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(54) Title: RECOMBINANT *VIBRIO CHOLERAE* EXOTOXINS

(57) Abstract: The invention features recombinant exotoxins from *Vibrio cholerae* are for the therapeutic treatment of a variety of human diseases, particularly diseases characterized by an abundance or excess of undesired cells.

RECOMBINANT VIBRIO CHOLERAE EXOTOXINS

THE FIELD OF THE INVENTION

The present invention relates to therapies based on *Vibrio cholerae* exotoxin (VCE), and methods and compositions to utilize the exotoxin and its derivatives as selective cytotoxic or cytostatic agents for selected target cells.

BACKGROUND OF THE INVENTION

Selective killing of particular types of cells is desirable in a variety of clinical settings, including the treatment of cancer, which is usually manifested through growth and accumulation of malignant cells. An established treatment for cancer is chemotherapy, which kills tumor cells by inhibiting DNA synthesis or damaging DNA (Chabner and Roberts, Nat. Rev. Cancer 5:65 (2005)). However, such treatments often cause severe systemic toxicity due to nondiscriminatory killing of normal cells. Because many cancer chemotherapeutics exert their efficacy through selective destruction of proliferating cells, increased toxicities to normal tissues with high proliferation rates, such as bone marrow, gastrointestinal tract, and hair follicles, have usually prevented their use in optimal doses. Such treatments often fail, resulting in drug resistance, disease relapse, and/or metastasis. To reduce systemic toxicity, different strategies have been explored to selectively target a particular cell population. Antibodies and other ligands that recognize tumor-associated antigens have been coupled with small molecule drugs or protein toxins, generating conjugates and fusion proteins that are often referred to as immunoconjugates and immunotoxins, respectively (Allen, Nat. Rev. Cancer 2:750 (2002)).

In addition to dose-limiting toxicities, another limitation for chemotherapy is its ineffectiveness for treatment of cancers that do not involve accelerated proliferation, but rather prolonged survival of malignant cells due to defective apoptosis (Kitada et al., Oncogene 21:3459 (2002)). For example, B cell chronic lymphocytic leukemia (B-CLL) is a disease characterized by slowly accumulating apoptosis-resistant neoplastic B cells, for which currently there is no cure (Munk and Reed, Leuk. Lymphoma 45:2365 (2004)).

Cancer stem cells (CSCs) are a small fraction of tumor cells that have a capacity for self-renewal and unlimited growth, and therefore are distinct from their

progeny in their capacity to initiate cancers (Schulenburg et al., Cancer 107:2512 (2006)). Current cancer therapies do not target these cancer stem cells specifically, and it is hypothesized that the persistence of CSCs results in an ineradicable subset of cells that can give rise to progeny cells exhibiting drug resistance and/or contributing to the formation of metastases. In those tumors which harbor CSCs it is highly desirable to be able to eliminate these cells. CSCs have been thought to possess many properties similar to that of normal stems cells, e.g., long life span, relative mitotic quiescence, and active DNA repair capacity, as well as resistance to apoptosis and to drug/toxins through high level expression of ATP-binding cassette drug transporters such as P-glycoprotein. Consequently, CSCs are thought to be difficult to target and destroy by conventional cancer therapies (Dean et al., Nat. Rev. Cancer 5:275 (2005)). Conversely, it is critically important to distinguish CSCs from normal stem cells because of the essential roles that normal stem cells play in the renewal of normal tissues.

To increase the selectivity of highly toxic anti-tumor agents, various attempts have been made to take advantage of specific features of the tumor microenvironment, such as the low pH, low oxygen tension, or increased density of tumor specific enzymes, that are not found in the vicinity of normal cells in well-perfused tissues. Environmentally sensitive anti-tumor agents have been developed that are hypothesized to exhibit increased toxicity in the solid tumor. For example "bioreductive prodrugs" are agents that can be activated to cytotoxic agents in the hypoxic environment of a solid tumor (Ahn and Brown, Front Biosci. 2007 May 1;12:3483-501.) Similarly Kohchi et al. describe the synthesis of chemotherapeutic prodrugs that can be activated by membrane dipeptidases found in tumors (Bioorg Med Chem Lett. 2007 Apr 15;17(8):2241-5.) The use of selective antibody conjugated enzymes to alter the tumor microenvironment has also been explored by many groups. In the strategy known as antibody-directed enzyme prodrug therapy (ADEPT), enzymes conjugated to tumor-specific antibodies are intended to be delivered to the patient, followed by a chemotherapeutic agent that is inactive until subject to the action of the conjugated enzyme (see for example Bagshawe, Expert Rev Anticancer Ther. 2006 Oct;6(10):1421-31 or Rooseboome et al. Pharmacol Rev. 2004 Mar;56(1):53-102) To date the clinical advantages of these strategies remain

undocumented and there remains a high interest in developing more selective and more potent agents that can show therapeutic utility.

SUMMARY OF THE INVENTION

The present invention features compositions and therapies based on a recombinant *Vibrio cholerae* Exotoxin (VCE). The invention includes mutant VCE fusion proteins that comprise the native ADP-ribosyltransferase activity, a modified cell binding domain that binds to one or more specific cell surface proteins, as well as a modified translocation domain cleavable by selected proteolytic activities.

In one aspect, the invention features a recombinant VCE including an amino acid sequence with greater than 70, 80%, 90%, 95%, 96%, 97%, 98%, 99%, sequence identity or has 100% sequence identity to SEQ ID NO:1.

In another aspect, the invention features a protein (e.g., a fusion protein) including a fragment of VCE, wherein the fragment includes an amino acid sequence with greater than 70, 80%, 90%, 95%, 96%, 97%, 98%, 99%, sequence identity or has 100% sequence identity to SEQ ID NO:2. This protein can contain ADP-ribosylating activity and/or cell-membrane translocation activity (e.g., by having a VCE cell-membrane translocation domain). In another aspect, this protein does not include the VCE cell binding domain.

In any of the forgoing aspects the VCE fragment or protein can be fused to a the non-native cell targeting moiety (e.g., an antibody, or functional fragment thereof, an artificially diversified polypeptide binder, or a ligand for a receptor). The non-native cell targeting moiety can target, for example, a cell surface target that is expressed on cancer cells, or can target a cell selected from the group consisting of a hematopoietic cell, lymphocyte, and a nociceptive neuron.

In any of the forgoing aspects, the native furin cleavage site of the VCE fragment or protein is replaced with a modifiable activation domain, wherein the modifiable activation domain includes a substrate for an exogenous enzyme (e.g., a substrate for granzyme B activity). The exogenous enzyme can be a protease, including an exogenous human protease or non-human (or non-mammalian) protease (e.g., a viral protease). The modifiable activation domain may include a post-translational modification of a protease cleavage site or a substrate for an enzyme capable of removing a post-translational modification.

In another aspect, the invention features a vector including a nucleic acid encoding any of the forgoing proteins or protein fusions. In another aspect, the invention features a host cell containing any of forgoing vectors.

In yet another aspect, the invention features an antibody (e.g., a monoclonal antibody) that specifically binds any of the forgoing proteins or protein fusions.

In another aspect, the invention features a method of destroying a target cell (e.g., a cancer cell, a hematopoietic cell, a lymphocyte, and a nociceptive neuron) by contacting the target cell with a protein including a fragment of VCE, where the fragment includes an amino acid sequence with greater than 70, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or has 100% sequence identity to SEQ ID NO:2 (or a protein encoded by SEQ ID NO:3). This protein can contain ADP-ribosylating activity and/or cell-membrane translocation activity (e.g., by having a VCE cell-membrane translocation domain). In another aspect, this protein does not include the VCE cell binding domain.

In any of the forgoing methods, the VCE fragment or protein can be fused to a the non-native cell targeting moiety (e.g., an antibody, or functional fragment thereof, an artificially diversified polypeptide binder, or a ligand for a receptor). The non-native cell targeting moiety can target, for example, a cell surface target that is expressed on cancer cells, or can target a cell selected from the group consisting of a hematopoietic cell, lymphocyte, and a nociceptive neuron.

Also, in any of the forgoing methods, the native furin cleavage site of the VCE fragment or protein is replaced with a modifiable activation domain, wherein the modifiable activation domain includes a substrate for an exogenous enzyme (e.g., a substrate for granzyme B activity). The exogenous enzyme can be a protease, including an exogenous human protease or non-human (or non-mammalian) protease (e.g., a viral protease). The modifiable activation domain may include a post-translational modification of a protease cleavage site or a substrate for an enzyme capable of removing a post-translational modification.

As used herein the specification, "a" or "an" may mean one or more; "another" may mean at least a second or more.

The term "polypeptide" or "peptide" as used herein refers to two or more amino acids linked by an amide bond between the carboxyl terminus of one amino acid and the amino terminus of another.

The term "amino acid" as used herein refers to a naturally occurring or unnatural alpha or beta amino acid, wherein such natural or unnatural amino acids may be optionally substituted by one to four substituents, such as halo, for example F, Br, Cl or I or CF_3, alkyl, alkoxy, aryl, aryloxy, aryl(aryl) or diaryl, arylalkyl, arylalkyloxy, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkylalkyl, cycloalkylalkyloxy, optionally substituted amino, hydroxy, hydroxyalkyl, acyl, alkanoyl, heteroaryl, heteroaryloxy, cycloheteroalkyl, arylheteroaryl, arylalkoxycarbonyl, heteroarylalkyl, heteroarylalkoxy, aryloxyalkyl, aryloxyaryl, alkylamido, alkanoylamino, arylcarbonylamino, nitro, cyano, thiol, haloalkyl, trihaloalkyl and/or alkylthio.

The term "modified" as used herein refers to a composition that has been operably changed from one or more predominant forms found naturally to an altered form by any of a variety of methods, including genetic alteration or chemical substitution or degradation and comprising addition, subtraction, or alteration of biological components or substituents such as amino acid or nucleic acid residues, as well as the addition, subtraction or modification of protein post-translational modifications such as, without limitation, glycan, lipid, phosphate, sulfate, methyl, acetyl, ADP-ribosyl, ubiquitinyl, sumoyl, neddoyl, hydroxyl, carboxyl, amino, or formyl. "Modified" also comprises alteration by chemical or enzymatic substitution or degradation to add, subtract, or alter chemical moieties to provide a form not found in the composition as it exists in its natural abundance comprising a proportion of greater than 10%, or greater than 1%, or greater than 0.1%. The term "modified" is not intended to refer to a composition that has been altered incidentally as a consequence of manufacturing, purification, storage, or expression in a novel host and for which such alteration does not operably change the character of the composition.

The term "Vibrio Cholerae exotoxin A" or "VCE" as used herein refers to a protein selected from the family of protoxins, the prototype of which is a diphthamide-specific toxin encoded by the toxA gene of Vibrio cholerae. The prototypical VCE possesses a conserved DT-like ADP-ribosylation domain, and adopts an overall domain structure very similar to that of Pseudomonas exotoxin A (PEA), with moderate amino acid sequence identity (~32%). Full length "Vibrio Cholerae exotoxin A" or "VCE" has 70%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% sequence identity with SEQ ID NO:1. At minimum, "Vibrio Cholerae exotoxin A" or

"VCE" has the ADP-ribosylating activity of full length VCE (SEQ ID NO:2, and the protein encoded by SEQ ID NO:3). Sequence alignment suggests, and crystal structure confirms, that like PEA, the VCE possesses an N-terminal cell-targeting moiety (residues Met1-Lys297 and residues Ala419-Asn457) followed by a translocation domain (Gly298 to Ala418) and a C-terminal ADP-ribosyltransferase (Arg458 to Lys666) comprising an ER retention signal ^{662}KDEL^{665} (SEQ ID NO:4). A putative furin cleavage site (^{321}RKPK↓DL^{326}) (SEQ ID NO:5) is located near the N-terminus of the putative translocation domain.

The term "modified VCE", "modified VCE", or "engineered VCE" are used interchangeably herein to describe a recombinant or synthetic VCE protein that is modified to confer amino acid sequence changes as compared with that of VCE, including adding, deleting, and replacing amino acid sequences within the original sequence. In particular, the terms may refer to VCE proteins with sequence changes at the furin cleavage site to provide a mutated sequence that is a recognition site for proteases other than furin, and/or VCE fusion proteins with their native cell-targeting moiety partially or completely removed, mutationally altered, or changed to comprise other cell-targeting moieties. The term may also refer to VCE with amino acid covalent modifications such as glycosylation and PEGylation.

The term "VCE fusion" as used herein refers to a fusion protein containing a VCE or modified VCE, fused directly or indirectly to a heterologous sequence, for example, and a polypeptide that can bind to a targeted cell surface. The VCE or modified VCE is preferably located at the C-terminus of the fusion protein and the cell-targeting polypeptide attached to the N-terminus of the VCE or modified VCE. When discussed in the context of fusion toxins, "modified VCE" may simply be referred to as "VCE." The term "cell targeting moiety" as used herein refers to one or more protein domains that can bind to one or more cell surface targets, and thus can direct VCE or modified VCE to those cells. Such cell targeting moieties include, among others, antibodies or antibody-like molecules such as monoclonal antibodies, polyclonal antibodies, antibody fragments, single antibody domains and related molecules, such as scFv, diabodies, engineered lipocalins, camelbodies, nanobodies and related structures. Also included are soluble mediators, cytokines, growth factors, soluble receptor fragments, matrix fragments, synthetic molecules, or other structures that are known to have cognate binding structures on the targeted cell. In addition,

protein domains that have been selected by diversification of an invariant or polymorphic scaffold, for example, in the formation of binding principles from fibronectin, anticalins, titin and other structures, are also included. Cell targeting moieties can also include combinations of moieties (e.g., an scFv with a cytokine and an scFv with a second scFv).

The terms "fusion protein", "protoxin fusion," "toxin fusion", "protoxin activator fusion" and "protease fusion" as used herein refer to a protein that has a peptide component operably linked to at least one additional component and that differs from a natural protein in the composition and/or organization of its domains. The additional component can be peptide or non-peptide in nature. Additional peptide components can be derived by natural production or by chemical synthesis, and in the case of a peptide component that acts as an inhibitor moiety, a cell-targeting moiety, or a cleavage site, the additional peptide components need not be based on any natural template but may be selected for the desired purpose from an artificial scaffold or random sequence or by diversification of an existing template such that substantially all of the primary sequence similarity is lost but the functional attributes are preserved. Non-peptide additional components can include one or more functional chemical species. The chemical species may comprise a linker or a cleavage site, each optionally substituted with one or more linkers that may provide flexible attachment of the chemical species to a polypeptide or to another chemical species.

The term "selectively modifiable activation moiety" refers to an unnatural or not naturally found moiety of a protoxin or protoxin activator that, upon modification, converts a protoxin to a toxin or natively activatable protoxin or activates a protoxin proactivator or modifies the protoxin proactivator so that it becomes natively activatable. When the selectively modifiable activation moiety is a component of the protoxin fusion protein, modification of the modifiable activation moiety by the protoxin activator can result directly in the protoxin becoming toxic to the target cell, or can result in the protoxin assuming a form that is natively activatable to become toxic to the target cell. When the selectively modifiable activation moiety is a component of the protoxin proactivator protein, modification of the modifiable activation moiety by the proactivator activator can result directly in the proactivator becoming activated to a form that can modify the protoxin, or can result in the

proactivator assuming a form that is natively activatable to become a form that can modify the protoxin. Natively activatable protoxins or proactivators comprise, for example, modification of the modifiable activation moiety such that it is sensitive to endogenous components of the target cell, or the environment surrounding the target cells (e.g., a target cell specific protease or a ubiquitous protease).

The terms "operably linked" or "operable linkage" encompass the joining of two or more peptide components covalently or noncovalently or both covalently and noncovalently as well as the joining of one or more peptide components with one or more chemical species covalently or noncovalently or both covalently and noncovalently, as well as the joining of two or more chemical species covalently. Among suitable forms of covalent linkage for peptide components are direct translational fusion, in which a single polypeptide is formed upon translation of mRNA, or post-translational fusion, achieved by operable linkage through chemical or enzymatic means or by operable linkage through natural intermolecular reactions such as the formation of disulfide bonds. Operable linkage may be performed through chemical or enzymatic activation of various portions of a donor molecule to result in the attachment of the activated donor molecule to a recipient molecule. Following operable linkage two moieties may have additional linker species between them, or no additional species, or may have undergone covalent joining that results in the loss of atoms from one or more moieties, for example as may occur following enzymatically induced operable linkage.

The term "artificially diversified polypeptide binder" as used herein refers to a peptide or polypeptide comprising at least one domain that has been made to comprise multiple embodiments as a result of natural or in vitro mutation, including addition, deletion and substitution, so as to provide an ensemble of peptides or polypeptides from which a high affinity variant capable of binding to the desired cell surface target can be selected. Such artificially diversified polypeptide binders can comprise peptides, for example as selected by phage display, ribosome display, RNA display, yeast display, cell surface display or related methods, or polypeptides, similarly selected, and typically diversified in flexible loops of robust scaffolds so as to provide antibody variable region mimetics or related binding molecules.

The term "cell surface target" as used herein refers to any structure operably exposed on the surface of a cell, including transient exposure as for example may be

the consequence of fusion of intracellular vesicles with the plasma membrane, and that can be specifically recognized by a cell targeting moiety. A cell surface target may include one or more optionally substituted polypeptide, carbohydrate, nucleic acid, sterol or lipid moieties, or combinations thereof, as well as modifications of polypeptides, carbohydrate, nucleic acid, sterol or lipid moieties separately or in combination. A cell surface target may comprise a combination of optionally substituted polypeptide and optionally substituted carbohydrate, an optionally substituted carbohydrate and optionally substituted lipid or other structures operably recognized by a cell-targeting moiety. A cell surface target may comprise one or more such optionally substituted polypeptides, carbohydrates, nucleic acid, sterol or lipids in complexes, for example heteromultimeric proteins, glycan-substituted heteromultimeric proteins, or other complexes, such as the complex of a peptide with a major histocompatibility complex antigen. A cell surface target may exist in a form operably linked to the target cell through another binding intermediary. A cell surface target may be created by some intervention to modify particular cells with an optionally substituted small molecule, polypeptide, carbohydrate, nucleic acid, sterol or lipid. For example a cell surface target may be created by the administration of a species that binds to a cell of interest and thereby affords a binding surface for the modified VCE of the present invention.

The term "combinatorial targeting" and "binary targeting" refer to the methods for treating various diseases through selective killing of targeted cells using a combinatorial targeting approach as described in PCT Application Publication No. 2008/011157, which is herein incorporated by reference in its entirety. Briefly, the strategy features protoxin fusion proteins containing a cell targeting domain, a modifiable activation moiety which is activated by an activation moiety not naturally operably found in, on, or in the vicinity of a target cell. These methods also include the combinatorial use of two or more therapeutic agents, at minimum comprising a protoxin and a protoxin activator, to target and destroy a specific cell population. Each agent contains at least one cell targeting moiety binding to an independent cell surface target of the targeted cells. The protoxin contains a modifiable activation moiety that may be acted upon by the protoxin activator. The protoxin activator comprises an enzymatic activity that upon acting on the modifiable activation moiety converts, or allows to be converted, the protoxin to an active toxin or a natively

activatable toxin. The targeted cells are then inhibited or destroyed by the activated toxin. In cases where only two agents are involved, the strategy is referred to as "binary targeting". One example of a protoxin and protoxin activator pair is a modified VCE fusion protein and a protease fusion protein.

The term "activatable AB toxin" as used herein refers to any protein that comprises a cell-targeting and translocation domain (B domain) as well as a biologically active domain (A domain) and that requires the action of an endogenous target cell protease on an activation sequence to substantially promote their toxic effect. AB toxins have the capability to intoxicate target cells without requirement for accessory proteins or protein-delivery structures such as the type III secretion system of gram negative bacteria. AB toxins typically contain a site that is sensitive to the action of ubiquitous furin/kexin-like proteases, and must undergo cleavage to become activated. According to the present invention, the term "activatable AB toxin" is meant to include modified AB toxins in which the endogenous cell-targeting domain is replaced by one or more heterologous cell-targeting moiety, or in which one or more heterologous cell-targeting moiety is added to an intact endogenous cell-targeting domain, and the activation sequence is replaced with a modifiable activation moiety that may be modified by an exogenous activator.

The term "ADP-ribosylating toxin" refers to enzymes that transfer the ADP ribose moiety of β-NAD^+ to a eukaryotic target protein. This process impairs essential functions of target cells, leading to cytostasis or cytotoxicity. Examples of bacterial ADP-ribosylating toxins include Diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, *P. aeruginosa* cytotoxic exotoxin S, pertussis toxin, cholera toxin, heat-labile enterotoxins LT-I and LT-II from *E. coli* (Krueger and Barbieri, Clin. Microbiol. Rev. 8:34-47 (1995)), and Cholix toxin (Jorgensen et al. J. Biol. Chem. 283 (16):10671-10678 (2008)). Examples of nonbacterial ADP-ribosylating toxins include the DNA ADP-ribosylating enzymes pierisin-1, pierisin-2, CARP-1 and the related toxins of the clams Ruditapes philippinarum and Corbicula japonica (Nakano et al. Proc Natl Acad Sci U S A. 103(37):13652-7 (2006)). In addition, the application of *in silico* analyses have allowed the prediction of putative ADP-ribosylating toxins (Pallen et al. Trends Microbiol. 9:302-307 (2001).

The term "activatable ADP-ribosylating toxin" or "activatable ADPRT" as used herein refers to toxins that are functionally conserved enzymes produced by a variety

of species that share the ability to transfer the ADP ribose moiety of β-NAD^+ to a eukaryotic target protein and that require the action of an endogenous target cell protease on an activation sequence to substantially promote their toxic effect. This process impairs essential functions of target cells, leading to cytostasis or cytotoxicity. Examples of activatable bacterial ADPRTs are VLE, Diphtheria toxin (DT), *Pseudomonas aeruginosa* exotoxin A (PEA), pertussis toxin, cholera toxin, and heat-labile enterotoxins LT-I and LT-II from *E. coli* (Krueger and Barbieri, Clin. Microbiol. Rev. 8:34-47 (1995); Holbourn et al. The FEBS J. 273:4579-4593(2006)). Examples of activatable nonbacterial ADP-ribosylating toxins include the DNA ADP-ribosylating enzymes from Cabbage butterfly, Pieris Rapae (Kanazawa et al Proc. Natl. Acad. Sci. 98:2226-2231 (2001)) and, by sequence homology, Pieris brassicae (Takamura-Enya et al., Biochem. Biophys. Res. Commun. 32:579-582 (2004)).

The terms "VCE protoxin" and "VCE related protoxin" refer to recombinant toxins comprising at least one functional domain from VCE, either the ADP-ribosylating domain or the translocation domain. In one embodiment, such a VCE protoxin comprises the ADP-ribosyltransferase domain of VCE (Arg458 to Lys666), a C-terminal ER retention signal, a cell binding moiety, and the translocation domain of another ADP-ribosylating toxin or activatable ADP-ribosylating toxin such as that of PEA, in which the proteolytic activation site may be altered to be a substrate of a selected protease. In another embodiment, a VCE protoxin comprises the translocation domain of VCE (Gly298 to Ala418), a C-terminal ER retention signal, a cell binding moiety, and a cytotoxic moiety such as the catalytic domain of an activatable ADPRT and the biologically active A domain of an activatable AB toxin.

The term "translocation domain" of a toxin as used herein refers to an optional domain of a toxin (for example, a naturally occurring or modified toxin) that has cell-membrane translocation activity. "Cell membrane translocation activity" is activity that is necessary for translocation into the cytoplasm or a cytoplasm-contiguous compartment an active domain of a toxin. Prior to translocation the active domain may be located on the cell surface, or may have been conveyed from the cell surface into an intracellular space excluded from the cytoplasm, for example a vesicular compartment such as the endosome, lysosome, Golgi, or endoplasmic reticulum. Examples of such domains are the translocation domain of DT (residues 187-389) and

the translocation domain of Pseudomonas exotoxin A (residues 253-364). Not all toxins contain translocation domains (e.g., pore forming toxins).

The term "substrate" as used herein refers to the specific molecule, or the portion of a molecule, that is recognized and chemically modified by a particular enzyme.

The term "protease" as used herein refers to compositions that possess proteolytic activity, and preferably those that can recognize and cleave certain peptide sequences specifically. In one particular embodiment, the specific recognition site is equal to or longer than that of the native furin cleavage sequence of four amino acids, thus providing activation stringency comparable to, or greater than, that of native toxins. A protease may be a native, engineered, or synthetic molecule having the desired proteolytic activity. Proteolytic specificity can be enhanced by genetic mutation, in vitro modification, or addition or subtraction of binding moieties that control activity.

The term "heterologous" as used herein refers to a composition or state that is not native or naturally found, for example, that may be achieved by replacing an existing natural composition or state with one that is derived from another source. Thus replacement of a naturally existing, for example, furin-sensitive, cleavage site with the cleavage site for another enzyme, constitutes the replacement of the native site with a heterologous site. Similarly the expression of a protein in an organism other than the organism in which that protein is naturally expressed constitutes a heterologous expression system and a heterologous protein.

The term "exogenous" as used herein refers to any protein that is not operably present in, on, or in the vicinity of, a targeted host cell. By operably present it is meant that the protein, if present, is not present in a form that allows it to act in the way that the therapeutically supplied protein is capable of acting. Examples of a protoxin-activating moiety that may be present but not operably present include, for example, intracellular proteases, phosphatases or ubiquitin C-terminal hydrolases, which are not operably present because they are in a different compartment than the therapeutically supplied protease, phosphatase or ubiquitin C-terminal hydrolase (which when therapeutically supplied is either present on the surface of the cell or in a vesicular compartment topologically equivalent to the exterior of the cell) and cannot act on the protoxin in a way that would cause its activation. A protein may also be

present but not operably present if it is found in such low quantities as not to significantly affect the rate of activation of the protoxin or protoxin proactivator, for example to provide a form not operably found in, on, or in the vicinity of, a targeted cell in a proportion of greater than 10%, or greater than 1%, or greater than 0.1% of the proportion that can be achieved by exogenous supply of a minimum therapeutically effective dose. As a further non-limiting example, replacement of a furin-sensitive site in a therapeutic protein with a site for a protease naturally found operably present on, in, or in the vicinity of a targeted host cell constitutes a heterologous replacement that can be acted on by an endogenous protease. Replacement of a furin-sensitive site in a therapeutic protein with a site for a protease not naturally found operably present in the vicinity of a targeted host cell constitutes a heterologous replacement that can be acted on by an exogenous protease.

The term "PEGylation" refers to covalent or noncovalent modifications of proteins with polyethylene glycol polymers of various sizes and geometries, such as linear, branched and dendrimer and may refer to block copolymers incorporating polyethylene glycol polymers or modified polymers with additional functionality, such as may be useful for the therapeutic action of a modified toxin. For example a polyethylene glycol moiety may join a modifiable activation sequence to an optional inhibitor sequence or may join one or more cell-targeting moieties to a modified toxin. Many strategies for PEGylating proteins in a manner that is consistent with retention of activity of the conjugated protein have been described in the art. These include conjugation to a free thiol such as a cysteine by alkylation or Michael addition, attachment to the N-terminus by acylation or reductive alkylation, attachment to the side chain amino groups of lysine residues, attachment to glutamine residues using transglutaminase, attachment to the N-terminus by native ligation or Staudinger ligation, or attachment to endogenous glycans, such as N-linked glycans or O-linked glycans. Numerous glycan addition strategies are known, including hydrazone formation with aldehydes generated by periodate oxidation, Staudinger ligation with glycan azides incorporated by metabolic labeling, and glycan substitution technology. Examples of noncovalent modification include the reaction of a high affinity ligand-substituted PEG with a protein domain binding such ligand, as for example the reaction of a biotin-substituted PEG moiety with a streptavidin or avidin fusion protein.

The term "PEG" refers to an optionally substituted polyethylene glycol moiety that may exist in various sizes and geometries, such as linear, branched or dendrimer and may refer to block copolymers or modified polymers with additional functionality, such as may be useful for the therapeutic action of a modified toxin. The number of optionally substituted or unsubstituted ethylene glycol moieties in a PEG moiety is at least two.

The term "PEGylated" refers to a composition that has undergone reversible or irreversible attachment of a PEG moiety.

The term "thiol-specific PEGylation" refers to attachment of an optionally substituted thiol-reactive PEG moiety to one or more thiol groups of a protein or protein substituent. The target of thiol-directed PEGylation can be a cysteine residue, or a thiol group introduced by chemical reaction, such as by the reaction of iminothiolane with lysine epsilon amino groups or N-terminal alpha amino or imino groups. A number of highly specific chemistries have been developed for thiol-directed PEGylation, i.e., PEG-ortho-pyridyl-disulfide, PEG-maleimide, PEG-vinylsulfone, and PEG-iodoacetamide. In addition to the type of thiol specific conjugation chemistry, commercially available thiol-reactive PEGs also vary in terms of size, linear or branched, and different end groups including hydroxyl, carboxylic acid, methoxy, or other alkoxy groups.

The term "carboxyl-reactive PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety capable of reacting with a carboxyl group, such as a glutamate or aspartate side chain or the C-terminus of a protein. The carboxyl groups of a protein can be subjected to carboxyl-reactive PEGylation using PEG-hydrazide when the carboxyl groups are activated by coupling agents such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) at acidic pH.

The term "amine-reactive PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety capable of reacting with an amine, such as a primary amine or a secondary amine. A common route for amine-reactive PEGylation of proteins is to use a PEG containing a functional group that reacts with lysines and/or an N-terminal amino or imino group (Roberts et al. Adv. Drug Deliv. Rev. 54(4):459-476 (2002)). Examples of amine-reactive PEGs include PEG dichlorotriazine, PEG tresylate, PEG succinimidyl carbonate, PEG benzotriazole

carbonate, PEG p-nitrophenyl carbonate, PEG carbonylimidazole, PEG succinimidyl succinate, PEG propionaldehyde, PEG acetaldehyde, and PEG N-hydroxysuccinimide.

The term "N-terminal PEGylation" refers to attachment of an optionally substituted PEG moiety to the amino terminus of a protein. Preferred protein fusions or protein hybrids for N-terminal PEGylation have at least one N-terminal amino group. N-terminal PEGylation can be carried out by reaction of an amine-reactive PEG with a protein, or by reaction of a thioester-terminated PEG with an N-terminal cysteine in the reaction known as native chemical ligation, or by reaction of a hydrazide, hydrazine or hydroxylamine terminated PEG with an N terminal aldehyde formed by periodate oxidation of an N-terminal serine or threonine residue. Preferably, a PEG-protein conjugate contains 1-5 PEG substituents, and may be optimized experimentally. Multiple attachments may occur if the protein is exposed to PEGylation reagents in excess. Reaction conditions, including protein:PEG ratio, pH, and incubation time and temperature may be adjusted to limit the number and/or sites of the attachments. Modification at active site(s) within a fusion protein may be prevented by conducting PEGylation in the presence of a substrate, reversible inhibitor, or a binding protein. A fusion protein with the desired number of PEG substitutions may also be obtained by isolation from a more complex PEGylated fusion protein mixture using column chromatography fractionation.

The term "unnatural amino acid-reactive PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety capable of reacting with unnatural amino acids bearing reactive functional groups that may be introduced into a protein at certain sites utilizing modified tRNAs. In particular, para-azidophenylalanine and azidohomoalanine may be specifically incorporated into proteins by expression in yeast (Deiters et al. Bioorg. Med. Chem. Lett. 14(23):5743-5 (2004)) and in E. coli (Kiick et al. Proc. Natl. Acad. Sci. U S A. 99(1):19-24 (2002)), respectively. These azide modified residues can selectively react with an alkyne derivatized PEG reagent to allow site specific PEGylation.

The term "glycan-reactive PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety capable of reacting with a glycosylated protein and the proteins containing N-terminus serine or threonine may be PEGylated followed by selective oxidation. Carbohydrate side chains may be

oxidized enzymatically, or chemically using sodium periodate to generate reactive aldehyde groups. N-terminus serine or threonine may similarly undergo periodate oxidation to afford a glyoxylyl derivative. Both aldehyde and glyoxylyl groups can selectively react with PEG-hydrazine or PEG-amine.

The term "enzyme-catalyzed PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety through one or more enzyme catalyzed reactions. One such approach is to use transglutaminases, a family of proteins that catalyze the formation of a covalent bond between a free amine group and the gamma-carboxamide group of protein- or peptide- bound glutamine. Examples of this family of proteins include transglutaminases of many different origins, including thrombin, factor XIII, and tissue transglutaminase from human and animals. A preferred embodiment comprises the use of a microbial transglutaminase, to catalyze a conjugation reaction between a protein substrate containing a glutamine residue embedded within a peptide sequence of LLQG and a PEGylating reagent containing a primary amino group (Sato Adv. Drug Deliv. Rev. 54(4):487-504 (2002)). Another example is to use a sortase to induce the same conjugation. Accordingly a substituted PEG moiety is provided that is endowed with LPXTG or NPQTN, respectively for sortase A and sortase B, and a second moiety such as a polypeptide containing the dipeptide GG or GK at the N-terminus, or a primary amine group, or the dipeptide GG or GK attached to a linker, and said sortase A or sortase B is then provided to accomplish the joining of the PEG moiety to the second moiety. Alternatively, said LPXTG or NPQTN can be provided at the C-terminus of a polypeptide to be modified and the PEG moiety can be supplied that is substituted with a GG or GK or a primary amine, and the sortase reaction performed.

