



(51) International Patent Classification:

A61K 31/55 (2006.01) **A61K 38/06** (2006.01)
A61K 31/167 (2006.01) **A61K 38/07** (2006.01)
A61K 31/225 (2006.01) **A61P 9/00** (2006.01)
A61K 31/365 (2006.01) **A61K 31/7088** (2006.01)
A61K 31/40 (2006.01) **A61K 38/17** (2006.01)
A61K 31/662 (2006.01) **A61K 39/395** (2006.01)
A61K 31/675 (2006.01)

(21) International Application Number:

PCT/CA2014/050261

(22) International Filing Date:

14 March 2014 (14.03.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/799,608 15 March 2013 (15.03.2013) US

(71) Applicant: **THE UNIVERSITY OF BRITISH COLUMBIA** [CA/CA]; University-Industry Liaison Office, #103 - 6190 Agronomy Road, Vancouver, British Columbia V6T 1Z3 (CA).

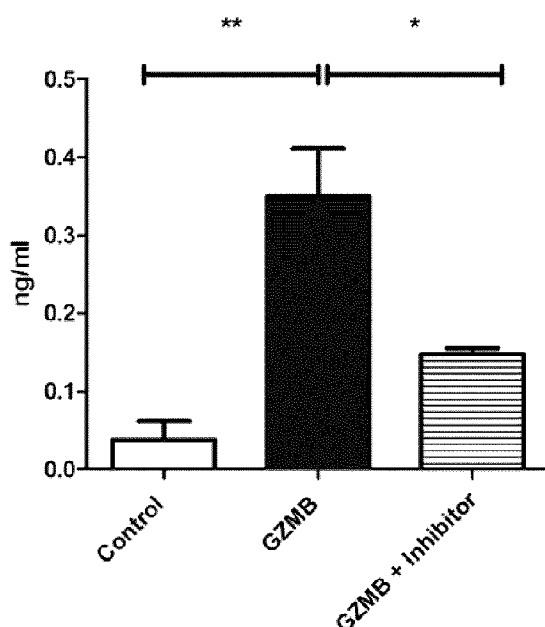
(72) Inventors: **HENDEL, Alon**; 7170 Camano Street, Vancouver, British Columbia V5S 4B9 (CA). **GRANVILLE, David J.**; 2513 Palisade Crescent, Port Coquitlam, British Columbia V3C 5Y1 (CA).

(74) Agent: **MBM INTELLECTUAL PROPERTY LAW LLP**; 275 Slater Street, 14th Floor, Ottawa, Ontario K1P 5H9 (CA).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,

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(54) Title: METHODS AND COMPOSITIONS FOR THE INHIBITION OF VASCULAR ENDOTHELIAL GROWTH FACTOR ACTIVITY AND VASCULAR PERMEABILITY

**FIG. 1**

(57) Abstract: Methods of altering microenvironmental levels of Vascular Endothelial Growth Factor in a subject are disclosed. The methods include providing a Granzyme B (GzmB) inhibitor to an area of inflammation. The area of inflammation can be associated with conditions that have an associated increase in vascular permeability, including for example, rheumatoid arthritis, myocardial infarction, ocular disease, skin conditions, such as psoriasis, and dermatitis, cancer, transplantation, and/or atherosclerosis, and the like. The Granzyme B inhibitor can be comprised of, but not limited to, a nucleic acid, a polypeptide, a Granzyme B specific antibody, a Granzyme B antibody derivative, or a small molecule.



TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

METHODS AND COMPOSITIONS FOR THE INHIBITION OF VASCULAR
ENDOTHELIAL GROWTH FACTOR ACTIVITY AND VASCULAR
PERMEABILITY

5 CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 61/799,608, filed March 15, 2013, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 The invention relates to compositions, methods, and uses for inhibitors of Granzyme B for inhibiting vascular permeability, edema, and/or accompanying inflammation.

BACKGROUND

Increased vascular permeability is one of the earliest manifestations of
15 inflammation, resulting in extravasation of protein-rich plasma into the effected tissue. Acute vascular permeability allows the deposition of circulating plasma matrix proteins including fibrin and fibronectin (FN) which facilitate cell migration in the inflamed area. This process also provides an access point for immune cells and immunoglobulins to enter the tissue and fight foreign antigens (Nagy *et al.*, *Cold Spring Harb. Perspect. Med.*
20 2:a006544, 2012). Conversely, chronic vascular hyperpermeability is suggested to sustain the inflammatory response and retard resolution, further promoting the development of chronic inflammation (Nagy *et al.*, *Cold Spring Harb. Perspect. Med.* 2:a006544, 2012; Costa *et al.*, *Angiogenesis* 10:149-166, 2007). This type of vascular hyperpermeability underlies the pathogenesis of a large number of chronic disorders
25 including rheumatoid arthritis (RA), psoriasis, ocular disease, cancer and chronic wounds (Nagy *et al.*, *Cold Spring Harb. Perspect. Med.* 2:a006544, 2012; Costa *et al.*, *Angiogenesis* 10:149-166, 2007).

Vascular endothelial growth factor (VEGF) is a potent vascular permeabilizing agent that is highly expressed during chronic inflammation (Nagy *et al.*, *Annu. Rev. Pathol.* 2:251-275, 2007). Low microenvironmental levels of VEGF are important in
30 order to maintain stable vascular integrity and promote endothelial cell survival through autocrine mechanisms (Lee *et al.*, *Cell* 130:691-703, 2007). Whereas elevated levels of VEGF induce vascular leakages by activating VEGF receptor 2 (VEGFR2) in endothelial

cells (EC) leading to the opening of intercellular and/or intracellular pathways that facilitate plasma extravasation (Koch and Claesson-Welsh, *Cold Spring Harb. Perspect. Med.* 2:a006502, 2012). Moreover, VEGF may serve as a pro-inflammatory mediator as it can enhance T cell (Xia *et al.*, *Blood* 102:161-168, 2003), and monocyte (Murakami *et al.*, *Blood* 108:1849-1856, 2006) migration as well as promote pro-inflammatory chemokines expression by EC including MCP-1, and IL-8, leading to further immune recruitment. Cellular sources for VEGF during inflammation can include macrophages and mast cells; however it can also be expressed by endothelial cells and acts in a paracrine and autocrine fashion. Thus, VEGF plays a major role in promoting chronic inflammation by inducing vascular permeability and contributing to immune cell recruitment.

