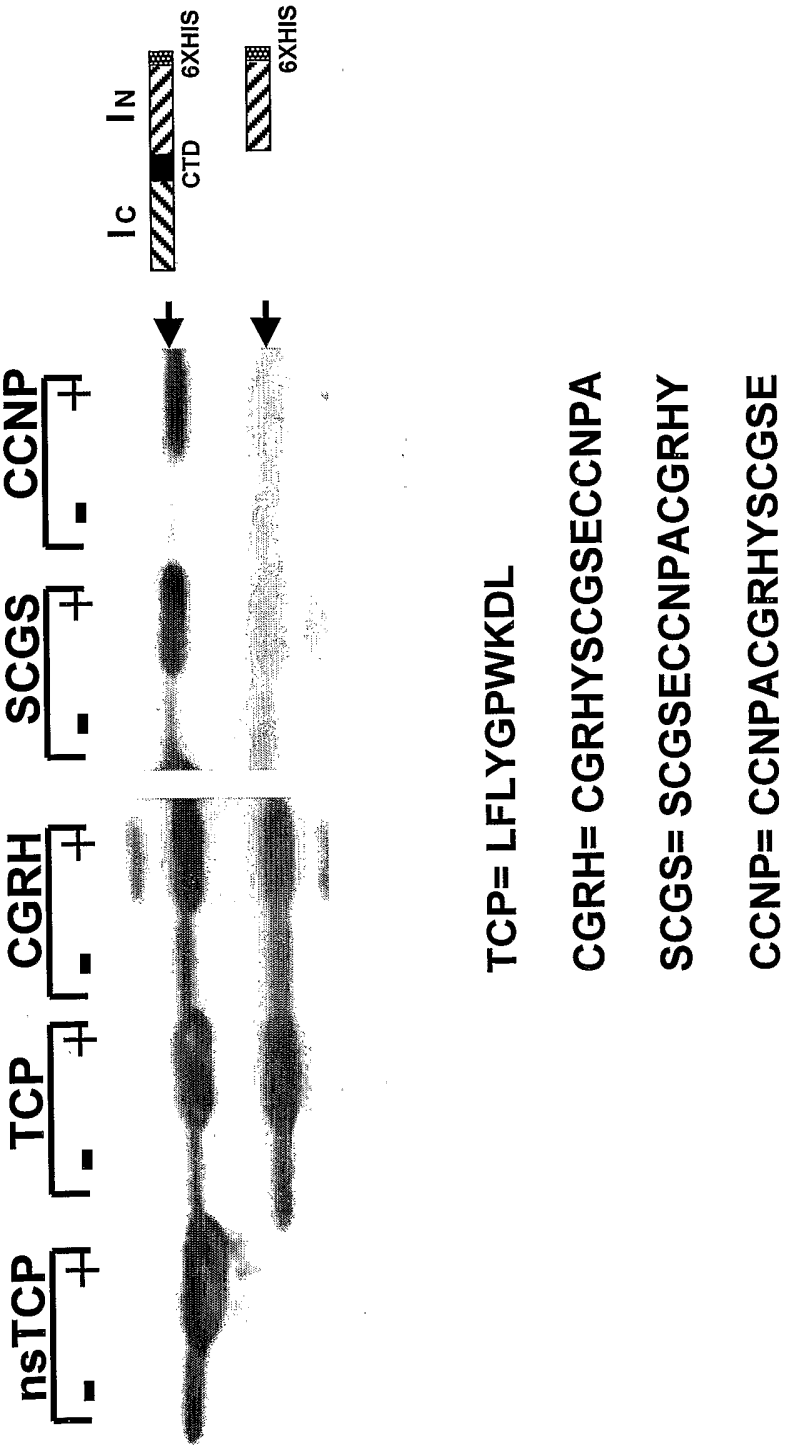
21/29
Fig. 21


GFP
I_C
I_N
I_C
I_N
= GFP or Cyclic Peptide
= Protease Cleavage Site
= 6X HIS Tag

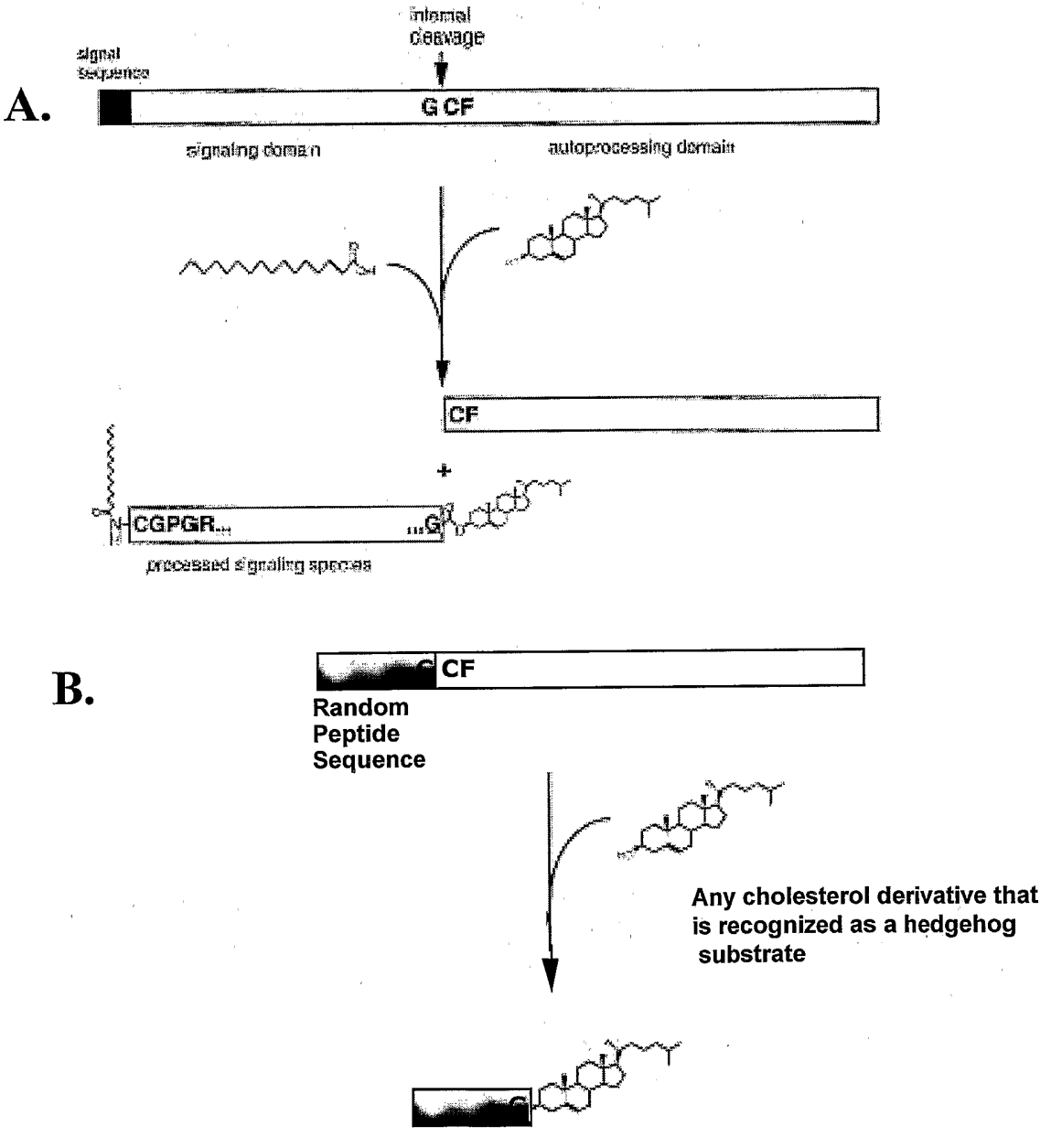
22/29
Fig. 22


Vector
sGFP
nsGFP
Vector
sGFP
nsGFP
EnteroKinase
- + - + - +
- + - + - +
kDa
250
145
60
45
30
22
17
6
4
Anti-HIS
Anti-GFP