The term "glycoPEGylation" refers to the reaction of a protein with an optionally substituted PEG moiety through enzymatic GalNAc glycosylation at specific serine and threonine residues in proteins expressed in a prokaryotic host, followed by enzymatic transfer of sialic acid conjugated PEG to the introduced GalNAc (Defrees et al. Glycobiology. 16(9):833-843 (2006)).

The term "intein-mediated PEGylation" refers to the reaction of a protein with an optionally substituted PEG moiety through an intein domain that may be attached to the C-terminus of the protein to be PEGylated, and is subsequently treated with a cysteine terminated PEG to afford PEGylated protein. Such intein-mediated protein

conjugation reactions are promoted by the addition of thiophenol or triarboxylethylphosphine (Wood, et al., Bioconjug. Chem. 15(2):366-372 (2004)).

The term "reversible PEGylation" refers to the reaction of a protein or protein substituent with an optionally substituted PEG moiety through a linker that can be cleaved or eliminated, liberating the PEG moiety. Preferable forms of reversible PEGylation involve the use of linkers that are susceptible to various activities present at the cell surface or in intracellular compartments, and allow the useful prolongation of plasma half-life and/or reduction of immunogenicity while still permitting the internalized or cell-surface-bound protoxin or protoxin proactivator or proactivator activator to carry out their desired action without inhibition or impediment by the PEG substitution. Examples of reversible PEGylation linkers include linkers susceptible to the action of cathepsins, furin/kexin proteases, and lysosomal hydrolases such as neuraminidases, nucleases and glycol hydrolases.

The term "administering" and "co-administering" as used herein refer to the administration of one or more proteins to an organism in need of treatment. The one or more proteins can be, for example, fusion proteins, and can be administered simultaneously and/or sequentially to an organism in need of treatment. The sequential order, time interval, and relative quantity of the application may be varied to achieve an optimized selective cytotoxic or cytostatic effect. It may be preferable to use one agent in large excess, or to use two agents in similar quantities. One agent may be applied significantly before the addition of the second agent, or they may be applied in closer intervals or at the same time. In addition administering and co-administering may include injection or delivery from more than one site, for example by injection into two different anatomical locations or by delivery by more than one modality, such as by aerosol and intravenous injection, or by intravenous and intramuscular injection.

The term "selective killing" is used herein to refer to the killing, destroying, or inhibiting of more cells of one particular population than another, e.g., by a margin of 99:1 or above, 95:5 or above, 90:10 or above, 85:15 or above, 80:20 or above, 75:25 or above, 70:30 or above, 65:35 or above, or 60:40 or above.

The term "destroying or inhibiting a target cell" is used herein to refer to reducing the rate of cellular division (cytostasis) or causing cell death (cytotoxicity) of a particular cell type (e.g., a cell expressing the desired cell surface targets).

Cytostasis or cytotoxicity may be achieved, for example, by the induction of differentiation of the cell, apoptosis of the cell, death by necrosis of the cell, or impairment of the processes of cellular division.

The term "glycosylation" refers to covalent modifications of proteins with carbohydrates. Glycosylation can be achieved through N-glycosylation or O-glycosylation. An introduction of consensus N-linked glycosylation sites may be preferred when the proteins are to be produced in a mammalian cell line or cell lines that create a glycosylation pattern that are innocuous to humans.

Human "granzyme B" (GrB) is a member of the granzyme family of serine proteases known to be involved in apoptosis. Specifically, GrB has been shown to cleave only a limited number of natural substrates, e.g., pro-caspase-3 and Bid. It has been shown that GrB is an enzyme with high substrate sequence specificity because of the requirement for interactions with an extended peptide sequence in the substrate for efficient catalysis, i.e., a consensus recognition sequence of IEPD. GrB is a single chain and single domain serine protease and is synthesized in a pro-form, which is activated by removal of the two amino acid pro-peptide by dipeptidyl peptidase I (DPPI). In the present invention, the term GrB for example refers to the mature form, i.e., the form without the propeptide.

Human "Granzyme M" (GrM) is another member of the granzyme family of serine proteases that is specifically found in granules of natural killer cells and is implicated in the induction of target cell death. It has been shown that GrM is an enzyme with high substrate sequence specificity because of the requirement for interactions with at least four amino acids in the peptide substrate for efficient catalysis, i.e., a preferred recognition sequence of KVPL.

The term "potyviral protease" refers to any of a variety of proteases encoded by members of the plant virus family Potyviridae and exhibiting high cleavage specificity. "Potyviral protease" encompasses the natural proteases as well as engineered variants generated by genetic mutation or chemical modification. The term "tobacco etch virus protease" or "TEV protease" refers to natural or engineered variants of a 27 kDa cysteine protease exhibiting stringent sequence specificity. It is widely used in biotechnology for removal of affinity tags of recombinant proteins. TEV protease recognizes a seven amino acid recognition sequence EXXYXQ↓S/G, where X is any residue.

The term "picornaviral protease" refers to any of a variety of proteases encoded by members of the animal virus family Picornaviridae and exhibiting high cleavage specificity. "picornaviral protease" encompasses the natural proteases as well as engineered variants generated by genetic mutation or chemical or enzymatic modification. The term "human Rhinovirus 3C consensus protease" refers to a synthetic picornaviral protease that is created by choice of a consensus sequence derived from multiple examples of specific rhinoviral proteases.

The term "retroviral protease" refers to any of a variety of proteases encoded by members of the virus family Retroviridae. "HIV protease" encompasses the natural proteases as well as engineered variants generated by genetic mutation or chemical or enzymatic modification.

The term "coronaviral protease" refers to any of a variety of proteases encoded by members of the animal virus family Coronaviridae and exhibiting high cleavage specificity. "coronaviral protease" encompasses the natural proteases as well as engineered variants generated by genetic mutation or chemical or enzymatic modification. The term "SARS protease" refers to a coronaviral protease encoded by any of the members of the family Coronaviridae inducing the human syndrome SARS.

By "substantially identical" is meant a nucleic acid or amino acid sequence that, when optimally aligned, for example using the methods described below, share at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with a second nucleic acid or amino acid sequence, e.g., a SAA sequence. "Substantial identity" may be used to refer to various types and lengths of sequence, such as full-length sequence, epitopes or immunogenic peptides, functional domains, coding and/or regulatory sequences, exons, introns, promoters, and genomic sequences. Percent identity between two polypeptides or nucleic acid sequences is determined in various ways that are within the skill in the art, for instance, using publicly available computer software such as Smith Waterman Alignment (Smith, T. F. and M. S. Waterman (1981) J Mol Biol 147:195-7); "BestFit" (Smith and Waterman, Advances in Applied Mathematics, 482-489 (1981)) as incorporated into GeneMatcher Plus™, Schwarz and Dayhof (1979) Atlas of Protein Sequence and Structure, Dayhof, M.O., Ed pp 353-358; BLAST program (Basic Local Alignment Search Tool; (Altschul, S. F., W. Gish, et al. (1990) J Mol Biol 215: 403-10),

BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, or Megalign (DNASTAR) software. In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. In general, for proteins or nucleic acids, the length of comparison can be any length, up to and including full length (e.g., 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%). Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By the term "cancer cell" is meant a component of a cell population characterized by inappropriate accumulation in a tissue. This inappropriate accumulation may be the result of a genetic or epigenetic variation that occurs in one or more cells of the cell population. This genetic or epigenetic variation causes the cells of the cell population to grow faster, die slower, or differentiate slower than the surrounding, normal tissue. The term "cancer cell" as used herein also encompasses cells that support the growth or survival of a malignant cell. Such supporting cells may include fibroblasts, vascular or lymphatic endothelial cells, inflammatory cells or co-expanded nonneoplastic cells that favor the growth or survival of the malignant cell. The term "cancer cell" is meant to include cancers of hematopoietic, epithelial, endothelial, or solid tissue origin. The term "cancer cell" is also meant to include cancer stem cells. The cancer cells targeted by the fusion proteins of the invention include those set forth in Table 1.

DESCRIPTION OF THE FIGURES

Fig. 1A is sequence alignment of PEA (SEQ ID NO:8) and VCE (SEQ ID NO:9) by BLAST. The PEA sequence is numbered from the mature N-terminus and the VCE is numbered from the N-terminus of the putative pre-protein.

Fig. 1B is an analysis of overall sequence identity and similarity between PEA and VCE as well as the sequence identity and similarity of individual domains of PEA and VCE.

Fig. 1C is the sequence of the putative furin cleavage site in VCE in comparison with the furin cleavage sites of PEA and DT. Residues that are known or

hypothesized to be important for efficient *in vitro* furin cleavage are highlighted in gray.

Fig. 2A is an immunoblot showing that the isolated ADPRT domain of VCE can modify a 100 kD protein in 293T cell lysate.

Figs. 2B –2D are immunoblots showing the result of affinity capture of biotin-ADP-ribosylated proteins followed by immunoblotting with anti-eEF2 antibody (Fig. 2B) or with anti-GrB2 antibody (Fig. 2C). The first two lanes of Fig. 2B show the presence of eEF2 in the lysate and the second two lanes demonstrate that eEF2 is selectively recovered by avidin beads following reaction of the lysate with VCE in the presence of biotin-NAD. Figs. 2C and 2D demonstrate that an antibody against an unrelated protein (Grb2) detects Grb2 in lysates (first two lanes) but no Grb2 is detected among the products released from avidin beads (last two lanes). This helps rule out the possibility that lysate proteins in general bind to avidin beads.

Fig. 3A is an immunoblot showing that 293T, CHO-K1 and Rel.22C express similar amount of eEF2 as detected by an eEF2-specific antibody.

Fig. 3B is an immunoblot showing that eEF2 from Rel.22C is resistant to the ADP-ribosylation activity of VCE, whereas eEF2 from 293T and CHO-K1 can be modified by VCE-ADPRT.

Fig. 4A is an immunoblot showing ADRPT activity of various N-GFD-VCE fusion proteins in comparison to PEA.

Fig. 4B is a graph showing cytotoxicity assay results using $CD19^+$ Jurkat cells.

Fig. 5A is schematic depiction of two VCE-based immunotoxins, CCPE-VCE and $CCPE^2$-VCE. In these immunotoxins, the cell targeting domain of the wild type VCE was replaced with one or two copies of C-terminal domain of Clostridium perfringens enterotoxin (CCPE) which has been shown to target cell surface proteins claudin3 and claudin4.

Figs. 5B and 5C are graphs showing the cytotoxicity assay results of CCPE-VCE on claudin3/4 positive cell lines, including HT29, MCF7, and MB231 and a claudin3/4 negative control cell line Nalm6. Nonlinear regression analysis was performed using the GraphPad Prism 4 program. The EC50 was calculated based on the calculated curves. $CCPE^2$-VCE exhibited 5-10 folds higher toxicity to claudin3/4 positive cells than did CCPE-VCE, while both immunotoxins exhibited similar toxicity to the negative cell line.

Fig. 6A is sequence alignment of the granzyme B cleavage site used in DT-anti-CD5, anti-CD5-PEA and anti-CD5-VCE. The arrow indicates the cleavage site.

Fig. 6B is an electrophoretic gel showing purified DT-anti-CD5, anti-CD5-PEA and anti-CD5-VCE.

Fig. 6C is an electrophoretic gel showing mouse granzyme B cleavage of purified DT-anti-CD5, anti-CD5-PEA and anti-CD5-VCE under different conditions.

Fig.7 is a graph showing cytotoxicity assay results of DT-, PEA-, VCE-based combinatorial targeting agents shown in Fig. 6. The cytotoxicity assay was carried out in the absence or presence of 1.0 nM GrB-anti-CD19. Nonlinear regression analysis was performed using the GraphPad Prism 4 program.

Fig. 8 is a graph showing cytotoxicity assay results of VCE-based combinatorial targeting agents using $CD5^+$Raji cells. The assays were performed with 1.0 nM GrB-anti-CD19 and various concentrations of anti-CD5-VCE. For comparison, we also measured cytotoxicity of anti-CD5-VCE bearing the endogenous furin cleavage sequence (anti–CD5-VCE_{wt}) and a mutant anti-CD5-VCE in which one the predicted active site residues glutamic acid 613 was replaced with alanine (anti-CD5-VCE_{E613A}). Nonlinear regression analysis was performed as described above.

Fig. 9A is a graph showing FACS analysis of purified PBMNC from a B-CLL patient with anti-CD5 and anti-CD19 antibodies.

Fig. 9B is a graph showing 1.0 nM GrB-anti-CD19 alone was not toxic to either PBMNC or $CD5^+$Raji.

Fig. 9C is a graph showing that anti-CD5-VCE selectively kill $CD5^+$Raji cells and a fraction of PBMNC only in the presence of GrB-anti-CD19.

Fig. 10 is a diagram showing mutations that to reduce the likelihood of vascular leak syndrome induced by recombinant VCE-based toxins.

Fig. 11 is a diagram showing mutations to reduce the immunogenicity of recombinant VCE-based toxins.

Fig. 12 is a graph showing cytotoxicity assay results of N-GFD-VCE, N-GFD-VCE_{E613A} and N-GFD-VE-PEA fusion proteins using $CD19^+$ Jurkat cells.

Fig. 13A is a graph showing ELISA results of anti-VCE polyclonal antibodies raised from 2 rabbits (9375 and 9376). Anti-VCE polyclonal antibodies from both animals react specifically to the fusion protein consisting of the ADPRT domain of

VCE antigen (MBP-VCE), but not to a control protein (MBP, maltose binding protein).

Fig. 13B is a commassie staining and immunoblot showing showing that anti-VCE polyclonal antibodies can be used for Western blot to detect denatured VCE. The left panel shows a commassie stained SDS-PAGE of the fusion protein containing specific antigen (lane 1, anti-CD19-VCE), cell lysate (lane2, 293T cell lysate) and a negative control protein (anti-CD5-Diphtheria toxin). Left panel shows the western blot results of the same set of proteins probed with anti-VCE polyclonal antibody from rabbit 9375, and visualized with HRP conjugated anti-rabbit Fc antibody.

Fig. 13C is a graph showing the results of affinity measurement of several anti-VCE monoclonal antibodies by surface plasma resonance. Affinity (K_D) of three represented clones was shown.

DETAILED DESCRIPTION OF THE INVENTION

Recombinant exotoxins from *Vibrio cholerae* are useful for the therapeutic treatment of a variety of human diseases, particularly diseases characterized by an abundance or excess of undesired cells. Vibrio exotoxins have superior biophysical properties, including robust folding and enhanced solubility when expressed at high levels in E. coli compared to similar toxins known in the art. Vibrio exotoxin fusion proteins comprising exogenous cell-targeting moieties bind to selected surface targets of cells of interest and induce intoxication. In addition, such fusion proteins in which the native activation sequence has been replaced with a modifiable activation moiety can be acted upon by a second proactivator or activator that can be made to be or is naturally specific to the cell that is to be targeted for intoxication. When Vibrio exotoxin activation sequences are replaced with those of exogenous proteases, the resulting engineered toxins are more easily activated by site-specific proteolysis than comparable toxins based on Pseudomonas exotoxin A or Diphtheria toxin. Vibrio exotoxin proteins are more potent as combinatorial toxins than either Pseudomonas or Diphtheria toxins.

The present invention provides a recombinant *Vibrio cholerae* Exotoxin (VCE), which has ~32% sequence identity with Pseudomonas exotoxin A (PEA), and demonstrates similar domain organization, furin activation, ADP-ribosyltransferase

(ADPRT) activity to EF-2, and related cytotoxicity. The present invention also provides mutant VCE fusion proteins that comprise the native ADP-ribosyltransferase activity, a modified cell binding domain that binds to one or more specific cell surface proteins, as well as a modified translocation domain cleavable by selected proteolytic activities.

In particular, the present invention provides modified VCE fusion proteins with an altered proteolytic activation site so that they are activated not by furin, but instead by another protease of choice. One preferred embodiment includes modified VCE fusion proteins activatable by endogenous proteases that are present or upregulated in targeted cells. Another preferred embodiment of the present invention includes modified VCE fusion proteins that are activated by exogenous proteases fusion proteins that are bound to a cell surface marker on a targeted cell.

One aspect of the present invention is that the cell surface targeting moiety of VCE may be replaced with alternative binding principles of different specificities to target specific cell surface target of the targeted cells. Another aspect of the present invention is that the translocation domain of VCE may be modified such that they are activated by enzymatic activities other than furin, including those of different proteases.

An additional aspect of the present invention is the native or modified translocation domain of VCE, which has only 31% sequence identity (35/112) to that of PEA. The translocation domain of VCE may be used to facilitate translocation other cell killing principles into targeted cells in conjunction with an ER retention sequence such as KDEL (SEQ ID NO:10). Such cytotoxic agents include, without limitation, small toxic molecules, oligonucleotides such as RNAi agents, and catalytic domains of protein toxins such as ADP-ribosyltransferase domain of PEA and N-glycosidase domain of ricin A.

The ADPRT domain of VCE is another embodiment of this invention. Only 86 out of 199 residues (43%) of this domain are identical to that of PEA. This catalytic domain may be used in combination with a translocation domain of different origin, such as that of PEA, as well as a different binding principle that binds to a specific cell surface marker of a targeted cell.

Antibodies generated against VCE or modified VCE of mouse, rat, goat, or other origin are also embodiments of this invention. Other biological or chemical probes specific for VCE are additional embodiments of the present invention.

I. Recombinant VCE

VCE is a recombinant product of *toxA* gene of *V. cholerae* TP strain. VCE was analyzed by X-ray crystallography and the resulting structural data confirmed that it is a member of the diphthamide-specific class of ADP-ribose transferases (Jorgensen et al. J. Biol. Chem. 283 (16):10671-10678 (2008)). VCE possesses an N-terminal cell-targeting moiety (residues Met1-Lys297 and residues Ala419-Asn457), followed by a translocation domain (Gly298 to Ala418) and a C-terminal ADP-ribosyltransferase domain (Arg458 to Lys666) comprising an ER retention signal ^{662}KDEL^{665} (SEQ ID NO:10). A putative furin cleavage site (^{321}RKPK↓DL^{326}) (SEQ ID NO:11) is located near the N-terminus of the putative translocation domain.

In comparison, the native cell binding domain of Pseudomonas Exotoxin A (PEA) is composed of Domain Ia (residues Ala1 to Glu252) and Domain Ib (residues Ala365-Gly404) (US Patent No. 4892827). Deletion of domain Ia from PEA have been found to reduce non-specific cytotoxicity (US Patent No. 4892827), and replacing it with another cell binding moiety such as a single chain antibody afforded modified PEA fusion proteins that are capable of effectively and selectively killing cells bearing appropriate antigens or receptors (US patent No. 5863745).

Furin has been identified as the intracellular protease responsible for cleaving modified PEA at ^{276}RQPR↓GW^{279} (SEQ ID NO:12)and activating translocation, and this furin-mediated cleavage occurs preferentially under acidic conditions (Chiron et al. J. Biol. Chem. 272(50):31701-31711 (1997)). Deletion of residues 1-28 of translocation domain comprising the furin cleavage site, i.e., residues Gly253 to Gly280, from native PEA substantially increased its toxicity (US Patent No. 5602095). It has also been disclosed that changing the furin cleavage site to a sequence recognized by a different protease, i.e., prostate specific antigen (PSA), enabling preferential inhibition of protein synthesis in PSA-expressing LNCap cells as compared to the DU145 cells that do not express PSA (US Patent No. 6426075). A similar approach was applied to diphtheria toxin (DT), where its furin cleavage site was replaced by a urokinase plasminogen activator (uPA) cleavage site, resulting in

selective killing of acute myeloid leukemia (AML) cells that overexpress uPA receptors (Abi-Habib et al. Blood 104 (7):2143-2148 (2004)).

Like the catalytic moieties of diphtheria toxin (DT) and Pseudomonas exotoxin A (PEA), the VCE catalytic moiety specifically ADP-ribosylates diphthamide on eEF2. ADP-ribosylation of diphthamide impairs the function of eEF2 and leads to inhibition of protein synthesis which results in profound physiological changes and ultimately cell death. In several regards, VCE resembles PEA more closely than it resembles DT. First, the domain organization of VCE appears similar to that of PEA, in which the cell-targeting domain is followed by the translocation domain and then the enzymatic domain. VCE and PEA both possess a masked ER retention signal at the C-terminus, suggesting that VCE and PEA enter the cytosol of target cells via endoplasmic reticulum. Both VCE and PEA have low lysine content, thought to be consistent with the mechanism of introduction of toxin into the cytoplasm through the endoplasmic reticulum associated degradation (ERAD) pathway. The present data support the view that the proteolytic event that activates PEA and VCE occurs in an acidic endosomal compartment, whereas furin cleavage of DT might take place in more neutral environment.

The C-terminus of VCE bears a characteristic endoplasmic reticulum retention signal (KDEL) (SEQ ID NO:10) followed by a lysine residue at the very C-terminus of the VCE which is removed by a ubiquitous carboxyl-peptidase activity such as carboxypeptidase B. VCE enters the cytosol of target cell in a manner similar to PEA and the C-terminal sequence of VCE is essential for full cytotoxicity. Thus, for maximum cytotoxic properties of a preferred VCE molecule, an appropriate carboxyl terminal sequence is preferred to translocate the molecule into the cytosol of target cells. Such preferred amino acid sequences include, without limitation, KDELK, RDELK, KDELR and RDELR (SEQ ID NOs:13-16).

The invention features compositions and methods including recombinant DNA constructs and expression of the modified VCE fusion proteins they encode. For example, modified VCE fusion comprising different cell binding specificity and altered protease recognition site can be produced from nucleic acid constructs encoding amino acid residues 1-666 of VCE, in which the native furin cleavage site ^{321}RKPK↓DL^{326} (SEQ ID NO:11) is replaced by a recognition sequence of an exogenous protease such as GrB, GrM, and TEV protease, and the cell binding

domain (residues 1-295) is replaced by a polypeptide that can bind to a specific cell surface target. Methods to introduce mutations into the nucleic acid sequence encoding VCE or to synthesize nucleic acid sequences that encode the mutant VCE are well known in the art (e.g., *Current Protocols in Molecular Biology* (Ausubel et al., eds., 2005)). The nucleic acid constructs can be generated using PCR. For example, the construct encoding the VCE fusion protein can be produced by mutagenic PCR, where primers encoding an alternative protease recognition site can be used to substitute the DNA sequence coding the furin cleavage site (residues 321-326: RKPK↓DL) (SEQ ID NO:11). Constructs containing the mutations can also be made through sequence assembly of oligonucleotides. Either approach can be used to introduce nucleic acid sequences encoding the granzyme B cleavage site IEPD↓DL (SEQ ID NO:17)in place of that which encodes RKPK↓DL(SEQ ID NO:11). In addition to IEPD (SEQ ID NO:18), GrB has been shown to recognize and cleave other similar peptide sequences with high efficiency, including IAPD and IETD (SEQ ID NOs:19 and 20). These and other sequences specifically cleavable by GrB may be incorporated. Genetically modified proteases of higher than natural specificity or displaying a different specificity than the naturally occurring protease may be of use in avoiding undesirable side effects attributable to the normal action of the protease.

DNA sequences encoding a cell-targeting polypeptide can be similarly cloned using PCR, and the full-length construct encoding the VCE fusion protein can be assembled by restriction digest of PCR products and the VCE construct followed by ligation. The construct may be designed to position a nucleic acid sequence encoding the modified VCE near the translation start site and the DNA sequence encoding the cell-targeting moiety close to the translation termination site. Such a sequence arrangement uses native VCE to confer optimal translocation efficiency of the catalytic domain of VCE to the cytosol.

VCE fusion proteins may be expressed in bacterial, insect, yeast, or mammalian cells, using established methods known to those skilled in the art, many of which are described, for example, in *Current Protocols in Protein Science* (Coligan et al., eds., 2006). DNA constructs intended for expression in each of these hosts may be modified to accommodate preferable codons for each host (Gustafsson et al., Trends Biotechnol. 22:346 (2004)), which may be achieved using established methods, for example, as described in *Current Protocols in Molecular Biology*

(Ausubel et al., eds., 2005), e.g., site-directed mutagenesis. To quickly identify an appropriate host system for the production of a particular VCE fusion, the Gateway cloning method (Invitrogen) may also be applied for shuffling a gene to be cloned among different expression vectors by *in vitro* site-specific recombination.

In addition to codon changes, other sequence modifications to the construct of a VCE fusion protein may include naturally occurring variations of VCE sequences that do not significantly affect its cytotoxicity and variants of the cell-targeting domain that do not abolish its ability to selectively bind to targeted cells.

Further, the sequence of the cell-targeting domain can be modified to select for variants with improved characteristics, e.g., reduced immunogenicity, higher binding affinity and/or specificity, superior pharmacokinetic profile, or improved production of the VCE fusion protein. Libraries of cell-targeting domains and/or VCE fusions can be generated using site-directed mutagenesis, error-prone PCR, or PCR using degenerate oligonucleotide primers. Sequence modifications may be necessary to remove or add consensus glycosylation sites, for maintaining desirable protein function or introducing sites of glycosylation to reduce immunogenicity.

For high yield expression of VCE fusion proteins, the encoding polynucleotide may be subcloned into one of many commercially available expression vectors, which typically contain a selectable marker, a controllable transcriptional promoter, and a transcription/translation terminator. In addition, signal peptides are often used to direct the localization of the expressed proteins, while other peptide sequences such as 6 His tags, FLAG tags, and myc tags may be introduced to facilitate detection, isolation, and purification of fusion proteins. To help successful folding of each domain within the VCE fusion, a flexible linker may be inserted between the modified VCE domain and the cell-targeting moiety in the expression construct.

VCE fusion proteins may be expressed in the bacterial expression system *Escherichia coli*. In this system a ribosome-binding site is used to enhance translation initiation. To increase the likelihood of obtaining soluble protein fusion, its expression construct may include DNA that encodes a carrier protein such as MBP, GST, or thioredoxin, either 5' or 3' to the VCE fusion, to assist protein folding. The carrier protein(s) may be proteolytically removed after expression. Proteolytic cleavage sites are routinely incorporated to remove protein or peptide tags and generate active fusion proteins. Most reports on successful *E. coli* expression of fusion proteins containing a

VCE moiety have been in the form of inclusion bodies, which may be refolded to afford soluble proteins.

VCE fusion proteins may be expressed in the methylotrophic yeast expression system *Pichia pastoris*. The expression vectors for this purpose may contain several common features, including a promoter from the *Pichia* alcohol oxidase (*AOX1*) gene, transcription termination sequences derived from the native *Pichia AOX1* gene, a selectable marker wild-type gene for histidinol dehydrogenase *HIS4*, and the 3'*AOX1* sequence derived from a region of the native gene that lies 3' to the transcription termination sequences, which is required for integration of vector sequence by gene replacement or gene insertion 3' to the chromosomal *AOX1* gene. Although *P. pastoris* has been used successfully to express a wide range of heterologous proteins as either intracellular or secreted proteins, secretion is more commonly used because *Pichia* secretes very low levels of native proteins. A secretion signal peptide MAT factor prepro peptide (MF-α1) is often used to direct the expressed protein to the secretory pathway.

Post-translational modifications such as N-linked glycosylation in *Pichia* occurs by adding approximately 8-14 mannose residues per side chain. Any consensus N-glycosylation sites NXS(T) within an expression construct can be mutated to avoid glycosylation.

VCE is potently toxic to eukaryotic cells if the catalytic domain translocates to or is localized to the cytosol. *Pichia* can support VCE expression because the expression of a toxic DT fusion by the secretory route has been successful (Woo et al., Protein Expr. Purif. 25:270 (2002)). Because the secretion of expressed heterologous protein in *Pichia* involves cleavage of signal peptide MF-α1 by Kex2, a furin-like protease, a DT or VCE fusion protein with its furin cleavage site replaced should be less toxic to *Pichia* than the corresponding wild type fusion proteins. Alternatively, VCE fusion proteins can be expressed in a mutant strain of *Pichia*, whose chromosomal EF-2 locus has been mutated to resist GDP ribosylation by catalytic domain of VCE (Liu et al., Protein Expr. Purif. 30:262 (2003)).

VCE fusion proteins may also be expressed in mammalian cells. Mutant cell lines that confer resistance to ADP-ribosylation have been described (Kohno and Uchida, J. Biol. Chem. 262 :12298 (1987); Liu et al., Protein Expr. Purif. 19:304 (2000); Shulga-Morskoy and Rich, Protein Eng. Des. Sel. 18:25 (2005)) and can be

used to express soluble VCE fusion proteins. For example, the toxin-resistant cell line CHO-K1 RE1.22c has been selected and used to express a DT-ScFv fusion protein (Liu et al., Protein Expr. Purif. 19:304 (2000)) and a mutant 293T cell line has been selected and used to express a DT-IL7 fusion protein (Shulga-Morskoy and Rich, Protein Eng. Des. Sel. 18:25 (2005)). It has been determined that a G-to-A transition in the first position of codon 717 of the EF-2 gene results in substitution of arginine for glycine and prevents post-translational modification of diphthamide at histidine 715 of EF-2, which is the target amino acid for ADP-ribosylation by DT. EF-2 produced by the mutant gene is fully functional in protein synthesis (Foley et al., Somat. Cell Mol. Genet. 18:227 (1992)). Based on this information and established methods such as described in *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 2005), different mammalian cells may be transfected with vectors containing G717A mutant of EF-2 gene and select for cells that are resistant to VCE.

Stable expression in mammalian cells also requires the transfer of the foreign DNA encoding the fusion protein and transcription signals into the chromosomal DNA of the host cell. A variety of vectors are commercially available, which typically contain phenotypic markers for selection in *E. coli* (Ap^r) and CHO cells (DHFR), a replication origin for E. coli, a polyadenylation sequence from SV40, a eukaryotic origin of replication such as SV40, and promoter and enhancer sequences. Based on methods described in *Current Protocols in Protein Science* (Coligan *et al.*, eds., 2006), and starting with the VCE-resistant cell lines, vectors containing DNA encoding VCE fusion proteins may be used to transfect host cells, which may be screened for high producers of the fusion proteins.

Because VCE is of bacterial origin, potential N-glycosylation sites within its sequence can be mutated in order to retain the cytotoxicity potential of native VCE. Further, glycosylation within cell-targeting domain can be avoided, maintaining its desirable binding characteristics. However, consensus N-glycosylation sites may be introduced to linkers or terminal sequences so that such glycosylation do not hamper the functions of VCE and cell-targeting moiety.

Various modifications have been described in the art that improved toxicity of PEA. These modifications are also useful for improving the toxicity of VCE immunotoxins.

Mere et al. J. Biol. Chem. 280: 21194-21201 (2005) teach that exposure to low endosomal pH during internalization of PEA triggers membrane insertion of its translocation domain, a process that is a prerequisite for PEA translocation to the cytosol where it inactivates protein synthesis. Membrane insertion is promoted by exposure of a key tryptophan residue (Trp 305). At neutral pH, this residue is buried in a hydrophobic pocket closed by the smallest α-helix (helix F) of the translocation domain. Upon acidification, protonation of the Asp that is the N-cap residue of the helix leads to its destabilization, enabling Trp side chain insertion into the endosome membrane. A mutant PEA in which the first two N-terminal amino acids (Asp 358 and Glu 359) of helix F replaced with non-acidic amino acids, showed destabilization of helix F, leading to exposure of tryptophan 305 to the outside of the molecule in the absence of an acidic environment and resulting in 7-fold higher toxicity than wild type PEA. Similarly, the mutant PEA in which entire helix F is removed was shown to exhibit 3- fold higher toxicity than wild type PEA. Although by sequence alignment, we did not find a helix corresponding to the helix F of PEA, we found that, similar to the proteolytic cleavage of PEA, cleavage of VCE by furin is favored in mildly acidic conditions, suggesting that a similar acid triggered conformational change might take place during membrane insertion of VCE. Mutations that facilitate membrane insertion of VCE, and thereby enhance cytotoxicity, might be found through means such as random mutagenesis.

Thus, preferable forms of VCE molecules for the present invention include those that exhibit more efficient membrane insertion, leading to higher toxicity.

One of the important factors determining the toxicity of the PEA-based or VCE-based immunotoxins depends on whether the immunotoxins are internalized by the target cell upon receptor binding. The internalization is considered the rate-limiting step in immunotoxin-mediated cytotoxicity (Li and Ramakrishnan. *J. Biol. Chem.* 269: 2652-2659 (1994)). He et al. fused Arg_9-peptide, a well known membrane translocational signal, to an anti-CEA (carcinoembryonic antigen) immunotoxin, PE35/CEA(Fv)/KDEL, at the position between the toxin moiety and the binding moiety. Strong binding and internalization of this fusion protein was observed in all detected cell lines, but little cytotoxicity to the cells that lack the CEA molecules on the cell surface was detected. However, the cytotoxicity beside the binding activity of the fusion protein to specific tumor cells expressing large amount of CEA molecules

on the cell surface was improved markedly, indicating that the Arg_9-peptide is capable of facilitating the receptor-mediated endocytosis of this immunotoxin, which leads to the increase of the specific cytotoxicity of this immunotoxin (He et al. *International Journal of Biochemistry and Cell Biology*, 37:192-205 (2005)). Accordingly, one preferred embodiment of protoxins that depend on translocation to the endoplasmic reticulum for intoxication includes the operable linkage of Arg9-peptide or related membrane translocation signals, such as, without limitation, those derived from HIV-Tat, Antennapedia, or Herpes simplex VP22, to such protoxins. A further preferred embodiment of the present invention includes modified PEA or VCE protoxins operably linked to Arg9-peptide or related membrane translocation signals, such as, without limitation, those derived from HIV-Tat, Antennapedia, or Herpes simplex VP22.