The extracellular matrix (ECM) has a major role in regulating VEGF bioavailability. VEGF contains a cluster of basic residues that facilitate the interaction with anionic ECM proteins (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992). VEGF interaction with the ECM greatly determines its bioavailability as the majority of VEGF is retained in the ECM after cell secretion (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992). Other proteases may alter VEGF interaction with the ECM including plasmin (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992) and MMPs (Lee *et al.*, *J. Cell Biol.* 169:681-691, 2005), giving rise to increased microvessel leakage and formation of aberrant neovasculature that is characteristic of pathological angiogenesis (Lee *et al.*, *J. Cell Biol.* 169:681-691, 2005). Thus, ECM-sequestered VEGF can be liberated by proteases and this process has significant implications on vascular integrity. Importantly, VEGF binds to the growth factor binding domain in fibronectin (Wijelath *et al.*, *Circ. Res.* 91:25-31, 2002; Wijelath *et al.*, *Circ. Res.* 99:853-860, 2006; Martino *et al.*, *Sci. Transl. Med.* 3:100ra89, 2011). Binding of VEGF to FN enhances EC migration, proliferation and promotes stable angiogenesis (Wijelath *et al.*, *Circ. Res.* 91:25-31, 2002; Wijelath *et al.*, *Circ. Res.* 99:853-860, 2006; Martino *et al.*, *Sci. Transl. Med.* 3:100ra89, 2011). Conversely, release of VEGF from ECM increases VEGF bioavailability and promotes the formation of aberrant, incomplete leaky neovasculature that is observed in chronic inflammatory disorders including RA, atherosclerosis and cancer (Nagy *et al.*, *Annu. Rev. Pathol.* 2:251-275, 2007). VEGF has also been associated with ocular disease, such as for example, age-related macular degeneration.

Granzyme B (GzmB or GranB) is a serine protease that is expressed and released by a variety of immune cells including lymphocytes, macrophages, neutrophils, and mast cells (Hendel *et al.*, *Cell Death Differ.* 17:596-606, 2010). Although traditionally viewed as a pro-apoptotic intracellular protease used by cytotoxic lymphocytes to induce target cell apoptosis, the importance of the role of extracellular GzmB activity in a number of chronic inflammatory disease has now been demonstrated (Boivin *et al.*, *Lab. Invest.* 89:1195-1220, 2009; Hiebert *et al.*, *Trends Mol. Med.* 8:732-741, 2012). GzmB accumulates in the ECM of inflamed tissues during chronic inflammation where it cleaves a number of ECM proteins, including fibronectin (FN). GzmB is also present in several bodily fluids in chronic diseases such as atherosclerosis, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA) (Kondo *et al.*, *Circ. J.* 73:503-507, 2009; Kummer *et al.*, *Clin. Immunol. Immunopathol.* 73:88-95, 1994). ECM proteolysis by GzmB leads to impaired tissue integrity in diseases. In a murine mouse model of abdominal aortic aneurysm (AAA), GzmB cleavage of fibrillin-1 and decorin results in increased AAA rupture (Ang *et al.*, *Cell Death Dis.* 2:e209, 2011; Chamberlain *et al.*, *Am. J. Pathol.* 176:1038-1049, 2010). In addition, degradation of decorin and FN by GzmB lead to increased skin thinning and delayed wound healing in apolipoprotein E knockout mice (Hiebert *et al.*, *Exp. Gerontol.* 46:489-499, 2011; Hiebert *et al.*, *Cell Death Differ.* 20:1404-1414, 2013). Moreover, extracellular GzmB is suggested to potentiate pro-inflammatory cytokine activities as it converts the pro form of IL-18 to its active form leading to increased IFN- γ release from human keratinocytes (Omoto *et al.*, *J. Dermatol. Sci.* 59:129-135, 2010). GzmB cleavage of IL-1 α results in enhanced pro-inflammatory activity of this cytokine both *in vitro* and *in vivo* (Afonía *et al.*, *Mol. Cell.* 44:265-278, 2011). Interestingly, GzmB cleavage of decorin, biglycan and betaglycan leads to release of TGF- β 1 from the matrix, suggesting that GzmB may indirectly affect normal cell function by altering growth factor bioavailability (Boivin *et al.*, *PLoS One* 7:e33163, 2012).

It has been previously demonstrated that GzmB cleavage of FN dysregulates angiogenesis by impairing EC adhesion, migration and capillary formation (Hendel *et al.*, *Matrix Biol.* 32:14-22, 2013). The present disclosure provides that GzmB cleavage of FN can result in increased VEGF bioavailability which in turn can promote significant vascular leakage *in vivo*. Importantly, using a GzmB inhibitor, in one embodiment a small molecule inhibitor, VEGF release from the matrix was reduced and its activity was

attenuated. A new mechanism by which extracellular GzmB can contribute to chronic inflammation by deregulating the angiogenic response and promoting vascular leakage is therefore provided. These findings will pave the way for the development of treatment approaches that aim to normalize neovessels and reduce vascular leakage in inflammatory diseases in which GzmB is highly evident including, but not limited to RA, ocular disease, myocardial infarct (MI), dermatitis (for example, psoriasis, allergic contact dermatitis, radiation induced dermatitis, and the like), cancer, transplantation, and atherosclerosis.

SUMMARY

The present disclosure provides a method for inhibiting Vascular Endothelial Growth Factor (VEGF) activity in a subject, the method comprising administering a Granzyme B (GzmB) inhibitor to the subject for a time and in an amount effective to inhibit VEGF activity. In certain embodiments the method comprises the inhibition of cleavage of an extracellular matrix protein. The extracellular matrix protein that the Granzyme B inhibitor is inhibited from cleaving can be fibronectin. In a particular embodiment the release of VEGF bound to the extracellular matrix is inhibited, and specifically the extracellular protein can be fibronectin.

In an additional embodiment the present disclosure provides a method for inhibiting vascular leakage in a subject, the method comprising administering a GzmB inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release. The method can inhibit the cleavage of an extracellular matrix protein, and particularly wherein the extracellular matrix protein is fibronectin. In certain embodiments of the method the release of VEGF bound to the extracellular matrix is inhibited, and in a particular embodiment the extracellular protein is fibronectin.