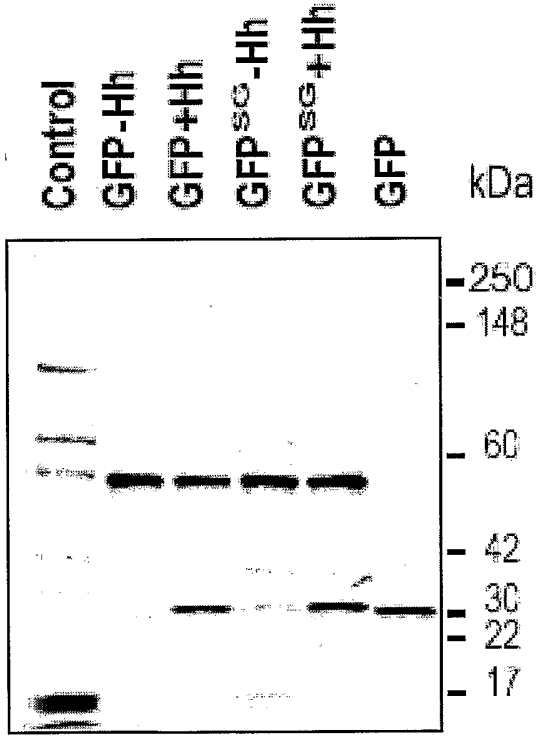
23/29
Fig. 23


nsTCP
TCP
CGRH
SCGS
CCNP
IC
IN
CTD
6XHIS
6XHIS
TCP= LFLYGPWKDL
CGRH= CGRHYSCGSECCNPA
SCGS= SCGSECCNPACGRHY
CCNP= CCNPACGRHYSCGSE

24/29
Fig. 24


A.
signal sequence
internal cleavage
GCF
signaling domain
autoprocessing domain
CF
+
N
CGPGR...
...G
processed signaling species
B.
CF
Random Peptide Sequence
Any cholesterol derivative that is recognized as a hedgehog substrate

25/29
Fig. 25



IP: anti-GFP
Blot: anti-GFP

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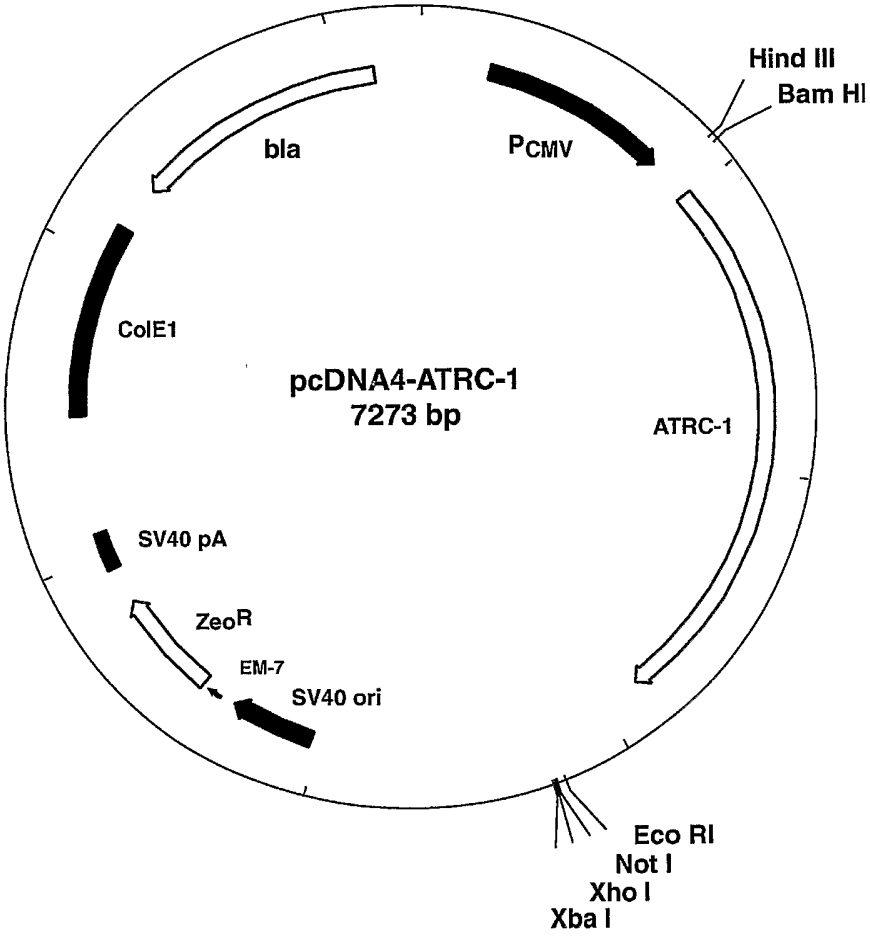
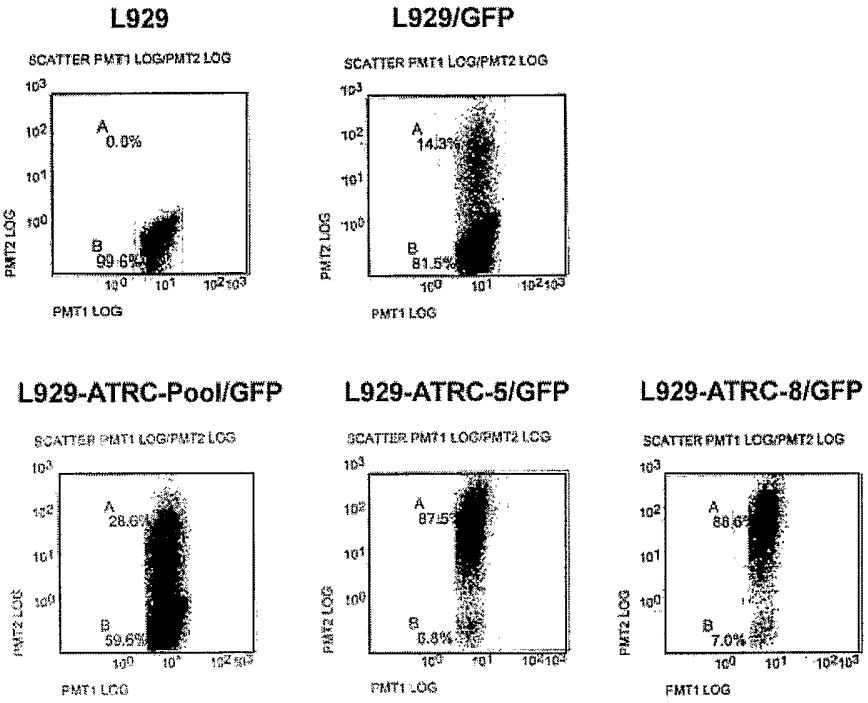
Fig. 26

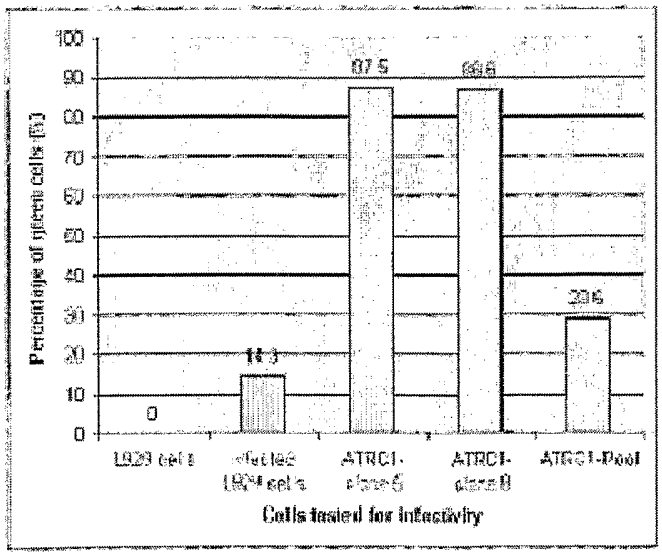

Hind III
Bam HI
bla
PCMV
ColE1
pcDNA4-ATRC-1
7273 bp
ATRC-1
SV40 pA
ZeoR
EM-7
SV40 ori
Eco RI
Not I
Xho I
Xba I