Toxicities that are independent of ligand binding have been observed with most targeted toxins. These include either hepatocyte injury causing abnormal liver function tests or vascular endothelial damage with resultant vascular leak syndrome (VLS). Both the hepatic lesion and the vascular lesion may relate to nonspecific uptake of targeted toxins by normal human tissues. U.S. Patent Application Publication No. 2006/0159708 A1 and U.S. Patent No. 6,566,500 describes methods and compositions relating to modified variants of diphtheria toxin and immunotoxins in general that reduce binding to vascular endothelium or vascular endothelial cells, and therefore, reduce the incidence of Vascular Leak Syndrome (VLS). In one example, variant of DT, V7AV29A, in which two of (X)D(Y) motifs are mutated is shown to maintain full cytotoxicity, but to exhibit reduced binding activity to human vascular endothelial cells (HUVECs), wherein the (X)D(Y) sequence is GDL, GDS, GDV, IDL, IDS, IDV, LDL,LDS, and LDV. U.S. Patent No. 5,705,156 teaches the use of modified PEA molecules in which 4 amino acids (57, 246, 247, 249) in domain I are mutated to glutamine or glycine to reduce nonspecific toxicity of PEA to animals. Hence one embodiment of the present invention includes modified VCE protoxins bearing sequence changes that favorably reduce toxicity to normal tissues.

The plasma half-lives of several therapeutic proteins have been improved using a variety of techniques such as those described by Collen et al., Bollod 71:216-219 (1998); Hotchkiss et al., Thromb. Haemostas. 60:255-261 (1988); Browne wt al., J. Biol. Chem. 263:1599-1602 (1988); Abuchowski et al., Cancer Biochem. Biophys.

7:175 (1984)). Antibodies have been chemically conjugated to toxins to generate immunotoxins which have increased half-lives in serum as compared with unconjugated toxins and the increased half-life is attributed to the native antibody. WO94/04689 teaches the use of modified immunotoxins in which the immunotoxin is linked to the IgG constant region domain having the property of increasing the half-life of the protein in mammalian serum. The IgG constant region domain is CH2 or a fragment thereof. Similar strategy can be applied to creating variants of VCE immunotoxin with increased serum half-life. In addition operable linkage to albumin, polyethylene glycol, or related nonimmunogenic polymers may promote the plasma persistence of therapeutic toxins.

Upon repeated treatment of immunotoxins, patients may develop antibodies that neutralize, hence lessen the effectiveness of immunotoxins. To circumvent the problem of high titer antibodies to a given immunotoxin, U.S. Patent No. 6,099,842 teaches the uses of a combination of immunotoxins bearing the same targeting principle, but differs in their cytotoxic moieties. In one example, anti-Tac(Fv)-PE40 and DT(1-388)-anti-Tac(Fv) immunotoxins are used in combination to reduced the possibility of inducing human anti-toxin antibodies. In principle, a similar strategy can be applied to the present invention wherein VCE fusions can be alternated with one or more other protoxins based on PEA and/or DT.

II. Translocation domain and catalytic domain of VCE

Extensive X-ray crystallographic and biochemical analyses have shown that ADP-ribosylating toxins such as PEA and VCE consist of well-defined domains including a cell binding domain, translocation domain, and ADP-ribosyl transferase domain (Wedekind et al. J. Mol. Biol. 314:823-837 (2001), Jorgensen et al. J. Biol. Chem. 283 (16):10671-10678 (2008), and references cited therein). It has been established that the cell-binding domain of these toxins may be deleted from the native toxin sequences and replaced with other cell targeting moieties such as antibodies, cytokines, and small molecules (Pastan et al. Annu. Rev. Med. 58:221-37 (2007) & Hilgenbrink and Low, J Pharm Sci. 94(10):2135-46 (2005)). The resulting immunotoxins have clinical utility in the treatment of oncologic diseases as first demonstrated by the successful development of diphtheria toxin-interleukin-2

conjugate (Ontak®) for the treatment of cutaneous T cell lymphoma (Foss, Clin. Lymphoma 1(2):110-6 (2000)).

Additionally, it has been disclosed that an ADPRT may be substituted by catalytic domains of other AB toxins. For example, it has been disclosed that a hybrid toxin may be generated by combining the catalytic ADP-ribosyltransferase domain of PEA (Gly405 to Lys613) with a ricin A chain, and appending it with C-terminus ER retention signal KDEL. This hybrid toxin showed similar cytoxicity to PEA. (Pitcher et al. Bioconj. Chem. 6:624-629 (1995)). The present invention provides the ADP-ribosyl transferase sequence of VCE as a catalytic, cytotoxic principle that may be used for applications in targeted cell killing in the context of the translocation domains of other toxins.

US Patent No. 6086900 disclosed the application of PEA lacking its intrinsic ADP-ribosyltransferase activity is useful for facilitating the transportation of a therapeutic agent that is attached to a non-terminal chemical binding site across a membrane into cytoplasm. Detoxification of PEA was achieved by deleting the glutamate residue in the active site of the catalytic domain at position 553. A cysteine residue was inserted into the deactivated catalytic domain to serve as a conjugation site for the molecule to be transported, a peptide nucleic acid (PNA). In another previously discussed example, the translocation domain of PEA (Gly253 to Asn364), along with a C-terminus ER retention signal KDEL(SEQ ID NO:10), enabled the observed cytoxicity of a recombinant hybrid toxin that utilizes ricin A chain as a cytotoxic agent (Pitcher et al. Bioconj. Chem. 6:624-629 (1995)). It has been shown that the translocation domain of PEA can support the introduction of barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, into the cytosol of mammalian cells (Prior et al. Biochemistry 31(14):3555-9 (1992)). Furthermore, the translocation domain of DT has been successfully utilized to transport Bad, a proapoptotic member of the Bcl-2 family, into human glioma cells and induce apoptosis (Ichinose et al. Cancer Res. 62(5):1433-8 (2002)).

The present invention provides the translocation domain sequence of VCE, which in combination with ER retention signal such as KDEL may be used to facilitate the transportation of various biological probes or therapeutic agents into the cytosol.

III. Disease Indications and Targeted Cell Surface Markers

The VCE protoxins disclosed in the present invention are useful for targeting and killing specific subsets of cells while sparing closely related cells, thereby providing a more specific and effective treatment for cancer. The targeting specificity of VCE protoxins can be conferred by progressively more selective cell targeting strategies: (i) replacing the native cell binding domain (residues 1-295) with a cell binding moiety that is operably linked and specifically targets a selected cell surface marker; (ii) replacing the native furin cleavage site (residues 321-326: RKPK↓DL) with a sequence recognized by a different protease that is overexpressed in the targeted cell; and (iii) combinatorial use of the modified VCE protoxin comprising both the selective cell surface binding moiety and an alternative proteolytic cleavage site with a protease fusion protein that is directed to the same cell by an operably linked cell surface binding moiety and that can specifically activate the protoxin.

The utility of the modified VCE protoxins lies in the selective elimination of subsets of cells to achieve a desired therapeutic effect. In particular the selectivity is provided by one or more cell-binding moieties, which can target cell surface targets on the targeted cancer cells, or on targeted noncancer cells that are preferably eliminated to achieve a therapeutic benefit.

A. Cell Surface Targets

Depending on the targeting strategy, modified VCE protoxins may be used alone, or in combination with protease fusion proteins. In any case, one or more of cell-targeting moieties can target a cell surface target typical of a specific type of cells, for example by recognizing lineage-specific markers found on subsets of cells and representing their natural origin, such as markers of the various organs of the body or specific cell types within such organs, or cells of the hematopoietic, nervous, or vascular systems. Alternatively one or more cell-targeting moieties can target cell surface markers aberrantly expressed on a diseased tissue, such as a cancer cell or a cell eliciting or effecting an autoimmune activity (e.g., B cells, T cells, dendritic cells, NK cells, neutrophils, leukocytes, macrophages, platelets, macrophages, myeloid cells, and granulocytes). One or more agents can target a cell surface marker that is aberrantly overexpressed by a cancer cell.

In particular, modified VCE protoxins, used alone or in combination with a protease fusion protein as heterologous activator, is used to destruct neoplastic or undesired cells selectively without severe damage to normal or desired cells, thereby providing treatments for cancers including leukemias and lymphomas, such as chronic B cell leukemia, mantle cell lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma, acute lymphoblastic leukemia, adult T-cell leukemia, Hodgkin's lymphoma, and non-Hodgkin's lymphoma; as well as solid tumors, including melanoma, colon cancer, breast cancer, prostate cancer, ovarian cancer, lung cancer, pancreatic cancer, kidney cancer, stomach cancer, liver cancer, bladder cancer, thyroid cancer, brain cancer, bone cancer, testicular cancer, uterus cancer, soft tissue tumors, nervous system tumors, and head and neck cancer.

Modified VCE protoxins can also be used to target non-cancerous cells, including autoreactive B or T cells, providing treatment for chronic inflammatory diseases including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, scleroderma, primary biliary cirrhosis, Graves' disease, Hashimoto's thyroiditis, type 1 diabetes, pernicious anemia, myasthenia gravis, Reiter's syndrome, immune thrombocytopenia, celiac disease, inflammatory bowel disease, and asthma and atopic disorders.

In addition the modified VCE protoxins can be used to ablate cells in the nervous system that are responsible for pathological or undesired activity, for example nociceptive neurons in the peripheral nervous system, or to treat sensory phantom sensation, or to control neuropathic pain, such as the pain caused by diabetic neuropathy or viral reactivation.

The modified VCE protoxins can also target cells infected by viral, microbial, or parasitic pathogens that are difficult to eradicate, providing treatment for acquired syndromes such as HIV, HBV, HCV or papilloma virus infections, tuberculosis, malaria, dengue, Chagas' disease, trypanosomiasis, leishmaniasis, or Lyme disease.

Furthermore, they can target specific cell types including, without limitation, parenchymal cells of the major organs of the body, as well as adipocytes, endothelial cells, cells of the nervous system , pneumocytes, B cells or T cells of specific lineage, dendritic cells, NK cells, neutrophils, leukocytes, macrophages, platelets,

macrophages, myeloid cells, granulocytes, adipocyte, and any other specific tissue cells.

The recombinant VCE fusion proteins and related protoxins can further target cells which produce disease through benign proliferation, such as prostate cells in benign prostatic hypertrophy, or in various syndromes leading to hyperproliferation of normal tissues or the expansion of undesired cellular compartments as for example of adipocytes in obesity.

It will be well recognized by those skilled in the art that there are many cell surface targets that may be used for targeting the modified VCE protoxins, and where applicable, its companion protease fusion protein to tumor tissues. For example, breast cancer cells may be targeted using overexpressed surface antigens such as claudin-3 (Soini, Hum. Pathol. 35:1531 (2004)), claudin-4 (Soini, Hum. Pathol. 35:1531 (2004)), MUC1 (Taylor-Papadimitriou et al., J. Mammary Gland Biol. Neoplasia 7:209 (2002)), EpCAM (Went et al., Hum. Pathol. 35:122 (2004)), CD24 (Kristiansen et al., J. Mol. Histol. 35:255 (2004)), and EphA2 (Ireton and Chen, Curr. Cancer Drug Targets 5:149 (2005); Zelinski et al.,Cancer Res. 61:2301 (2001)), as well as HER2 (Stern, Exp. Cell Res. 284:89 (2003)), EGFR (Stern, Cell Res. 284:89 (2003)), CEA, and uPAR (Han et al., Oncol. Rep. 14:105 (2005)). Colorectal cancer may be targeted using upregulated surface antigens such as A33 (Sakamoto et al., Cancer Chemother. Pharmacol. 46:S27 (2000)), EpCAM (Went et al., Hum. Pathol. 35:122 (2004)), EphA2 (Ireton and Chen, Curr. Cancer Drug Targets 5:149 (2005); Kataoka et al., Cancer Sci. 95:136 (2004)), CEA (Hammarstrom, Semin. Cancer Biol. 9:67 (1999)), CSAp, EGFR (Wong,Clin. Ther. 27:684 (2005)), and EphB2 (Jubb et al., Clin. Cancer Res. 11:5181 (2005)). Non-small cell lung cancer may be targeted using EphA2 (Kinch et al., Clin. Cancer Res. 9:613 (2003)), CD24 (Kristiansen et al., Br. J. Cancer 88:231 (2003)), EpCAM (Went et al., Hum. Pathol. 35:122 (2004)), HER2 (Hirsch et al., Br. J. Cancer 86:1449 (2002)), and EGFR (Dacic et al., Am. J. Clin. Pathol. 125:860 (2006)). Mesothelin has been targeted by a PEA based immunotoxin for the treatment of NSCLC (Ho et al., Clin. Cancer Res. 13(5):1571 (2007)). Ovarian cancer may be targeted using upregulated claudin-3 (Morin, Cancer Res. 65:9603 (2005)), claudin-4 (ibid.), EpCAM (Went et al., Hum. Pathol. 35:122 (2004)), CD24 (Kristiansen et al., J. Mol. Histol. 35:255 (2004)), MUC1 (Feng et al., Jpn. J. Clin. Oncol. 32:525 (2002)), EphA2 (Ireton and Chen, Curr. Cancer Drug

Targets 5:149 (2005)), B7-H4 (Simon et al., Cancer Res. 66:1570 (2006)), and mesothelin (Hassan et al., Appl. Immunohistochem Mol. Morphol. 13:243 (2005)), as well as CXCR4 (Jiang et al., Gynecol. Oncol. 20:20 (2006)) and MUC16/CA125. Pancreatic cancer may be targeted using overexpressed mesothelin (Rodriguez et al., World J. Surg. 29:297 (2005)), PSCA (Rodriguez et al., World J. Surg. 29:297 (2005)), CD24 (Kristiansen et al., J. Mol. Histol. 35:255 (2004)), HER2 (Garcea et al., Eur. J. Cancer 41:2213 (2005)), and EGFR (Garcea et al., Eur. J. Cancer 41:2213 (2005)). Prostate cancer may be targeted using PSMA (Kinoshita et al., World J. Surg. 30:628 (2006)), PSCA (Han et al., J. Urol. 171:1117 (2004)), STEAP (Hubert et al., Proc. Natl. Acad. Sci. USA 96:14523 (1999)), and EphA2 (Ireton and Chen, Curr. Cancer Drug Targets 5:149 (2005)). EpCAM is also upregulated in prostate cancer and has been targeted for the antibody-based treatment (Oberneder et al., Eu. J. Cancer 42:2530 (2006)). The expression of activated leukocyte cell adhesion molecule (ALCAM, as known as CD166) is a prognostic and diagnostic marker for prostate cancer (Kristiansen et al., J. Pathol. 205:359 (2005)), colorectal cancer (Weichert et al., J. Clin. Pathol. 57:1160 (2004)), and melanoma (van Kempen et al. Am. J. Pathol. 156(3):769 (2000)). All cancers that have been treated with chemotherapy and developed multidrug resistance (MDR) can be targeted using the transmembrane transporter proteins involved, including P-glycoprotein (P-gp), the multidrug resistance associated protein (MRP1), the lung resistance protein (LRP), and the breast cancer resistance protein (BCRP) (Tan et al., Curr. Opin. Oncol. 12:450 (2000)).

Significant advances have been made during the past decade in the identification of unique cell surface marker profiles of cancer stem cells from various cancers, distinguishing them from the bulk of corresponding tumor cells. For example, in acute myeloid leukemia (AML) it has been observed that the CD133+/CD38- AML cells, which constitute a small fraction of CD34+/CD38- AML cells, are responsible for initiating human AML in animal models (Yin et al., Blood 12:5002 (1997)). In addition, CD133 has been recently determined as a cancer stem cell surface marker for several solid tumors as well, including brain tumor (Singh et al., Nature 432:395 (2004) and Bao et al., Nature 444:756 (2006)), colon cancer (O'Brien et al., Nature 445:106 (2007) and Ricci-Vitiani et al, Nature 445:111 (2007)), prostate cancer (Rizzo et al., Cell Prolif. 38:363 (2005)), and heptocellular

carcinoma (Suetsugu et al., Biochem. Biophys. Res. Commun. 351:820 (2006) and Yin et al., Int. J. Cancer 120:1444 (2007)). In the case of colon cancer, the CD133+ tumorgenic cells were found to bind antibody Ber-EP4 (Ricci-Vitiani et al, Nature 445:111 (2007)), which recognizes the epithelial cell adhesion molecules (EpCAM), also known as ESA and CD326. More recently, it was reported that CD44+ may more accurately define the CSC population of colorectal cancer than CD133+ does, and the CSCs for colorectal cancer have been identified as $EpCAM^{high}$/CD44+/CD166+ (Dalerba et al., Proc. Natl. Acad. Sci. USA 104(24):10158 (2007)). Based on this information, EpCAM/CD133, EpCAM/CD44, EpCAM/CD166, and CD44/CD166 are possible combinations for combinatorial targeting of colon cancer CSCs. In addition to CD133, prostate cancer stem cells have been separately identified to be CD44+ (Gu et al. Cancer Res. 67:4807 (2007)), thus they may be targetable by using the CD44/CD133 pair of surface markers. Furthermore, CXCR4 was detected in the CD44+/CD133+ putative prostate CSCs, suggesting that the combination of CXCR4 with either CD44 or CD133 may provide useful pairs of targets for combinatorial targeting strategy. In other CSCs where the only currently known surface antigen is CD133, additional surface antigens may be identified through comprehensive antibody screening and then used to complement CD133 in a combinatorial targeting scheme. Likewise, tumorigenic cells for breast cancer have been identified as CD44+/CD24- subpopulation of breast cancer cells. Further analysis revealed that the CD44+/CD24-/EpCAM+ fraction has even higher tumorigenicity (Al-Hajj et al., Proc. Natl. Acad. Sci. USA 100:3983 (2003)). A combinatorial targeting approach using CD44+ and EpCAM+ as targeted surface markers could specifically kill these CSCs while leaving normal CD44+ leukocytes/erythrocytes and normal EpCAM+ epithelial cells unharmed. Another recent study has shown that pancreatic CSCs are CD44+/CD24+/EpCAM+ (Li et al., Cancer Res. 67:1030 (2007)). Consequently, the pancreatic CSCs may be targeted using a combination of CD44/CD24, CD44/EpCAM, or CD24/EpCAM.

B cell chronic lymphocytic leukemia (B-CLL) is characterized by slowly accumulating $CD5^{+}$ B cells (Guipaud et al., Lancet Oncol. 4:505 (2003)). CD5 is a cell surface protein found on normal T cells and a small fraction of B cells, known as B1 cells. Immunotoxins that target CD5 have shown high efficacy in killing T cells (Better et al., J. Biol. Chem. 270:14951 (1995)). The combinatorial targeting strategy

described in this invention makes it possible to use CD5 in combination with a B cell marker such as CD19, CD20, CD21, or CD22, thereby distinguishing B-CLL cells or other B cells in the B1 subset from T cells. The B1 subset is thought to give rise to low affinity polyreactive antibodies that are frequently found in the setting of autoimmune disorders, hence ablation of this population without significantly impairing the remainder of B cells could favorably impact the course of autoimmune disease without comprising the immune response of an individual to the same extent that ablation of all B cells would induce.

Examples of surface antigens that can be useful targets for the protease fusion and toxin fusion proteins of the invention are set forth in Table 1 of PCT Application Publication No. 2008/011157, which is herein incorporated by reference in its entirety. For combinatorial targeting each antigen may be targeted in combination with one or more other antigens. Examples for combinatorial use of two surface antigens for binary targeting are also shown.

B. Cell Targeting Moieties

The invention provides recombinant VCE fusion proteins and related protoxins containing a cell-targeting moiety. Such cell targeting moieties of the invention include proteins derived from antibodies, antibody mimetics, ligands specific for certain receptors expressed on a target cell surface, carbohydrates, and peptides that specifically bind cell surface molecules.

One embodiment of the cell-targeting moiety is a protein that can specifically recognize a target on the cell surface. The most common form of target recognition by proteins is antibodies. One embodiment employs intact antibodies in all isotypes, such as IgG, IgD, IgM, IgA, and IgE. Alternatively, the cell-targeting moiety can be a fragment or reengineered version of a full length antibody such as Fabs, Fab', Fab2, or scFv fragments (Huston, et al. 1991. Methods Enzymol. 203:46-88, Huston, et al. 1988. Proc Natl Acad Sci U S A. 85:5879-83). In one embodiment the binding antibody is of human, murine, goat, rat, rabbit, or camel antibody origin. In another embodiment the binding antibody is a humanized version of animal antibodies in which the CDR regions have grafted onto a human antibody framework (Queen and Harold. 1996. U.S. Patent No. 5530101). Human antibodies to human epitopes can be isolated from transgenic mice bearing human antibodies as well as from phage display

libraries based on human antibodies (Kellermann and Green. 2002. Curr Opin Biotechnol. 13:593-7, Mendez, et al. 1997. Nat Genet. 15:146-56, Knappik, et al. 2000. J Mol Biol. 296:57-86). The binding moiety may also be molecules from the immune system that are structurally related to antibodies such as reengineered T-cell receptors, single chain T-cell receptors, CTLA-4, monomeric Vh or Vl domains (nanobodies), and camelized antibodies (Berry and Davies. 1992. J Chromatogr. 597:239-45, Martin, et al. 1997. Protein Eng. 10:607-14, Tanha, et al. 2001. J Biol Chem. 276:24774-80, Nuttall, et al. 1999. Proteins. 36:217-27). A further embodiment may contain diabodies which are genetic fusions of two single chain variable fragments that have specificity for two distinct epitopes on the same cell. As an example, a diabody with an anti-CD19 and anti-CD22 scFv can be fused to protoxins in order to increase the affinity to B-cell targets (Kipriyanov. 2003. Methods Mol Biol. 207:323-33).

In another embodiment the cell-targeting moiety can also be diversified proteins that act as antibody mimetics. Diversified proteins have portions of their native sequence replaced by sequences that can bind to heterologous targets. Diversified proteins may be superior to antibodies in terms of stability, production, and size. One example is fibronectin type III domain, which has been used previously to isolate affinity reagents to various targets (Lipovsek and Pluckthun. 2004. J Immunol Methods. 290:51-67, Lipovsek, et al. 2007. J Mol Biol. 368:1024-41, Lipovsek, Wagner, and Kuimelis. 2004. U.S. Patent 20050038229) . Lipocalins have been used for molecular diversification and selection (Skerra et al. 2005. U.S. Patent 20060058510). Lipocalins are a class of proteins that bind to steroids and metabolites in the serum. Functional binders to CTLA4 and VEGF have been isolated using phage display techniques (Vogt and Skerra. 2004. Chembiochem. 5:191-9). C-type lectin domains, A-domains and ankyrin repeats provide frameworks that can be oligomerized in order to increase the binding surface of the scaffold (Mosavi, et al. 2004. Protein Sci. 13:1435-48). Other diversified proteins include and are not limited to human serum albumin, green fluorescent protein, PDZ domains, Kunitz domains, charybdotoxin, plant homeodomain, and β-lactamase. A comprehensive review of protein scaffolds is described in (Hosse, et al. 2006. Protein Sci. 15:14-27, Lipovsek. 2005.). Those skilled in the art understand that many diverse proteins or protein

domains have the potential to be diversified and may be developed and used as affinity reagents, and these may serve as cell-binding moieties in protoxins.

In another embodiment, the cell-targeting moiety can be a naturally occurring ligand, adhesion molecule, or receptor for an epitope expressed on the cell surface. Compositions of the ligand may be a peptide, lectin, hormone, fatty acid, nucleic acid, or steroid. For example, human growth hormone could be used as a cell-targeting moiety for cells expressing human growth hormone receptor. Solubilized receptor ligands may also be used in cases in which the natural ligand is an integral membrane protein. Such solubilized integral membrane proteins are well-known in the art and are easily prepared by the formation of a functional fragment of a membrane protein by removing the transmembrane or membrane anchoring domains to afford a soluble active ligand; for example, soluble CD72 may be used as a ligand to localize engineered protoxins to CD5 containing cells. Another example is the binding of urokinase type plasminogen activator (uPA) to its receptor uPAR. It has been shown that the region of u-PA responsible for high affinity binding ($K_d \approx 0.5$ nM) to uPAR is entirely localized within the first 46 amino acids called N-terminal growth factor like domain (N-GFD) (Appella, et al. 1987. J Biol Chem. 262:4437-40). Avimers refer to multiple receptor binder domains that have been shuffled in order to increase the avidity and specificity to specific targets (Silverman, et al. 2005. Nat Biotechnol. 23:1556-61). These receptor binding domains and ligands may be genetically fused and produced as a contiguous polypeptide with protoxins or they can be isolated separately and then chemically or enzymatically attached. They may also be non-covalently associated with protoxins.

In a previously reported example, Denileukin difitox is a fusion protein of DT and human interleukin (IL)-2 (Fenton and Perry. 2005 Drugs 65:2405). Denileukin difitox targets any cells that express IL-2 receptor (IL2R), including the intended target CTCL cells. Acute hypersensitivity-type reactions, vascular leak syndrome, and loss of visual acuity have been reported as side effects. Because human normal non-hematopoietic cells of mesenchymal and neuroectodermal origin may express functional IL2R, some cytotoxic effects observed could be due to a direct interaction between IL-2 and non-hematopoietic tissues. In order to overcome this toxicity, the invention features, for example, addition of a T cell marker as a second targeting element, e.g., CD3.

If the moiety is a carbohydrate such as mannose, mannose 6-phosphate, galactose, N-acetylglucosamine, or sialyl-Lewis X, it can target the mannose receptor, mannose 6-phosphate receptor, asialoglycoprotein receptor, N-acetylglucosamine receptor, or E-selectin, respectively. If the moiety comprises a sialyl-Lewis X glycan operably linked to a tyrosine sulfated peptide or a sulfated carbohydrate it can target the P-selectin or L-selectin, respectively.

As another example, the binding partners may be from known interactions between different organisms, as in a pathogen host interaction. The C-terminal domain of the *Clostridium perfringens* enterotoxin (C-CPE) binds with high affinity and specificity to the mammalian claudin3/4 adhesion molecules. Although claudins are components of most cells tight junctions, they are not typically exposed on the apical surface. The C-CPE can be appended to protoxins in order to localize them to cells overexpressing unengaged claudin3/4, a condition of many types of cancers (Takahashi, et al. 2005. J Control Release. 108:56-62, Ebihara, et al. 2006. J Pharmacol Exp Ther. 316:255-60).

An example of a peptide is the use of angiotensin to localize complexes to cells expressing angiotensin receptor. In another embodiment, the binding peptide could be an unnatural peptide selected from a random sequence library. One group has identified a peptide using phage display, termed YSA, which can specifically recognize EphA2 receptors. EphA2 is overexpressed in many breast cancers (Koolpe, et al. 2005. J Biol Chem. 280:17301-11, Koolpe, et al. 2002. J Biol Chem. 277:46974-9). In order to increase binding affinity, peptides could be multimerized through sequential repeated fusions or attaching them to a dendrimer which can then be attached to protoxins.

In another embodiment, the cell-targeting moiety can be a nucleic acid that consists of DNA, RNA, PNA or other analogs thereof. Nucleic acid aptamers have been identified to many protein targets and bind with very high affinity through a process of in vitro evolution (Gold. 1991. U.S. Patent 5475096, Wilson and Szostak. 1999. Annu Rev Biochem. 68:611-47). RNA aptamers specific for PSMA were shown to specifically localized conjugated gelonin toxin to cells overexpressing PSMA (Chu, et al. 2006. Cancer Res. 66:5989-92). The nucleic acid can be chemically synthesized or biochemically transcribed and then modified to include an attachment group for conjugation to the reengineered toxin. The nucleic acid may be

directly conjugated using common crosslinking reagents or enzymatically coupled by processes known in the art. The nucleic acid can also be non-covalently associated with protoxins.

The cell-targeting moiety may be identified using a number of techniques described in the art. Typically natural hormones and peptide ligands can be identified through reported interactions in the reported literature. Additionally, antibody mimics and nucleic acid aptamers can be identified using selection technologies that can isolate rare binding molecules toward epitopes of interest, such as those expressed on cancer cells or other diseased states. These techniques include SELEX, phage display, bacterial display, yeast display, mRNA display, in vivo complementation, yeast two-hybrid system, and ribosome display (Roberts and Szostak. 1997. Proc Natl Acad Sci U S A. 94:12297-302, Boder and Wittrup. 1997. Nat Biotechnol. 15:553-7, Ellington and Szostak. 1990. Nature. 346:818-22, Tuerk and MacDougal-Waugh. 1993. Gene. 137:33-9, Gyuris, et al. 1993. Cell. 75:791-803, Fields and Song. 1989. Nature. 340:245-6, Mattheakis, et al. 1994. Proc Natl Acad Sci U S A. 91:9022-6). Antibodies can be generated using the aforementioned techniques or in a traditional fashion through immunizing animals and isolating the resultant antibodies or creating monoclonal antibodies from plasma cells.

The targets of the cell-targeting moieties can be protein receptors, carbohydrates, and lipids on or around the cell surface. Examples of polypeptide modifications known in the art that may advantageously comprise elements of a cell surface target include glycosylation, sulfation, phosphorylation, ADP-ribosylation, and ubiquitination. Examples of carbohydrate modifications that may be distinctive for a specific lineage of cells include sulfation, acetylation, dehydrogenation and dehydration. Examples of lipid modification include glycan substitution and sulfation. Examples of lipids that may be distinctive for a specific targeted cell include sphingolipids and their derivatives, such as gangliosides, globosides, ceramides and sulfatides, or lipid anchor moieties, such as the glycosyl phosphatidyl inositol-linked protein anchor.

The cell-targeting moiety may indirectly bind to the target cell through another binding intermediary that directly binds to a cell surface epitope, as long as the cell-targeting moiety acts to localize the reengineered toxin to the cell surface. The targets of these binding modules may be resident proteins, receptors, carbohydrates, lipids,

cholesterol, and other modifications to the target cell surface. The cell-targeting moiety can be joined to protoxins either through direct translational fusions if the DNA encoding both species is joined. Alternatively, chemical coupling methods and enzymatic crosslinking can also join the two components. The cell-targeting moiety may contain sequences not involved in the structure or binding of the agent, but involved with other processes such as attachment or interaction with protoxins.

IV. Modification of Activation Sequence

VCE protoxins according to the present invention comprise modifications of the naturally occurring activation sequence of the modified VCE permitting activation of the modified VCE in a variety of different cancer types. The modified activation sequence comprises one or more general cleavage site modifications, or a plurality of specific cleavage site modifications, resulting in a single modified VCE that is capable of being activated to kill numerous types of cancer cells.

A. Endogenous protease activity present in targeted cells

Modified VCE protoxins with one or more general cleavage site modifications comprise a modification of the naturally occurring activation sequence to provide one or more cleavage sites for a general activating agent, a general activating agent is an enzyme, the presence of which is associated with a variety of different cancer types. For example, the expression of the enzyme can be up-regulated in a cancer cell compared to a normal cell, or the enzyme can be localized to cancer cells as compared to normal cells, or the enzyme may be produced and/or activated by cancer associated tissue or cells. A general activating agent may be, for example, a protease.

In one embodiment, the modified VCE protoxin comprises an activation sequence modified to include two or more general cleavage sites, each of the general cleavage sites can be cleaved by the same general activating agent. Alternatively, each of the general cleavage sites can be cleaved by a different activating agent. When more than one general cleavage site is present, these cleavage sites may either be adjacent to each other, may overlap or may be separated by intervening sequences of varying lengths as is known in the art. In another embodiment, the modified VCE protoxin comprises an activation sequence modified to include one general cleavage site. In still another embodiment, the modified VCE protoxin comprises an activation

sequence modified to include two general cleavage sites. In yet another embodiment, the modified VCE protoxin comprises an activation sequence modified to include less than five general cleavage sites.

The one or more general cleavage site modifications to the naturally occurring activation sequence may be achieved as is known in the art. This modification results in functional deletion of the naturally occurring activation sequence, or of one or more naturally occurring cleavage sites in the activation sequence. Functional deletion is achieved by mutation, which can result in, for example, partial or complete deletion, insertion, or other variation made to the naturally occurring activation sequence that renders it inactive. In one embodiment, the native activation sequence of VCE may be functionally deleted by insertion of one or more general cleavage site. In another embodiment, functional deletion of the naturally occurring activation sequence, or of one or more naturally occurring cleavage sites in the activation sequence is achieved via mutations in one or more amino acid residues of the native activation sequence which result in the creation of one or more general cleavage sites, each of which can be cleaved by a general activating agent. In an alternate embodiment, the native activation sequence of VCE is functionally deleted by replacing the naturally occurring activation sequence, or one or more naturally occurring cleavage sites in the activation sequence with one or more general cleavage sites, each of which can be cleaved by a general activating agent. As described above, the modified VCE protoxin according the present invention comprise one or more general cleavage site modifications that provide one or more cleavage sites, each recognized by a general activating agent that is a protease, the presence of which is associated with a variety of different cancer types. In one embodiment of the invention, the general activating agent is a protease that is associated with cancer invasion and metastasis in general.