In yet another embodiment of the disclosure provides a method for inhibiting VEGF release from the extracellular matrix in a subject, the method comprising administering a GzmB inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release. In a particular embodiment the cleavage of an extracellular matrix protein is inhibited, and more specifically the extracellular matrix protein is fibronectin.

In still yet another embodiment the present disclosure provides a method for inhibiting VEGF release from fibronectin in a subject, the method comprising administering a GzmB inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release.

The methods provided above can use a Granzyme B inhibitor selected from the group consisting of a nucleic acid molecule, a polypeptide, an antibody, and a small molecule. The antibody can be a monoclonal antibody, a humanized antibody, an antigen binding fragment of an antibody, a single chain antibody, a diabody, or a chimeric antibody. In certain embodiments of the disclosed methods the Granzyme B inhibitor is selected from one or more of the following:

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-

- ((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- 5 (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- 25 (R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-

yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; Bio-x-IEPO^P-(OPh)₂; azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-benzo[b]thien-3-ylacetyl) amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-ylmethyl)-, 5 ((2S,5S)- ; (4S)-4-[[[(2S)-2-acetamido-4-methylpentanoyl]amino]-5-[2-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbonyl]pyrrolidin-1-yl]-5-oxopentanoic acid; (4S)-4-[[[(2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[[(2S,3S)-3-hydroxy-1-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid, (Ac-IEPD-CHO); 5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino)propanoylamino] 10 propanoylamino] pentanoic acid; 5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino)propanoylamino] propanoylamino] pentanoic acid; (2S,5S)-4-oxo-5-{[N-(phenylacetyl)-L-isoleucyl]amino}-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboximide; or a salt or solvate of any of the above; ZINC05723764 (NCI 644752); ZINC05723787 (NCI 644777); ZINC05316154 (NCI 641248); ZINC05723499 (NCI 641235); ZINC05723646 (NCI 642017); ZINC05398428 (NCI 641230); ZINC05723503 (NCI 641236); ZINC05723446 (NCI 640985); ZINC05317216 (NCI 618792); ZINC05315460 (NCI 630295); ZINC05316859 (NCI 618802); ZINC05605947 (NCI 623744); an isocoumarin; a peptide chloromethyl ketone; a peptide phosphonate; a GzmB inhibitory nucleic acid 20 molecule; an anti-Granzyme B antibody, or an antigen specific binding fragment or derivative thereof; an inhibitory GzmB polypeptide; a SerpB9 polypeptide, or a GzmB inhibitory fragment thereof; a Serp2 polypeptide, or a GzmB inhibitory fragment thereof; a CrmA polypeptide, or a GzmB inhibitory fragment thereof; or a Serpin A3 polypeptide, or a GzmB inhibitory fragment thereof.

25 In certain embodiments disclosed herein the GzmB inhibitor can be formulated for topical administration, lavage, epidermal administration, sub-epidermal administration, dermal administration, sub-dermal administration, sub-cutaneous administration, systemic administration, injection, inhalation, or oral administration.

The methods can be used wherein the subject is a mammal and in particular 30 wherein the subject is a human.

Still further the present disclosure provides for the use of a GzmB inhibitor in the preparation of a medicament for use in inhibiting VEGF activity in a subject, for use in inhibiting vascular leakage, for use in inhibiting VEGF release from the extracellular

matrix, particularly wherein the extracellular matrix protein is fibronectin. The GzmB inhibitors for each of these uses can be selected from the group consisting of a nucleic acid molecule, a polypeptide, an antibody, and a small molecule. In a typical embodiment of any of these uses the GzmB inhibitor can be selected from one or more of the following:

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-

- hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- 5 (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-
- 10 1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi] indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-
- 15 1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-
- 20 oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-
- 25 carboxamide; (R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl) methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-
- 30 oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-

hexahydroazepino[3,2,1-hi]indole-2-carboxamide; Bio-x-IEPO^P-(OPh)₂; azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-benzo[b]thien-3-ylacetyl) amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-ylmethyl)-, ((2S,5S)- ; (4S)-4-[[2S)-2-acetamido-4-methylpentanoyl] amino]-5-[2-[[2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbamoyl]pyrrolidin-1-yl]-5-oxopentanoic acid; (4S)-4-[[2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[2S,3S)-3-hydroxy-1-[[2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid; 5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoylamino] pentanoic acid; 5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoylamino] pentanoic acid; (2S,5S)-4-oxo-5-{[N-(phenylacetyl)-L-isoleucyl]amino}-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboximide; or a salt or solvate of any of the above; ZINC05723764 (NCI 644752); ZINC05723787 (NCI 644777); ZINC05316154 (NCI 641248); ZINC05723499 (NCI 641235); ZINC05723646 (NCI 642017); ZINC05398428 (NCI 641230); ZINC05723503 (NCI 641236); ZINC05723446 (NCI 640985); ZINC05317216 (NCI 618792); ZINC05315460 (NCI 630295); ZINC05316859 (NCI 618802); ZINC05605947 (NCI 623744); an isocoumarin; a peptide chloromethyl ketone; a peptide phosphonate; a GzmB inhibitory nucleic acid molecule; an inhibitory GzmB polypeptide; a SerpB9 polypeptide, or a GzmB inhibitory fragment thereof; a Serp2 polypeptide, or a GzmB inhibitory fragment thereof; a CemA polypeptide of a GzmB inhibitory fragment thereof; or a Serpin A3 polypeptide, or a GzmB inhibitory fragment thereof.

For the above disclosed uses the GzmB inhibitor can be formulated for topical administration, lavage, epidermal administration, sub-epidermal administration, dermal administration, sub-dermal administration, sub-cutaneous administration, systemic administration, injection, inhalation, or oral administration. In a typical embodiment the subject is a mammal, and more particularly, the subject is a human.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 demonstrates that GzmB releases VEGF from human plasma FN. VEGF (50 ng/ml) was added to FN coated wells and incubated for 2 hrs at 37 °C.