27/29
Fig. 27

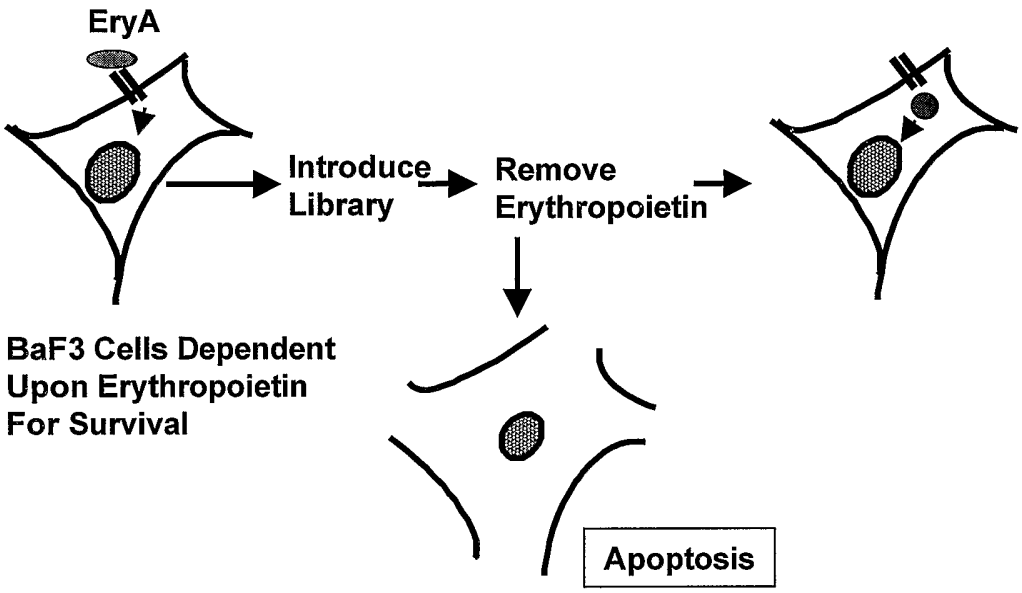
A



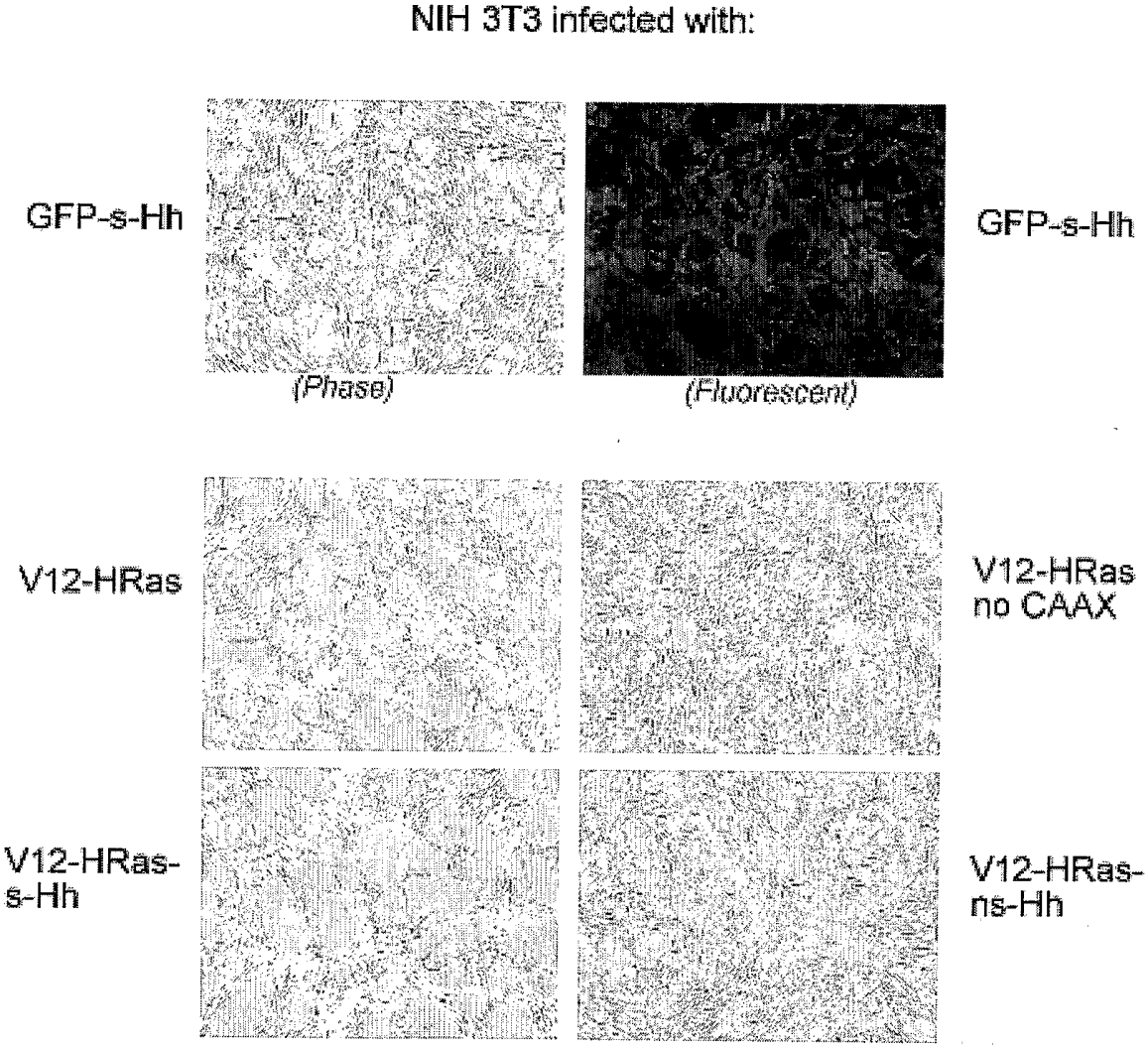
B



28/29
Fig. 28


EryA
Introduce Library
Remove Erythropoietin
BaF3 Cells Dependent Upon Erythropoietin For Survival
Apoptosis

29/29
Fig. 29


NIH 3T3 infected with:
GFP-s-Hh
(Phase)
(Fluorescent)
GFP-s-Hh
V12-HRas
V12-HRas no CAAX
V12-HRas-s-Hh
V12-HRas-ns-Hh

SEQUENCE LISTING

<110> ENANTA PHARMACEUTICALS, INC. *et al.*

<120> METHODS AND COMPOSITIONS FOR IDENTIFYING PEPTIDE APTAMERS CAPABLE OF ALTERING A CELL PHENOTYPE

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aatgttaatt ccattttaaa tgcacagatg tttttatttc ataagggttt caatgtgcat 180

gaatggcaat attcctgtta ccaaagctag tataaataaa aatagataaa cgtggaaatt 240

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tcaattagtt gatttttatt tttgacatat acatgtgaaa gaccccacct gtaggtttgg 420

caagctagct taagtaacgc cattttgcaa ggcatggaaa aatacataac tgagaataga 480

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tccccagatg cggtccagcc ctcagcagtt tctagagaac catcagatgt ttccagggtg 720

ccccaaggac ctgaaatgac cctgtgcctt atttgaacta accaatcagt tcgcttctcg 780

cttctgttcg cgcgcttctg ctccccgagc tcaataaaag agcccacaac ccctcactcg 840

gggcgccagt cctccgattg actgagtcgc ccgggtaccc gtgtatccaa taaaccctct 900

tgcagttgca tccgacttgt ggtctcgctg ttccttggga gggtctcctc tgagtgattg 960

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gccctcactc cttctctagg cgcccccata tggccatatg agatcttata tggggcaccc 1920

ccgccccttg taaacttccc tgaccctgac atgacaagag ttactaacag cccctctctc 1980

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ataatttttct gctagccaat ggggcgatcg cccacaattg ttttaacgga tcccccgggc 2400