Examples of such proteases include the matrix metalloproteinase (MMP) family, the caspases, elastase, and the plasminogen activator family, as well as fibroblast activation protein. Members of the MMP family include collagenases, stromelysin, gelatinases, and 5 membrane-type metalloproteases. In particular, MMP-2 (gelatinase A), MMP-9 (gelatinase B), and membrane-type 1 MMP (MTl-MMP) have been reported to be most related to invasion and metastasis in various human cancers. Examples of proteases of the plasminogen activator family include uPA (urokinase-type plasminogen activator) and tPA (tissue-type plasminogen activator).

In another embodiment, the protease is up-regulated and/or secreted by cancer cells.

Examples of these proteases include matrix metalloproteases, some cathepsins, tPA, some caspases, kallikreins, elastase, plasmin, thrombin, and uPAIn a further embodiment, the protease is activated by enzymes expressed by cancer cells. In still another embodiment, the protease is activated by receptors expressed by cancer cells. A non-limiting example of such a protease is uPA (e.g., with a cleavage site of: SGRSAQ), which is activated by the receptor uPAR (urokinase-type plasminogen activator receptor).

In another embodiment, the general activating agent is a protease that is associated with angiogenesis in general (e.g., matrix metalloproteases and caspases).

Modified VCE protoxin with a plurality of specific cleavage site modifications comprise modification of the naturally occurring activation sequence to include two or more different types of specific cleavage sites, each type capable of being cleaved by a specific activating agent. The two or more different types of cleavage sites may further comprise a cleavage site for a general activating agent. A specific activating agent is an enzyme, the presence of which is associated with a specific type of cancer. For example, expression of the enzyme can be up-regulated in a specific type of cancer cell, or the enzyme can be localized to a specific type of cancer cell, or the enzyme may be produced by a cell that is associated with a specific type of cancer. A specific activating agent may be, for example, a protease.

Modifications comprising a plurality of specific cleavage sites may be achieved as is known in the art, and described above. This modification also results in functional deletion of the naturally occurring activation sequence, or of one or more naturally occurring cleavage sites in the activation sequence. In one embodiment, the native activation sequence of the VCE is functionally deleted by insertion of a plurality of specific cleavage sites. In another embodiment, functional deletion of the naturally occurring activation sequence is achieved via mutations in the amino acid sequence of the naturally occurring activation sequence, resulting in the addition of two or more specific cleavage sites, each of which can be cleaved by a specific activating agent. In an alternate embodiment, the native activation sequence of the VCE may be replaced with two or more specific cleavage sites, each of which is capable of being cleaved by a specific activating agent. As is known in the art, the

specific cleavage sites may either be adjacent to each other, may overlap or may be separated by intervening sequences of varying lengths as is known in the art.

In another embodiment of the invention, the plurality of specific cleavage site modifications adds two or more cleavage sites, each of which is recognized by a specific activating agent that is a protease. In another embodiment of the invention, the specific activating agent is a protease that is associated with invasion and metastasis of a specific cancer. In a further embodiment of the invention, the specific activating agent is a protease, the expression of which is up-regulated in a specific cancer. In still another embodiment, the specific activating agent is a protease that is produced by a cell that is associated with a specific cancer.

In another embodiment, the specific activating agent is a protease that is associated with colon cancer.

B. Proteolytic activity delivered to the targeted cells

A major limitation of previously described approaches to targeting cells is their reliance on endogenous proteases, which may not be present on all tumors, or may be present in inadequate abundance, or may be shed in substantial quantities, leading to nonspecific activation of the toxin. The present invention also provides VCE protoxins that are activatable by protease fusions that are independently brought to the targeted cells through specific binding to the cell surface; these VCE protoxins are useful for targeted destruction of tumor cells or other undesired cells that have no appropriate endogenous protease activity.

An exogenous protease and corresponding cleavage site may be chosen for the present invention based on the following considerations. The protease is preferably capable of cleaving a protoxin activation moiety without significantly inactivating the protoxin or itself. The protease is preferably not naturally found in or on cells that are desired to be spared, with the exception that the protease can be naturally found in such cells if its natural location does not allow it to activate an externally administered protoxin. For example, an intracellular protease such as a caspase may be used if the toxin must be activated at the surface of the cell or in some intracellular vesicular compartment that does not naturally contain the intracellular protease, such as the endosome, golgi, or endoplasmic reticulum. In such cases the cells that are desired to be spared could contain the protease but the protease would not activate the protoxin.

The catalytic activity of the protease is preferably stable to in vivo conditions for the time required to exert its therapeutic effect in vivo. If the therapeutic program requires the repeat administration of the protease, the protease is preferably resistant to interference by the formation of antibodies that impair its function, for example neutralizing antibodies. In some embodiments the protease has low immunogenicity or can be optionally substituted to reduce immunogenicity or can be optionally substituted to reduce the effect of antibodies on its activity. The protease preferably has low toxicity itself or has low toxicity in the form of its operable linkage with one or more cell surface binding moieties. The protease is preferably stable or can be made to be stable to conditions associated with the manufacturing and distribution of therapeutic products. The protease is preferably a natural protease, a modified protease, or an artificial enzyme.

Desirable proteases of the present invention include those known to have highly specific substrate selectivities, either by virtue of an extended catalytic site or by the presence of specific substrate-recognition modules that endow a relatively nonselective protease with appropriate specificity. Proteases of limited selectivity can also be made more selective by genetic mutation or chemical modification of residues close to the substrate-binding pocket.

As is known in the art, many proteases recognize certain cleavage sites, and some specific, non-limiting examples are given below. One of skill in the art would understand that cleavage sites other than those listed are recognized by the listed proteases, and can be used as a general protease cleavage site according to the present invention.

Proteases of human origin are preferred embodiments of the present invention due to reduced risk of immunogenicity. A human protease utilizing any catalytic mechanism, i.e., the nature of the amino acid residue or cofactor at the active site that is involved in the hydrolysis of the peptides and proteins, including aspartic proteases, cysteine proteases, metalloproteases, serine proteases, and threonine proteases, may be useful for the present invention.

Because model studies of a potential therapeutic agent must be conducted in animals to determine such properties as toxicity, efficacy, and pharmacokinetics prior to clinical trials in human, the presence of proteinase inhibitors in the plasma of animals could also limit the development of therapeutics comprising proteolytic

activities. The proteinase inhibitors in animal plasma can possess inhibitory properties that are different from their human counterparts. For example human GrB has been found to be inhibited by mouse serpina3n, which is secreted by cultured Sertoli cells and is the major component of serpina3 (α_1-antichymotrypsin) present in mouse plasma (Sipione et al., J. Immunol. 177:5051–5058 (2006)). However, the human α_1-antichymotrypsin has not been shown to be an inhibitor of human GrB. The difference between mouse and human plasma protease inhibitors may be traced to their genetic differences. Whereas the major human plasma protease inhibitors, α_1-antitrypsin and α_1-antichymotrypsin, are each encoded by a single gene, in the mouse they are represented by clusters of 5 and 14 genes, respectively. Even though there is a high degree of overall sequence similarity within these clusters of inhibitors, the reactive-center loop (RCL) domain, which determines target protease specificity, is markedly divergent. To overcome inhibition by mouse proteases, the screening and mutagenesis strategies described herein can be applied to identify mutant proteases that are resistant to inhibition by inhibitors present in the animal model of choice.

Human granzymes

Recombinant human granzyme B (GrB) may be used as an exogenous protease within the protease fusion protein. GrB has high substrate sequence specificity with a consensus recognition sequence of IEPD and is known to cleave only a limited number of natural substrates. GrB is found in cytoplasmic granules of cytotoxic T-lymphocytes and natural killer cells, and thus should be useful for the present invention provided these cells are not the targeted cells. The optimum pH for GrB activity is around pH 8, but it retains its activity between pH 5.5 and pH 9.5 (Fynbo et al., Protein Expr. Purif. 39:209 (2005)). GrB cleaves peptides containing IEPD (SEQ ID NO:18) with high efficiency and specificity (Harris et al., J. Biol. Chem. 273:27364 (1998)). Because GrB is involved in regulating programmed cell death, it is tightly regulated *in vivo*. In addition, GrB is a single chain and single domain serine protease, which could contribute to a simpler composite structure of the fusion protein. Moreover, GrB has recently been found to be very stable in general, and it performs very well in the cleavage of different fusion proteins (Fynbo et al., Protein Expr. Purif. 39:209 (2005)).

Any member of the granzyme family of serine proteases, e.g., granzyme A and granzyme M, may be used as the recombinant protease component of the protease fusion in this invention. For example, granzyme M (GrM) is specifically found in the granules of natural killer cells and can hydrolyze the peptide sequence KV(Y)PL(M) (SEQ ID NO:21)with high efficiency and specificity (Mahrus et al., J. Biol. Chem. 279:54275 (2004)).

In designing and utilizing protease fusions of the invention, it should be noted that proteinase inhibitors may hamper the proteolytic activities of protease fusion proteins. For example, GrB is specifically inhibited by intracellular proteinase inhibitor 9 (PI-9), a member of the serpin superfamily that primarily exists in cytotoxic lymphocytes (Sun et al., J. Biol. Chem. 271:27802 (1996)) and has been detected in human plasma. GrB can also be inhibited by α_1-protease inhibitor (α_1PI) that is present in human plasma (Poe et al., J. Biol. Chem. 266:98 (1991)). GrM is inhibited by α_1-antichymotrypsin (ACT) and α_1PI (Mahrus et al., J. Biol. Chem. 279:54275 (2004)), and GrA is inhibited *in vitro* by protease inhibitors antithrombin III (ATIII) and α2-macroglobulin (α_2M) (Spaeny-Dekking et al., Blood 95:1465 (2000)). These proteinase inhibitors are also present in human plasma (Travis and Salvesen, Annu. Rev. Biochem. 52:655 (1983)).

One approach to preserve proteolytic activities of granzymes is to utilize complexation with proteoglycan, since the mature and active form of GrA has been observed in human plasma as a complex with serglycin, a granule-associated proteoglycan (Spaeny-Dekking et al., Blood 95:1465 (2000)). Glycosaminglycan complexes of GrB have also been found proteolytically active (Galvin et al., J. Immunol. 162:5345 (1999)). Thus, it is possible to keep granzyme fusion proteins active in plasma through formulations using chondroitin sulfates.

Cathepsins and Caspases

Any member of the cathepsins (Chwieralski et al., Apoptosis 11:143 (2006)), e.g., cathepsin A, B, C, D, E, F, G, H, K, L, S, W, and X, may also be used as the recombinant protease for the present invention. Cathepsins are proteases that are localized intralysosomally under physiologic conditions, and therefore have optimum activity in acidic environments. Cathepsins comprise proteases of different enzyme classes; e.g., cathepsins A and G are serine proteases, cathepsins D and E are aspartic

proteases. Certain cathepsins are caspases, a unique family of cysteine proteases that play a central role in the initiation and execution phases of apoptosis. Among all known mammalian proteases, only the serine protease granzyme B has substrate specificity similar to the caspases.

A cathepsin or caspase can be used as an exogenous activator or proactivator only if the protoxin to be activated is not exposed to that cathepsin or caspase prior to internalization (in the case of toxins that must be internalized) or during the course of the natural formation of the active toxin. For example, the protoxins of pore-forming toxins are activated at the cell surface, followed by oligomerization and pore formation. Because pore forming toxins do not localize to lysosome, cathepsins and caspases can be applied as exogenous activators. On the other hand, because the A-B toxin DT is known to be translocated directly into the cytosol through the endosome and/or lysosome, where cathepsins naturally reside, cathepsins should not be used as exogenous activators for DT-based protoxins. VCE-based toxins may be compatible with the use of lysosomal proteases as exogenous activators, because they are transported to the trans-Golgi network and the ER before the translocation into cytosol. All caspases, including caspase-1, -2, -3, -4, -5, -6, -7, -8, -9 and more, show high selectivity and cleave proteins adjacent to an aspartate residue (Timmer and Salvesen, Cell Death Diff. 14:66-72 (2007)). The preferred cleavage site for caspase-1, 4, -5, and -14 are (W/Y)EXD↓Φ, where X is any residue and Φ represents a Gly, Ala, Thr, Ser, or Asn (SEQ ID NO:22). The preferred substrate for caspase-8, -9, and -10 contains the sequence of (I/L)EXD↓Φ (SEQ ID NO:23), and that of caspase-3 and -7 contains DEXD↓Φ (SEQ ID NO:24). Caspase-6 preferably cleaves at VEXD↓Φ (SEQ ID NO:25), while caspase-2 selectively targets (V/L)DEXD↓Φ (SEQ ID NO:26). Because the naturally occurring inhibitors of caspases, e.g., IAPs, are usually located intracellularly (LeBlanc, Prog. Neuropsychopharmacol. Biol. Psychiatry 27:215 (2003)), the probability of inhibition in plasma is dramatically reduced. Although caspase-1 and caspase-4 can be inhibited by PI-9 at moderate rates, it does not inhibit caspase-3 (Annand et al., Biochem. J. 342:655 (1999)).

Other Human Proteases

Many human proteases, including those have been identified as certain disease markers secreted by diseased cells, or associated with cancer invasion and metastasis, may be useful for the present invention as the heterologous protease. These proteases are well studied and detailed information on proteolytic activity and sequence selectivity is available. Examples of such proteases include urokinase plasminogen activator (uPA), which recognizes and cleaves GSGR↓SA (SEQ ID NO:27); prostate-specific antigen (PSA), which prefers substrate sequence SS(Y/F)Y↓SG (SEQ ID NO:28); renin, which cleaves at HPFHL↓VIH (SEQ ID NO:29); and MMP-2, which can cleave at HPVG↓LLAR (SEQ ID NO:30). Additional examples include the caspases, elastase, kallikreins, the matrix metalloprotease (MMP) family, the plasminogen activator family, as well as fibroblast activation protein.

In certain cases, the protease involved in one disease may be useful for the treatment of another disease that does not usually involve its overexpression. In other instances, the concentration of the secreted protease at native level may not be sufficient to activate corresponding toxin fusion to the extent that is necessary for targeted cell killing, i.e., is not operably present on the targeted cells. Additional proteolytic activity delivered to the cells through targeted protease fusion would provide desired toxin activation. In one embodiment, the protease fusion could have the same sequence specificity as the protease secreted by the diseased cells. In another embodiment, it may be desirable to use a combination of multiple, different, proteolytic cleavage activities to increase overall cleavage efficiency, with at least one of the proteolytic activity being provided by a targeted protease fusion.

Additional examples of endogenous proteases include those have been identified as certain disease markers, which are upregulated in certain disease. Non-limiting examples of such proteases include urokinase plasminogen activator (uPA), which recognizes and cleaves GSGR↓SA (SEQ ID NO:31); prostate-specific antigen (PSA), which prefers substrate sequence SS(Y/F)Y↓SG (SEQ ID NO:32); renin, which cleaves at HPFHL↓VIH (SEQ ID NO:33); and MMP-2, which can cleave at HPVG↓LLAR (SEQ ID NO:34). Although the use of such designed zymogens may not be optimal for protease such as GrB, which requires a specific N-terminus amino acid Ile to be active, such in vivo activation process may be applicable to other proteases and activating enzymes.

Alternatively, potential candidate proteases may be screened *in vitro* by interactions with known proteinase inhibitors in plasma or with human plasma directly to avoid potential complications posed by these proteinase inhibitors. Alternatively, proteases for which cognate inhibitors are found in plasma can be engineered to provide mutant forms that resist inhibition. For example, *in vitro* *E. coli* expression-screening methods have been developed to select mutant proteases that are resistant to known HIV-1 protease inhibitors (Melnick et al., Antimicrob. Agents Chemother. 42:3256 (1998)).

Retroviral proteases may also be used for the present invention. Human retroviral proteases, including that of human immunodeficiency virus type 1 (HIV-1) (Beck et al., 2002), human T cell leukemia viruses (HTLV) (Shuker et al., Chem. Biol. 10:373 (2003)), and have been extensively studied as targets of anti-viral therapy. These proteases often have long recognition sequences and high substrate selectivity.

Picornaviral proteases may also be used for the present invention. Such picornaviral proteases have been studied as targets of anti-viral therapy, for example human Rhinovirus (HRV) (Binford et al., Antimicrob. Agents Chemother. 49:619 (2005)),

Recombinant heterologous proteases of any origin may be engineered to possess the aforementioned qualities and be used for the present invention. For example, tobacco etch virus (TEV) protease has very high substrate specificity and catalytic efficiency, and is used widely as a tool to remove peptide tags from recombinant proteins (Nunn et al., J. Mol. Biol. 350:145 (2005)). TEV protease recognizes an extended seven amino acid residue long consensus sequence E-X-X-Y-X-Q↓S/G (where X is any residue) (SEQ ID NO:35) that is present at protein junctions. Those skilled in the art would recognize that it is possible to engineer a particular protease such that its sequence specificity is altered to prefer another substrate sequence (Tozser et al., FEBS J. 272:514 (2005)).

Further modifications can be engineered to increase the activity and/or specificity of proteases. These modifications include PEGylation to increase stability to serum or to lower immunogenicity, and genetic engineering/selection may produce mutant proteases that possess altered properties such as resistance to certain inhibitors, increased thermal stability, and improved solubility.

Retroviral Proteases

Recombinant human retroviral proteases may also be used for the present invention. Human retroviral proteases, including that of human immunodeficiency virus type 1 (HIV-1) (Beck et al., 2002), human T cell leukemia viruses (HTLV) (Shuker et al., Chem. Biol. 10:373 (2003)), and severe acute respiratory syndrome coronavirus (SARS), have been extensively studied as targets of anti-viral therapy. These proteases often have long recognition sequences and high substrate selectivity. For example, SQNY↓PIV (SEQ ID NO:36) was determined as a preferred cleavage sequence of HIV-1 protease (Beck et al. Curr. Drug Targets Infect. Disord. 2(1):37-50 (2002), the preferred cleavage sequence for HTLV protease has been determined to be PVIL↓PIQA (SEQ ID NO:37) (Naka et al. Bioorg. Med. Chem. Lett. 16(14):3761-3764 (2006).

Coronaviral Proteases

Coronaviral or toroviral proteases are encoded by members of the animal virus family Coronaviridae and exhibit high cleavage specificity. Such proteases are another preferred embodiment for the present invention. The SARS 3C-like protease has been found to selectively cleave at AVLQ↓SGF (SEQ ID NO:38) (Fan et al. Biochem. Biophys. Res. Commun. 329(3):934-940 (2005)).

Picornaviral Proteases

Picornaviral proteases may also be used for the present invention. Such picornaviral proteases have been studied as targets of anti-viral therapy, for example human Rhinovirus (HRV) (Binford et al., Antimicrob. Agents Chemother. 49:619 (2005)). HRV 3C protease recognizes and cleaves ALFQ↓GP (SEQ ID NO:39) (Cordingley et al. J. Biol. Chem. 265(16):9062-9065 (1990)).

Potyviral Proteases

Potyviral proteases are encoded by members of the plant virus family Potyviridae and exhibiting high cleavage specificity, and are another preferred embodiment for the present invention. For example, tobacco etch virus (TEV) protease has very high substrate specificity and catalytic efficiency, and is used widely as a tool to remove peptide tags from overexpressed recombinant proteins

(Nunn et al., J. Mol. Biol. 350:145 (2005)). TEV protease recognizes an extended seven amino acid residue long consensus sequence E-X-X-Y-X-Q↓S/G (where X is any residue) that is present at protein junctions (SEQ ID NO:40). Those skilled in the art would recognize that it is possible to engineer a particular protease such that its sequence specificity is altered to prefer another substrate sequence (Tozser et al., FEBS J. 272:514 (2005)).

Proteases of Other Origins

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide range of sources such as plants, animals, and microorganisms (Rao et al. Microbiol. Mol. Biol. Rev. 62(3):597-635 (1998)). All these proteases are potential candidates for the present invention. In a preferred embodiment, PEGylation may be utilized to reduce the immunological potential of fusion proteases for the present invention, particularly for those that are of non-human origins. PEGylation may confer additional benefits to protease fusion proteins, such as improved plasma persistence and reduced non-specific cell binding.

Additional proteases can be found, for example, in PCT Application Publication No. 2008/011157, which is herein incorporated by reference in its entirety.

V. Linkages

According to the present invention, each moiety within a protoxin fusion protein(e.g., one or more cell targeting moieties, one or more selectively modifiable activations domains, one or more natively activatable domain, and one or more toxin domains) or a protoxin activator fusion, (e.g., one or more cell targeting moieties, one or more modification domains, one or more natively activatable domain, and one or more toxin domains) may function independently but each is operably linked. Within each fusion protein the operable linkage between the two functional moieties acts as a molecular bridge, which may be covalent or non-covalent. The moieties of each fusion protein may be operably linked in any orientation with respect to each other, that is, C-terminal of one to N-terminal of the other, or C-terminal of one to C-terminal of the other, or N-terminal of one to N-terminal of the other, or by internal residues to terminal residues or internal residues to internal residues. An optional

linker can serve as a glue to physically join the two moieties, as a separator to allow spatial independence, or as a means to provide additional functionality to each other, or a combination thereof. For example, it may be desirable to separate the cell-targeting moiety from the operably linked enzyme moiety to prevent them from interfering with each other's activity. In this case the linker provides freedom from steric conflict between the operably linked moieties. The linker may also provide, for example, lability to the connection between the two moieties, an enzyme cleavage site (e.g., a cleavage site for protease or a hydrolytic site for esterase), a stability sequence, a molecular tag, a detectable label, or various combinations thereof.

Chemical activation of amino acid residues can be carried out through a variety of methods well known in the art that result in the joining of the side chain of amino acid residues on one molecule with side chains of residues on another molecule, or through the joining of side chains to the alpha amino group or by the joining of two or more alpha amino groups. Typically the joining induced by chemical activation is accomplished through a linker which may be a small molecule, an optionally substituted branched or linear polymer of identical or nonidentical subunits adapted with specific moieties at two or more termini to attach to polypeptides or substitutions on polypeptides, or an optionally substituted polypeptide. Examples of common covalent protein operable linkage agents may be found in various vendor offerings, including those offered for sale by Pierce Chemical Corporation. In general it is preferable to be able to induce operable linkage of components in a site-specific manner, to afford a simple reproducibly manufactured substance. Operable linkage by chemical activation can be the result of chemical activation targeted to specific residues that are functionally unique i.e. are present only once in the moiety to be activated or are preferentially activatable because of a unique chemical environment, for example, such as would produce a reduction in pK of an epsilon amino unit of a lysine residue. Potential groups for chemical activation can be made functionally unique by genetic removal of all other residues having the same properties, for example to remove all but a single cysteine residue, or all but a single lysine reside. Amino terminal residues can be favorably targeted by virtue of the low pK of the alpha amino group, or by suitable chemistry exploiting the increased reactivity of the alpha amino group in close proximity to another activatable group. Examples of the latter include native chemical ligation, Staudinger ligation, and oxidation of amino

terminal serine to afford an aldehyde substituent. Chemical activation can also be carried out through reactions that activate naturally occurring protein substituents, such as oxidation of glycans, or other naturally occurring protein modifications such as those formed by biotin or lipoic acid, or can be based on chemical reactions that convert the functionality of one side chain into that of another, or that introduce a novel chemical reactive group that can subsequently activated to produce the desired operable linkage. Examples of the latter include the use of iminodithiolane to endow a lysine residue with a sulfhydryl moiety or the reaction of a cysteine moiety with an appropriate maleimide or haloacetamide to change the functionality of the thiol to another desired reactive moiety. Chemical activation can also be carried out on both species to be operably linked to provide reactive species that interact with one another to provide an operable linkage, for example the introduction of a hydrazide, hydrazine or hydroxylamine on one moiety and an aldehyde on the other.

Noncovalent operable linkage can be obtained by providing a complementary surface between one moiety and another to provide a complex which is stable for the intended useful persistence of the operably linked moieties in therapeutic use. Such noncovalent linkages can be created from either two or more polypeptides that may be the same or dissimilar or one or more polypeptide and a small molecule or ligand attached to the second moiety. Attachment of the small molecule or ligand can take place through in vitro or in vivo processes, such as the incorporation of biotin or lipoic acid into their specific acceptor sequences which may be natural or artificial biotin or lipoic acid acceptor domains and which may be achieved either by natural incorporation in vivo or by enzymatic biotinylation or lipoylation in vitro. Alternatively, the protein may be substituted with biotin or other moieties by chemical reaction with biotin derivatives. Common examples of biotin derivatives used to couple with proteins include aldehydes, amines, haloacetamides, hydrazides, maleimides, and activated esters, such as N-hydroxysuccinimide esters. Examples of commonly employed noncovalent linkage include the linkage induced by binding of biotin and its derivatives or biotin-related substituents such as iminobiotin or diaminobiotin or thiobiotin to streptavidin or avidin or variants thereof, the binding of enzymes to their covalent or noncovalent specific inhibitors, such as the binding of methotrexate to mammalian dihydrofolate reductase, the binding of natural or synthetic leucine zippers to one another, the binding of enzymes to specific or

nonspecific inhibitors, such as antitrypsin or leupeptin or alpha-2-macroglobulin, the binding of aryl bis-arsenates to alpha helices bearing appropriately positioned cysteine residues, the binding between a nucleic acid aptamer and its target; between a peptide and a nucleic acid such as Tat-TAR interaction.

Enzymatic activation of one polypeptide to afford coupling with another polypeptide can also be employed. Enzymes or enzyme domains that undergo covalent modification by reaction with substrate-like molecules can also be used to create fusions. Examples of such enzymes or enzyme domains include O6-alkylguanine DNA-alkyltransferase (Gronemeyer et al. Protein Eng Des Sel. 2006 19(7):309-16), thymidylate synthase, or proteases that are susceptible to covalent or stable noncovalent modification of the active site, as for example DPPIV (SEQ ID NO:41).

The present invention also features the use of bifunctional or multifunctional linkers, which contain at least two interactive or reactive functionalities that are positioned near or at opposite ends, each can bind to or react with one of the moieties to be linked. The two or more functionalities can be the same (i.e., the linker is homobifunctional) or they can be different (i.e., the linker is heterobifunctional). A variety of bifunctional or multifunctional cross-linking agents are known in the art are suitable for use as linkers. For example, cystamine, m-maleimidobenzoyl-N-hydroxysuccinimide-ester, N-succinimidyl-3-(2-pyridyldithio)-propionate, methylmercaptobutyrimidate, dithiobis(2-nitrobenzoic acid), and many others are commercially available, e.g., from Pierce Chemical Co. Rockford, IL. Additional chemically orthogonal reactions suitable for such specific operable linkage reactions include, for example, Staudinger ligation, Cu[I] catalyzed [2+3] cycloaddition, and native ligation.

The bifunctional or multifunctional linkers may be interactive but non-reactive. Such linkers include the composite use of any examples of non-covalent interactions discussed above.

The length and composition of the linker can be varied considerably provided that it can fulfill its purpose as a molecular bridge. The length and composition of the linker are generally selected taking into consideration the intended function of the linker, and optionally other factors such as ease of synthesis, stability, resistance to certain chemical and/or temperature parameters, and biocompatibility. For example,

the linker should not significantly interfere with the regulatory ability of the cell-targeting moiety relating to targeting of the toxin, or with the activity of the toxin or enzyme relating to activation and/or cytotoxicity.

Linkers suitable for use according to the present invention may be branched, unbranched, saturated, or unsaturated hydrocarbon chains, including peptides as noted above.

Furthermore, if the linker is a peptide, the linker can be attached to the toxin moiety and enzyme moiety and/or the cell-targeting moiety using recombinant DNA technology.

In one embodiment of the present invention, the linker is a branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 100 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is H, or Cl to C6 alkyl), and wherein the chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C3-C6) cycloalkyl, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

Examples of suitable linkers include, but are not limited to, peptides having a chain length of 1 to 100 atoms, and linkers derived from groups such as ethanolamine, ethylene glycol, polyethylene with a chain length of 6 to 100 carbon atoms, polyethylene glycol with 3 to 30 repeating units, phenoxyethanol, propanolamide, butylene glycol, butyleneglycolamide, propyl phenyl, and ethyl, propyl, hexyl, steryl, cetyl, and palmitoyl alkyl chains.

In one embodiment, the linker is a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is as defined above), and wherein the chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, hydroxy, oxo (=0), carboxy, aryl and aryloxy.

In another embodiment, the linker is an unbranched, saturated hydrocarbon chain having from 1 to 50 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is as defined above), and wherein the

chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, hydroxy, oxo (=O), carboxy, aryl and aryloxy.

In a specific embodiment of the present invention, the linker is a peptide having a chain length of 1 to 50 atoms. In another embodiment, the linker is a peptide having a chain length of 1 to 40 atoms.

As known in the art, the attachment of a linker to a protoxin moiety (or of a linker element to cell-targeting moiety or a cell-targeting moiety to a protoxin moiety) need not be a particular mode of attachment or reaction. Various non-covalent interactions or reactions providing a product of suitable stability and biological compatibility are acceptable.

One preferred embodiment of the present invention relies on enzymatic reaction to provide an operable linkage between the moieties of a protoxin, protoxin activator, or protoxin proactivator. Among the enzymatic reactions that produce such operable linkage, it is well-known in the art that transglutaminase ligation, sortase ligation, and intein-mediated ligation provide for high specificity.

The preferred peptide substrate sequences listed above are for example and non-limiting. It is known in the art that these families of enzymes can recognize and utilize different sequences as substrates, and those sequences are included here as embodiments for the present invention.

In some aspects, the invention features the use of natively activatable linkers. Such linkers are cleaved by enzymes of the complement system, urokinase, tissue plasminogen activator, trypsin, plasmin, or another enzyme having proteolytic activity may be used in one embodiment of the present invention. According to another embodiment of the present invention, a protoxin is attached via a linker susceptible to cleavage by enzymes having a proteolytic activity such as a urokinase, a tissue plasminogen activator, plasmin, thrombin or trypsin. In addition, protoxins may be attached via disulfide bonds (for example, the disulfide bonds on a cystine molecule) to the cell-targeting moiety. Since many tumors naturally release high levels of glutathione (a reducing agent) this can reduce the disulfide bonds with subsequent release of the protoxin at the site of delivery.

In one embodiment, the cell-targeting moiety is linked to a protoxin by a cleavable linker region. In another embodiment of the invention, the cleavable linker region is a protease-cleavable linker, although other linkers, cleavable for example by small molecules, may be used. Examples of protease cleavage sites are those cleaved by factor Xa, thrombin and collagenase. In one embodiment of the invention, the protease cleavage site is one that is cleaved by a protease that is up-regulated or associated with cancers in general. Examples of such proteases are uPA, the matrix metalloproteinase (MMP) family, the caspases, elastase, and the plasminogen activator family, as well as fibroblast activation protein. In still another embodiment, the cleavage site is cleaved by a protease secreted by cancer-associated cells. Examples of these proteases include matrix metalloproteases, elastase, plasmin, thrombin, and uPA. In another embodiment, the protease cleavage site is one that is up-regulated or associated with a specific cancer. In yet another embodiment, the proteolytic activity may be provided by a protease fusion targeted to the same cell. Various cleavage sites recognized by proteases are known in the art and the skilled person will have no difficulty in selecting a suitable cleavage site. Non-limiting examples of cleavage sites are provided elsewhere in this document. As is known in the art, other protease cleavage sites recognized by these proteases can also be used. In one embodiment, the cleavable linker region is one which is targeted by endocellular proteases.

Chemical linkers may also be designed to be substrates for carboxylesterases, so that they may be selectively cleaved by these carboxyltransferases or corresponding fusion proteins with a cell-targeting moiety. One preferred embodiment comprises the use of a carboxyl transferase activity to activate the cleavage of an ester linker. For example but without limitation, secreted human carboxyltransferase-1, -2, and -3 may be used for this purpose. Additional examples include carboxyl transferase of other origins.

Another embodiment of the cleavable linkers comprises nucleic acid units that are specifically susceptible to endonucleases. Endonucleases are known to be present in human plasma at high levels.

In another embodiment, the modifiable activation moiety is not a peptide, but a cleavable linker that may be acted upon by a cognate enzymatic activity provided by the activator or proactivator. The cleavable linker is preferably situated at the same

location as the furin-like cleavage sequence in an activatable protoxin, or at the location of the zymogen inhibitory peptide in an activatable proactivator. The cleavable linker may replace the furin-like cleavage sequence or be attached in parallel to the furin-like cleavage or another modifiable activation moiety, providing a protoxin that requires both a furin-like cleavage or other proteolytic event and a linker cleavage for activation. In one embodiment the cleavable linker joins the ADP ribosyltransferase domain of a VCE-based protoxin to the translocation domain of that or another protoxin. In another embodiment the cleavable linker joins the translocation domain of a PEA or VCE-based protoxin to the ADP ribosyltransferase domain of the same or a different toxin. In yet another embodiment the cleavable linker joins the pore-forming domain of a pore-forming toxin with the C-terminal inhibitory peptide.