Unbound VEGF was removed by washing with DPBS. GzmB (50 nM) with vehicle control (DMSO) or GzmB with inhibitor (Compound 20, 50 μ M) were added to the wells and incubated for 2 hrs at 37 °C. Supernatants were removed and analyzed for VEGF by ELISA. Results are presented as mean $\pm$ SEM of n = 3 with three replicates per treatment (*P<0.05; ** P<0.01).

FIGURE 2 demonstrates that GzmB releases VEGF from HUVEC matrix. HUVEC were grown to confluence and maintained in serum-reduced media for 9 days. Cells were removed by adding NH₄OH followed by extensive washing. Remaining ECM was incubated with VEGF (50 ng/ml) for 2 hrs at 37 °C. Unbound VEGF was removed by washing with DPBS. GzmB (50 nM) or GzmB with inhibitor (Compound 20, 50 μ M) were added and incubated for additional 2 hrs at 37 °C. Supernatants were removed and analyzed for VEGF by ELISA. Results are presented as mean $\pm$ SEM of n = 3 with three replicates per treatment (*P<0.05; ** P<0.01).

FIGURES 3A and 3B demonstrate that GzmB degrades FN but does not cleave VEGF. Figure 3A depicts FN cleavage. FN coated culture wells were treated with either GzmB or plasmin (50 nM) with or without inhibitors (A is aprotinin) for 2 hrs at 37 °C. Supernatants were analyzed by Western blot using anti-FN antibody. Figure 3B depicts VEGF cleavage. The same enzyme preparations used for FN were incubated with 100 ng VEGF in microtubes for 2 hrs at 37 °C. Samples were analyzed by Western blot using anti-VEGF antibody (arrow denotes VEGF fragment).

FIGURES 4A and 4B demonstrate that GzmB-mediated VEGF release from FN activates VEGFR2. FN coated wells were incubated with VEGF and treated with GzmB or GzmB with inhibitor as in Figure 1. Supernatants were removed and added to a HUVEC monolayer culture for 7 min. Cell lysates were analyzed by Western blot using (A) phospho Y1214 VEGFR2 antibody (pVEGFR2 Y1214), and (B) phospho Y1175 VEGFR2 antibody (pVEGFR2 Y1175). HUVEC treated directly with VEGF (50 ng/ml) were used as a positive control (+ve ctr.). Total VEGFR2 and β -tubulin antibodies were used as loading controls. Quantification is presented as the densitometry ratio of phosphorylated VEGFR2 (pVEGFR2) to total VEGFR2 normalized to β -tubulin. Results are presented as mean $\pm$ SEM of n = 3 with three replicates per treatment (*P < 0.05).

FIGURES 5A and 5B demonstrate that GzmB induces VEGF-dependent increase in vascular permeability *in vivo*. Evan's blue (0.5 %) was injected via the tail vein prior to ear injections of (Figure 5A) mouse granzyme B (mGzmb) (100 ng) or saline control,

or (Figure 5B) mGzmb with anti-mouse VEGF antibody (1.5 μ g) or mGzmb and IgG control antibody. Ear tissues were excised as indicated using a 7 mm punch biopsy (areas in circle). Evan's blue extraction was performed by drying the tissue and immersing it in formamide at 55°C for 24 hrs. Results are presented as absorbance (610 nm) normalized to tissue weight (mg). Results are presented as mean $\pm$ SEM of n = 5 for each experimental group; *P < 0.05).

FIGURE 6A, 6B, and 6C demonstrate endogenous VEGF expression in mouse ear and mGzmb cleavage of murine FN (mFN). Figure 6A. Untreated mouse ears were analyzed for endogenous mouse VEGF expression using immunohistochemistry with anti-mouse VEGF antibody. (i) Mouse kidney sections were used as the positive control for VEGF staining (iii). Ear section (ii) or kidney section (iv) stained without primary antibody were used as the negative controls. Scale bar = 100 μ m. Figure 6B. Double immunofluorescence using anti-mouse-VEGF antibody (i, grey) and anti-FN antibody (ii, light grey) demonstrating colocalization of VEGF and FN (iii, overlay, arrows) in the deep dermis. Scale bar = 47 μ m. Figure 6C. Culture wells coated with mFN were treated with either mGzmb or human Gzmb (hGzmb) and incubated for 2 hrs at 37 °C. Supernatants were removed and analyzed by Western blot using anti-FN antibody.

FIGURE 7 demonstrates vascular permeability is reduced in GzmB knockout mice (GzmB-KO) mice after delayed type hypersensitivity- (DTH-) induced inflammation. Wild-type (WT) and GzmB-KO mice were challenged with topical administration of oxazolone to both ears 7 days post initial sensitization leading to the development of a DTH inflammatory reaction in the ear. At the indicated time points Evans blue was injected and ears were harvested followed by dye extraction to assess vascular permeability as indicated below. Results are presented as $\pm$ SEM. 24 hrs WT n = 8; GzmB-KO n = 11; 72 hrs WT n = 11; 72 hrs; GzmB-KO n = 5, (=P<0.05, **=P<-.1). The absorbance of extract solution was read at 610 nm and normalized to the weight of baked ear.

DETAILED DESCRIPTION

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

Until recently Granzyme B (also referred to herein as GzmB or GranB) was thought to act within cells to mediate cell destruction. This cytotoxic enzyme effectively

kills virally infected and malignant cells. However, as described herein, it has shown that GzmB when present external to cells releases Vascular Endothelial Growth Factor (VEGF) from extracellular matrix (ECM) stores, including fibronectin, and induces vascular permeability *in vivo*. As also described herein, once GzmB activity is inhibited,
5 the release of VEGF from the extracellular matrix is slowed or halted and the vascular permeability that can contribute to neovessel leakage during chronic inflammation is halted.

GzmB has been known as a pro-apoptotic serine protease found in the granules of cytotoxic lymphocytes (CTL) and natural killer (NK) cells. GzmB is released towards
10 target cells, along with the pore-forming protein, perforin, resulting in its perforin-dependent internalization into the cytoplasm and subsequent induction of apoptosis (see, for *e.g.*, Medema *et al.*, *Eur. J. Immunol.* 27:3492-3498, 1997). However, during aging, inflammation and chronic disease, GzmB can also be expressed and secreted by other types of immune (*e.g.*, mast cell, macrophage, neutrophils, and dendritic cells) or non-
15 immune (keratinocyte, chondrocyte) cells and has been shown to possess extracellular matrix remodeling activity (Choy *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 24:2245-2250, 2004 and Buzza *et al.*, *J. Biol. Chem.* 280:23549-23558, 2005).