tgcaggaatt cgatatcaag ctttggcacc gaaatttaa ccgttgagta cggcccattg 2460

cccattggca aaattgtgag tgaagaaatt aattgttctg tgtacagtgt tgatccagaa 2520

gggagagttt acacccaggc gatcgcccaa tggcatgacc ggggagagca ggaagtattg 2580

gaatatgaat tggaagatgg ttcagtaatc cgagctacct ctgaccaccg ctttttaacc 2640

accgattatc aactgttggc gatcgaagaa atttttgcta ggcaactgga cttgttgact 2700

ttagaaaata ttaagcaaac tgaagaagct cttgacaacc atcgtcttcc ctttccatta 2760

cttgacgctg ggacaattaa ataagtcgac ctcgagatcc cccagatccc gcgattagtc 2820

caatttgtta aagacaggat atcagtggtc caggctctag ttttgactca acaatatcac 2880

cagctgaagc ctatagagta cgagccatag ataaaataaa agattttatt tagtctccag 2940

aaaaaggggg gaatgaaaga ccccacctgt aggtttggca agctagctta agtaacgcca 3000

ttttgcaagg catggaaaaa tacataactg agaatagaga agttcagatc aaggtcagga 3060

acagatggaa cagctgaata tgggccaaac aggatatctg tggtaagcag ttcctgcccc 3120

ggctcagggc caagaacaga tggaacagct gaatatgggc caaacaggat atctgtggta 3180

agcagttcct gccccggctc agggccaaga acagatggtc cccagatgcg gtccagccct 3240

cagcagtttc tagagaacca tcagatgttt ccagggtgcc ccaaggacct gaaatgaccc 3300

tgtgccttat ttgaactaac caatcagttc gcttctcgct tctgttcgcg cgcttctgct 3360

ccccgagctc aataaaagag cccacaaccc ctcactcggg gcgccagtcc tccgattgac 3420

tgagtcgccc gggtacccgt gtatccaata aaccctcttg cagttgcatc cgacttgtgg 3480

tctcgctgtt ccttgggagg gtctcctctg agtgattgac tacccgtcag cgggggtctt 3540

tcacacatgc agcatgtatc aaaattaatt tggtttttt tcttaagtat ttacattaaa 3600

tggccatagt acttaaagtt acattggctt ccttgaaata aacatggagt attcagaatg 3660

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ttttttttg tcctctgtct tccatttgtt gttgttgttg tttgtttgtt tgtttgttgg 3780

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ctctgtaacc cagggtgacc ttgaagtcat gggtagcctg ctgttttagc cttcccacat 3900

ctaagattac aggtatgagc tatcattttt ggtatattga ttgattgatt gattgatgtg 3960

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atgataataa tggtttctta gacgtcaggt ggcacttttc ggggaaatgt gcgcggaacc 4740

cctatttgtt tatttttcta aatacattca aatatgtatc cgctcatgag acaataaccc 4800

tgataaatgc ttcaataata ttgaaaaagg aagagtatga gtattcaaca tttccgtgtc 4860

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ctcggtcgcc gcatacacta ttctcagaat gacttggttg agtactcacc agtcacagaa 5160

aagcatctta cggatggcat gacagtaaga gaattatgca gtgctgccat aaccatgagt 5220

gataacactg cggccaactt acttctgaca acgatcggag gaccgaagga gctaaccgct 5280

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ccagatggta agccctcccg tatcgtagtt atctacacga cggggagtca ggcaactatg 5640

gatgaacgaa atagacagat cgctgagata ggtgcctcac tgattaagca ttggtaactg 5700

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gcaccgccta catacctcgc tctgctaatc ctgttaccag tggctgctgc cagtggcgat 6120

aagtcgtgtc ttaccgggtt ggactcaaga cgatagttac cggataaggc gcagcggtcg 6180

ggctgaacgg ggggttcgtg cacacagccc agcttggagc gaacgaccta caccgaactg 6240

agatacctac agcgtgagct atgagaaagc gccacgcttc ccgaagggag aaaggcggac 6300

aggtatccgg taagcggcag ggtcggaaca ggagagcgca cgagggagct tccagggga 6360

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accgagcgca gcgagtcagt gagcgaggaa gcggaagagc gcccaatacg caaaccgcct 6660

ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc cgactggaaa 6720

gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc accccaggct 6780

ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata acaatttcac 6840

acaggaaaca gctatgacca tgattacgcc 6870

<210> 3
<211> 1391
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<220>
<221> CDS
<222> (1)..(1296)

<220>
<221> CDS
<222> (1300)..(1302)

<220>
<221> CDS
<222> (1306)..(1389)

<400> 3

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tct aga gcc gcc atg gtt aaa gtt atc ggt cgt cgt tcc ctc gga gtg   48
Ser Arg Ala Ala Met Val Lys Val Ile Gly Arg Arg Ser Leu Gly Val
  1               5                  10                  15

caa aga ata ttt gat att ggt ctt ccc caa gac cat aat ttt ctg cta   96
Gln Arg Ile Phe Asp Ile Gly Leu Pro Gln Asp His Asn Phe Leu Leu
            20                  25                  30

gcc aat ggg gcg atc gcc cac aat tgt ttt aac gga tcc cat cat cat   144
Ala Asn Gly Ala Ile Ala His Asn Cys Phe Asn Gly Ser His His His
        35                  40                  45

cat cat cat ggt atg gct agc atg act ggt gga cag caa atg ggt cgg   192
His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg
    50                  55                  60

gat ctg tac gac gat gac gat aag gta cct agg atc gaa caa aaa ctc   240
Asp Leu Tyr Asp Asp Asp Asp Lys Val Pro Arg Ile Glu Gln Lys Leu
65                  70                  75                  80

atc tca gaa gag gat ctg ggc atg gtg agc aag ggc gag gag ctg ttc   288
Ile Ser Glu Glu Asp Leu Gly Met Val Ser Lys Gly Glu Glu Leu Phe
                85                  90                  95

acc ggg gtg gtg ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc   336
Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly
            100                 105                 110

cac aag ttc agc gtg tct ggc gag ggc gag ggc gat gcc acc tac ggc   384
His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly
        115                 120                 125

aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc   432
Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro
    130                 135                 140

tgg ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc   480
Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser
145                 150                 155                 160

cgc tac ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg   528
Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met
                165                 170                 175

ccc gaa ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc   576
Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly
            180                 185                 190

aac tac aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg   624
Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val
        195                 200                 205

aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc   672
Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile
    210                 215                 220
```

```
ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc   720
Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile
225                 230                 235                 240

atg gcc gac aag cag aag aac ggc atc aag gcg aac ttc aag atc cgg   768
Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg
                245                 250                 255

cac aac atc gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag   816
His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln
            260                 265                 270

aac acc ccc atc ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac   864
Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr
        275                 280                 285

ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat   912
Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp
    290                 295                 300

cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc   960
His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly
305                 310                 315                 320

atg gac gag ctg tac gga tcc ccc ggg ctg cag gaa ttc gat atc aag  1008
Met Asp Glu Leu Tyr Gly Ser Pro Gly Leu Gln Glu Phe Asp Ile Lys
                325                 330                 335

ctt gat tgc ctc agt ttt ggc acc gaa att tta acc gtt gag tac ggc  1056
Leu Asp Cys Leu Ser Phe Gly Thr Glu Ile Leu Thr Val Glu Tyr Gly
            340                 345                 350

cca ttg ccc att ggc aaa att gtg agt gaa gaa att aat tgt tct gtg  1104
Pro Leu Pro Ile Gly Lys Ile Val Ser Glu Glu Ile Asn Cys Ser Val
        355                 360                 365

tac agt gtt gat cca gaa ggg aga gtt tac acc cag gcg atc gcc caa  1152
Tyr Ser Val Asp Pro Glu Gly Arg Val Tyr Thr Gln Ala Ile Ala Gln
    370                 375                 380

tgg cat gac cgg gga gag cag gaa gta ttg gaa tat gaa ttg gaa gat  1200
Trp His Asp Arg Gly Glu Gln Glu Val Leu Glu Tyr Glu Leu Glu Asp
385                 390                 395                 400

ggt tca gta atc cga gct acc tct gac cac cga ttt tta acc acg att  1248
Gly Ser Val Ile Arg Ala Thr Ser Asp His Arg Phe Leu Thr Thr Ile
                405                 410                 415

atc aac tgt tgg cga tcg aag aaa ttt ttg cta ggc aac tgg act tgt  1296
Ile Asn Cys Trp Arg Ser Lys Lys Phe Leu Leu Gly Asn Trp Thr Cys
            420                 425                 430

tga ctt tag aaa ata tta agc aaa ctg aag aag ctc ttg aca acc atc  1344
    Leu     Lys Ile Leu Ser Lys Leu Lys Lys Leu Leu Thr Thr Ile
                435                 440                 445