Preferable cleavable linkers are those which are stable to in vivo conditions but susceptible to the action of an activator. Many examples of suitable linkers have been provided in the context of attempts to develop antibody-directed enzyme prodrug therapy. For example a large class of enzyme substrates that lead to release of an active moiety, such as a fluorophore, have been devised through the use of what are known as self-immolative linkers. Self-immolative linkers are designed to liberate an active moiety upon release of an upstream conjugation linkage, for example between a sugar and an aryl moiety. Such linkers are often based on glycosides of aryl methyl ethers, for example the phenolic glycosides of 3-nitro, 4-hydroxy benzyl alcohol; see for example Ho et al. Chembiochem, 2007 Mar 26;8(5):560-6, or the phenolic amides of 4-amino benzyl alcohol, for example Niculescu-Duvaz et al. J Med Chem. 1998 Dec 17;41(26):5297-309 or Toki et al. J Org Chem. 2002 Mar 22;67(6):1866-72.

To create self-immolative linkers based on glycosides the phenolic hydroxyl is glycated by reaction with a 1-Br-substituted sugar such as alpha-1-Br galactose or alpha-1-Br glucuronic acid to provide the substrate for the activating enzyme, and the benzyl alcohol moiety is then activated with a carbonylation reagent such as phosgene or carbonyl diimidazole and reacted with a primary amine to afford a carbamate linkage. Upon scission of the aryl glycosidic bond or the aryl ester, the aryl moiety eliminates, leaving a carbamoyl moiety that in turn eliminates, affording CO2 and the

regenerated amine. The amine may be the alpha amino group of a polypeptide chain or the epsilon amino of a lysine side chain.

To create self-immolative linkers based on amide bonds the phenyl amine of 4-amino benzyl alcohol is reacted with an activated carboxyl group of a suitable peptide or amino acid to create a phenyl amide that can be a substrate for an appropriate peptidase, for example carboxypeptidase G2 Niculescu-Duvaz et al. J Med Chem. 41(26):5297-309 (1998). The benzyl alcohol moiety is then activated with a carbonylation reagent such as phosgene or carbonyl diimidazole and reacted with a primary amine to afford a carbamate linkage. Upon scission of the aryl amide bond, the aryl moiety eliminates, leaving a carbamoyl moiety that in turn eliminates, affording CO2 and the regenerated amine. Said amine may be the alpha amino group of a polypeptide chain or the epsilon amino of a lysine side chain.

For the creation of an appropriate self-immolating activation moiety according to the present invention the aryl group is substituted with a reactive moiety that provides a linkage to one element of the protoxin or proactivator, such as the toxin moiety or the translocation moiety or the inhibitory peptide moiety.

Similar forms of self-immolative linker are also well-known in the art. For example Papot et al. Bioorg Med Chem Lett. 8(18):2545-8 (1998) teach the creation of glucuronide prodrugs based on aryl malonaldehydes that undergo elimination of the aryl linker moiety upon cleavage by a glucuronidase. Suitable linkers based on aryl malonaldehydes in the context of the present invention provide a modifiable activation moiety in which the aryl substituent is operably linked to one terminus of the toxin moiety, for example at the location of the furin cleavage site, and the carbamoyl functionality is operably linked to the translocation moiety or inhibitory moiety. In the system devised by Papot et al, cleavage by glucuronidase will result in elimination of the aryl malonaldehyde and activation of the protoxin. Similar elimination events are known to take place following hydrolysis of the lactam moiety of linkers based on 7-aminocephalosporanic acid, and enzymatically activated prodrugs based on beta-lactam antibiotics or related structures are well known in the art. For example Alderson et al. Bioconjug Chem. 17(2):410-8 (2006) teach the creation of a 7-aminocephalosporanic acid-based linker that undergoes elimination and scission of a carbamate moiety in similar fashion to that of the aryl malonaldehydes disclosed by Papot et al.. In addition, Harding et al. Mol Cancer

Ther. 4(11):1791-800 (2005) teach a beta-lactamase that has reduced immunogenicity that can be favorably applied as an activator for a prodrug moiety based on a 7-aminocephalosporanic acid nucleus.

In yet another embodiment the modifiable activation moiety is a peptide but is operably linked by a flexible nonpeptide linker at either or both termini in the same location as the natural furin-like protease cleavage site, or in parallel to the natural furin-like cleavage site. In such embodiments the activator is a cognate protease or peptide hydrolase recognizing the peptide of the modifiable activation moiety. In a doubly triggered protoxin, the furin-like cleavage site is replaced by a modifiable activation moiety and a cleavable linker is attached in parallel to the modifiable activation moiety. In such a protoxin the action of two activators is required to activate the protoxin.

VI. Isolation and Purification of Toxin Fusion and Protease Fusion Proteins

A. General Strategies for Recombinant Protein Purification

There are many established strategies to isolate and purify recombinant proteins known to those skilled in the art, such as those described in *Current Protocols in Protein Science* (Coligan *et al.*, eds. 2006). Conventional chromatography such as ion exchange chromatography, hydrophobic-interaction (reversed phase) chromatography, and size-exclusion (gel filtration) chromatography, which exploit differences of physicochemical properties between the desired recombinant protein and contaminants, are widely used. HPLC can also been used.

To facilitate the purification of recombinant proteins, a variety of vector systems have been developed to express the target protein as part of a fusion protein appended by an N-terminal or C-terminal polypeptide (tag) that can be subsequently removed using a specific protease. Using such tags, affinity chromatography can be applied to purify the proteins. Examples of such tags include proteins and peptides for which there is a specific antibody (e.g., FLAG fusion purified using anti-FLAG antibody columns), proteins that can specifically bind to columns containing a specific ligand (e.g., GST fusion purified by glutathione affinity gel), polyhistidine tags with affinity to immobilized metal columns (e.g., 6 His tag immobilized on Ni^{2+} column and eluted by imidazole), and sequences that can be biotinylated by the host

during expression or *in vitro* after isolation and enable purification on an avidin column (e.g., BirA).

B. Isolation and Purification of Fusion Proteins Expressed in Insoluble Form

Many recombinant fusion proteins are expressed as inclusion bodies in *Escherichia coli*, i.e., dense aggregates that consist mainly of a desired recombinant product in a nonnative state. In fact, most reported DT-ScFv fusion proteins expressed in *E. coli* are obtained in insoluble forms. Usually, the inclusion bodies form because (a) the target protein is insoluble at the concentrations being produced, (b) the target protein is incapable of folding correctly in the bacterial environment, or (c) the target protein is unable to form correct disulfide bonds in the reducing intracellular environment.

Those skilled in the art recognize that different methods that can be used to obtain soluble, active fusion proteins from inclusion bodies. For example, inclusion bodies can be separated by differential centrifugation from other cellular constituents to afford almost pure insoluble product located in the pellet fraction. Inclusion bodies can be partially purified by extracting with a mixture of detergent and denaturant, either urea or guanidine·HCl, followed by gel filtration, ion exchange chromatography, or metal chelate chromatography as an initial purification step in the presence of denaturants. The solubilized and partially purified proteins can be refolded by controlled removal of the denaturant under conditions that minimize aggregation and allow correct formation of disulfide bonds. To minimize nonproductive aggregation, low protein concentrations should be used during refolding. In addition, various additives such as nondenaturing concentrations of urea or guanidine·HCl, arginine, detergents, and PEG can be used to minimize intermolecular associations between hydrophobic surfaces present in folding intermediates.

C. Isolation and Purification of Fusion Proteins Expressed in Soluble Form

Recombinant proteins can also be expressed and purified in soluble form. Recombinant proteins that are not expressed in inclusion bodies either will be soluble

inside the cell or, if using an excretion vector, will be extracellular (or, if *E. coli* is the host, possibly periplasmic). Soluble proteins can be purified using conventional methods afore described.

VI. Assays for Measuring Inhibition of Cell Growth

Various assays well known in the art are useful for determining the efficacy of the protein preparations of the invention, including those assays that measure cell proliferation and death. The following are examples of many assays that can be used for analyzing the cytotoxicity of the reagents in the present invention.

A. Protein Synthesis Inhibition Assays

Because many toxins (e.g., VCE) exert their cytotoxicity through inhibition of protein synthesis, an assay that directly quantifies protein being synthesized by the cell after its exposure to the toxin is especially useful. In this assay, cells are exposed to a toxin and then incubated transiently with radioactive amino acids such as [^{3}H]-Leu, [^{35}S]-Met or [^{35}S]-Met-Cys. The amount of radioactive amino acid incorporated into protein is subsequently determined, usually by lysing cells and precipitating proteins with 10% trichloroacetic acid (TCA), providing a direct measure of how much protein is synthesized. Using such an assay, it was demonstrated that, although the entry of DT into a cell is not associated with an immediate block in protein synthesis, prolonged action (4-24 hours) of single DT catalytic fragment molecules in the cytosol is sufficient to obtain complete protein synthesis inhibition at low toxin concentrations (Falnes et al., J. Biol. Chem. 275:4363 (2000)).

An extension of this method is a luciferase-based assay (Zhao and Haslam, J. Med. Microbiol. 54:1023 (2005)). Luciferase cDNA was incorporated into a wide variety of dividing or non-dividing mammalian cells using an adenoviral expression system, and the resulting cells allowed to constitutively transcribe the luciferase cDNA, which had been engineered to contain an additional PEST sequence for a short intracellular half-life. The assay measures the level of protein synthesis in cells through the light output from D-luciferin reaction catalyzed by the short-lived luciferase. In cells constitutively expressing the luciferase mRNA, inhibition of protein synthesis results in diminished luciferase translation and proportionately reduced light output.

B. Thymidine Incorporation Assay

The rate of proliferation of cells can be measured by determining the incorporation of [^{3}H]-thymidine into cellular nucleic acids. This assay may be used for analyzing cytotoxicity of toxins (e.g., DT-based immunotoxins). Using this method a DT-IL3 immunotoxin was shown to be active in inhibiting growth of IL3-receptor bearing human myeloid leukemia cell lines (Frankel et al., Leukemia. 14:576 (2000)). The toxin fusion and protease fusion proteins of the present invention may be tested using such an assay, individually or combinatorially.

C. Colony Formation Assay

Colony formation may provide a much more sensitive measure of toxicity than certain other commonly employed methods. The reason for this increased sensitivity may be the fact that colony formation is assessed while the cells are in a state of proliferation, and thus more susceptible to toxic effects. The sensitivity of the colony-formation assay, and the fact that dose and time-dependent effects are detectable, enables acute and chronic exposure periods to be investigated as well as permitting recovery studies. For example, the cytotoxicity of a recombinant DT-IL6 fusion protein towards human myeloma cell lines was investigated using methylcellulose colony formation by U266 myeloma cells. In cultures containing both normal bone marrow and U266 cells DT-IL-6 effectively inhibited the growth of U266 myeloma colonies but had little effect on normal bone marrow erythroid, granulocyte and mixed erythroid/granulocyte colony growth (Chadwick et al., Haematol. 85:25 (1993)).

D. MTT Cytotoxicity Assay

The cytotoxicity of a particular fusion protein or a combination of fusion proteins can be assessed using an MTT cytotoxicity assay. The specific cytotoxicity of a DT-GMCSF fusion protein against human leukemia cell lines bearing high affinity receptors for human GMCSF was demonstrated using such an MTT assay, colony formation assay, and protein inhibition assay (Bendel et al., Leuk. Lymphoma. 25:257 (1997)). In a typical MTT assay, the yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent and quantified by UV-VIS spectrometry.

After cells are grown to 80-100% confluence, they are washed with serum-free buffer and treated with cytotoxic agent(s). After incubation of the cells with the MTT reagent for approximately 2 to 4 hours, a detergent solution is added to lyse the cells and solubilize the colored crystals. The samples are analyzed at a wavelength of 570 nm and the amount of color produced is directly proportional to the number of viable cells.

VII. Functional Assays for VCE Fusion Proteins

A. *In Vitro* Protein Synthesis Inhibition Assay

In eukaryotic cells, VCE inhibits protein synthesis because its catalytic domain can inactivate elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation after endocytosis to cytosol. *In vitro* eukaryotic translation systems, e.g., using rabbit reticulocyte lysate and wheat germ extract, are potentially suited for examining the catalytic function of recombinant VCE fusion proteins. For example, TNT-coupled wheat germ extract, supplemented by NAD^+, amino acids, [^{35}S]-Met, DNA template, and an RNA polymerase, has been used to test the inhibition of protein synthesis by a recombinantly expressed catalytic fragment of DT (Epinat and Gilmore, Biochim. Biophys. Acta. 1472:34 (1999)). The level of ^{35}S-labeled translated protein is an indicator of the extent of DT toxicity.

These *in vitro* assays can be used to screen inhibitory effects of VCE fusions in the absence or presence of certain proteolytic activity, providing a facile assay to analyze the functional integrity of engineered VCE fusion proteins as well as that of protease fusion proteins.

B. *In Vitro* EF-2 ADP-ribosylation Assay

VCE inhibits protein synthesis by catalyzing the transfer of ADP-ribose moiety of NAD to a post-translationally modified His715 of EF-2 called diphthamide. Thus the function of VCE fusions can also be directly assayed *in vitro* by correlating its catalytic activity to rate of transfer of radiolabeled ADP-ribose to recombinant EF-2 as was previously shown for DT (Parikh and Schramm, Biochemistry 43:1204 (2004)). This assay has been applied for testing the inhibition of ADP-ribosyltransferase activity, and is often used as one of the assays for DT-based

immunotoxins (Frankel et al., Leukemia. 14:576 (2000)). Non-radioactively labeled NAD, such as biotinylated NAD or etheno-NAD, may also be used as a substrate (Zhang. Method Enzymol. 280:255-265 (1997))..

C. *In Vitro* Proteolytic Activity Assay

The functional activity of recombinant protease fusion proteins may be assayed *in vitro* either using a peptide or protein substrate containing the recognition sequence of the protease. Various protocols are well known to those skilled in the art.

VIII. Administration of Proteins

The proteins of the invention are typically administered to the subject by means of injection using any route of administration such as by intrathecal, subcutaneous, submucosal, or intracavitary injection as well as by intravenous or intraarterial injection. Thus, the proteins may be injected systemically, for example, by the intravenous injection of the proteins into the patient's bloodstream or alternatively, the proteins can be directly injected at a specific site. The proteins of the invention can be administered alone, or in combination with other proteins or therapies.

The protoxin of the invention can be administered prior to, simultaneously with, or following the administration of the protoxin activator or protoxin proactivator and optionally administered prior to, simultaneously with, for following the administration of the proactivator activator of the invention. In preferred embodiments the components are administered in such a way as to minimize spontaneous activation during administration. When administered separately, the administration of two or more fusion proteins can be separated from one another by, for example, one minute, 15 minutes, 30 minutes, one hour, two hours, six hours, 12 hours, one day, two days, one week, or longer. Furthermore, one or more of the fusion proteins of the invention may be administered to the subject in a single dose or in multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one day, two days, one week, two weeks, or one month. For example, the proteins may be administered once a week for, e.g., 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the

individual need and the professional judgment of the person administering or supervising the administration of the proteins. For example, the dosage of the proteins can be increased if the lower dose does not sufficiently destroy or inhibit the growth of the desired target cells. Conversely, the dosage of the proteins can be decreased if the target cells are effectively destroyed or inhibited.

While the attending physician ultimately will decide the appropriate amount and dosage regimen, a therapeutically effective amount of the proteins may be, for example, in the range of about 0.0035 μg to 20 μg/kg body weight/day or 0.010 μg to 140 μg/kg body weight/week. A therapeutically effective amount may be in the range of about 0.025 μg to 10 μg/kg, for example, about 0.025, 0.035, 0.05, 0.075, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 μg/kg body weight administered daily, every other day, or twice a week. In addition, a therapeutically effective amount may be in the range of about 0.05, 0.7, 0.15, 0.2, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 14.0, 16.0, or 18.0 μg/kg body weight administered weekly, every other week, or once a month. Furthermore, a therapeutically effective amount of the proteins may be, for example in the range of about 100 $\mu g/m^2$ to 100,000 $\mu g/m^2$ administered every other day, once weekly, or every other week. The therapeutically effective amount may be in the range of about 1000 $\mu g/m^2$ to 20,000 $\mu g/m^2$, for example, about 1000, 1500, 4000, or 14,000 $\mu g/m^2$ of the proteins administered daily, every other day, twice weekly, weekly, or every other week.

In some cases it may be desirable to modify the plasma half-life of a component alone or in the combinatorial therapeutic agent of the present invention. The plasma half-lives of therapeutic proteins have been extended using a variety of techniques such as those described by Collen et al., Bollod 71:216-219 (1998); Hotchkiss et al., Thromb. Haemostas. 60:255-261 (1988); Browne wt al., J. Biol. Chem. 263:1599-1602 (1988); Abuchowski et al., Cancer Biochem. Biophys. 7:175 (1984)). Antibodies have been chemically conjugated to toxins to generate immunotoxins which have increased half-lives in serum as compared with unconjugated toxins and the increased half-life is attributed to the native antibody. WO94/04689 teaches the use of modified immunotoxins in which the immunotoxin is linked to IgG constant region domain having the property of increasing the half-life of the protein in mammalian serum. The IgG constant region domain is CH2 or a fragment thereof.

The administration the proteins of the invention may be by any suitable means that results in a concentration of the proteins that, combined with other components, effectively destroys or inhibits the growth of target cells. The proteins may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is suitable for any parenteral (e.g., subcutaneous, intravenous, intramuscular, or intraperitoneal) administration route. The pharmaceutical compositions are formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. Gennaro, Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. Swarbrick and Boylan, 1988-1999, Marcel Dekker, New York).

Experimental Results

A. Construction of Fusion Proteins and Cell Lines

*Construction of anti-CD19 ScFv VCE (*anti-CD*19 VCE) fusion gene*

A synthetic gene encoding VCE was prepared by Codon Devices (Cambridge, MA) using codons optimized for expression in *E. Coli*. To facilitate the purification of proteins derived from VCE and to expose the ER retention signal of VCE, the sequence encoding the last 5 amino acids of wild type VCE (Lys-Asp-Glu-Leu-Lys) (SEQ ID NO:42) was replaced with a sequence encoding a Hisx6 tag and an ER retention signal (His-His-His-His-His-His-Lys-Asp-Glu-Leu) (SEQ ID NO:43). The genes encoding anti-CD5-VCE and anti-CD19-VCE fusion were prepared by genetic fusion of anti-CD5 ScFv or anti-CD19 ScFv coding regions with sequences encoding domain II and domain III of VCE, joined by a DNA sequence encoding a flexible linker (Gly-Ser-Gly-Ala-Ser) (SEQ ID NO:44). The granzyme B activable protoxin was prepared by replacing the sequence encoding the furin recognition sequence (RKPRDL) (SEQ ID NO:11) of VCE with a consensus granzyme B recognition sequence (IEPDDL) (SEQ ID NO:17).

Construction of a Human Granzyme B-anti-CD19 ScFv (GrB-anti-CD19) Fusion Gene

The sequence corresponding to the mature human Granzyme B (amino acids 21 to 247) was amplified from a full length Granzyme B cDNA clone obtained from OriGene Inc. and inserted into the pEAK15 vector together with a synthetic anti-CD19 ScFv DNA fragment by a three-piece ligation (pEAK15 GrB-anti-CD19L). The promoter for the fusion gene in the resulting expression construct is a CMV/chicken β-actin hybrid promoter. The open reading frame encoding the fusion protein directs the formation of a signal peptide derived from the Gaussia princeps luciferase, a synthetic N-linked glycosylation site, a FLAG tag and an enterokinase cleavage sequence followed by the mature human granzyme B sequence, a flexible linker $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$(SEQ ID NO:45), the anti-CD19 ScFv, and a C-terminal 6 His tag. The desired structure of the resulting fusion protein coding sequence was confirmed by DNA sequencing.

Construction of diphtheria toxin anti-CD5 ScFv (DT-anti-CD5) fusion gene

A DT-anti-CD5 fusion gene was prepared by chemical synthesis by Retrogen Co. (San Diego) using codons jointly optimized for expression in *Pichia Pastoris* and in human cell lines. The sequence encoding the furin recognition site ($_{190}$RVRRSVG$_{196}$) (SEQ ID NO:46) was replaced with a consensus granzyme B recognition sequence ($_{190}$IEPDSG$_{195}$) (SEQ ID NO:47). Two potential N-linked glycosylation sites were mutated as described (Thompson et al. Protein Eng. 14(12):1035-41 (2001)) and a 6 His tag sequence was added to the C -terminus of the fusion gene for detection and purification. The fusion gene was cloned into XhoI and NotI sites of the pPIC9 vector (*Invitrogen*) while maintaining the α-factor signal peptide and the Kex2 cleavage site.

Generation of $CD5^+$ Raji, and cells

Raji cells (ATCC) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% iron supplemented calf serum (Hyclone), 2 mM L-Glutamax.

To prepare recombinant retroviral transducing particles, the GFP coding sequence found in the retroviral vector M3P-GFP was replaced with full length cDNA encoding human CD5. To produce vesicular stomatitis virus envelope glycoprotein

(VSVG)-pseudotyped viral particles, linearized M3P-CD5 plasmid was cotransfected with pMD-MLV pMD-VSVG to 293 ETN cells, which were seeded at $5x10^6$ per 10 cm^2 plate a day before transfection. M3P-CD5, pMD-MLV-G/P and pMD-VSVG DNAs were mixed and transfected at 10 μg, 7 μg and 3 μg, respectively, per 10 cm^2 plate, using 2.5μl of TransFectin (Bio-Rad) per μg of DNA. Viral particles were collected 48 hours after transfection and filtered through a 0.45 μm filter (Corning).

For transduction, 5 $x10^5$ Raji cells were suspended in 1.5 ml of culture medium and mixed with 1.5 ml of filtered virus in a 6-well plate. Three μl of 8 mg/ml polybrene was added to the mixture to the final concentration of 8 μg/ ml. The plate was centrifuged at 2000 rpm for 1 hour before initiation of culture in a 37°C incubator containing 5% CO_2. To isolate Raji cells expressing CD5, the infected cells were sorted after staining with FITC conjugated anti-human CD5 antibody (eBioscience). Raji cells expressing high concentrations of CD5 were collected and used for the cytotoxicity assay.

Flow Cytometric Analysis

The presence of CD5 and CD19 on cell surface was analyzed using indirect immunofluorescence staining. Cells were incubated with mouse anti-human CD5 or mouse anti-human CD19 (Pharmingen, San Diego, CA) at a concentration of 0.5 μg per one million cells. Goat F $(ab')_2$ anti-mouse IgG1 conjugated with RPEA (Southern Biotechnology) was used as secondary antibody at a concentration of 0.25 μg per million of cells. The stained cells were analyzed by flow cytometry (FAXCaliber).

B. Expression and Purification GrB-anti-CD19 Fusion from 293ETN cells

293ETN cells were seeded at 5 10^6-6 10^6 cells per 10 cm^2 plate and were transfected with 12 μg of pEAK15 GrB-anti-CD19L and 25 μl of TransFectin (Bio-Rad) according to the manufacturer's protocol. Transfected cells were cultured in Opti-MEM (Invitrogen) for 3 days to allow fusion protein to accumulate. Supernatants were collected and incubated with pre-equilibrated Ni-NTA resin (Qiagen) and the fusion protein was eluted with buffer containing 50 mM HEPES pH7.5, 150 mM NaCl, 250 mM imidazole and 5% glycerol. The purified GrB-anti-CD19 fusion protein was incubated with enterokinase (New England Biolabs) at room

temperature overnight to activate the proteolytic activity of Granzyme B. To remove enterokinase and N-terminal peptide released by enterokinase, the reaction mixture was subjected to affinity purification with Ni-NTA resin. In another form of preparation, the enterokinase and N-terminal peptide released by enterokinase, were removed by gel filtration purification (superdex 200, G E Healthcare). The proteolytic activity of the granzyme B-anti-CD19 ScFv was measured by incubating the purified protein with a fluorogenic peptide substrate (Ac-IEPD-AMC, Sigma Aldrich). Accumulation of fluorescent product was monitored every 30 s at excitation and emission wavelengths of 380 and 460 nm respectively for 15 min.

C. Expression and Purification of DT-anti-CD5 Fusion from *P. Pastoris*

Pichia Pastoris KM71 cells (Invitrogen) were transformed with the expression plasmid by electroporation. Positive clones were selected according to manufacturer's protocol. For large scale purification, a single colony was cultured at 28 °C overnight in 10 ml Buffer Minimal Glycerol medium (BMG) pH 6.0. The overnight culture was transferred to 1L BMG pH 6.0 and cultured at 28 °C until OD600 reached 6.0. To induce protein expression, the culture was spun down and resuspended with 100 ml Buffered (pH 6.6) Methanol-complex Medium containing 1% casamino acids (BMMYC) and cultured at 15 °C for 48 hours. Supernatants were collected and adjusted to pH 7.6 with 5% NaOH. Clarified supernatants were subjected to affinity purification as described above for the purification of the GrB-anti-CD19 fusion protein.

D. Expression and Purification of anti-CD5-VCE and anti-CD19 VCE Fusion Proteins from *E. coli*

DNA sequence corresponding to anti-CD5-VCE, anti-CD19-VCE and various variants thereof were cloned between the NcoI and NotI sites of the pET22b vector (Novagen). Transformed bacterial cells (BL21) were cultured in LB medium at 37 °C . Protein expression was induced with 0.2 mM IPTG for overnight at 17 °C at OD_{600}=0.3-0.5. The bacterial periplasmic fraction was collected as described (Malik et al. Protein Expr Purif. 55(1):100-11 (2007)) and fusion protein was purified with Ni-NTA resin.

E. Expression and Purification of N-GFD-VCE, CCPE-VCE and CCPE^2-VCE Fusion Proteins from *E. coli*

The DNA sequences corresponding to N-GFD-VCE, CCPE-VCE and CCPE^2-VCE and their variants were cloned between the NcoI and NotI sites of the pET28a vector (Novagen). Transformed bacterial cells (BL21) were cultured in LB medium at 37 °C. Protein expression was induced with 0.2 mM IPTG for overnight at 17 °C at OD_{600}=0.5-0.8. Soluble protein fraction was extracted with B-PERII (Pierce Biotechnology) and subjected to Ni-NTA purification.

F. Identification of Putative *Vibrio cholerae* Exotoxins using BLAST Analysis

BLAST analysis indicates that the hypothetical *toxA* gene product from an aquatic strain of *Vibrio cholerae* (GI: 58615288) possesses a conserved diphtheria toxin (DT) like ADP-ribosylation domain, and adopts an overall domain structure very similar to that of Pseudomonas exotoxin A (PEA), with moderate amino acid sequence identity (33%). Similar to PEA, the Vibrio exotoxin A (VCE) possesses an N-terminal cell binding domain, an ADP-ribosylation catalytic domain near C-terminus, and a translocation domain in the middle. BLAST analysis also identified two additional hypothetical Vibrio exotoxins in the NCBI protein database, *i.e.*, hypothetical exotoxin A from *Vibrio cholerae* strain 1587 (GI: 124114053) and hypothetical protein from *Vibrio cholerae* strain V51 (GI:116219709 & 116219710), which have ~97% amino acid sequence identity with the aquatic VCE and low sequence identity to PEA.

Although VCE only shares moderate protein sequence homology to PEA (33% sequence identity, Fig. 1B), residues thought to be critical for the function of PEA are conserved in VCE, including hypothesized active site residues (H440, Y481, E553 in PEA), a furin cleavage site in the domain II, and an ER retention signal at the C-terminus (Fig. 1A and 1C). Furthermore, the predicted VCE catalytic domain sequence could be threaded onto the structure of the PEA catalytic domain, consistent with the notion that VCE folds into a structure similar to that of PEA and thus might possess similar enzymatic activity (Yates S.P., TIBS 31, 123-133, 2006).

G. Characterization of VCE

Upon cell entry, DT and PEA specifically ribosylate an exposed histidine of eukaryotic elongation factor 2 (eEF2) that has undergone extensive post-translational modification to form a residue known as diphthamide. ADP-ribosylation of diphthamide interferes with the function of eEF2 causing an arrest of protein synthesis which results in profound physiological changes and ultimately cell death. To determine whether VCE intoxicates target cells with the same toxic principle used by PEA and DT, purified ADPRT domain of VCE was incubated with human 293T cell lysate in the presence of biotin-NAD. Biotin-NAD has been shown to be a substrate for ADPRT and, as a result of ADPRT reaction, the target proteins are biotinylated (Zhang, Method Enzymol. 280:255-265 (1997)). We found that in the presence of VCE and biotin-NAD, a protein with apparent molecular weight of 100 kD was biotinylated (Fig. 2A, lane 2). A protein with identical molecular weight was also found to be modified by PEA (Fig. 4A, lane 2), suggesting that VCE and PEA may target the same protein, known in the PEA case to be eEF2. To further investigate the target protein of VCE, the modified target protein was subjected to affinity capture using monomeric avidin beads. The captured proteins were then probed with antibodies recognizing human eEF2 (Fig. 2B) or human GrB2 (Fig. 2C). eEF2, but not GrB2, can be captured by avidin beads upon modification by the ADPRT domain of VCE, supporting the view that eEF2 is the endogenous target of VCE.

Several DT-resistant cell lines have been developed, one of which, Rel.22C, has been derived from a Chinese Hamster Ovary (CHO) cell line (Moehring et al. *Somat. Cell Genet.* 5:453-468 (1979)). The specific mutation that confers DT-resistance upon the cell line Rel.22C is located on eEF2, resulting a mutant eEF2 devoid of diphthamide side-group modification (Foley et al. *J. Biol. Chem.* 270:23218-25,1995). Although VCE modified eEF2 from wild type CHO cells and 293T cells, VCE failed to modified eEF2 from Rel.22C (Fig. 3B, compare lanes 1 and 2 to 3). These data strongly support the view that VCE, like PEA and DT, specifically modifies eEF2.

H. Replacing the Cell Binding Domain of VCE

To address if VCE can be made to function as conventional immunotoxins, we replaced the DNA sequences encoding domain I of VCE with those encoding the N-

terminal growth factor-like domain of urokinase-like plasminogen activator to afford VCE fusion protein N-GFD-VCE. The N-terminal growth factor-like domain of urokinase-like plasminogen activator has been shown to bind urokinase-like plasminogen activator receptor (uPAR/CD87) with very high affinity (~0.1 nM) (Appella et al. J. Biol. Chem. 262:4437-4440 (1987)), and immunotoxins bearing the N-terminal growth factor-like domain of urokinase-like plasminogen activator have been reported to be highly toxic to target cells expressing uPAR/CD87 (Rajagopal and Kreitman. J. Biol. Chem. 275:7566-7573 (2000); Ramage et al. Leuk. Res. 27:79-84 (2003)). The N-GFD-VCE was prepared in E. coli, purified, and incubated with Jurkat T cells, which have previously been shown to express a low level of uPAR/CD87 (800+/- 50/cell). N-GFD-VCE efficiently killed Jurkat cells with IC_{50}=0.4 nM (Fig. 4B triangles). In contrast, N-GFD-VCE has little or no toxicity to Raji cells, which have little or no detectable uPAR/CD87 . These results suggest that domain I, but not domain II or III, of VCE is responsible for cell binding and that a fusion protein comprising domain II and III of VCE and a targeting principle replacing domain I can function as a selective cytotoxic agent. In another example domain I of VCE was replaced with a single chain Fv (scFv) domain. scFv-VCE fusion protein targeting CD5 or CD19 efficiently killed cell lines expressing CD5 and CD19, respectively (Fig. 8 triangles and data not shown).

In yet another example domain I of VCE was replaced with a cell targeting domain derived from C-terminal domain of Clostridium perfringens enterotoxin (CCPE), known to target the tight junction proteins Claudin3 and 4. CCPE-VCE fusion protein efficiently killed cell lines expressing Claudin3 and/or 4, including HT29, MCF7 and MB231, and was ineffective in killing a negative control cell line Nalm6 (Fig 5). To increase the affinity of CCPE based immunotoxins to cells expressing Claudin3/4, we replaced domain I of VCE with tandem CCPE domains, resulting in the fusion protein $CCPE^2$-VCE. The EC50 of $CCPE^2$-VCE for the inhibition of protein synthesis by claudin3/4 positive cell lines was about 5-10 times lower than that of CCPE-VCE, whereas toxicity to a negative cell line was relatively unchanged (compare Figures 6B to 6C). These results support the notion that VCE can be linked to a number of cell targeting moieties, such as N-GFD of u-PA, ScFv, one of two copies of CCPE and other naturally occurring or artificially created

affinity reagents, creating VCE fusion proteins that can selectively target cells of interest.