In rheumatoid arthritis (RA), a constant state of chronic inflammation contributes to synovial tissue degradation which ultimately leads to significant pain, discomfort and
20 joint dysfunction (Yoo *et al.*, *Mediators Inflamm.* 2008:129873, 2008). GzmB is highly elevated in RA, and it accumulates in the ECM where it contributes to tissue degradation by ECM proteolysis (Tak *et al.*, *Clin. Exp. Immunol.* 116:366-370, 1999; Froelich *et al.*, *J. Immunol.* 151:7161-7171, 1993). VEGF is highly expressed in RA and contributes to pathological angiogenesis that promotes immune cell infiltration and retards resolution
25 and healing (Yoo *et al.*, *Mediators Inflamm.* 2008:129873, 2008). Therefore, limiting VEGF bioavailability may normalize neovessel formation, reduce inflammation and favor the induction of an appropriate healing response. Although several treatment approaches aiming to reduce the inflammatory response in RA have been explored, including anti-VEGF treatment, these treatments are often accompanied by serious side effects including
30 hypertension, bleeding, and arterial thromboembolism (Yoo *et al.*, *Mediators Inflamm.* 2008:129873, 2008), which may occur due to indiscriminate deactivation of VEGF activities. VEGF is important for the normal maintenance of blood vessels and supports stable angiogenesis when it binds to the ECM. Using an anti-GzmB treatment approach

may prove more beneficial as currently there are no physiological roles for extracellular GzmB as elevated extracellular GzmB levels are evident only in pathological conditions. Therefore, the risk for side effects due to extracellular GzmB inhibition is limited. Moreover, by inhibiting GzmB, VEGF-matrix interaction is preserved, which will allow
5 the ECM to sequester high levels of VEGF in the tissue, and also maintain low microenvironmental levels of free VEGF that are required for preserving neovessel stability and integrity. The reduction in ECM proteolysis due to GzmB inhibition will also contribute to a reduction in the chemotactic, pro-inflammatory activities of VEGF as it will be kept bound to the ECM.

10 In atherosclerosis, intra-plaque neovessel leakage and hemorrhage is a major contributor to increased plaque instability (Di Stefano *et al.*, *Curr. Pharm. Des.* 15:1095-1106, 2009; Virmani *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 25:2054-2061, 2005). Degradation of red blood cells within the plaque is a major source for cholesterol, derived from the red blood cell membrane, which may further potentiate plaque
15 expansion (Virmani *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 25:2054-2061, 2005). Moreover, increased plaque leakiness and formation of intra-plaque thrombus may further augment the inflammatory response and promote plaque instability. Previous work has demonstrated that while GzmB is not present in healthy, non-atherosclerotic arteries, increased GzmB expression is evident in advanced lesions (Choy *et al.*, *Mod.*
20 *Pathol.* 16:460-470, 2003). Several clinical studies support a correlation between elevated plasma GzmB levels and atherosclerotic lesion severity. GzmB plasma levels are significantly higher in patients with unstable carotid plaques compared to patients with stable lesions, corresponding to increased incidences of cerebrovascular events (Skjelland *et al.*, *Artherosclerosis* 195:e142-e146, 2007). Extracellular GzmB may thus
25 promote neovessel leakage by releasing VEGF from the matrix, contributing to enhanced intra-plaque inflammation and expansion of the necrotic core resulting in a rupture prone plaque. Treatment approaches aiming to inhibit VEGF activity may impair VEGF beneficial attributes by promoting collateral neovessel growth that aims to compensate for the limited blood flow within the affected artery. However, inhibiting GzmB will
30 directly prevent VEGF release within the plaque itself which in turn will minimize intra-plaque hemorrhaging, without losing the beneficial effect of matrix-bound VEGF and its effect on promoting collateral neovessel growth. Thus, GzmB inhibition will attenuate pathological angiogenesis in atherosclerotic lesions, reduce inflammation and promote

stable neovessel formation that will support immune cell emigration from the plaque and support inflammatory resolution.

In myocardial infarction (MI), the ischemic episode results in an increased expression of VEGF that promotes re-vascularization of the tissue and enhances long-term survival of the affected area (Fukuda, *et al.*, *J. Mol. Cell. Cardiol.* 36:547-559, 2004). However, increased vascular permeability and the development of edema in the myocardium exacerbates tissue injury, prevents recovery and increases infarct size (Weis, *et al.*, *J. Clin. Invest.* 113:885-894, 2004). Inhibition of VEGF in mice after ischemic injury in the brain reduced edema formation and attenuated tissue damage (van Bruggen *et al.*, *J. Clin. Invest.* 104:1613-1620, 1999). Thus, strategies aiming to inhibit VEGF-mediated vascular leakage are suggested to be beneficial in reducing edema formation post MI (Weis, *et al.*, *J. Clin. Invest.* 113:885-894, 2004). Direct VEGF inhibition reduces the extent of neovascularisation that is required for myocardium tissue recovery. A better approach would be to minimize the vascular permeability effect of VEGF and maximize its ability to promote stable neovascularization. Interestingly, in a clinical study in patients that experience acute MI, it was reported that plasma GzmB levels were significantly increased after the acute phase, indicating that GzmB may also be involved in ventricular remodelling after ischemic injury (Kondo *et al.*, *Circ. J.* 73:503-507, 2009). Therefore, inhibition of extracellular GzmB in the myocardium may provide a beneficial strategy to inhibit VEGF-mediated vascular permeability, while retaining the beneficial effect of matrix-bound VEGF in promoting stable neovascularisation that will support infarct healing.

GzmB cleavage of the ECM therefore alters VEGF bioavailability and promotes vascular leakage and inhibition of GzmB can provide a useful strategy to control the microenvironmental levels of VEGF in diseased tissue. While high levels of VEGF promote pathological angiogenesis and increase vascular leakage, low VEGF levels promote stable neovascularisation and support healing. GzmB inhibition will therefore retain the beneficial effects of matrix-bound VEGF on the development of functional neovessels, while attenuating the pro-inflammatory effect of VEGF-mediated vascular permeability.