gtc ttc cct ttc cat tac ttg acg ctg gga caa tta aat aag tcg ac   1391
Val Phe Pro Phe His Tyr Leu Thr Leu Gly Gln Leu Asn Lys Ser
            450                 455                 460
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<210> 4
<211> 432
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 4
Ser Arg Ala Ala Met Val Lys Val Ile Gly Arg Arg Ser Leu Gly Val
1 5 10 15
Gln Arg Ile Phe Asp Ile Gly Leu Pro Gln Asp His Asn Phe Leu Leu
20 25 30
Ala Asn Gly Ala Ile Ala His Asn Cys Phe Asn Gly Ser His His His
35 40 45
His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg
50 55 60
Asp Leu Tyr Asp Asp Asp Asp Lys Val Pro Arg Ile Glu Gln Lys Leu
65 70 75 80
Ile Ser Glu Glu Asp Leu Gly Met Val Ser Lys Gly Glu Glu Leu Phe
85 90 95
Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly
100 105 110
His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly
115 120 125
Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro
130 135 140
Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser
145 150 155 160
Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met
165 170 175
Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly
180 185 190
Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val
195 200 205
Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile
210 215 220
Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile
225 230 235 240
Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg
245 250 255
His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln
260 265 270
Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr
275 280 285
Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp
290 295 300
His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly
305 310 315 320
Met Asp Glu Leu Tyr Gly Ser Pro Gly Leu Gln Glu Phe Asp Ile Lys
325 330 335
Leu Asp Cys Leu Ser Phe Gly Thr Glu Ile Leu Thr Val Glu Tyr Gly
340 345 350
Pro Leu Pro Ile Gly Lys Ile Val Ser Glu Glu Ile Asn Cys Ser Val
355 360 365
Tyr Ser Val Asp Pro Glu Gly Arg Val Tyr Thr Gln Ala Ile Ala Gln
370 375 380
Trp His Asp Arg Gly Glu Gln Glu Val Leu Glu Tyr Glu Leu Glu Asp
385 390 395 400

Gly Ser Val Ile Arg Ala Thr Ser Asp His Arg Phe Leu Thr Thr Ile
405 410 415
Ile Asn Cys Trp Arg Ser Lys Lys Phe Leu Leu Gly Asn Trp Thr Cys
420 425 430

<210> 5
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<400> 5
tgyytaagct t 11

<210> 6
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<220>
<221> misc_feature
<222> (7)..(9)
<223> n may be any nucleotide

<400> 6
aattsynnnt gyyta 15

<210> 7
<211> 108
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<220>
<221> misc_feature
<222> (8)..(97)
<223> n may be any nucleotide; the triplet nnn may be reiterated from 1-30 times

<400> 7
gaattctnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnntgc ttaagctt 108

<210> 8
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<221> UNSURE
<222> (3)..(32)
<223> Xaa may be any amino acid and may be reiterated from 1-30 times

<220>
<223> Description of Artificial Sequence: Synthetic construct

<400> 8
Asn Ser Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20 25 30

Cys Leu Ser
35

<210> 9
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer/probe

<400> 9
gccgtctaga gccgccatgg ttaaagttat cggtcgtcgt tccctc 46

<<210> 10
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 10
gctagtcgac ttatttaatt gtcccagcgt caagtaatgg aaaggg 46

<210> 11
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 11
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
1 5 10

<210> 12
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> misc_feature
<222> (1)...(36)
<223> n= any nucleotide

<400> 12
aatnnnnnnn nnnnnggncc nnnnnnnnnn nnnnnn 36

<210> 13
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> misc_feature
<222> (1)...(36)
<223> n = any nucleotide

<400> 13
agctnnnnnn nnnnnnnnnn ggnccnnnnn nnnnnn 36

<210> 14
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> VARIANT
<222> (1)...(10)
<223> Xaa = any amino acid

<400> 14
Xaa Xaa Xaa Xaa Gly Pro Xaa Xaa Xaa Xaa
1 5 10

<210> 15
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 15
aatttgttcc tgtacgggcc ctggaaagac ttgtta 36

<210> 16
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 16
agcttaacaa gtctttccag ggcccgtaca ggaaca 36

<210> 17
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 17
Leu Phe Leu Tyr Gly Pro Trp Lys Asp Leu
1 5 10

<210> 18
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 18
aatttgttcc tgtacgccgc ctggaaagac ttgtta 36

<210> 19
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 19
aaagcttaac aagtctttcc aggcggcgta caggaaca 38

<210> 20
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> VARIANT
<222> 5
<223> Xaa may be Ala or Gly

<221> VARIANT
<222> 6
<223> Xaa may be Ala or Pro

<400> 20
Leu Phe Leu Tyr Xaa Xaa Trp Lys Asp Leu Leu
1 5 10

<210> 21
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 21
aattcgttcc tgtacgccgc ctggaaagac tgctta 36

<210> 22
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 22
agcttaagca gtctttccag gcggcgtaca ggaacg 36

<210> 23
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> VARIANT
<222> 5
<223> Xaa may be Ala or Gly

<221> VARIANT
<222> 6
<223> Xaa may be Ala or Pro

<400> 23
Ser Phe Leu Tyr Xaa Xaa Trp Lys Asp Cys Leu
1 5 10

<210> 24
<211> 1251
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<220>
<221> CDS
<222> (1)..(1248)

<400> 24

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atg gtt aaa gtt atc ggt cgt cgt tcc ctc gga gtg caa aga ata ttt   48
Met Val Lys Val Ile Gly Arg Arg Ser Leu Gly Val Gln Arg Ile Phe
  1               5                  10                  15

gat att ggt ctt ccc caa gac cat aat ttt ctg cta gcc aat ggg gcg   96
Asp Ile Gly Leu Pro Gln Asp His Asn Phe Leu Leu Ala Asn Gly Ala
             20                  25                  30

atc gcc cac aat tct gaa gag gac ttg gga ggc ggt gcc atg gtg agc  144
Ile Ala His Asn Ser Glu Glu Asp Leu Gly Gly Gly Ala Met Val Ser
         35                  40                  45

aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg  192
Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu
     50                  55                  60

gac ggc gac gta aac ggc cac aag ttc agc gtg tct ggc gag ggc gag  240
Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu
 65                  70                  75                  80

ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc acc acc  288
Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr
                 85                  90                  95

ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg acc tac  336
Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr
            100                 105                 110

ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag cag cac gac  384
Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp
        115                 120                 125

ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc  432
Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile
    130                 135                 140

ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc  480
Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe
145                 150                 155                 160

gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc  528
Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe
                165                 170                 175

aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac  576
Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn
            180                 185                 190

agc cac aac gtc tat atc atg gcc gac aag cag aag aac ggc atc aag  624
Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys
        195                 200                 205
```

```
gcg aac ttc aag atc cgg cac aac atc gag gac ggc agc gtg cag ctc   672
Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu
    210                 215                 220

gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg   720
Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu
225                 230                 235                 240

ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac   768
Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
                245                 250                 255

ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc   816
Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala
            260                 265                 270

gcc ggg atc act ctc ggc atg gac gag ctg tac aag gga ggt ggc ggt   864
Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Gly Gly Gly Gly
        275                 280                 285

gaa cag aag ctc atc tgc tta agc ttt ggc acc gaa att tta acc gtt   912
Glu Gln Lys Leu Ile Cys Leu Ser Phe Gly Thr Glu Ile Leu Thr Val
    290                 295                 300

gag tac ggc cca ttg ccc att ggc aaa att gtg agt gaa gaa att aat   960
Glu Tyr Gly Pro Leu Pro Ile Gly Lys Ile Val Ser Glu Glu Ile Asn
305                 310                 315                 320

tgt tct gtg tac agt gtt gat cca gaa ggg aga gtt tac acc cag gcg  1008
Cys Ser Val Tyr Ser Val Asp Pro Glu Gly Arg Val Tyr Thr Gln Ala
                325                 330                 335

atc gcc caa tgg cat gac cgg gga gag cag gaa gta ttg gaa tat gaa  1056
Ile Ala Gln Trp His Asp Arg Gly Glu Gln Glu Val Leu Glu Tyr Glu
            340                 345                 350

ttg gaa gat ggt tca gta atc cga gct acc tct gac cac cgc ttt tta  1104
Leu Glu Asp Gly Ser Val Ile Arg Ala Thr Ser Asp His Arg Phe Leu
        355                 360                 365

acc acc gat tat caa ctg ttg gcg atc gaa gaa att ttt gct agg caa  1152
Thr Thr Asp Tyr Gln Leu Leu Ala Ile Glu Glu Ile Phe Ala Arg Gln
    370                 375                 380

ctg gac ttg ttg act tta gaa aat att aag caa act gaa gaa gct ctt  1200
Leu Asp Leu Leu Thr Leu Glu Asn Ile Lys Gln Thr Glu Glu Ala Leu
385                 390                 395                 400

gac aac cat cgt ctt ccc ttt cca tta ctt gac gct ggg aca att aaa  1248
Asp Asn His Arg Leu Pro Phe Pro Leu Leu Asp Ala Gly Thr Ile Lys
                405                 410                 415

taa                                                             1251
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<210> 25
<211> 416
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