I. Identification of residues critical for cytotoxicity of VCE

Mutational analyses of DT and PEA have identified several residues critical for various aspects of toxin function. By sequence alignment, we identified corresponding residues in VCE. A putative catalytically inactive mutant was created by replacing glutamic acid 613 (E613A) with alanine. In vitro ADPRT assay with human eEF2 indicated that ADPRT activity of E613A mutant was significantly impaired compared with the wild type counterpart (Fig. 4A, compare lanes 4 and 5), and cell culture experiments showed that N-GFD-VCE_{E613A} was not toxic to Jurkat cells (Fig. 4B). Tryptophan 305 of PEA has been shown to play an important role in initiating membrane insertion of PEA (Mere et al. J. Biol. Chem 280:21194-21201 (2005)). Mutation of W305 of PEA to phenylalanine or alanine reduced the toxicity of PEA by 3- or 30-fold, respectively. Sequence analysis suggested that the residue functionally corresponding to W305 of PEA might be phenylalanine 343 (F343) in VCE. We mutated F343 of VCE to tryptophan (F343W) and alanine (F343A) and found that although mutations at F343 did not alter the ADPRT activity (Fig. 4A compare lanes 7 and 8 to 5), the cytotoxicity of the mutant N-GFD-VCE forms was reduced by 1.6 fold (F343W) or 60 fold (F343A) (Fig. 4B).

In addition, a mutant N-GFD-VCE fusion protein in which the furin cleavage site was replaced with a granzyme B cleavage site exhibited functional ADPRT activity (Fig. 4A, compare lane 3 and 4), but was not cytotoxic to target cells (Fig. 4B). Together, this series of experiments identified a residue critical for catalytic activity of ADPRT, a residue critical for membrane insertion, and the furin cleavage sequence critical for activation of VCE.

J. Engineering VCE for Specific Cell Surface Targeting and Proteolytic Activation

To address whether VCE-based immunotoxins can be adapted as the protoxin component of a combinatorial targeting strategy, the furin cleavage sequence of VCE was replaced with a granzyme B cleavage sequence or with candidate cleavage sequences for a mutant granzyme B. Substitution of the furin cleavage sequence

(RKPR↓DL) (SEQ ID NO:48)with a granzyme B cleavage (IEPD↓SG, IEPD↓DL, IAPD↓SG and IAPD↓DL) (SEQ ID NOs:49-52)sequence significantly reduced the toxicity of N-GFD-VCE to Jurkat cells expressing human CD19, although the modified toxin retained full enzymatic activity (Fig. 4A compare lane 3 and 4). In the presence of GrB-anti-CD19, N-GFD-VCE was activated and exhibited cytotoxicity to target cells (Fig 4B), suggesting that GrB-anti-CD19 was able to proteolytically activate N-GFD-VCE bearing a granzyme B cleavage site in place of the endogenous furin recognition sequence.

Surprisingly, when the ability of granzyme B to cleave three anti-CD5- based immunotoxins, anti-CD5-PE, anti-CD5-VCE and DT-anti-CD5, was compared, the engineered granzyme B sites were not equally susceptible to cleavage. Under conditions leading to the cleavage of a majority of VCE fusion protein, only a small fraction of DT and PEA fusion proteins was cleaved (Fig. 6A-C). These results suggested that VCE might exhibit better specific toxicity in the presence of granzyme B fusion protein. The ability of the three toxin fusion proteins to kill target cells in the presence or absence of GrB-anti-CD19 was explored. Like DT-anti-CD5 and anti-CD5-PEA fusion proteins bearing a granzyme B cleavage sequence, anti-CD5-VCE fusion protein bearing a granzyme B cleavage sequence alone was not toxic to target cells and selectively killed target cells only in the presence of GrB-anti-CD19 fusion protein (Fig. 7). The ability of GrB-anti-CD19 to activate cytotoxicity of modified immunotoxins was found to correlate with the ability of GrB-anti-CD19 to cleave modified immunotoxins *in vitro*. The relative potency illustrated by observed EC_{50} values were: anti-CD5-VCE (~1.3 nM) < DT-anti-CD5 (~3.0 nM) < anti-CD5-PEA (~4.8 nM).

To further assess the effectiveness of a potential combinatorial targeting strategy, we also compared the anti-CD5-VCE bearing a granzyme B site to an anti-CD5-VCE fusion protein with endogenous furin cleavage site and an anti-CD5-VCE fusion protein in which one of the active sites was mutated (glutamic acid 613 to alanine). As expected, fusion proteins bearing the active site mutation failed to kill target cells at all concentrations tested (Fig. 7, diamonds). Replacing the furin cleavage site with a granzyme B cleavage site substantially reduced the toxicity of anti-CD5-VCE fusion protein (Fig. 8, squares). However, in the presence of 1.0 nM GrB- anti-CD19, the cytotoxicity of anti-CD5-VCE fusion protein was fully restored

(Fig. 8, compare triangles with inverted triangles). These results demonstrate that binary targeting agents are highly selective and can be as effective as conventional immunotoxins.

K. Selective killing of PBMNC from a CLL patient by the combination of anti-CD5-VCE and anti-CD19-GrB

To test whether the binary targeting agents can specifically kill B cell-chronic lymphocytic leukemia cells, we carried out a cytostasis assay with purified peripheral blood mononuclear cells (PBMNC) from a patient with B cell chronic lymphocytic leukemia (B-CLL). FACS analysis indicated that about 30% of the PBMNC from the patient were $CD5^+$ B cells (Fig. 9A). Individual components of the binary targeting agents were not toxic to PBMNC (Fig. 9B and C). At the concentrations at which the binary targeting agents completely inhibited protein synthesis activity of a reporter cell line ($CD5^+$ Raji), about 30% of total protein synthesis activity from PBMNC was arrested. No further inhibition of protein synthesis was observed as the concentration of DT- anti-CD5 was increased, consistent with the notion that the binary targeting agents only arrest the protein synthetic activity of the target cell population. These data suggested that combinatorial targeting agents can be deployed to eliminate specific cell populations from heterogeneous mixture of cells with minimal toxicity to other cell types.

L. Mutations that reduce vascular leak syndrome (VLS)

The clinical utility of immunotoxins has been limited by a variety of toxic syndromes, manifest as hepatotoxicity, neurotoxicity, and vascular leak syndrome (VLS). VLS is characterized by hypoalbuminemia, pleural effusion, weight gain, edema, hypotension, increase in hematocrit and organ failure. Dose escalation and hence effectiveness of immunotoxins, including that are PEA-based (Pai, et al., Nat. Med., 2: 350-353,1996), has been limited by VLS. Using a rat model, the toxin component of a PEA-based immunotoxin was found to be responsible for inducing VLS, and nonsteroidal anti-inflammatory agents were found to block VLS in rats receiving PEA immunotoxin. (Seegall et al., Clin. Cancer Res. 3(3):339-45 (1997)). The mechanisms underlying VLS are unclear and are likely to involve a cascade of events that are initiated by endothelial cell (EC) damage and involve inflammatory

reaction. It has been proposed that a structural motif in VLS-inducing molecules may be responsible for binding to endothelial cells and initiating VLS. The proposed motif is (x)D(y) where x = L, I, G, or V and y = V, L, or S. Previous studies suggested that deletions or mutations within this motif and/or its flanking sequences might prevent VLS (Baluna et al., Proc. Natl. Acad. Sci. USA 96:3957–3962 (1999) and Smallshaw JE et al. Nat. Biotechnol. 21:387-391 (2003)).

A recent study has shown that mutations within the translocation and catalytic domains of PEA that are adjacent to, but not specifically at, the three (x)D(y) motifs, i.e., GDL (323-325), GDV (405-407) and GDV (580-582), may also reduce VLS. In particular, a triple mutant R293K/N416Q/R576K was shown to maintain cytotoxicity to targeted cells while exhibiting much weakened VLS induction (Wang et al. Cancer Immunol Immunother. 56(11):1775-83(2007)). The residues within PEA are shown in Figure 10 with corresponding residues in VCE. To illustrate the similarity of the mutated regions between PEA and VCE, the nearest neighboring consensus residues are shown underlined in orange color.

Because VCE folds into a structure similar to that of PEA (Yates S.P., TIBS 31, 123-133, 2006), corresponding mutations on VCE, i.e., N338K/N468Q/N636K, may reduce potential VLS induction by VCE. It is noteworthy that the translocation and catalytic domains of VCE do not contain any (x)D(y) motifs. This may render VCE less toxic. On the other hand, the observed reduction in VLS by R293K/N416Q/R576K PEA mutant may not necessarily be related to these motifs. Corresponding VCE mutations N338K, N468Q, and/or N636K are preferred embodiments for VLS reduction.

M. Mutations that reduce antigenicity of VCE

When PEA-based immunotoxins are administered to patients, neutralizing antibodies often develop within 3 weeks. These antibodies, which almost always react with PEA and very infrequently with the cell-targeting domain, limit the number of treatment cycles that can be applied (Roscoe et al. Eur. J. Immunol. 27(6):1459-68 (1997)). The major human B cell epitopes of the translocation and catalytic domains of PEA have been mapped, and the corresponding antibodies characterized (Onda et al., J. Immunol. 177(12):8822-34 (2006)). The location of each epitope on PEA was determined by preparing 41 mutants of PEA in which bulky surface residues were

mutated to either alanine or glycine. All 7 major epitope groups and 9 of 13 epitope subgroups were identified by 14 different mutants and these retained high cytotoxic activity. The 14 mutants reported with diminished binding to antibodies are predicted to be less antigenic forms of PEA in an epitope basis (Onda et al., J. Immunol. 177(12):8822-34 (2006)). The positions of these mutations within PEA, as well as the corresponding residues within VCE, are shown in Figure 10 (identified by arrows). To illustrate the similarity of the mutated regions between PEA and VCE, the nearest neighboring consensus residues (including itself if it is a consensus) are shown underlined in orange color. The closest consensus residue to R576 of PEA and Y636 of VCE are four residues away (see also Fig. 10 for illustration of this position).

The reported mutations of PEA include: R313A, D324A, E327A, Q332A, R412A, E431A, R432G, R467A, R490A, R513A, R538A, R576A, K590A. Corresponding residues in VCE are shown in Fig. 11. Potential mutations in VCE include: T358, D374, R377, N381, N382, Q464, R483, E484, G522, A552, R575, R598, Y636, and K648. Mutations may be made to small residues such as Gly and Ala to reduce antigenicity. Some of these, including N381, N382, Q464, G522, and A552, are already small residues and may not need to be mutated.

Additional mutations, including many shown in Fig. 11, have been disclosed in WO27016150A2.

N. Transduction of heterologous proteins by the translocation domain of VCE

The translocation domain of PEA has been shown to be able to shuttle heterologous proteins or small molecules into the cytosol of target cells (Theuer et al., J. Biol. Chem 267:16872-16877 (1992); Prior et al., Cell 64:1017-1023 (1991); US patent number US6086900). To address if the translocation domain of VCE also is capable of shuttling heterologous proteins into the cytosol of target cells, we replaced the ADPRT domain of VCE with the ADPRT domain of PEA, which only shares about 39% identity to the ADPRT domain of VCE. We found that the fusion protein bearing the VCE translocation domain and ADPRT domain of PEA (N-GFD-VCE-PEA) is toxic to Jurkat cells, indicating that the ADPRT domain of PEA was shuttled to the cytosol of target cells by the translocation domain of VCE (Fig 12). These results support the idea that the translocation domain of VCE can be employed to

carry proteins, or molecules that are not permeable to lipid bilayer, across cell membranes.

O. Generation of polyclonal and monoclonal antibodies against VCE

Polyclonal antibodies to VCE were prepared by immunization of rabbits with domain III (the ADPRT domain) of VCE. To reduce any potential toxicity of the antigen to the host animals, a mutant VCE ADPRT domain in which the active site glutamic acid residue was replaced with an alanine (E613A) was used as the antigen. The production of anti-VCE polyclonal antibodies was carried out by the Affinity Bioreagents (Golden, CO). Sera from immunized rabbits was affinity purified and titered by ELISA assay. Polyclonal antibodies raised in two rabbits (9375 and 9376) reacted specifically to a fusion protein consisting of the maltose binding protein (VCE antigen) and ADPRT domain of VCE, but not to a maltose binding protein alone (control antigen) (Fig. 13A). The polyclonal antibodies were also used as immunoblot reagents to detect the ADPRT domain of VCE. As shown in Fig. 13B, polyclonal antibodies raised from rabbit 9375 specifically reacted with anti-CD19-VCE fusion protein determinants, but did not recognize anti-CD5-diphtheria toxin fusion protein nor any protein from 293T cell lysate. Similar results were obtained with antibodies from rabbit 9376. These results indicated that the affinity-purified polyclonal antibodies are highly specific to the ADPRT domain of VCE and to fusion proteins containing ADPRT domain of VCE. To create monoclonal antibodies against VCE, the spleen of the animal 9376 was retrieved and sent to Epitomics Inc. (Burlingame, CA) for the production of hybridomas. Several high affinity monoclonal antibodies that react with native VCE antigen as well as antibodies that react with both native VCE and blot-transferred VCE. Fig. 13C shows SPR results of several monoclonal antibodies identified from the screening of about 3000 hybridomas.

Table 3

Sequences

VCE (SEQ ID NO:1)

Wild type sequence

gi|58615288|gb|AAW80252.1| hypothetical exotoxin A [Vibrio cholerae]
MYLTFYLEKVMKKMLLIAGATVISSMAHPTFAVEDELNIFDECRSPCSLTPEPGKPIQSKLSIPSDVVL
DEGVLYYSMTINDEQNDIKDEDKGESIITIGEFATVRATRHYVNQDAPFGVIHLDITTENGTKTYSYNR
KEGEFAINWLVPIGEDSPASIKISVDELDQQRNIIEVPKLYSIDLDNQTLEQWKTQGNVSFSVTRPEHN
IAISWPSVSYKAAQKEGSRHKRWAHWHTGLALCWLVPMDAIYNYITQQNCTLGDNWFGGSYETVAGTPK
VITVKQGIEQKPVEQRIHFSKGNAMSALAAHRVCGVPLETLARSRKPRDLTDDLSCAYQAQNIVSLFVA
TRILFSHLDSVFTLNLDEQEPEVAERLSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSA
GAQAADILSLFCPDADKSCVASNNDQANINIESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTR
EGYVFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRA
ERDARGVMLRVYIPRASLERFYRTNTPLENAEEHITQVIGHSLPLRNEAFTGPESAGGEDETVIGWDMA
IHAVAIPSTIPGNAYEELAIDEEAVAKEQSISTKPPYKERKDELK

SEQ ID NO:2

Protein sequence corresponding to ADPRT domain of VCE

mgpenravitpqgvtnwtyqeleathqaltregyvfvgyhgtnhvaaqtivnriapvprgnnteneekw
gglyvathaevahgyarikegtgeyglptraerdargvmlrvyipraslerfyrtntplenaeehitqv
ighslplrneaftgpesaggedetvigwdmaihavaipstipgnayeelaideeavakeqsistkppyk
erhhhhhhkdel

SEQ ID NO:3

Synthetic gene encoding ADPRT domain of VCE

ATGGGCCCTGAAAATCGCGCGGTTATCACCCCGCAAGGCGTCACGAACTGGACCT
ATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGTTACGTGTTTGTAGGGT
ATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCGGTCCCAC
GTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGG
AAGTAGCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGC
GTGCAGAACGCGACGCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGG
AGCGCTTCTATCGTACCAACACTCCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTA
TCGGCCACTCTCTGCCACTGCGCAACGAAGCATTTACGGGTCCTGAAAGTGCGGGGGGAG
AGGATGAAACCGTGATTGGCTGGGACATGGCTATCCATGCCGTAGCAATTCCGTCAACTA
TTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTCGCGAAAGAACAAT
CCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGATGAAC
TGTAA

SEQ ID NO:53

N-CFD-VCE

Synthetic gene encoding N-CFD-VCE with endogenous furin cleavage site

ATGGGCTCCAACGAACTGCATCAGGTGCCGAGCAACTGCGATTGTCTGAACGGCGGTACCTGCGTTTCC
AACAAATATTTTTCTAACATTCACTGGTGTAACTGCCCGAAAAAATTCGGTGGACAACATTGTGAAATC
GACGGCGGTGGTGGTTCGGGCGGTGGCGGTTCGGGTGGCGGTGGCAGCTCTAGCAAAGGCAATGCCATG
AGTGCACTGGCTGCGCACCGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCACGCAAACCACGT
GACCTGACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGT
ATCCTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAG
CGCCTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCG
CGTCAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCA
CAAGCTGCGGATATTCTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACGAT
CAGGCTAATATCAACATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACC
CCGCAAGGCGTCACGAACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGT
TACGTGTTTGTAGGGTATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCG
GTCCCACGTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGGAA
GTAGCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGC
GACGCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAAC
ACTCCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAA
GCATTTACGGGTCCTGAAAGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCCAT
GCCGTAGCAATTCCGTCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTC

GCGAAAGAACAATCCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGAT
GAACTGTAAGCGGCCGC

SEQ ID NO:54

Protein sequence corresponding to synthetic N-GFD-VCE with endogenous furin cleavage site

MSNELHQVPSN CDCLNGGTCV SNKYFSNIHW CNCPKKFGGQ HCEID
GGGGSGGGGSGGGGSSSKGNAMSALAAHRVCGVPLETLARSRKPRDLTDDLSCAYQAQNIVSLFVATRI
LFSHLDSVFTLNLDEQEPEVAERLSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQ
AADILSLFCPDADKSCVASNNDQANINIESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTREGY
VFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERD
ARGVMLRVYIPRASLERFYRTNTPLENAEEHITQVIGHSLPLRNEAFTGPESAGGEDETVIGWDMAIHA
VAIPSTIPGNAYEELAIDEEAVAKEQSISTKPPYKERHHHHHHKDEL

SEQ ID NO:55

N-GFD-VCE

Synthetic gene encoding N-GFD-VCE with a granzyme B cleavage site

CCATGGGCTCCAACGAACTGCATCAGGTGCCGAGCAACTGCGATTGTCTGAACGGCGGTACCTGCGTTT
CCAACAAATATTTTTCTAACATTCACTGGTGTAACTGCCCGAAAAAATTCGGTGGACAACATTGTGAAA
TCGACGGCGGTGGTGGTTCGGGCGGTGGCGGTTCGGGTGGCGGTGGCAGCTCTAGCAAAGGCAACGCGA
TGAGCGCGCTGGCCGCACATCGTGTGTGCGGCGTTCCGCTGGAAACCCTGGCTCGCTCT**ATTGAGCCAG**
ATAGTGGTACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGC
GTATCCTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTG
AGCGCCTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCG
CGCGTCAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGG
CACAAGCTGCGGATATTCTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACG
ATCAGGCTAATATCAACATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCA
CCCCGCAAGGCGTCACGAACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAG
GTTACGTGTTTGTAGGGTATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCC
CGGTCCCACGTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGG
AAGTAGCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAAC
GCGACGCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCA
ACACTCCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACG
AAGCATTTACGGGTCCTGAAAGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCC
ATGCCGTAGCAATTCCGTCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAG
TCGCGAAAGAACAATCCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAG
ATGAACTGTAAGCGGCCGC

Sequences replacing bolded underlined region have been made, including sequence encoding IEPDDL SEQ ID NO:56 (**ATTGAGCCAGATGACCTG**) SEQ ID NO:57, IAPDSG SEQ ID NO:58 (**ATTGCTCCAGATAGTGGT**) SEQ ID NO:59, and IAPDDL SEQ ID NO:60 (**ATTGCTCCAGATGACCTG**) SEQ ID NO:61.

SEQ ID NO:62

Protein sequence corresponding to synthetic N-GFD-VCE with a granzyme B cleavage site

MSNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDGGGGSGGGGSGGGGSSSKGNAMS
ALAAHRVCGVPLETLARSIEPDSGTDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAER
LSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQ
ANINIESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPV
PRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNT
PLENAEEHITQVIGHSLPLRNEAFTGPESAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVA
KEQSISTKPPYKERHHHHHHKDEL

Sequences in place of underlined region have been made, including IEPDDL SEQ ID NO:56, IAPDSG SEQ ID NO:58 and IAPDDL SEQ ID NO:60.

N-GFD-VCE

Synthetic gene encoding N-GFD-VCE with a granzyme B cleavage site and a amino acid substitution at Phenylalanine 343 (with alanine F343A, or with tryptophan F343W)

SEQ ID NO:63

CCATGGGCTCCAACGAACTGCATCAGGTGCCGAGCAACTGCGATTGTCTGAACGGCGGTACCTGCGTTT
CCAACAAATATTTTTCTAACATTCACTGGTGTAACTGCCCGAAAAAATTCGGTGGACAACATTGTGAAA
TCGACGGCGGTGGTGGTTCGGGCGGTGGCGGTTCGGGTGGCGGTGGCAGCTCTAGCAAAGGCAACGCGA
TGAGCGCGCTGGCCGCACATCGTGTGTGCGGCGTTCCGCTGGAAACCCTGGCTCGCTCTATTGAGCCAG
ATAGTGGTACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTG**TTT**GTTGCAACGC
GTATCCTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTG
AGCGCCTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCG
CGCGTCAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGG
CACAAGCTGCGGATATTCTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACG
ATCAGGCTAATATCAACATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCA
CCCCGCAAGGCGTCACGAACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAG
GTTACGTGTTTGTAGGGTATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCC
CGGTCCCACGTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGG
AAGTAGCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAAC
GCGACGCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCA
ACACTCCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACG
AAGCATTTACGGGTCCTGAAAGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCC
ATGCCGTAGCAATTCCGTCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAG
TCGCGAAAGAACAATCCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAG
ATGAACTGTAAGCGGCCGC

Underlined TTT was replaced with TGG to yield F343W mutant or with GCG to yield F343A mutant

Protein sequence corresponding to synthetic N-GFD-VCE with a granzyme B cleavage site and a amino acid substitution at Phenylalanine 343 (with alanine F343A, or with tryptophan F343W)

SEQ ID NO:64

MSNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDGGGGSGGGGSGGGGSSSKGNAMS
ALAAHRVCGVPLETLARSIEPDDLTDDLSCAYQAQNIVSLFVATRIL**F**SHLDSVFTLNLDEQEPEVAER
LSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQ
ANINIESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPV
PRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNT
PLENAEEHITQVIGHSLPLRNEAFTGPESAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVA
KEQSISTKPPYKERHHHHHHKDEL

Underlined F was replaced with W to yield F343W mutant or with A to yield F343A mutant

N-GFD-VCE

SEQ ID NO:65

Synthetic gene encoding N-GFD-VCE with endogenous furin cleavage site and substitution of Glu 613 with Ala (E613A)

ATGGGCTCCAACGAACTGCATCAGGTGCCGAGCAACTGCGATTGTCTGAACGGCGGTACCTGCGTTTCC
AACAAATATTTTTCTAACATTCACTGGTGTAACTGCCCGAAAAAATTCGGTGGACAACATTGTGAAATC
GACGGCGGTGGTGGTTCGGGCGGTGGCGGTTCGGGTGGCGGTGGCAGCTCTAGCAAAGGCAATGCCATG
AGTGCACTGGCTGCGCACCGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCACGCAAACCACGT
GACCTGACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGT
ATCCTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAG
CGCCTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCG
CGTCAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCA
CAAGCTGCGGATATTCTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACGAT
CAGGCTAATATCAACATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACC
CCGCAAGGCGTCACGAACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGT
TACGTGTTTGTAGGGTATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCG

GTCCCACGTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGGAA
GTAGCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGC
GACGCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAAC
ACTCCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAA
GCATTTACGGGTCCTGAAAGTGCGGGGGGAGAGGATGCAACCGTGATTGGCTGGGACATGGCTATCCAT
GCCGTAGCAATTCCGTCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTC
GCGAAAGAACAATCCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGAT
GAACTGTAAGCGGCCGC

Underlined TTT was replaced with TGG to yield F343W mutant or with GCG to yield F343A mutant

SEQ ID NO:66

Protein sequence corresponding to synthetic N-GFD-VCL with endogenous furin cleavage site and substitution of Glu 613 with Ala (E613A)

MSNELHQVPSN CDCLNGGTCV SNKYFSNIHW CNCPKKFGGQ HCEID
GGGGSGGGGSGGGGSSSKGNAMSALAAHRVCGVPLETLARSRKPRDLTDDLSCAYQAQNIVSLFVATRI
LFSHLDSVFTLNLDEQEPEVAERLSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQ
AADILSLFCPDADKSCVASNNDQANINIESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTREGY
VFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERD
ARGVMLRVYIPRASLERFYRTNTPLENAEEHITQVIGHSLPLRNEAFTGPESAGGADETVIGWDMAIHA
VAIPSTIPGNAYEELAIDEEAVAKEQSISTKPPYKERHHHHHHKDEL

Synthetic gene encoding GrB-anti-CD19

SEQ ID NO:67

ATGGGCGTGAAGGTGCTGTTCGCCCTGATCTGCATCGCCGTGGCGctcgccgacaactcgagctacaag
gacgacgacgacaagATCATCGGGGGACATGAGGCCAAGCCCCACTCCCGCCCCTACATGGCTTATCTT
ATGATCTGGGATCAGAAGTCTCTGAAGAGGTGCGGTGGCTTCCTGATACAAGACGACTTCGTGCTGACA
GCTGCTCACTGTTGGGGAAGCTCCATAAATGTCACCTTGGGGGCCCACAATATCAAAGAACAGGAGCCG
ACCCAGCAGTTTATCCCTGTGAAAAGACCCATCCCCCATCCAGCCTATAATCCTAAGAACTTCTCCAAC
GACATCATGCTACTGCAGCTGGAGAGAAAGGCCAAGCGGACCAGAGCTGTGCAGCCCCTCAGGCTACCT
AGCAACAAGGCCCAGGTGAAGCCAGGGCAGACATGCAGTGTGGCCGGCTGGGGGCAGACGGCCCCCCTG
GGAAAACACTCACACACACTACAAGAGGTGAAGATGACAGTGCAGGAAGATCGAAAGTGCGAATCTGAC
TTACGCCATTATTACGACAGTACCATTGAGTTGTGCGTGGGGGACCCAGAGATTAAAAAGACTTCCTTT
AAGGGGGACTCTGGAGGCCCTCTTGTGTGTAACAAGGTGGCCCAGGGCATTGTCTCCTATGGACGAAAC
AATGGCATGCCTCCACGAGCCTGCACCAAAGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAA
CGCTACGCCATGGGAGGCGGAGGCTCCGGAGGAGGAGGGTCCGGGGGCGGCGGAAGCATGGCCCAGGTG
CAGCTGCAGCAGTCCGGCGCTGAGCTGGTGCGCCCTGGCTCCTCCGTGAAAATCTCCTGCAAGGCTTCC
GGCTACGCTTTCTCCTCCTACTGGATGAACTGGGTGAAGCAGCGCCCTGGCCAGGGCCTGGAGTGGATC
GGCCAAATCTGGCCGGGCGACGGCGACACCAACTACAACGGCAAGTTCAAGGGCAAGGCTACCCTGACC
GCTGACGAGTCCTCCTCCACCGCTTACATGCAGCTGTCCTCCCTGGCTTCCGAGGACTCCGCTGTGTAC
TTCTGCGCTCGCCGCGAGACCACCACCGTGGGCCGCTACTACTACGCTATGGACTACTGGGGCCAGGGC
ACCTCGGTGACCGTGTCCTCCGGCGGCGGCGGCTCCGGCGGCGGCGGCTCCGGCGGCGGGAGCTCCGAC
ATCCTGCTGACCCAGACCCCGGCTTCCCTGGCTGTGTCCCTGGGCCAGCGCGCTACCATCTCCTGCAAG
GCTTCCCAGTCCGTGGACTACGACGGCGACTCCTACCTGAACTGGTACCAGCAGATCCCGGGCCAGCCG
CCGAAGCTGCTGATCTACGACGCTTCCAACCTGGTGTCCGGCATCCCGCCGCGCTTCTCCGGCTCCGGC
TCCGGCACCGACTTCACCCTGAACATCCACCCGGTGGAGAAGGTGGACGCTGCTACCTACCACTGCCAG
CAGTCCACCGAGGACCCGTGGACCTTCGGCGGCGGCACCAAGCTGGAGATCAAGCGCGGTGGTGACATG
CATCACCATCACCATCACTGA

GrB-anti-CD19 Protein sequence

SEQ ID NO:68

MGVKVLFALICIAVALADNSSYKDDDDKIIGGHEAKPHSRPYMAYLMIWDQKSLKRCGGFLIQDDFVLT
AAHCWGSSINVTLGAHNIKEQEPTQQFIPVKRPIPHPAYNPKNFSNDIMLLQLERKAKRTRAVQPLRLP
SNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTVQEDRKCESDLRHYYDSTIELCVGDPEIKKTSF
KGDSGGPLVCNKVAQGIVSYGRNNGMPPRACTKVSSFVHWIKKTMKRYAMGGGGSGGGGSGGGGSMAQV
QLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLT
ADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTSVTVSSGGGGSGGGGSGGGSSD
ILLTQTPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSG
SGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIKRGGDMHHHHHH

anti-CD5-VCE

synthetic gene encoding antiCD5-VCE with endogenous furin cleavage site

SEQ ID NO:69

ATGgccaacatccagctggtgcagtctggtcctgagctgaagaagcctggtgagactgtcaaaatctcc
tgcaaggcttctgggtataccttcactaactatggtatgaactgggtgaagcaggctcctggtaagggt
ctgcgttggatgggctggattaacacccacactggtgagcctacttatgctgatgacttcaagggacgt
tttgccttctctctggaaacttctgccagcactgcctatctccagatcaacaacctcaaaaatgaggac
actgctacttacttctgtacacgtcgtggttacgactggtacttcgatgtctggggtgctgggaccacg
gtgaccgtgttctccgggggaggtggcagcgggggaggtggcagcggcggcgggagctccgacatcaag
atgacccagtctccttcttccatgtatgcttctctgggtgagcgtgtcactatcacttgcaaggccagc
caggacattaatagctatctgagctggttccatcataaacctgggaaatctcctaagaccctgatctat
cgtgctaaccgtctggttgatggggtcccttctcgtttcagcggctctggttctgggcaagattattct
ctcaccatcagcagcctggactatgaagatatgggtatttattattgtcaacagtatgatgagtctcct
tggactttcggtggtggcaccaagctggagatgaaaggctctggcGCTAGCAAAGGCAATGCCATGAGT
GCACTGGCTGCGCACCGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCA**CGCAAACCACGTGAC**
CTGACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGTATC
CTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAGCGC
CTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCGCGT
CAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCACAA
GCTGCGGATATTCTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACGATCAG
GCTAATATCAACATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACCCCG
CAAGGCGTCACGAACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGTTAC
GTGTTTGTAGGGTATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCGGTC
CCACGTGGCAATAACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGGAAGTA
GCTCACGGTTATGCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGCGAC
GCGCGTGGTGTGATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAACACT
CCGCTGGAAAATGCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAAGCA
TTTACGGGTCCTGAAAGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCCATGCC
GTAGCAATTCCGTCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTCGCG
AAAGAACAATCCATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGATGAA
CTGTAA

Genes with altered underlined sequences encoding various granzyme B cleavage sequences (**ATTGAGCCAGATGACCTG—IEPDDL** SEQ ID NO:70; **ATTGCTCCAGATAGTGGT—IAPDSG)** SEQ ID NO:71; **ATTGAGCCAGATAGTGGT—IEPDSG** SEQ ID NO:72; **and ATTGCTCCAGATGACCTG--IAPDDL** SEQ ID NO:73)were also made.

Protein sequence of anti-CD5-VCE with a 15 amino acid linker

SEQ ID NO:74

MANIQLVQSGPELKKPGETVKISCKASGYTFTNYGMNWVKQAPGKGLRWMGWINTHTGEPTYADDFKGR
FAFSLETSASTAYLQINNLKNEDTATYFCTRRGYDWYFDVWGAGTTVTVFSGGGGSGGGGSGGGGGSSD
IKMTQSPSSMYASLGERVTITCKASQDINSYLSWFHHKPGKSPKTLIYRANRLVDGVPSRFSGSGSGQD
YSLTISSLDYEDMGIYYCQQYDESPWTFGGGTKLEMKGSGASKGNAMSALAAHRVCGVPLETLARS**RKP**
RDLTDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAERLSDLRRINENNPGMVTQVLTV
ARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQANINIESRSGRSYLPENRAVI
TPQGVTNWTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHA
EVAHGYARIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNTPLENAEEHITQVIGHSLPLRN
EAFTGPESAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVAKEQSISTKPPYKERHHHHHHK
DEL

Proteins with altered underlined sequence, including IEPDDL SEQ ID NO:75, IAPDSG SEQ ID NO:76, IEPDSG SEQ ID NO:77, and IAPDDL SEQ ID NO:78were also made.