I. Methods of Use

In some embodiments, the methods and uses described herein are based, in part, on the discovery that GzmB cleaves fibronectin (FN) and releases Vascular Endothelial

Growth Factor (VEGF) *in vivo*. The release of VEGF is specific to the cleavage of FN by GzmB as VEGF is not released in the absence of GzmB or when GzmB activity is inhibited by a GzmB specific inhibitor. It has further been found that inhibition of the release of VEGF from extracellular matrix can control the microenvironmental levels of VEGF in diseased tissue, which in turn can decrease vascular leakage, promote stable neovascularization and support healing.

Accordingly, the present disclosure provides, among others, methods for inhibiting VEGF activity, inhibiting VEGF release from extracellular matrix and/or fibronectin, and methods for reducing vascular leakage or vascular permeability in inflamed tissue, *e.g.*, rheumatoid arthritis, myocardial infarction, skin conditions, such as psoriasis, dermatitis (for example, allergic contact dermatitis, radiation induced dermatitis, and the like), cancer, transplantation, or ocular disease (for example, macular degeneration, and the like), and other inflammatory conditions.

Use of an "effective amount" of a GzmB inhibitor of the present invention (and therapeutic compositions comprising such agents) is an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, an effective amount of a Granzyme B inhibitor may vary according to factors such as the disease state, age, sex, reproductive state, and weight, and the ability of the inhibitor to elicit a desired response in the subject. Dosage regimens can be adjusted to provide the optimum response. For example, several divided doses can be provided daily or the dose can be proportionally reduced as indicated by the exigencies of the situation.

An "effective amount" or "therapeutically effective amount" of a GzmB inhibitor, *e.g.*, which inhibits VEGF activity in a subject, *e.g.*, is an amount sufficient to produce the desired effect, *e.g.*, a decrease in the amount of VEGF released from the extracellular matrix, a decreased amount of vascular leakage, improved blood flow, normalization of neovessel formation, decreased pro-inflammatory cytokine expression, promotion of stable neovascularization. A decrease in the amount of VEGF released from the extracellular matrix or an inhibition of vascular leakage, in comparison to the normal level of VEGF or vascular leakage, is achieved when the value obtained with a Granzyme B inhibitor relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring and determining a decrease in the amount of VEGF released from the extracellular matrix or an inhibition of vascular leakage, in comparison to the normal

level of VEGF or vascular leakage, are known in the art and described herein and include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blot, Northern blot, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays described herein and known to those of
5 ordinary skill in the art.

In certain embodiments of the invention, the methods and uses for inhibiting vascular leakage in a subject having chronic inflammation include administering or applying a GzmB inhibitor for a time and in an amount sufficient such that a decrease in the amount of VEGF released from the extracellular matrix or an inhibition of vascular
10 leakage, in comparison to the normal level of VEGF or vascular leakage, is inhibited. In other embodiments, the methods and uses for inhibiting vascular leakage in a subject having chronic inflammation include administering or applying a GzmB inhibitor for a time and in an amount sufficient such that release of VEGF.

The GzmB inhibitor for use in the methods, uses and compositions described
15 herein may be a nucleic acid, a polypeptide, an antibody, such as a humanized antibody, chimeric antibody, antigen binding antibody and derivatives thereof, or a small molecule. GzmB inhibitors for use in any of the methods, uses, and compositions are described in detail below.

The term "subject" or "patient" is intended to include mammalian organisms.
20 Examples of subjects or patients include humans and non-human mammals, *e.g.*, nonhuman primates, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In specific embodiments of the invention, the subject is a human.

The term "administering" includes any method of delivery of a GzmB inhibitor or
25 a pharmaceutical composition comprising a GzmB inhibitor into a subject's system or to a particular region in or on a subject. In certain embodiments, a moiety is administered topically, intravenously, intramuscularly, subcutaneously, intradermally, intranasally, orally, transcutaneously, intrathecal, intravitreally, intracerebral, or mucosally.

In one embodiment, the administration of the GzmB inhibitor is a local
30 administration, *e.g.*, administration to the site, *e.g.*, an inflamed microenvironment, *e.g.*, an inflamed joint, an area of skin, a site of an myocardial infarct, an eye, a neovascularized tumor, and the like. In one embodiment the administration of the GzmB

inhibitor is topical administration to the site of inflammation, *e.g.*, a site of chronic inflammation; the eye, an area of inflammation, and the like.

As used herein, the term "applying" refers to administration of a GzmB inhibitor that includes spreading, covering (at least in part), or laying on of the inhibitor. For example, a GzmB inhibitor may be applied to an area of inflammation on a subject or applied to, for example the eye or an area of inflammation by spreading or covering the surface of the eye with an inhibitor, injection, oral or nasal administration, and the like.

As used herein, the term "contacting" (*i.e.*, contacting a protein, a cell, *e.g.*, a host cell, or a subject with a GzmB inhibitor) includes incubating the GzmB inhibitor and the, *e.g.*, cell, together *in vitro* (*e.g.*, adding the moiety to cells in culture) as well as administering the moiety to a subject such that the moiety and cells or tissues of the subject are contacted *in vivo*.

As used herein, the terms "treating" or "treatment" refer to a beneficial or desired result including, but not limited to, alleviation or amelioration of one or more symptoms, diminishing the extent of a disorder, stabilized (*i.e.*, not worsening) state of a disorder, amelioration or palliation of the disorder, whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival in the absence of treatment.

II. Granzyme B Inhibitors

A GzmB inhibitor for use in any of the compositions, methods and uses of the present invention may be a nucleic acid molecule, a peptide, an antibody, such as a humanized antibody, a chimeric antibody, a nanobody, or a camelid antibody, or a small molecule.

Many GzmB inhibitors are known to a person of skill in the art and are, for example, described in international patent application published under WO 2012/076985, WO 2003/065987 and United States patent application published under US 2003/0148511; Willoughby *et al.*, *Bioorg. Med. Chem. Lett.* 12:2197-2200, 2002; Hill *et al.*, *J. Thorac. Cardiovasc. Surg.* 110:1658-1662, 1995; Sun *et al.*, *J. Biol. Chem.* 271:27802-27809, 1996; Sun *et al.*, *J. Biol. Chem.* 272:15434-15441, 1997; Bird *et al.*, *Mol. Cell Biol.* 18:6387-6398, 1998; Kam *et al.*, *Biochim. Biophys. Acta* 1477:307-323, 2000; and Mahrus and Craik, *Chem. Biol.* 12:567-577, 2005, all of which are incorporated herein in their entirety.