<400> 25
Met Val Lys Val Ile Gly Arg Arg Ser Leu Gly Val Gln Arg Ile Phe
1 5 10 15

Asp Ile Gly Leu Pro Gln Asp His Asn Phe Leu Leu Ala Asn Gly Ala
20 25 30

Ile Ala His Asn Ser Glu Glu Asp Leu Gly Gly Gly Ala Met Val Ser
35 40 45

Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu
50 55 60

Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu
65 70 75 80

Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr
85 90 95

Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr
100 105 110

Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp
115 120 125

Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile
130 135 140

Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe
145 150 155 160

Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe
165 170 175

Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn
180 185 190

Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys
195 200 205

Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu
210 215 220

Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu
225 230 235 240

Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
245 250 255

Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala
260 265 270

Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Gly Gly Gly Gly
275 280 285

Glu Gln Lys Leu Ile Cys Leu Ser Phe Gly Thr Glu Ile Leu Thr Val
290 295 300

Glu Tyr Gly Pro Leu Pro Ile Gly Lys Ile Val Ser Glu Glu Ile Asn
305 310 315 320

Cys Ser Val Tyr Ser Val Asp Pro Glu Gly Arg Val Tyr Thr Gln Ala
325 330 335

Ile Ala Gln Trp His Asp Arg Gly Glu Gln Glu Val Leu Glu Tyr Glu
340 345 350

Leu Glu Asp Gly Ser Val Ile Arg Ala Thr Ser Asp His Arg Phe Leu
355 360 365

Thr Thr Asp Tyr Gln Leu Leu Ala Ile Glu Glu Ile Phe Ala Arg Gln
370 375 380

Leu Asp Leu Leu Thr Leu Glu Asn Ile Lys Gln Thr Glu Glu Ala Leu
385 390 395 400

Asp Asn His Arg Leu Pro Phe Pro Leu Leu Asp Ala Gly Thr Ile Lys
405 410 415

<210> 26
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 26
Glu Cys Cys Asn Pro Ala Cys Gly Arg His Tyr Ser Cys
1 5 10

<210> 27
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(45)

<400> 27
tgc tgc aac ccg gcg tgc ggt cgt cat tat agc tgc ggt agc gaa 45
Cys Cys Asn Pro Ala Cys Gly Arg His Tyr Ser Cys Gly Ser Glu
1 5 10 15

<210> 28
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 28
Cys Cys Asn Pro Ala Cys Gly Arg His Tyr Ser Cys Gly Ser Glu
1 5 10 15

<210> 29
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(45)

<400> 29
tgc ggt cgt cat tat agc tgc ggt agc gaa tgc tgc aac ccg gcg 45
Cys Gly Arg His Tyr Ser Cys Gly Ser Glu Cys Cys Asn Pro Ala
1 5 10 15

<210> 30
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 30
Cys Gly Arg His Tyr Ser Cys Gly Ser Glu Cys Cys Asn Pro Ala
1 5 10 15

<210> 31
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(45)

<400> 31
tcg tgc ggt agc gaa tgc tgc aac ccg gcg tgc ggt cgt cat tat 45
Ser Cys Gly Ser Glu Cys Cys Asn Pro Ala Cys Gly Arg His Tyr
1 5 10 15

<210> 32
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 32
Ser Cys Gly Ser Glu Cys Cys Asn Pro Ala Cys Gly Arg His Tyr
1 5 10 15

<210> 33
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic construct

<400> 33
Ile Cys Cys Asn Pro Ala Cys Gly Pro Leu Tyr Ser Cys
1 5 10

<210> 34
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> misc_feature
<222> (4)...(9)
<223> N equals 3 or 6 nucleotides

<221> misc_feature
<222> (46)...(57)
<223> N equals 0,3,6,9 or 12 nucleotides

<221> misc_feature
<222> (1)...(60)
<223> N = any nucleotide

<221> CDS
<222> (1)...(57)

<400> 34
atg nnn nnn tgt tgt nnn ccc gcc tgt ggg nnn nnn nnn nnn tgt nnn 48
Met Xaa Xaa Cys Cys Xaa Pro Ala Cys Gly Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15

nnn nnn nnn tga 60
Xaa Xaa Xaa

<210> 35
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(19)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 35
Met Xaa Xaa Cys Cys Xaa Pro Ala Cys Gly Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15
Xaa Xaa Xaa

<210> 36
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(69)

<221> misc_feature
<222> (1)...(66)
<223> N = any nucleotide

<400> 36
atg nnn nnn tgt tgt nnn nnn ccc ccc tgt nnn nnn nnn nnn tgt nnn 48
Met Xaa Xaa Cys Cys Xaa Xaa Pro Pro Cys Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15

ccc nnn nnn tgt tgt nnn tga 69
Pro Xaa Xaa Cys Cys Xaa *
20

<210> 37
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(22)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 37
Met Xaa Xaa Cys Cys Xaa Xaa Pro Pro Cys Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15

Pro Xaa Xaa Cys Cys Xaa
20

<210> 38
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(102)

<221> misc_feature
<222> (1)...(105)
<223> N = any nucleotide

<221> misc_feature
<222> (27)...(44)
<223> N equals 15 or 18 nucleotides

<221> misc_feature
<222> (51)...(59)
<223> N equals 6 or 9 nucleotides

<221> misc_feature
<222> (63)...(80)
<223> N equals 12, 15 or 18 nucleotides

<221> misc_feature
<222> (84)...(102)
<223> N equals 0, 3, 6, 9, 12, 15 or 18 nucleotides

<400> 38
atg tgt nnn nnn nnn nnn nnn nnn ctt nnn nnn nnn nnn nnn nnn tgt 48
Met Cys Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Cys
1 5 10 15

tgt nnn nnn nnn tgt nnn nnn nnn nnn nnn nnn tgt nnn nnn nnn nnn 96
Cys Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
20 25 30

nnn nnn tga 105
Xaa Xaa

<210> 39
<211> 34
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(34)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 39
Met Cys Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Cys
1 5 10 15
Cys Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
20 25 30
Xaa Xaa

<210> 40
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(57)

<221> misc_feature
<222> (1)...(60)
<223> N = any nucleotide

<221> misc_feature
<222> (4)...(9)
<223> N equals 3 or 6 nucleotides

<221> misc_feature
<222> (46)...(7)
<223> N equals 0,3,6,9 or 12 nucleotides

<400> 40
atg nnn nnn gan ara nnn ccc gcc aaa ggg nnn nnn nnn nnn rrn nnn 48
Met Xaa Xaa Xaa Xaa Xaa Pro Ala Lys Gly Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

nnn nnn nnn tga 60
Xaa Xaa Xaa

<210> 41
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> 4
<223> Xaa may be Glu or Asp

<221> VARIANT
<222> 5
<223> Xaa may be Arg or Lys

<221> VARIANT
<222> 9
<223> Xaa may be Arg or Lys

<221> VARIANT
<222> 15
<223> Xaa may be Glu or Asp

<221> VARIANT
<222> 2,3,6,11-14,16-19
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 41
Met Xaa Xaa Xaa Xaa Xaa Pro Ala Lys Gly Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15
Xaa Xaa Xaa

<210> 42
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(57)

<221> misc_feature
<222> (1)...(57)
<223> N = any nucleotide

<221> misc_feature
<222> (4)...(9)
<223> N equals 3 or 6 nucleotides

<221> misc_feature
<222> (46)...(57)
<223> N equals 0,3,6,9 or 12 nucleotides

<400> 42
atg nnn nnn tgg tgt nnn ccc gcc tgg ggg nnn nnn nnn nnn tgt nnn 48
Met Xaa Xaa Trp Cys Xaa Pro Ala Trp Gly Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15

nnn nnn nnn tga 60
Xaa Xaa Xaa

<210> 43
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(19)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 43
Met Xaa Xaa Trp Cys Xaa Pro Ala Trp Gly Xaa Xaa Xaa Xaa Cys Xaa
1 5 10 15
Xaa Xaa Xaa