Anti-CD19-VCE

SEQ ID NO:79

ATGGCCCAGGTGCAGCTGCAGCAGTCCGGCGCTGAGCTGGTGCGCCCTGGCTCCTCCGTGAAAATCTCC
TGCAAGGCTTCCGGCTACGCTTTCTCCTCCTACTGGATGAACTGGGTGAAGCAGCGCCCTGGCCAGGGC
CTGGAGTGGATCGGCCAAATCTGGCCGGGCGACGGCGACACCAACTACAACGGCAAGTTCAAGGGCAAG
GCTACCCTGACCGCTGACGAGTCCTCCTCCACCGCTTACATGCAGCTGTCCTCCCTGGCTTCCGAGGAC
TCCGCTGTGTACTTCTGCGCTCGCCGCGAGACCACCACCGTGGGCCGCTACTACTACGCTATGGACTAC
TGGGGCCAGGGCACCTCGGTGACCGTGTCCTCCGGCGGCGGCGGCTCCGGCGGCGGCGGCTCCGGCGGC
GGGTCCGGGAGCTCCGACATCCTGCTGACCCAGACCCCGGCTTCCCTGGCTGTGTCCCTGGGCCAGCGC
GCTACCATCTCCTGCAAGGCTTCCCAGTCCGTGGACTACGACGGCGACTCCTACCTGAACTGGTACCAG
CAGATCCCGGGCCAGCCGCCGAAGCTGCTGATCTACGACGCTTCCAACCTGGTGTCCGGCATCCCGCCG
CGCTTCTCCGGCTCCGGCTCCGGCACCGACTTCACCCTGAACATCCACCCGGTGGAGAAGGTGGACGCT
GCTACCTACCACTGCCAGCAGTCCACCGAGGACCCGTGGACCTTCGGCGGCGGCACCAAGCTGGAGATC
AAGCGCGGCTCTGGCGCTAGCAAAGGCAATGCCATGAGTGCACTGGCTGCGCACCGCGTATGCGGTGTG
CCGCTGGAGACACTGGCCCGTTCACGCAAACCACGTGACCTGACCGATGACCTGAGCTGCGCGTATCAG
GCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGTATCCTGTTCAGTCATCTGGATTCAGTCTTTACT
CTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAGCGCCTGTCCGATCTGCGTCGCATTAATGAAAAC
AATCCAGGCATGGTGACACAAGTTCTGACCGTCGCGCGTCAGATCTACAACGACTATGTAACGCACCAT
CCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCACAAGCTGCGGATATTCTGAGCCTGTTCTGTCCA
GATGCCGACAAATCTTGCGTGGCAAGTAATAACGATCAGGCTAATATCAACATTGAGTCACGCTCCGGA
CGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACCCCGCAAGGCGTCACGAACTGGACCTATCAGGAG
CTGGAAGCCACTCACCAGGCACTGACACGTGAAGGTTACGTGTTTGTAGGGTATCATGGAACGAATCAC
GTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCGGTCCCACGTGGCAATAACACTGAGAATGAAGAG
AAATGGGGTGGCCTGTACGTTGCAACACATGCGGAAGTAGCTCACGGTTATGCCCGCATTAAAGAAGGG
ACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGCGACGCGCGTGGTGTGATGCTGCGCGTCTACATC
CCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAACACTCCGCTGGAAAATGCCGAAGAGCATATTACA
CAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAAGCATTTACGGGTCCTGAAAGTGCGGGGGGAGAG
GATGAAACCGTGATTGGCTGGGACATGGCTATCCATGCCGTAGCAATTCCGTCAACTATTCCAGGTAAT
GCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTCGCGAAAGAACAATCCATTTCGACAAAACCGCCT
TATAAAGAGCGTCACCATCATCACCATCACAAAGATGAACTGTAA

Genes with altered underlined sequences encoding various granzyme B cleavage sequences (**ATTGAGCCAGATGACCTG—IEPDDL; ATTGCTCCAGATAGTGGT—IAPDSG); ATTGAGCCAGATAGTGGT—IEPDSG; and ATTGCTCCAGATGACCTG--IAPDDL)** were also made.

Anti-CD19-VCE protein sequence

SEQ ID NO:80

MAQVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGK
ATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTSVTVSSGGGGSGGGGSGG
GSGSSDILLTQTPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPP
RFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIKRGSGASKGNAMSALAAHRVCGV
PLETLARS**RKPRDL**TDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAERLSDLRRINEN
NPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQANINIESRSG
RSYLPENRAVITPQGVTNWTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEE
KWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNTPLENAEEHIT
QVIGHSLPLRNEAFTGPESAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVAKEQSISTKPP
YKERHHHHHHKDEL

Proteins with altered underlined sequence, including IEPDDL, IEPDSG, IAPDDL, IAPDSG, were also made.

anti-CD5-PEA

Synthetic gene encoding anti-CD5-PEA

SEQ ID NO:81

ATGGACTACAAGGACGACGACGACAAGGGCATGgccaacatccagctggtgcagtctggtcctgagctg
aagaagcctggtgagactgtcaaaatctcctgcaaggcttctgggtataccttcactaactatggtatg
aactgggtgaagcaggctcctggtaagggtctgcgttggatgggctggattaacacccacactggtgag
cctacttatgctgatgacttcaagggacgttttgccttctctctggaaacttctgccagcactgcctat
ctccagatcaacaacctcaaaaatgaggacactgctacttacttctgtacacgtcgtggttacgactgg
tacttcgatgtctggggtgctgggaccacggtgaccgtgttctccgggggaggtggcagcgggggaggt
ggcagcggcggcgggagctccgacatcaagatgacccagtctccttcttccatgtatgcttctctgggt
gagcgtgtcactatcacttgcaaggccagccaggacattaatagctatctgagctggttccatcataaa

cctgggaaatctcctaagaccctgatctatcgtgctaaccgtctggttgatggggtcccttctcgtttc
agcggctctggttctgggcaagattattctctcaccatcagcagcctggactatgaagatatgggtatt
tattattgtcaacagtatgatgagtctccttggactttcggtggtggcaccaagctggagatgaaagga
ggcggaggctccggaggaggaggcgggtccgctagcctGATCGCCCTGACCGCCCACCAGGCCTGCCAC
CTGCCGCTGGAGACCTTCACCGCTAGCATCGAGCCGGACGGCTGGGAGCAGCTGGAGCAGTGCGGCTAC
CCGGTGCAGCGCCTGGTGGCCCTGTACCTGGCCGCCCGCCTGTCCTGGAACCAGGTGGACCAGGTGATC
CGCAACGCCCTGGCCTCCCCGGGCTCCGGCGGCGACCTGGGCGAGGCCATCCGCGAGCAGCCGGAGCAG
GCCCGCCTGGCCCTGACCCTGGCCGCCGCCGAGTCCGAGCGCTTCGTGCGCCAGGGCACCGGCAACGAC
GAGGCCGGCGCCGCCAACGCCGACGTGGTGTCCCTGACCTGCCCGGTGGCCGCCGGCGAGTGCGCCGGC
CCGGCCGACTCCGGCGACGCCCTGCTGGAGCGCAACTACCCGACCGGCGCCGAGTTCCTGGGCGACGGC
GGCGACGTGTCCTTCTCCACCCGCGGCACCCAGACCTGGACCGTGGAGCGCCTGCTGCAGGCCCACCGC
CAGCTGGAGGAGCGCGGCTACGTGTTCGTGGGCTACCACGGCACCTTCCTGGAGGCCGCCCAGTCCATC
GTGTTCGGCGGCGTGCGCGCCCGCTCCCAGGACCTGGACGCCATCTGGCGCGGCTTCTACATCGCCGGC
GACCCGGCCCTGGCCTACGGCTACGCCCAGGACCAGGAGCCGGACGCCCGCGGTCGCATCCGCAACGGC
GCCCTGCTGCGCGTGTACGTGCCGCGCTCCTCCCTGCCGGGCTTCTACCGCACCTCCCTGACCCTGGCC
GCCCCGGAGGCCGCCGGCGAGGTGGAGCGCCTGATCGGCCACCCGCTGCCGCTGCGCCTGGACGCCATC
ACCGGCCCGGAGGAGGAGGGCGGTCGCCTGGAGACCATCCTGGGCTGGCCGCTGGCCGAGCGCACCGTG
GTGATCCCGTCCGCCATCCCGACCGACCCGCGCAACGTGGGCGGCGACCTGGACCCGTCCTCCATCCCG
GACAAGGAGCAGGCCATCTCCGCCCTGCCGGACTACGCCTCTCAGCCGGGCAAGCCGCCGCACCACCAC
CACCACCACAAGGACGAGCTGTAG

anti-CD5 PEA protein sequence

SEQ ID NO:82

MDYKDDDDKGMANIQLVQSGPELKKPGETVKISCKASGYTFTNYGMNWVKQAPGKGLRWMGWINTHTGE
PTYADDFKGRFAFSLETSASTAYLQINNLKNEDTATYFCTRRGYDWYFDVWGAGTTVTVFSGGGGSGGG
GSGGGSSDIKMTQSPSSMYASLGERVTITCKASQDINSYLSWFHHKPGKSPKTLIYRANRLVDGVPSRF
SGSGSGQDYSLTISSLDYEDMGIYYCQQYDESPWTFGGGTKLEMKGGGGSGGGGGSASLIALTAHQACH
LPLETFTASIEPDGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPEQ
ARLALTLAAAESERFVRQGTGNDEAGAANADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDG
GDVSFSTRGTQTWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAG
DPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAI
TGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPHHH
HHHKDEL

GENE ENCODING N-GFD-VC-PEA FUSION PROTEIN

SEQ ID NO:83

ATGGGCTCCAACGAACTGCATCAGGTGCCGAGCAACTGCGATTGTCTGAACGGCGGTACCTGCGTTTCC
AACAAATATTTTTCTAACATTCACTGGTGTAACTGCCCGAAAAAATTCGGTGGACAACATTGTGAAATC
GACGGCGGTGGTGGTTCGGGCGGTGGCGGTTCGGGTGGCGGTGGCAGCTCTAGCAAAGGCAATGCCATG
AGTGCACTGGCTGCGCACCGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCACGCAAACCACGT
GACCTGACCGATGACCTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGT
ATCCTGTTCAGTCATCTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAG
CGCCTGTCCGATCTGCGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCG
CGTCAGATCTACAACGACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCA
CAAGCTGCCGACGTGGTGTCCCTGACCTGCCCGGTGGCCGCCGGCGAGTGCGCCGGCCCGGCCGACTCC
GGCGACGCCCTGCTGGAGCGCAACTACCCGACCGGCGCCGAGTTCCTGGGCGACGGCGGCGACGTGTCC
TTCTCCACCCGCGGCACCCAGACCTGGACCGTGGAGCGCCTGCTGCAGGCCCACCGCCAGCTGGAGGAG
CGCGGCTACGTGTTCGTGGGCTACCACGGCACCTTCCTGGAGGCCGCCCAGTCCATCGTGTTCGGCGGC
GTGCGCGCCCGCTCCCAGGACCTGGACGCCATCTGGCGCGGCTTCTACATCGCCGGCGACCCGGCCCTG
GCCTACGGCTACGCCCAGGACCAGGAGCCGGACGCCCGCGGTCGCATCCGCAACGGCGCCCTGCTGCGC
GTGTACGTGCCGCGCTCCTCCCTGCCGGGCTTCTACCGCACCTCCCTGACCCTGGCCGCCCCGGAGGCC
GCCGGCGAGGTGGAGCGCCTGATCGGCCACCCGCTGCCGCTGCGCCTGGACGCCATCACCGGCCCGGAG
GAGGAGGGCGGTCGCCTGGAGACCATCCTGGGCTGGCCGCTGGCCGAGCGCACCGTGGTGATCCCGTCC
GCCATCCCGACCGACCCGCGCAACGTGGGCGGCGACCTGGACCCGTCCTCCATCCCGGACAAGGAGCAG
GCCATCTCCGCCCTGCCGGACTACGCCTCTCAGCCGGGCAAGCCGCCGCACCACCACCACCACCACAAG
GACGAGCTGTAGCGGCCGC

Protein sequence corresponding to synthetic N-GFD-VC-PEA FUSION PROTEIN

SEQ ID NO:84

MSNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCEIDGGGGSGGGGSGGGGSSSKGNAMS
ALAAHRVCGVPLETLARSRKPRDLTDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAER
LSDLRRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADVVSLTCPVAAGECAGPADSG
DALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGV
RARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYRTSLTLAAPEAA
GEVERLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQA
ISALPDYASQPGKPPHHHHHHKDEL

GENE ENCODING
CCPE-VCE FUSION PROTEIN
SEQ ID NO:85
ATGGGTCATCACCACCATCATCACAAGGGCGAGCTCGAAAGATCCGTTTTAACAGTTCCATCTACAGAT
ATAGAAAAAGAAATCCTTGATTTAGCTGCTGCTACAGAAAGATTAAATTTAACTGATGCATTAAACTCA
AATCCAGCTGGTAATTTATATGATTGGCGTTCTTCTAACTCATACCCTTGGACTCAAAAGCTCAATTTA
CACTTAACAATTACAGCTACTGGACAAAAATATAGAATCTTAGCAAGCAAAATTGTTGATTTTAATATT
TATTCAAATAATTTTAATAATCTAGTGAAATTAGAACAGTCCTTAGGTGATGGAGTAAAAGATCATTAT
GTTGATATAAGTTTAGATGCTGGACAATATGTTCTTGTAATGAAAGCTAATTCATCATATAGTGGAAAT
TACCCTTATTCAATATTATTTCAAAAATTTAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGT
CTCGATTCTACGCGTACCGGTGGCTCTGGCGCTAGCAAAGGCAATGCCATGAGTGCACTGGCTGCGCAC
CGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCACGCAAACCACGTGACCTGACCGATGACCTG
AGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGTATCCTGTTCAGTCATCTG
GATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAGCGCCTGTCCGATCTGCGT
CGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCGCGTCAGATCTACAACGAC
TATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCACAAGCTGCGGATATTCTG
AGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACGATCAGGCTAATATCAACATT
GAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACCCCGCAAGGCGTCACGAAC
TGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGTTACGTGTTTGTAGGGTAT
CATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCGGTCCCACGTGGCAATAAC
ACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGGAAGTAGCTCACGGTTATGCC
CGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGCGACGCGCGTGGTGTGATG
CTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAACACTCCGCTGGAAAATGCC
GAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAAGCATTTACGGGTCCTGAA
AGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCCATGCCGTAGCAATTCCGTCA
ACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTCGCGAAAGAACAATCCATT
TCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGATGAACTGTAA

Protein sequence corresponding to synthetic CCPE-VCE FUSION PROTEIN
SEQ ID NO:86
MGHHHHHHKGELERSVLTVPSTDIEKEILDLAAATERLNLTDALNSNPAGNLYDWRSSNSYPWTQKLNL
HLTITATGQKYRILASKIVDFNIYSNNFNNLVKLEQSLGDGVKDHYVDISLDAGQYVLVMKANSSYSGN
YPYSILFQKFKLEGKPIPNPLLGLDSTRTGGSGASKGNAMSALAAHRVCGVPLETLARSRKPRDLTDDL
SCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAERLSDLRRINENNPGMVTQVLTVARQIYND
YVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQANINIESRSGRSYLPENRAVITPQGVTN
WTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHAEVAHGYA
RIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNTPLENAEEHITQVIGHSLPLRNEAFTGPE
SAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVAKEQSISTKPPYKERHHHHHHKDEL

GENE ENCODING CCPE-VCE FUSION PROTEIN
SEQ ID NO:87
ATGGGTCTTGATTTAGCTGCTGCTACAGAAAGATTAAATTTAACTGATGCATTAAACTCAAATCCAGCT
GGTAATTTATATGATTGGCGTTCTTCTAACTCATACCCTTGGACTCAAAAGCTCAATTTACACTTAACA
ATTACAGCTACTGGACAAAAATATAGAATCTTAGCAAGCAAAATTGTTGATTTTAATATTTATTCAAAT
AATTTTAATAATCTAGTGAAATTAGAACAGTCCTTAGGTGATGGAGTAAAAGATCATTATGTTGATATA
AGTTTAGATGCTGGACAATATGTTCTTGTAATGAAAGCTAATTCATCATATAGTGGAAATTACCCTTAT
TCAATATTATTTCAAAAATTTGAAGGTGGCGGTTCCGAAGGTGGTGGGTCCGAAGGTGGTGGCTCCGGC
GGTTCCATCCTTGATTTAGCTGCTGCTACAGAAAGATTAAATTTAACTGATGCATTAAACTCAAATCCA
GCTGGTAATTTATATGATTGGCGTTCTTCTAACTCATACCCTTGGACTCAAAAGCTCAATTTACACTTA
ACAATTACAGCTACTGGACAAAAATATAGAATCTTAGCAAGCAAAATTGTTGATTTTAATATTTATTCA
AATAATTTTAATAATCTAGTGAAATTAGAACAGTCCTTAGGTGATGGAGTAAAAGATCATTATGTTGAT
ATAAGTTTAGATGCTGGACAATATGTTCTTGTAATGAAAGCTAATTCATCATATAGTGGAAATTACCCT
TATTCAATATTATTTCAAAAATTTGACGGTTCCGCTAGCAAAGGCAATGCCATGAGTGCACTGGCTGCG

CACCGCGTATGCGGTGTGCCGCTGGAGACACTGGCCCGTTCACGCAAACCACGTGACCTGACCGATGAC
CTGAGCTGCGCGTATCAGGCCCAAAATATTGTGTCTCTGTTTGTTGCAACGCGTATCCTGTTCAGTCAT
CTGGATTCAGTCTTTACTCTGAACCTGGACGAACAGGAGCCGGAAGTAGCTGAGCGCCTGTCCGATCTG
CGTCGCATTAATGAAAACAATCCAGGCATGGTGACACAAGTTCTGACCGTCGCGCGTCAGATCTACAAC
GACTATGTAACGCACCATCCTGGTCTGACTCCGGAACAGACATCGGCCGGGGCACAAGCTGCGGATATT
CTGAGCCTGTTCTGTCCAGATGCCGACAAATCTTGCGTGGCAAGTAATAACGATCAGGCTAATATCAAC
ATTGAGTCACGCTCCGGACGTTCGTACCTGCCTGAAAATCGCGCGGTTATCACCCCGCAAGGCGTCACG
AACTGGACCTATCAGGAGCTGGAAGCCACTCACCAGGCACTGACACGTGAAGGTTACGTGTTTGTAGGG
TATCATGGAACGAATCACGTTGCTGCGCAAACCATTGTGAACCGCATCGCCCCGGTCCCACGTGGCAAT
AACACTGAGAATGAAGAGAAATGGGGTGGCCTGTACGTTGCAACACATGCGGAAGTAGCTCACGGTTAT
GCCCGCATTAAAGAAGGGACCGGAGAGTATGGCCTGCCTACGCGTGCAGAACGCGACGCGCGTGGTGTG
ATGCTGCGCGTCTACATCCCGCGTGCTTCGCTGGAGCGCTTCTATCGTACCAACACTCCGCTGGAAAAT
GCCGAAGAGCATATTACACAGGTTATCGGCCACTCTCTGCCACTGCGCAACGAAGCATTTACGGGTCCT
GAAAGTGCGGGGGGAGAGGATGAAACCGTGATTGGCTGGGACATGGCTATCCATGCCGTAGCAATTCCG
TCAACTATTCCAGGTAATGCGTACGAGGAACTGGCCATCGATGAAGAGGCAGTCGCGAAAGAACAATCC
ATTTCGACAAAACCGCCTTATAAAGAGCGTCACCATCATCACCATCACAAAGATGAACTGTAA

Protein sequence corresponding to synthetic CGPF-VCF FUSION PROTEIN
SEQ ID NO:88
MGLDLAAATERLNLTDALNSNPAGNLYDWRSSNSYPWTQKLNLHLTITATGQKYRILASKIVDFNIYSN
NFNNLVKLEQSLGDGVKDHYVDISLDAGQYVLVMKANSSYSGNYPYSILFQKFEGGGSEGGGSEGGGSG
GSILDLAAATERLNLTDALNSNPAGNLYDWRSSNSYPWTQKLNLHLTITATGQKYRILASKIVDFNIYS
NNFNNLVKLEQSLGDGVKDHYVDISLDAGQYVLVMKANSSYSGNYPYSILFQKFDGSASKGNAMSALAA
HRVCGVPLETLARSRKPRDLTDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAERLSDL
RRINENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVASNNDQANIN
IESRSGRSYLPENRAVITPQGVTNWTYQELEATHQALTREGYVFVGYHGTNHVAAQTIVNRIAPVPRGN
NTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERDARGVMLRVYIPRASLERFYRTNTPLEN
AEEHITQVIGHSLPLRNEAFTGPESAGGEDETVIGWDMAIHAVAIPSTIPGNAYEELAIDEEAVAKEQS
ISTKPPYKERHHHHHHKDEL

Other Embodiments

Various modifications and variations of the described methods and compositions of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific desired embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the fields of medicine, immunology, pharmacology, endocrinology, or related fields are intended to be within the scope of the invention.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually incorporated by reference. The nucleic acid and amino acid sequences relevant to this application are listed above in Table 3.

What is claimed is:

Claims

1. A recombinant *Vibrio cholerae* exotoxin (VCE) comprising an amino acid sequence with greater than 80% sequence identity to SEQ ID NO:1.

2. The VCE of claim 1, wherein said amino acid sequence has greater than 90% sequence identity to SEQ ID NO:1.

3. The VCE of claim 1, wherein said amino acid sequence has greater than 95% identity to SEQ ID NO:1

4. The VCE of claim 1, wherein said amino acid sequence has 100% sequence identity to SEQ ID NO:1.

5. A vector comprising a sequence encoding the VCE of claim 1.

6. A host cell comprising a sequence encoding the VCE of claim 1.

7. An antibody that specifically binds the VCE of claim 1.

8. The antibody of claim 7, wherein said antibody is a monoclonal antibody.

9. A protein comprising a fragment of VCE, wherein said fragment comprises an amino acid sequence with greater than 80% sequence identity to SEQ ID NO:2.

10. The protein of claim 9, wherein said fragment comprises an amino acid sequence with greater than 90% sequence identity to SEQ ID NO:2.

11. The protein of claim 9, wherein said fragment comprises an amino acid sequence with greater than 95% sequence identity to SEQ ID NO:2.

12. The protein of claim 9, wherein said fragment comprises an amino acid sequence with 100% sequence identity to SEQ ID NO:2.

13. The protein of claim 9, wherein said fragment comprises ADP-ribosylating activity.

14. The protein of claim 13, wherein protein comprises cell-membrane translocation activity.

15. The protein of claim 14, wherein said fragment comprises a VCE cell-membrane translocation domain having said cell-membrane translocation activity.

16. The protein of claim 15, wherein the native furin cleavage site of said fragment is replaced with a modifiable activation domain, wherein said modifiable activation domain comprises a substrate for an exogenous enzyme.

17. The protein of claim 16, wherein said modifiable activation domain comprises a substrate for granzyme B activity.

18. The protein of claim 14, wherein said protein does not comprise the VCE cell binding domain.

19. A vector comprising a sequence encoding the protein of claim 9.

20. A host cell comprising a sequence encoding the protein of claim 9.

21. An antibody that specifically binds the the protein of claim 9.

22. The protein of claim 9, wherein said protein is a fusion protein.

23. The fusion protein of claim 22 comprising a non-native cell targeting moiety.

24. The fusion protein of claim 23, wherein said non-native cell targeting moiety is an antibody, or functional fragment thereof.

25. The fusion protein of claim 23, wherein said non-native cell targeting moiety is an artificially diversified polypeptide binder.

26. The fusion protein of claim 23, wherein said non-native cell targeting moiety is a ligand for a receptor.

27. The fusion protein of claim 23, wherein said non-native cell targeting moiety recognizes a cell surface target that is expressed on cancer cells.

28. The fusion protein of claim 23, wherein said non-native cell targeting moiety recognizes a cell selected from the group consisting of a hematopoietic cell, lymphocyte, and a nociceptive neuron.

29. The fusion protein of claim 22, further comprising a modifiable activation domain, wherein said modifiable activation domain comprises a substrate for an exogenous enzyme.

30. The fusion protein of claim 29, wherein said enzyme is a protease.

31. The fusion protein of claim 30, wherein said protease is an exogenous human protease.

32. The fusion protein of claim 30, wherein said protease is a non-human protease.

33. The fusion protein of claim 32, wherein said protease is a non-mammalian protease.

34. The fusion protein of claim 33, wherein said protease is a viral protease.

35. The fusion protein of claim 29, wherein said modifiable activation domain comprises a post-translational modification of a protease cleavage site.

36. The fusion protein of claim 29, wherein said modifiable activation domain comprises a substrate for an enzyme capable of removing a post-translational modification.

37. The fusion protein of claim 29, wherein said modifiable activation domain comprises a substrate for granzyme B activity.

38. A method of destroying a target cell, the method comprising contacting said target cell with a protein comprising a fragment of VCE, wherein said fragment comprises an amino acid sequence with greater than 80% sequence identity to SEQ ID NO:2.

39. The method of claim 38, wherein said fragment comprises an amino acid sequence with greater than 90% sequence identity to SEQ ID NO:2.

40. The method of claim 38, wherein said fragment comprises an amino acid sequence with greater than 95% sequence identity to SEQ ID NO:2.

41. The method of claim 38, wherein said fragment comprises an amino acid sequence with 100% sequence identity to SEQ ID NO:2.

42. The method of claim 38, wherein said fragment comprises ADP-ribosylating activity.

43 The method of claim 42, wherein protein comprises cell-membrane translocation activity.

44. The method of claim 43, wherein said fragment comprises a VCE cell-membrane translocation domain having said cell-membrane translocation activity.

45. The method of claim 44 wherein the native furin cleavage site of said fragment is replaced with a modifiable activation domain, wherein said modifiable activation domain comprises a substrate for an exogenous enzyme.

46. The method of claim 45, wherein said modifiable activation domain comprises a substrate for granzyme B activity.

47. The method of claim 43, wherein said protein does not comprise the VCE cell binding domain.

48. The method of claim 38, wherein said protein is a fusion protein.

49. The method of claim 48, wherein said fusion protein comprises a non-native cell targeting moiety.

50. The method of claim 49, wherein said said non-native cell targeting moiety is an antibody, or functional fragment thereof.

51. The method of claim 49, wherein said non-native cell targeting moiety is an artificially diversified polypeptide binder.

52. The method of claim 49, wherein said non-native cell targeting moiety is a ligand for a receptor.

53. The method of claim 49, wherein said non-native cell targeting moiety recognizes a cell surface target that is expressed on cancer cells.

54. The method of claim 49, wherein said non-native cell targeting moiety recognizes a cell selected from the group consisting of a hematopoietic cell, lymphocyte, and a nociceptive neuron.

55. The method of claim 48, further comprising a modifiable activation domain, wherein said modifiable activation domain comprises a substrate for an exogenous enzyme.

56. The method of claim 55, wherein said enzyme is a protease.

57. The method of claim 56, wherein said protease is an exogenous human protease.

58. The fusion protein of claim 56, wherein said protease is a non-human protease.

59. The method of claim 58, wherein said protease is a non-mammalian protease.

60. The method of claim 59, wherein said protease is a viral protease.

61. The method of claim 55, wherein said modifiable activation domain comprises a post-translational modification of a protease cleavage site.

62. The method of claim 55, wherein said modifiable activation domain comprises a substrate for an enzyme capable of removing a post-translational modification.

63. The method of claim 55, wherein said modifiable activation domain comprises a substrate for granzyme B activity.

A

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PEA   2   EEAFDLWNECAKACVLDLKDGVR-SSRMSVDPAIADTNGQGVLHYSMVLEGGNDALKLAI  60
          E+  ++++EC   C L  + G    S++S+   +     +GVL+YSM +     + +K
VCE  34   EDELNIFDECRSPCSLTPEPGKPIQSKLSIPSDVV--LDEGVLYYSMTINDEQNDIKDE-  90

PEA  61   DNALSITSDG---------------------LTIRLEGGVEPNKPVRYSYTRQARGSWS  98
          D   SI + G                      L I  E G +      YSY R+  G ++
VCE  99   DKGESIITIGEFATVRATRHYVNQDAPFGVIHLDITTENGTKT-----YSYNRK-EGEFA 144

PEA  99   LNWLVPIGHEKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLA--KLARDATFFVRAH 156
          +NWLVPIG + P++IK+ + EL+    +  +  +Y+I++ ++ L   K   + +F V
VCE 145   INWLVPIGEDSPASIKISVDELDQQRNIIEVPKLYSIDLDNQTLEQWKTQGNVSFSVTRP 204

PEA 157   ESNEMQPTLAISHAGVSVVMAQTQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNL 216
          E N     +AIS   VS   AQ +  R KRW+ W +G  LC L P+D +YNY+ QQ C L
VCE 205   EHN-----IAISWPSVSYKAAQKEGSRHKRWAHWHTGLALCWLVPMDAIYNYITQQNCTL 259

PEA 217   DDTWEGKIYRVLAGNP----AKHDLDIKPTVISHRLHFPEGGSLAALTAHQACHLPLETF 272
           D W G  Y  +AG P     K  ++ KP  +  R+HF +G +++AL AH+ C +PLET
VCE 260   GDNWFGGSYETVAGTPKVITVKQGIEQKP--VEQRIHFSKGNAMSALAAHRVCGVPLETL 317

PEA 273   TRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSG-----GDLGE 327
           R R+PR       C Y  Q +V+L++A R+  ++D V    L              DL
VCE 318   ARSRKPRDLTDDLSCAYQAQNIVSLFVATRILFSHLDSVFTLNLDEQEPEVAERLSDLRR 377

PEA 328   AIREQPEQARLALTLAAAESERFVRQGTG----NDEAGAANADVVSLTCPVAAGECAGPA 383
               P      LT+A      +V    G       AGA  AD++SL CP A   C   +
VCE 378   INENNPGMVTQVLTVARQIYNDYVTHHPGLTPEQTSAGAQAADILSLFCPDADKSCVA-S 436

PEA 384   DSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGTF 443
          ++  A +     +G  +L +   V  + +G  NWT + L   H+ L   GYVFVGYHGT
VCE 437   NNDQANINIESRSGRSYLPENRAV-ITPQGVTNWTYQELEATHQALTREGYVFVGYHGTN 495

PEA 444   LEAAQSIV---FGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQE-------PDARGRI 493
            AAQ+IV       R  + + +  W G Y+A     +A+GYA+ +E        P    R
VCE 496   HVAAQTIVNRIAPVPRGNNTENEEKWGGLYVATHAEVAHGYARIKEGTGEYGLPTRAERD 555

PEA 494   RNGALLRVYVPRSSLPGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLE 553
            G +LRVY+PR+SL  FYRT+  L    A   + ++IGH LPLR +A TGPE  GG  E
VCE 556   ARGVMLRVYIPRASLERFYRTNTPL--ENAEEHITQVIGHSLPLRNEAFTGPESAGGEDE 613