In one embodiment, a GzmB inhibitor for use in any of the compositions, uses and methods of the invention is a nucleic acid molecule. As used herein, the term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and any chemical modifications thereof. Such modifications
5 include, but are not limited to backbone modifications, methylation, and unusual base-pairing combinations. As detailed herein, the term "nucleic acid" includes, without limitation, RNAi technologies. for example, RNA compounds used to inhibit Granzyme B may be small interfering RNA (siRNA) compounds.

In one embodiment, a GzmB inhibitor for use in the compositions, uses and
10 methods of the invention is an interfering or inhibiting nucleic acid molecule. The terms "inhibiting nucleic acid molecule", "interfering nucleic acid molecule" or "interfering nucleic acid" as used herein include single-stranded RNA (ssRNA) (*e.g.*, mature miRNA, ssRNAi oligonucleotides, ssDNAi oligonucleotides), double-stranded RNA (*i.e.*, duplex RNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, or pre-miRNA), self-
15 delivering RNA (sdRNA; see, *e.g.*, U.S. Patent Publication Nos. 200913120341, 200913120315, and 201113069780, the entire contents of all of which are incorporated herein by reference), a DNA-RNA hybrid (see, *e.g.*, WO 2004/078941), or a DNA-DNA hybrid (see, *e.g.*, WO 2004/104199) that is capable of reducing or inhibiting the expression (and, thus, the activity) of a target gene or sequence (*e.g.*, by mediating the
20 degradation or inhibiting the translation of mRNAs which are complementary to the inhibiting or interfering RNA sequence) when the inhibiting or interfering nucleic acid is in the same cell as the target gene or sequence. An inhibiting or interfering nucleic acid thus refers to a single-stranded nucleic acid molecule that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands
25 or by a single, self-complementary strand. An inhibiting or interfering nucleic acid can have substantial or complete identity to the target gene or sequence, or can comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the inhibiting or interfering nucleic acid can correspond to the full-length target gene, or a subsequence thereof (*e.g.*, the gene for Granzyme B, the nucleotide and amino acid sequence of which is known and
30 may be found in, for example, GenBank Accession No, GI:221625527, the entire contents of which are incorporated herein by reference, and SEQ ID NO:1 and SEQ ID NO:2). Preferably, the inhibiting or interfering nucleic acid molecule is chemically

synthesized. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an inhibiting or interfering nucleic acid (*e.g.*, siRNA) sequence that does not have 100% complementarity to its target sequence. An inhibiting or interfering nucleic acid can have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions can be contiguous or can be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions can comprise a single nucleotide or can comprise, for example, two, three, four, five, or more nucleotides.

An inhibiting or interfering nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an inhibiting or interfering nucleic acid is an antisense nucleic acid and can hydrogen bond to the sense nucleic acid.

In one embodiment, an inhibiting or interfering nucleic acid useful in the present method is a "small interfering RNA" or "an siRNA" molecule. In another embodiment, an inhibiting or interfering nucleic acid molecule useful in the present method is a "self-delivering RNA" or "sdRNA" molecule. In one embodiment, an inhibiting or interfering nucleic acid useful in the present method mediates RNAi. RNA interference (RNAi) is a post-transcriptional, targeted gene-silencing technique that uses double-stranded RNA (dsRNA) to degrade messenger RNA (mRNA) containing the same sequence as the dsRNA (Sharp and Zamore, *Science* 287:2431-2432, 2000; Zamore, *et al.*, *Cell* 101:25-33, 2000; Tuschl, *et al.*, *Genes Dev.* 13:3191-3197, 1999; Cottrell and Docring, *Trends Microbiol.* 11:37-43, 2003; Bushman, *Mol. Therapy* 7:9-10, 2003; McManus and Sharp *Nat. Rev. Gene* 3:737-747, 2002). The process occurs when an endogenous ribonuclease cleaves the longer dsRNA into shorter, *e.g.*, 21- or 22-nucleotide-long RNAs, termed small interfering RNAs or siRNAs. The smaller RNA segments then mediate the degradation of the target mRNA. Kits for synthesis of RNAi are commercially available from, *e.g.*, New England Biolabs or Ambion. In one embodiment one or more of the chemistries described herein for use in antisense RNA can be employed in molecules that mediate RNAi.

An inhibiting or interfering nucleic acid can include, *e.g.*, siRNA and sdRNA, of about 10-60, 10-50, or 10-40 (duplex) nucleotides in length, more typically about 8-15, 10-30, or 10-25 (duplex) nucleotides in length, about 10-24, (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 10-60, 10-50, 10-40, 10-30, or 10-25 nucleotides in length, about 10-24, 11-22, or 11-23 nucleotides in length, and the double-stranded siRNA is about 10-60, 10-50, 10-40, 10-30, or 10-25 base pairs in length). siRNA and sdRNA duplexes may comprise 3'-overhangs of about 1, 2, 3, 4, 5, or about 6 nucleotides and 5'-phosphate termini. Examples of siRNA and sdRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA (or sdRNA) molecule. As used herein, the terms "siRNA" and "sdRNA" include RNA-RNA duplexes as well as DNA-RNA hybrids (see, *e.g.*, WO 2004/078941).

Preferably, siRNA and sdRNA are chemically synthesized. siRNA and sdRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA about 5, about 10, about 15, about 20, about 25, or greater nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (see, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA* 99:9942-9947, 2002; Calegari *et al.*, *Proc. Natl. Acad. Sci. USA* 99:14236, 2002; Byrom *et al.*, *Ambion TechNotes*. 10:4-6, 2003; Kawasaki *et al.*, *Nucl. Acids Res.* 31:981-987, 2003; Knight *et al.*, *Science* 293:2269-2271, 2001; and Robertson *et al.*, *J. Biol. Chem.* 243:82, 1968). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript.