<210> 44
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(57)

<221> misc_feature
<222> (1)...(60)

<221> misc_feature
<222> (4)...(9)
<223> N equals 3 or 6 nucleotides

<221> misc_feature
<222> (46)...(57)
<223> N equals 0,3,6,9 or 12 nucleotides

<400> 44
atg nnn nnn tgt tgg nnn ccc gcc tgt ggg nnn nnn nnn nnn tgg nnn 48
Met Xaa Xaa Cys Trp Xaa Pro Ala Cys Gly Xaa Xaa Xaa Xaa Trp Xaa
1 5 10 15

nnn nnn nnn tga 60
Xaa Xaa Xaa

<210> 45
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(19)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 45
Met Xaa Xaa Cys Trp Xaa Pro Ala Cys Gly Xaa Xaa Xaa Xaa Trp Xaa
1 5 10 15
Xaa Xaa Xaa

<210> 46
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic construct

<221> CDS
<222> (1)...(57)

<221> misc_feature
<222> (1)...(60)
<223> N = any nucleotide

<221> misc_feature
<222> (4)...(9)
<223> N equals 3 or 6 nucleotides

<221> misc_feature
<222> (46)...(57)
<223> N equals 0,3,6,9 or 12 nucleotides

<400> 46
atg nnn nnn tgg tgg nnn ccc gcc tgg ggg nnn nnn nnn nnn tgg nnn 48
Met Xaa Xaa Trp Trp Xaa Pro Ala Trp Gly Xaa Xaa Xaa Xaa Trp Xaa
1 5 10 15

nnn nnn nnn tga 60
Xaa Xaa Xaa

<210> 47
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(19)
<223> Xaa may be any amino acid

<223> Synthetic construct

<400> 47
Met Xaa Xaa Trp Trp Xaa Pro Ala Trp Gly Xaa Xaa Xaa Xaa Trp Xaa
1 5 10 15
Xaa Xaa Xaa

<210> 48
<211> 795
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(792)

<400> 48

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gga tcc ttc ccg gcc acg gtg cac ctg gag cag ggc ggc acc aag ctg   48
Gly Ser Phe Pro Ala Thr Val His Leu Glu Gln Gly Gly Thr Lys Leu
  1               5                  10                  15

gtg aag gac ctg agc ccc ggg gac cgc gtg ctg gcg gcg gac gac cag   96
Val Lys Asp Leu Ser Pro Gly Asp Arg Val Leu Ala Ala Asp Asp Gln
             20                  25                  30

ggc cgg ctg ctc tac agc gac ttc ctc act ttc ctg gac cgc gac gac  144
Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr Phe Leu Asp Arg Asp Asp
         35                  40                  45

ggc gcc aag aag gtc ttc tac gtg atc gag acg cgg gag ccg cgc gag  192
Gly Ala Lys Lys Val Phe Tyr Val Ile Glu Thr Arg Glu Pro Arg Glu
     50                  55                  60

cgc ctg ctg ctc acc gcc gcg cac ctg ctc ttt gtg gcg ccg cac aac  240
Arg Leu Leu Leu Thr Ala Ala His Leu Leu Phe Val Ala Pro His Asn
 65                  70                  75                  80

gac tcg gcc acc ggg gag ccc gag gcg tcc tcg ggc tcg ggg ccg cct  288
Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser Ser Gly Ser Gly Pro Pro
                 85                  90                  95

tcc ggg ggc gca ctg ggg cct cgg gcg ctg ttc gcc agc cgc gtg cgc  336
Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu Phe Ala Ser Arg Val Arg
            100                 105                 110

ccg ggc cag cgc gtg tac gtg gtg gcc gag cgt gac ggg gac cgc cgg  384
Pro Gly Gln Arg Val Tyr Val Val Ala Glu Arg Asp Gly Asp Arg Arg
        115                 120                 125

ctc ctg ccc gcc gct gtg cac agc gtg acc cta agc gag gag gcc gcg  432
Leu Leu Pro Ala Ala Val His Ser Val Thr Leu Ser Glu Glu Ala Ala
    130                 135                 140

ggc gcc tac gcg ccg ctc acg gcc cag ggc acc att ctc atc aac cgg  480
Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg
145                 150                 155                 160

gtg ctg gcc tcg tgc tac gcg gtc atc gag gag cac agc tgg gcg cac  528
Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Glu His Ser Trp Ala His
                165                 170                 175

cgg gcc ttc gcg ccc ttc cgc ctg gcg cac gcg ctc ctg gct gca ctg  576
Arg Ala Phe Ala Pro Phe Arg Leu Ala His Ala Leu Leu Ala Ala Leu
            180                 185                 190
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gcg ccc gcg cgc acg gac cgc ggc ggg gac agc ggc ggc ggg gac cgc   624
Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp Ser Gly Gly Gly Asp Arg
        195                 200                 205

ggg ggc ggc ggc ggc aga gta gcc cta acc gct cca ggt gct gcc gac   672
Gly Gly Gly Gly Gly Arg Val Ala Leu Thr Ala Pro Gly Ala Ala Asp
    210                 215                 220

gct ccg ggt gcg ggg gcc acc gcg ggc atc cac tgg tac tcg cag ctg   720
Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile His Trp Tyr Ser Gln Leu
225                 230                 235                 240

ctc tac caa ata ggc acc tgg ctc ctg gac agc gag gcc ctg cac ccg   768
Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp Ser Glu Ala Leu His Pro
                245                 250                 255

ctg ggc atg gcg gtc aag tcc agc tga                              795
Leu Gly Met Ala Val Lys Ser Ser
            260
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<210> 49
<211> 264
<212> PRT
<213> Homo sapiens

<400> 49

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Gly Ser Phe Pro Ala Thr Val His Leu Glu Gln Gly Gly Thr Lys Leu
  1               5                  10                  15

Val Lys Asp Leu Ser Pro Gly Asp Arg Val Leu Ala Ala Asp Asp Gln
            20                  25                  30

Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr Phe Leu Asp Arg Asp Asp
        35                  40                  45

Gly Ala Lys Lys Val Phe Tyr Val Ile Glu Thr Arg Glu Pro Arg Glu
    50                  55                  60

Arg Leu Leu Leu Thr Ala Ala His Leu Leu Phe Val Ala Pro His Asn
65                  70                  75                  80

Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser Ser Gly Ser Gly Pro Pro
                85                  90                  95

Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu Phe Ala Ser Arg Val Arg
            100                 105                 110

Pro Gly Gln Arg Val Tyr Val Val Ala Glu Arg Asp Gly Asp Arg Arg
        115                 120                 125

Leu Leu Pro Ala Ala Val His Ser Val Thr Leu Ser Glu Glu Ala Ala
    130                 135                 140

Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg
145                 150                 155                 160

Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Glu His Ser Trp Ala His
                165                 170                 175
```

Arg Ala Phe Ala Pro Phe Arg Leu Ala His Ala Leu Leu Ala Ala Leu
180 185 190

Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp Ser Gly Gly Gly Asp Arg
195 200 205

Gly Gly Gly Gly Gly Arg Val Ala Leu Thr Ala Pro Gly Ala Ala Asp
210 215 220

Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile His Trp Tyr Ser Gln Leu
225 230 235 240

Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp Ser Glu Ala Leu His Pro
245 250 255

Leu Gly Met Ala Val Lys Ser Ser
260

<210> 50
<211> 242
<212> DNA
<213> Gallus gallus

<400> 50
agggacagcc ccccccaaa gcccccaggg atgtaattac gtccctcccc cgctagggca 60

gcagcgagcc gcccggggct ccggtccggt ccggcgctcc cccgcatccc cgagccggca 120

gcgtgcgggg acagcccggg cacggggaag gtggcacggg atcgctttcc tctgaacgct 180

tctcgctgct ctttgagcct gcagacacct ggggggatac ggggaaaaaa gctttaggct 240

ga 242

<210> 51
<211> 1869
<212> DNA
<213> Mus musculus

<400> 51
atgggctgca aaaacctgct cggtctgggc cagcagatgc tgcgccggaa ggtggtggac 60

tgcagccggg aggagagccg gctgtcccgc tgcctcaaca cctatgacct ggtagctctt 120

ggggtgggca gcaccttggg cgctggtgtc tatgtcctag ccggtgccgt ggcccgtgaa 180

aatgctggcc ctgccatcgt catctccttc ttgattgctg ctctcgcctc cgtgctggcc 240

ggcctgtgct acggcgagtt tggtgcccgt gtccccaaga cgggctcagc ctacctctac 300

agctacgtga cggtggggga gctttgggcc ttcatcactg gctggaacct gattctctcc 360

tacatcatcg gtacttcaag cgtggcaaga gcctggagtg cgacttttga cgagctgata 420

ggcaagccca tcggagagtt ctcacgtcag cacatggccc tgaatgctcc tggggtgctg 480

gcccaaaccc cggacatatt tgctgtgatt ataattatca tcttaacagg actgttaact 540

cttggcgtga aggagtcagc catggtcaac aaaattttca cctgtatcaa tgtcctggtc 600

ttgtgcttca tcgtggtgtc cgggttcgtg aaaggctcca ttaaaaactg gcagctcacg 660

gagaaaaatt tctcctgtaa caacaacgac acaaacgtga aatacggtga gggagggttt 720

atgcccttg gattctctgg tgtcctgtca ggggcagcga cctgctttta tgccttcgtg 780

ggctttgact gcatcgccac cacaggggaa gaagtcaaga acccccagaa ggccattcct 840

gtgggcatcg tggcgtccct cctcatttgc ttcatagcgt actttggcgt gtccgccgct 900

ctcacgctca tgatgcctta cttctgcctg gacatcgaca gcccgctgcc tggtgccttc 960

aagcaccagg gctgggaaga agctaagtac gcagtggcca ttggctctct ctgcgcactt 1020

tccaccagtc tcctaggctc catgtttccc atgccccgag ttatctatgc catggctgaa 1080

gatggactac tgtttaaatt tttggccaaa atcaacaata ggaccaaaac acccgtaatc 1140

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ctggtggacc tcatgtccat tggcactctc ctggcttact ctttggtggc tgcctgtgtt 1260

ttggtcttac ggtaccagcc agaacaacct aatctggtat accagatggc cagaaccacc 1320

gaggagctag atcgagtaga tcagaatgag ctggtcagtg ccagtgaatc acagacaggc 1380

tttttaccgg tagccgagaa gttttctctg aaatccatcc tctcacccaa gaacgtggag 1440

ccctccaaat tctcagggct aattgtgaac atttcagccg gcctcctagc cgctcttatc 1500

atcaccgtgt gcattgtggc cgtgcttgga agagaggccc tggccgaagg gacactgtgg 1560

gcagtctttg taatgacagg gtcagtcctc ctctgcatgc tggtgacagg catcatctgg 1620

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cggtttgcag tgtggatgct gataggtttc accatctatt tcggttatgg gatctggcac 1800

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tgcaaatga 1869

<210> 52
<211> 622
<212> PRT
<213> Mus musculus

<400> 52
Met Gly Cys Lys Asn Leu Leu Gly Leu Gly Gln Gln Met Leu Arg Arg
1 5 10 15

Lys Val Val Asp Cys Ser Arg Glu Glu Ser Arg Leu Ser Arg Cys Leu
20 25 30

Asn Thr Tyr Asp Leu Val Ala Leu Gly Val Gly Ser Thr Leu Gly Ala
35 40 45

Gly Val Tyr Val Leu Ala Gly Ala Val Ala Arg Glu Asn Ala Gly Pro
50 55 60

Ala Ile Val Ile Ser Phe Leu Ile Ala Ala Leu Ala Ser Val Leu Ala
65 70 75 80

Gly Leu Cys Tyr Gly Glu Phe Gly Ala Arg Val Pro Lys Thr Gly Ser
85 90 95

Ala Tyr Leu Tyr Ser Tyr Val Thr Val Gly Glu Leu Trp Ala Phe Ile
100 105 110

Thr Gly Trp Asn Leu Ile Leu Ser Tyr Ile Ile Gly Thr Ser Ser Val
115 120 125

Ala Arg Ala Trp Ser Ala Thr Phe Asp Glu Leu Ile Gly Lys Pro Ile
130 135 140

Gly Glu Phe Ser Arg Gln His Met Ala Leu Asn Ala Pro Gly Val Leu
145 150 155 160

Ala Gln Thr Pro Asp Ile Phe Ala Val Ile Ile Ile Ile Ile Leu Thr
165 170 175

Gly Leu Leu Thr Leu Gly Val Lys Glu Ser Ala Met Val Asn Lys Ile
180 185 190

Phe Thr Cys Ile Asn Val Leu Val Leu Cys Phe Ile Val Val Ser Gly
195 200 205

Phe Val Lys Gly Ser Ile Lys Asn Trp Gln Leu Thr Glu Lys Asn Phe
210 215 220

Ser Cys Asn Asn Asn Asp Thr Asn Val Lys Tyr Gly Glu Gly Gly Phe
225 230 235 240

Met Pro Phe Gly Phe Ser Gly Val Leu Ser Gly Ala Ala Thr Cys Phe
245 250 255

Tyr Ala Phe Val Gly Phe Asp Cys Ile Ala Thr Thr Gly Glu Glu Val
260 265 270

Lys Asn Pro Gln Lys Ala Ile Pro Val Gly Ile Val Ala Ser Leu Leu
275 280 285

Ile Cys Phe Ile Ala Tyr Phe Gly Val Ser Ala Ala Leu Thr Leu Met
290 295 300

Met Pro Tyr Phe Cys Leu Asp Ile Asp Ser Pro Leu Pro Gly Ala Phe
305 310 315 320

Lys His Gln Gly Trp Glu Glu Ala Lys Tyr Ala Val Ala Ile Gly Ser
325 330 335

Leu Cys Ala Leu Ser Thr Ser Leu Leu Gly Ser Met Phe Pro Met Pro
340 345 350

Arg Val Ile Tyr Ala Met Ala Glu Asp Gly Leu Leu Phe Lys Phe Leu
355 360 365

Ala Lys Ile Asn Asn Arg Thr Lys Thr Pro Val Ile Ala Thr Val Thr
370 375 380

Ser Gly Ala Ile Ala Ala Val Met Ala Phe Leu Phe Glu Leu Lys Asp
385 390 395 400

Leu Val Asp Leu Met Ser Ile Gly Thr Leu Leu Ala Tyr Ser Leu Val
405 410 415

Ala Ala Cys Val Leu Val Leu Arg Tyr Gln Pro Glu Gln Pro Asn Leu
420 425 430

Val Tyr Gln Met Ala Arg Thr Thr Glu Glu Leu Asp Arg Val Asp Gln
435 440 445

Asn Glu Leu Val Ser Ala Ser Glu Ser Gln Thr Gly Phe Leu Pro Val
450 455 460

Ala Glu Lys Phe Ser Leu Lys Ser Ile Leu Ser Pro Lys Asn Val Glu
465 470 475 480

Pro Ser Lys Phe Ser Gly Leu Ile Val Asn Ile Ser Ala Gly Leu Leu
485 490 495

Ala Ala Leu Ile Ile Thr Val Cys Ile Val Ala Val Leu Gly Arg Glu
500 505 510

Ala Leu Ala Glu Gly Thr Leu Trp Ala Val Phe Val Met Thr Gly Ser
515 520 525

Val Leu Leu Cys Met Leu Val Thr Gly Ile Ile Trp Arg Gln Pro Glu
530 535 540

Ser Lys Thr Lys Leu Ser Phe Lys Val Pro Phe Val Pro Val Leu Pro
545 550 555 560

Val Leu Ser Ile Phe Val Asn Ile Tyr Leu Met Met Gln Leu Asp Gln
565 570 575

Gly Thr Trp Val Arg Phe Ala Val Trp Met Leu Ile Gly Phe Thr Ile
580 585 590

Tyr Phe Gly Tyr Gly Ile Trp His Ser Glu Glu Ala Ser Leu Ala Ala
595 600 605

Gly Gln Ala Lys Thr Pro Asp Ser Asn Leu Asp Gln Cys Lys
610 615 620

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