PEA 554   TILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPREDLK 613
          T++GW +A   V IPS IP +        +D  ++  KEQ+IS  P Y     K  +++LK
VCE 614   TVIGWDMAIHAVAIPSTIPGNAYEELA-IDEEAVA-KEQSISTKPPY-----KERKDELK 666
```

Fig. 1

B

PEA domains	% identities/positives
Domain I (1-252)	31/48
Domain II (253-364)	31/46
Domain Ib (365-404)	28/50
Domain III (405-613)	39/54
Overall	33/49

C

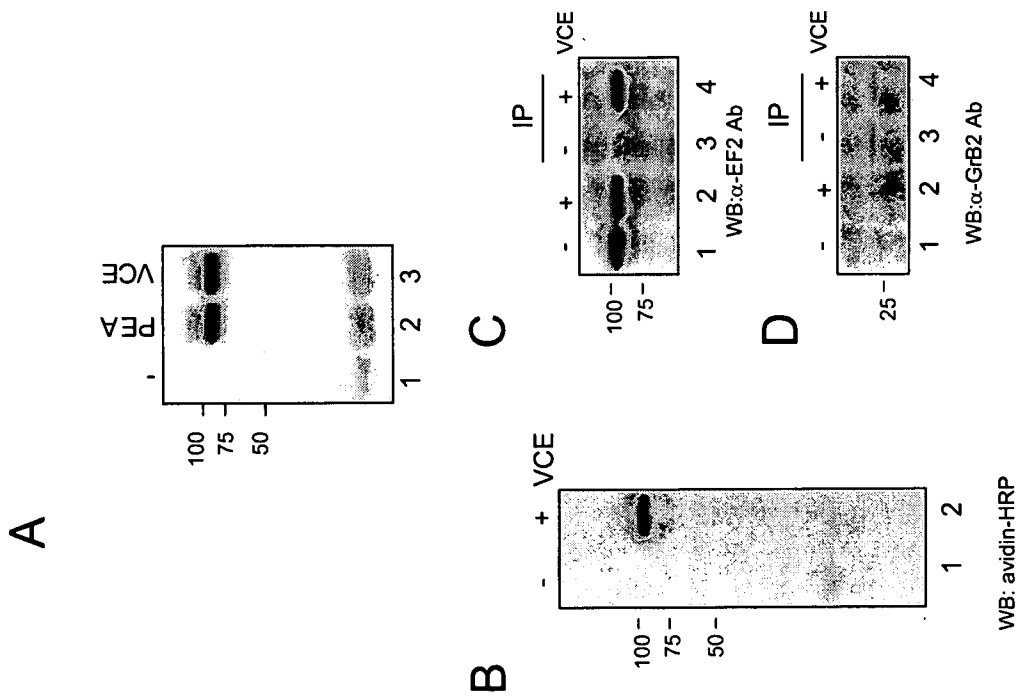

A
-
PEA
VCE
1 2 3
100
75
50
B
- + VCE
1 2
100
75
50
WB: avidin-HRP
C
IP
- + - + VCE
1 2 3 4
100
75
WB:α-EF2 Ab
D
IP
- + - + VCE
1 2 3 4
25
WB:α-GrB2 Ab

Fig. 2

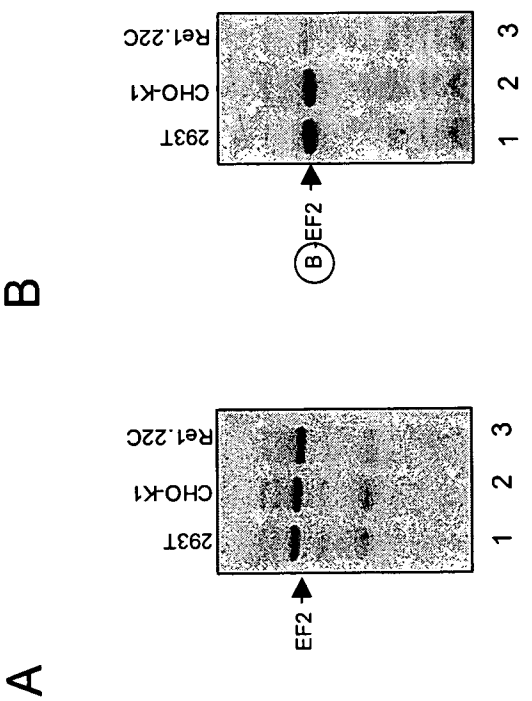


Fig. 3

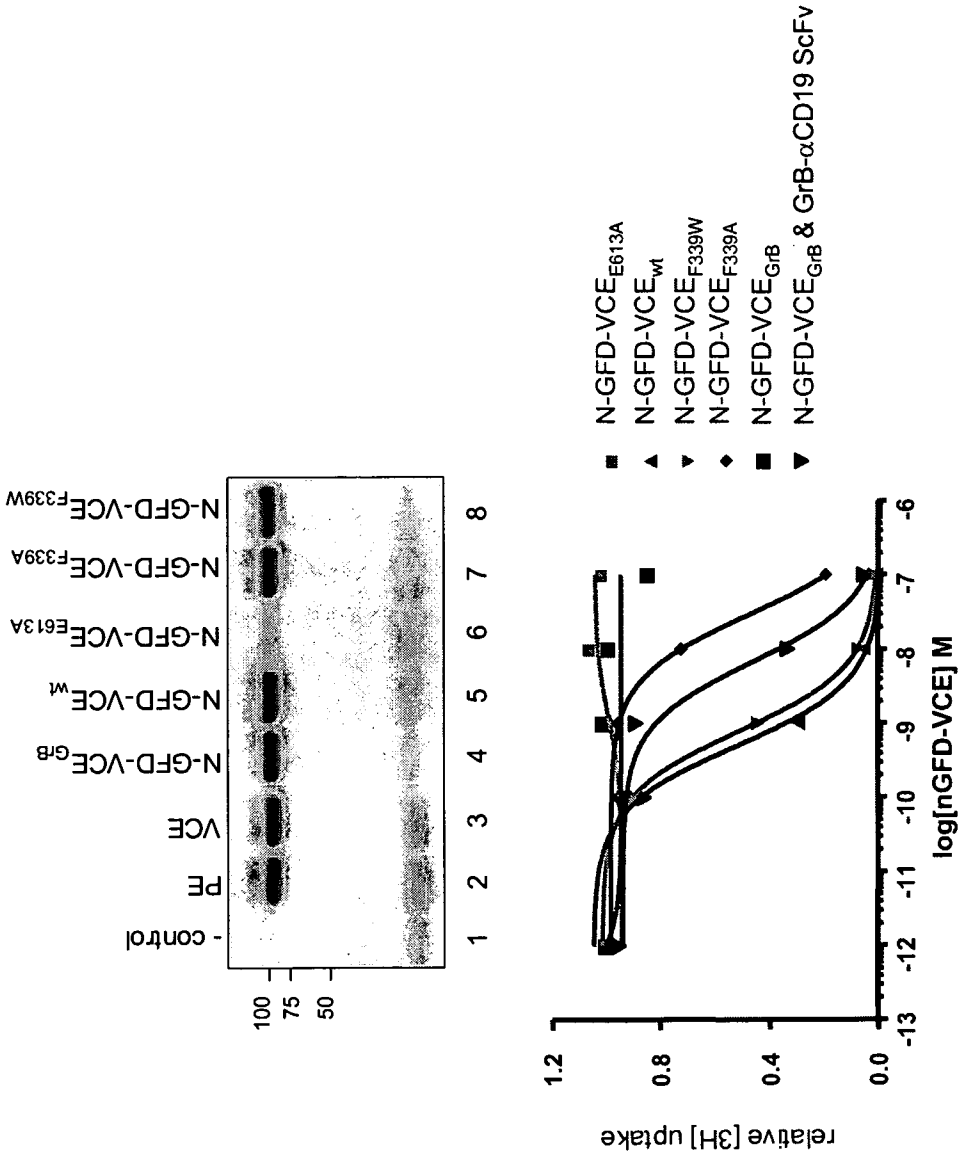

A
- control
PE
VCE
N-GFD-VCE$_{GrB}$
N-GFD-VCE$_{wt}$
N-GFD-VCE$_{E613A}$
N-GFD-VCE$_{F339A}$
N-GFD-VCE$_{F339W}$
100
75
50
1 2 3 4 5 6 7 8
B
N-GFD-VCE$_{E613A}$
N-GFD-VCE$_{wt}$
N-GFD-VCE$_{F339W}$
N-GFD-VCE$_{F339A}$
N-GFD-VCE$_{GrB}$
N-GFD-VCE$_{GrB}$ & GrB-αCD19 ScFv
relative [3H] uptake
1.2
0.8
0.4
0.0
-13 -12 -11 -10 -9 -8 -7 -6
log[nGFD-VCE] M

Fig. 4

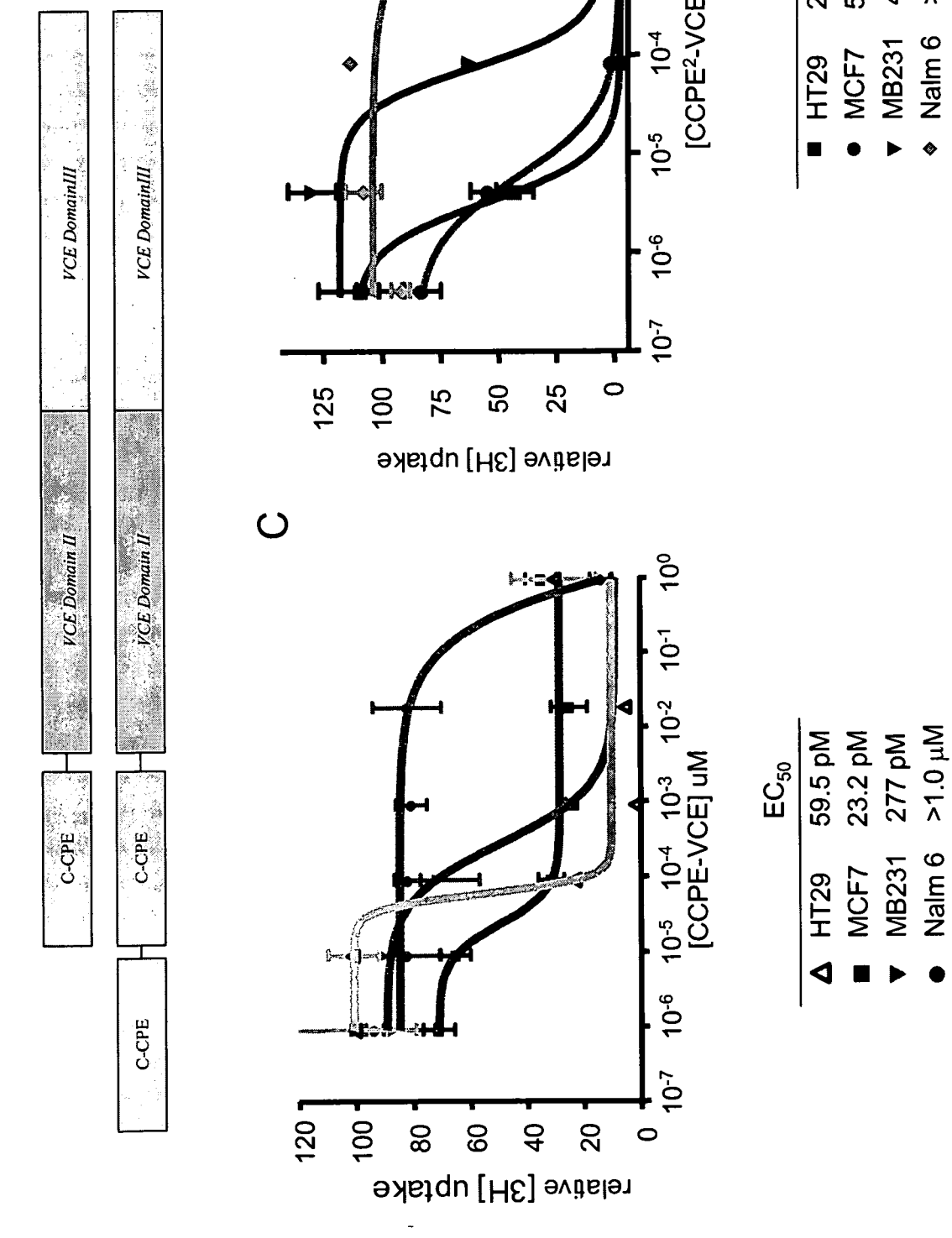

A
C-CPE
VCE Domain II
VCE DomainIII
C-CPE
C-CPE
VCE Domain II
VCE DomainIII
C-CPE
B
relative [3H] uptake
[CCPE-VCE] uM
EC50
HT29 59.5 pM
MCF7 23.2 pM
MB231 277 pM
Nalm 6 >1.0 µM
C
relative [3H] uptake
[CCPE2-VCE] uM
EC50
HT29 2.87 pM
MCF7 5.76 pM
MB231 47.6 pM
Nalm 6 >1.0 µM

Fig. 5

A
GrB site P4 P3 P2 P1 P1' P2'
Consensus I E P D X G
DT-αCD5 I E P D T G
αCD5-PE I E P D G W
αCD5-VCE I E P D S G
B
M
DT–αCD5
αCD5-PE
αCD5 -VC E
75
50
C
αCD5-PE
αCD5-VC
DT- αCD5
mGrB
pH (7.4-4.0)

Fig. 6

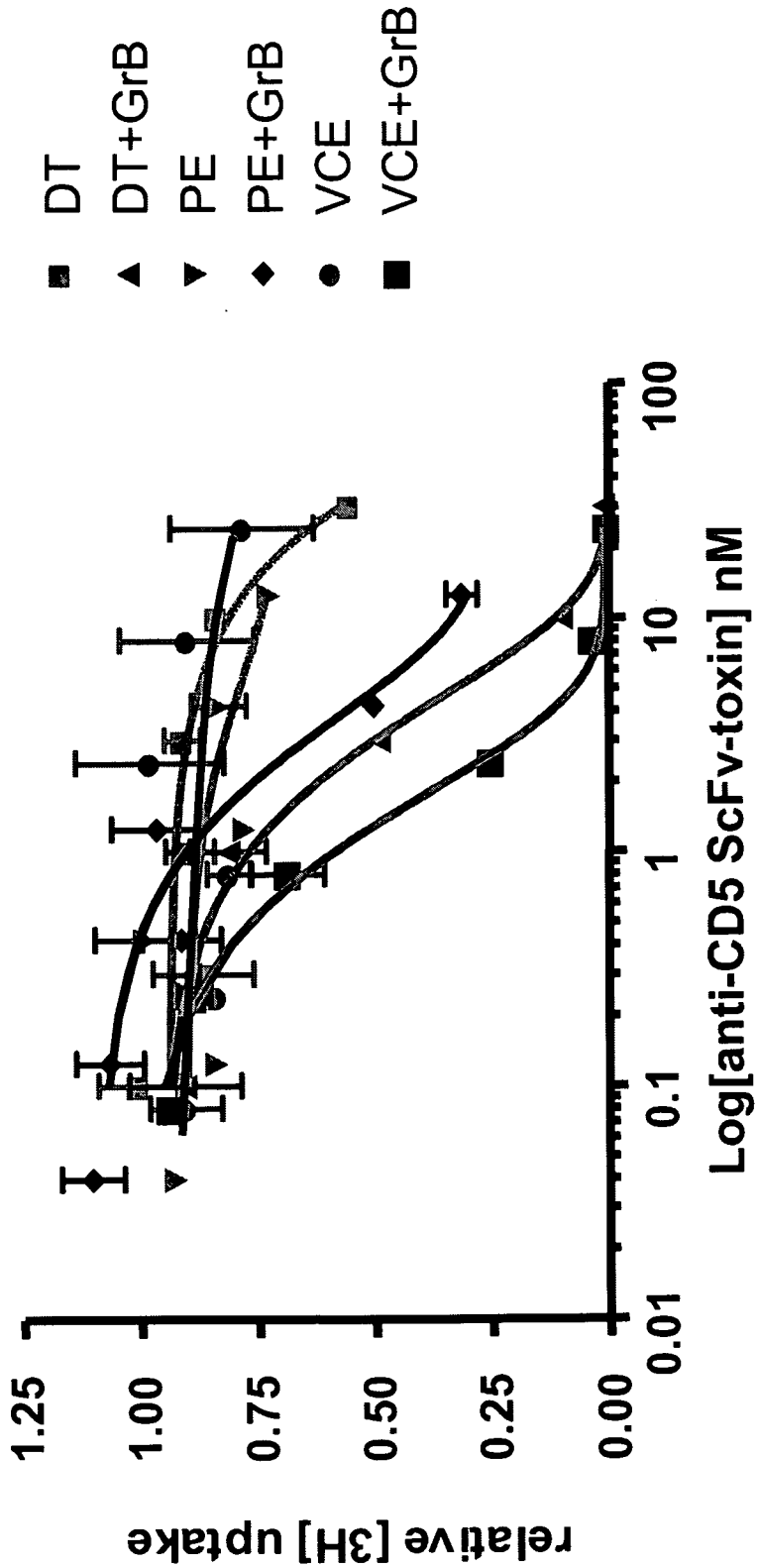

DT
DT+GrB
PE
PE+GrB
VCE
VCE+GrB
relative [3H] uptake
1.25
1.00
0.75
0.50
0.25
0.00
0.01
0.1
1
10
100
Log[anti-CD5 ScFv-toxin] nM

Fig. 7

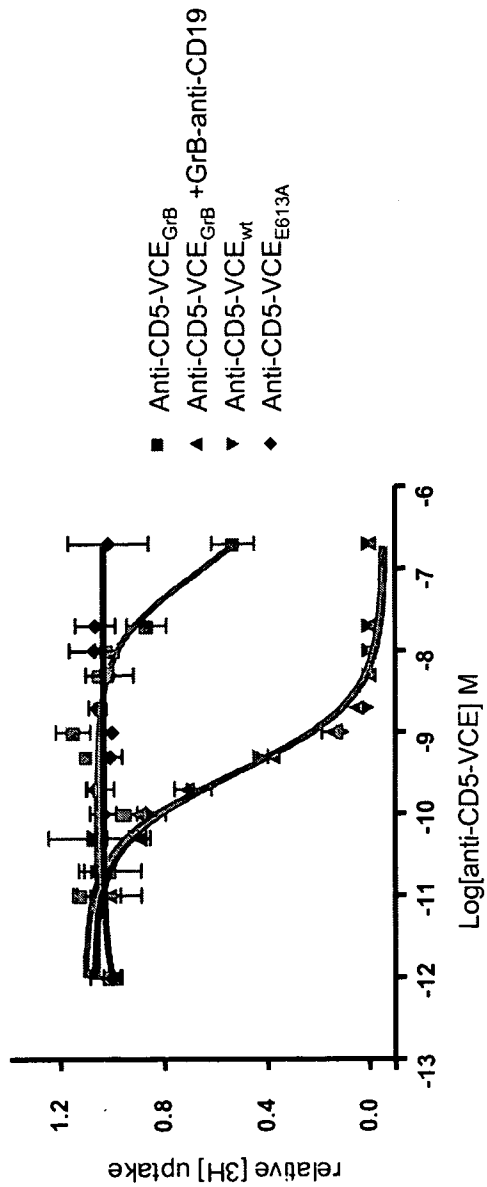

Anti-CD5-VCE$_{GrB}$
Anti-CD5-VCE$_{GrB}$ +GrB-anti-CD19
Anti-CD5-VCE$_{wt}$
Anti-CD5-VCE$_{E613A}$
relative [3H] uptake
1.2
0.8
0.4
0.0
-13
-12
-11
-10
-9
-8
-7
-6
Log[anti-CD5-VCE] M

Fig. 8

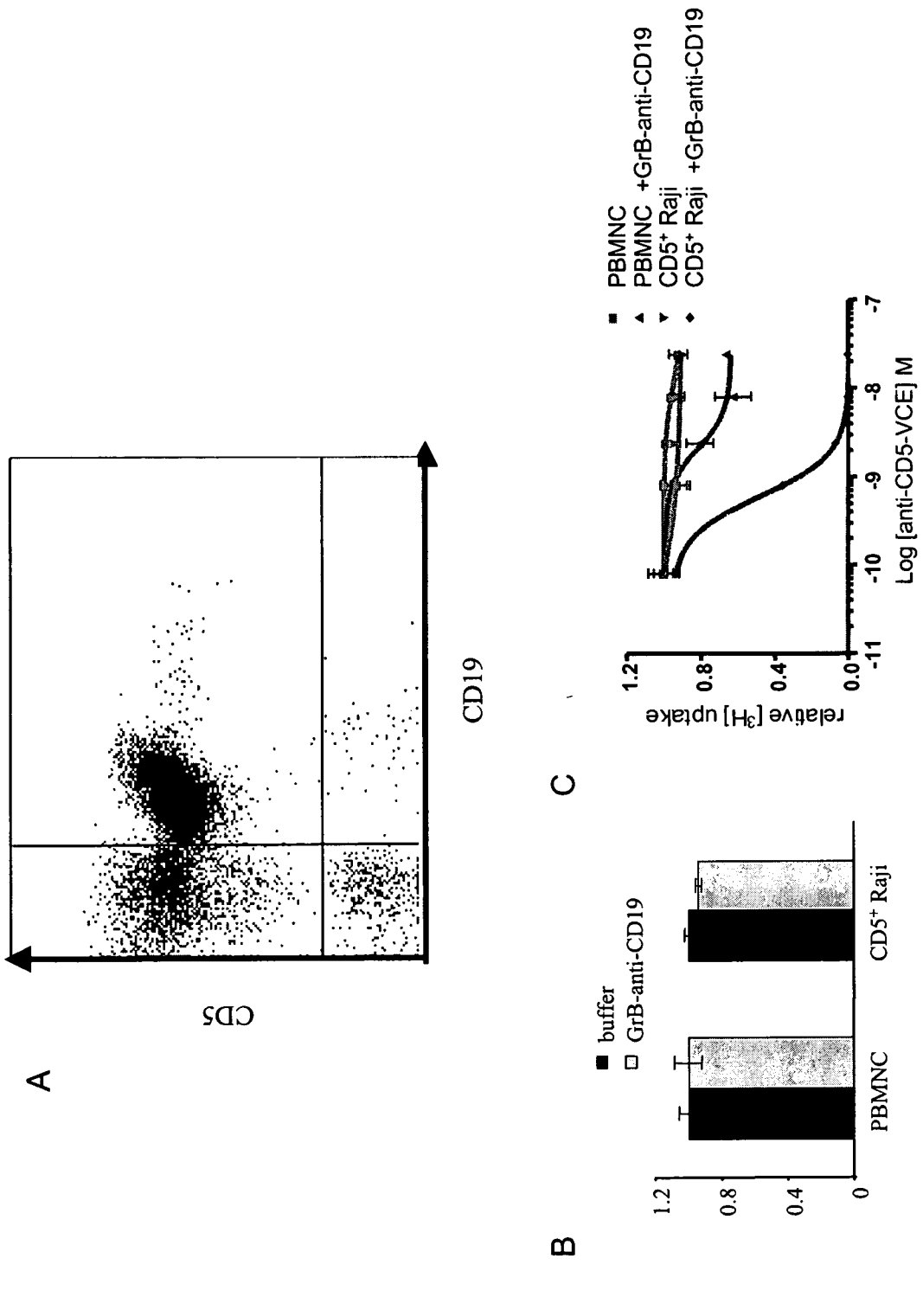
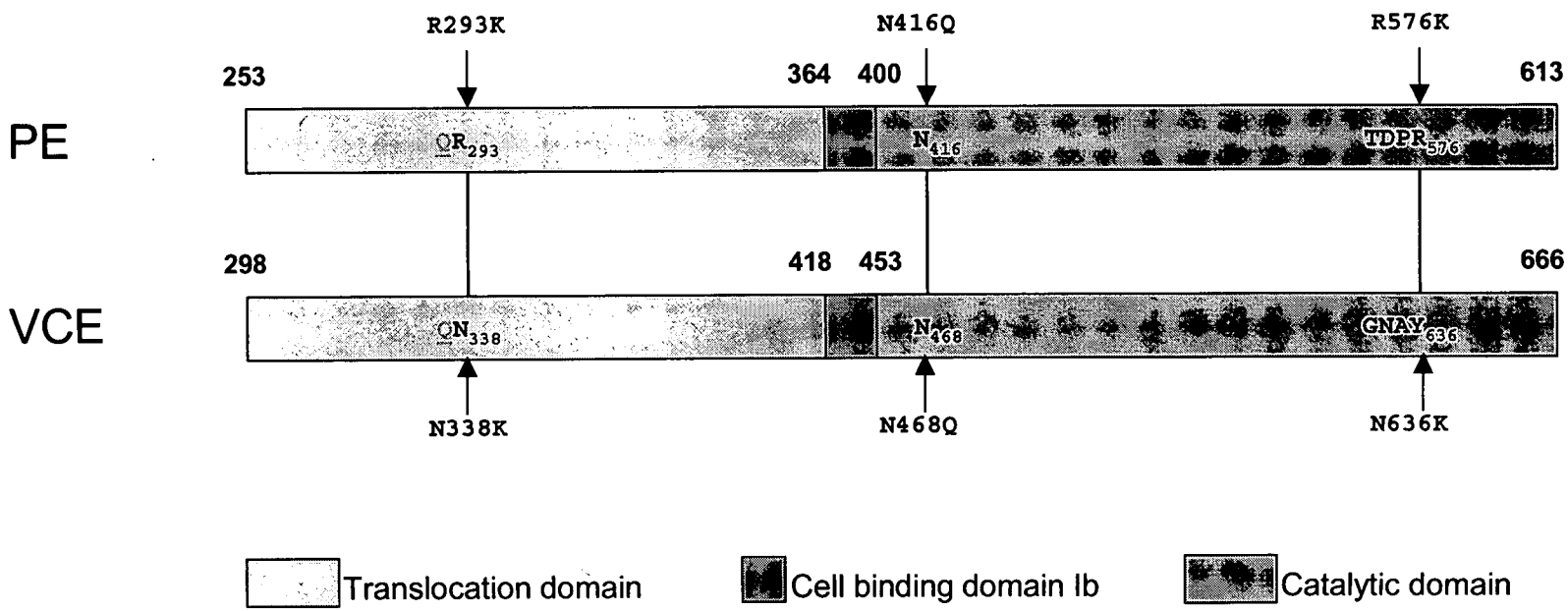

A
CD5
CD19
B
buffer
GrB-anti-CD19
1.2
0.8
0.4
0
PBMNC
CD5+ Raji
C
PBMNC
PBMNC +GrB-anti-CD19
CD5+ Raji
CD5+ Raji +GrB-anti-CD19
relative [3H] uptake
1.2
0.8
0.4
0.0
-11
-10
-9
-8
-7
Log [anti-CD5-VCE] M

Fig. 9

VLS Reducing Mutations



X_{nnn}: Residues that are mutated in PE and to be mutated in VCE

$\underline{X}$: Consensus residues closest to the mutated residue

Fig10

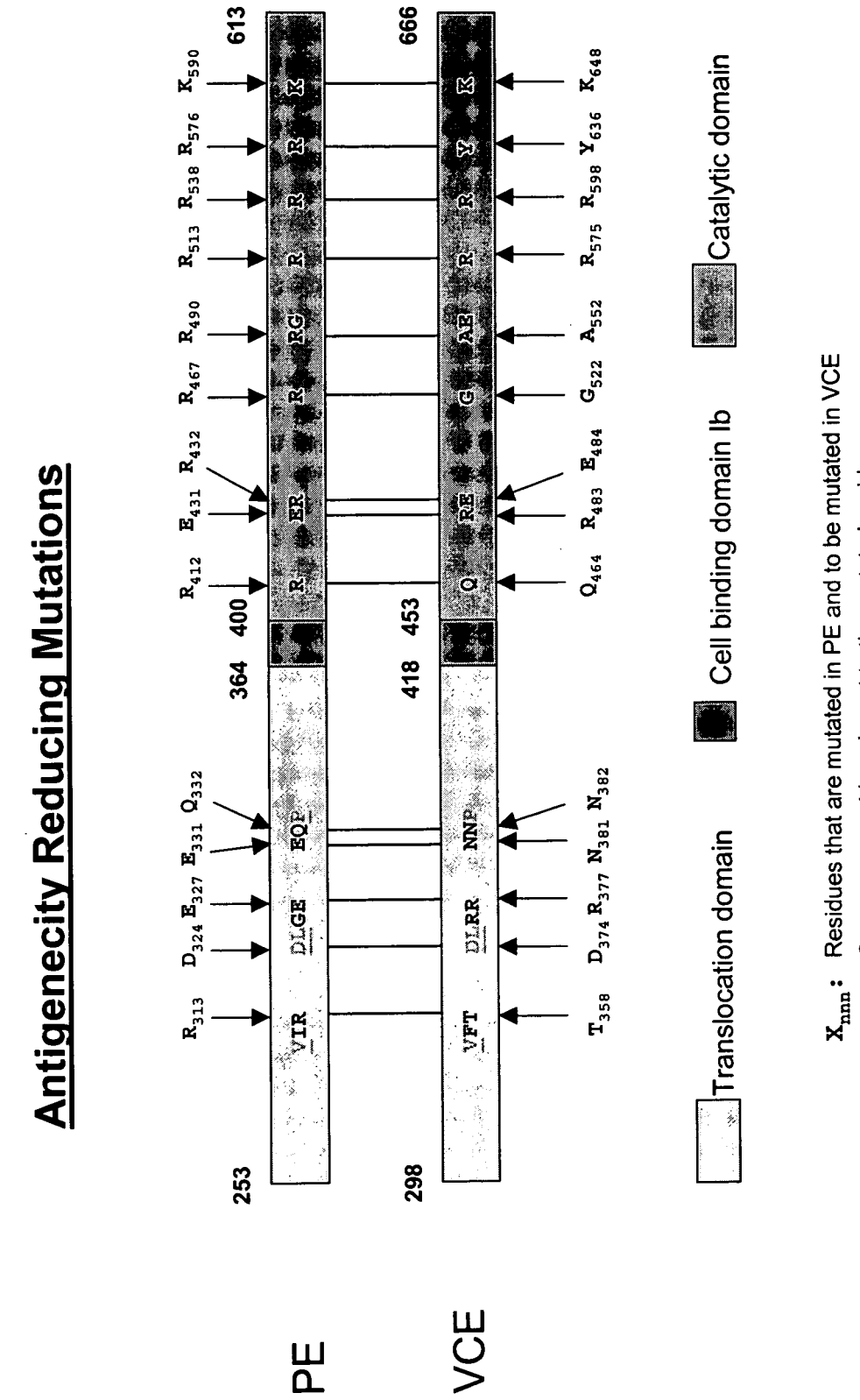

Antigenecity Reducing Mutations
PE
VCE
253
298
364
400
418
453
613
666
R313
D324
E327
E331
Q332
T358
D374
R377
N381
N382
R412
E431
R432
R467
R490
R513
R538
R576
K590
Q464
R483
E484
G522
A552
R575
R598
Y636
K648
VIR
DLGE
EQP
VFT
DLRR
NNP
Translocation domain
Cell binding domain Ib
Catalytic domain
Xnnn: Residues that are mutated in PE and to be mutated in VCE
X: Consensus residue closest to the mutated residue

Fig 11

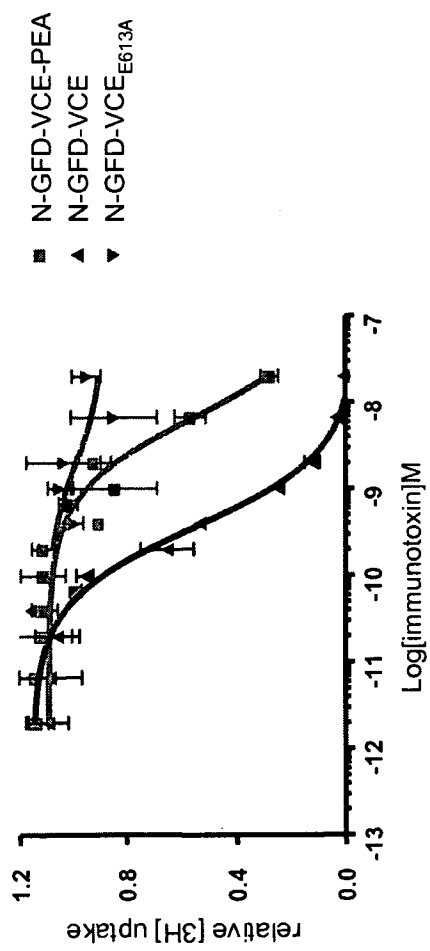

N-GFD-VCE-PEA
N-GFD-VCE
N-GFD-VCE$_{E613A}$
relative [3H] uptake
1.2
0.8
0.4
0.0
-13
-12
-11
-10
-9
-8
-7
Log[immunotoxin]M

Fig. 12

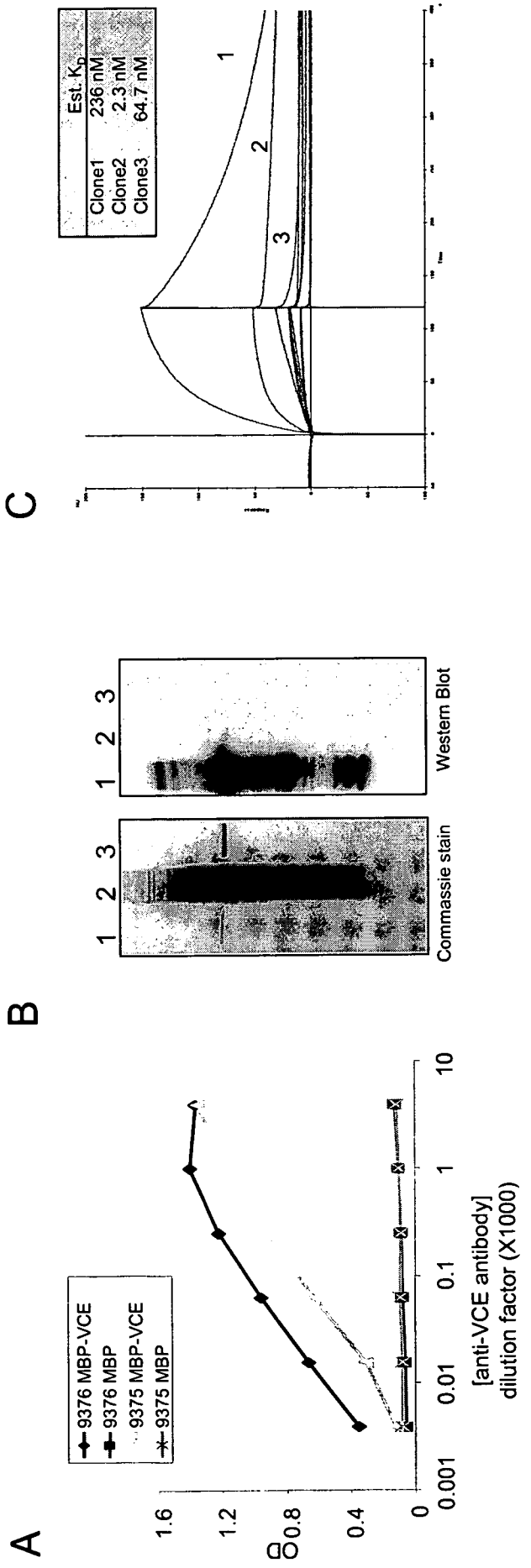

A
9376 MBP-VCE
9376 MBP
9375 MBP-VCE
9375 MBP
OD
1.6
1.2
0.8
0.4
0
0.001
0.01
0.1
1
10
[anti-VCE antibody]
dilution factor (X1000)
B
1 2 3
Commassie stain
1 2 3
Western Blot
C
Est. K_D
Clone1 236 nM
Clone2 2.3 nM
Clone3 64.7 nM
1
2
3

Fig. 13