In certain instances, siRNA or sdRNA can be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

Given the coding strand sequences encoding GzmB known in the art and disclosed herein (SEQ ID NO:8), an interfering nucleic acid of the invention can be designed according to the rules of Watson and Crick base pairing. The inhibiting or interfering nucleic acid molecule can be complementary to the entire coding region of GzmB mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of GzmB mRNA. For example, an inhibiting or interfering oligonucleotide can be complementary to the region surrounding the processing site of ubiquitin and GzmB mRNA. An interfering RNA oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An inhibiting or interfering nucleic acid useful in the present methods can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an interfering nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the interfering nucleic acids include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. To inhibit expression in cells, one or more interfering nucleic acid molecules can be used. Alternatively, an interfering nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation

(i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest).

The inhibiting or interfering nucleic acid can include any RNA compound which has sequence homology to the GzmB gene and which are capable of modulating the expression of GzmB protein. Examples inhibiting or interfering nucleic acids which are capable of modulating expression of GzmB are found for example in US6,159,694; US6,727,064; US7,098,192; and US7,307,069, the entire contents of all of which are incorporated herein by reference.

Antisense oligonucleotides directed against Granzyme B have been designed and manufactured by Biagnostik (Euromedex, Mundolshei, France) and are described in, for example, Hernandez-Pigeon, *et al.*, *J. Biol Chem.* 281:13525-13532, 2006 and Gruno, *et al.*, *Blood* vol. 96:1914-1920, 2000.

In another embodiment, a GzmB inhibitor for use in the compositions, methods and uses of the disclosure is an inhibitory polypeptide or a peptide.

As used herein, the term "polypeptide" refers to a polymer of amino acids having no specific length, unless otherwise specified joined by a peptide bond. It is well known to the skilled artisan that a peptide bond is also known in the art as an amide bond, and is a covalent chemical bond formed between two molecules when the carboxyl group of one molecule reacts with the amine group of the other molecule, thereby releasing a molecule of water (H₂O). Thus, "peptides" and "proteins" are included in the definition of "polypeptide" and these terms are used interchangeably throughout the specification, as well as in the claims. The term "polypeptide" does not exclude posttranslational modifications, such as modification of the terminal amino group and/or carboxyl group of the peptide and/or amino acid side chains by alkylation, amidation, or acylation to provide esters, amides or substituted amino groups; or covalent attachment to the polypeptide of glycosyl groups, acetyl groups, phosphate or phosphonate groups, lipid groups, hydroxylation of proline or lysine, and the like. In certain embodiments the polypeptide can be a chloromethyl ketone derivative. In yet another embodiment the peptide can be an inhibitory GzmB peptide. Also encompassed by this definition of "polypeptide" are homologs thereof.

In another aspect, one or both, usually one terminus of the peptide, can be substituted with a lipophilic group, usually aliphatic or aralkyl group, which can include a heteroatom. Chains can be saturated or unsaturated. Conveniently, commercially

available aliphatic fatty acids, alcohols and amines can be used, such as caprylic acid, capric acid, lauric acid, myristic acid and myristyl alcohol, palmitic acid, palmitoleic acid, stearic acid and stearyl amine, oleic acid, linoleic acid, docosahexaenoic acid, and the like. (see, for *e.g.*: US6,225,444). Preferred are unbranched, naturally occurring fatty acids between 14-22 carbon atoms in length. Other lipophilic molecules include glyceryl lipids and sterols, such as cholesterol. The lipophilic groups can be reacted with the appropriate functional group on the oligopeptide in accordance with conventional methods, frequently during the synthesis on a support, depending on the site of attachment of the oligopeptide to the support. Lipid attachment is useful where oligopeptides may be introduced into the lumen of the liposome, along with other therapeutic agents for administering the polypeptide or peptide and agents into a host.

Depending upon their intended use, particularly for administration to mammalian hosts, the subject polypeptides or peptides can also be modified by attachment to other compounds for the purposes of incorporation into carrier molecules, changing polypeptide or peptide bioavailability, extending or shortening half-life, controlling distribution to various tissues or the blood stream, diminishing or enhancing binding to blood components, and the like. The prior examples serve as examples and are non-limiting.

Peptides can be prepared in a number of ways. Chemical synthesis of peptides is well known in the art. Solid phase synthesis is commonly used and various commercial synthetic apparatuses are available, for example automated synthesizers by Applied Biosystems Inc., Foster City, Calif.; Beckman; and the like. Solution phase synthetic methods can also be used, particularly for large-scale production.

Peptides can also be present in the form of a salt, generally in a salt form which is pharmaceutically acceptable. These include inorganic salts of sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, and the like. Various organic salts of the peptide can also be made with, including, but not limited to, acetic acid, propionic acid, pyruvic acid, maleic acid, succinic acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, salicylic acid, and the like.

Polypeptides can also be made intracellularly in cells by introducing into the cells an expression vector encoding the polypeptide. Such expression vectors can be made by standard techniques. The polypeptide can also be expressed extracellularly as a fusion with another protein or peptide (*e.g.*, a glutathione S-transferase (GST) fusion).

Synthesized polypeptides can then be introduced into cells by a variety of means known in the art for introducing polypeptides into cells (*e.g.*, liposome and the like).

In one embodiment, a polypeptide for use in the methods, compositions, and uses described herein is a serpin. Serpins are a group of naturally occurring proteins that inhibit serine proteases. In one embodiment, the serpin binds to GzmB and has GzmB inhibitory function. In another embodiment, the GzmB inhibitor is a Serp2 peptide, or a GzmB inhibitory fragment thereof. The amino acid and nucleotide sequence of Serp2 are known and may be found in, for example, Genbank Accession No. GI:58219011, the entire contents of which are incorporated herein by reference, and SEQ ID NOs: 3 and 4.

In one embodiment the GzmB inhibitor is a protease inhibitor-9 (PI9) peptide, or a GzmB inhibitory fragment thereof (see, *e.g.*, US Patent Publication No. 2003/0148511, the entire contents of which are incorporated herein by reference). PI9, also known as SerpinB9 is a human serpin that inhibits Granzyme B (see, *e.g.*, review in Bird, *Immunol. Cell Biol.* 77:47-57, 1999). The amino acid and nucleotide sequence of SerpinB9 are known and may be found in, for example, Genbank Accession No. G I :223941859, the entire contents of which are incorporated herein by reference, and SEQ ID NOs:5 and 6. In one embodiment, the peptide is SerpinB9 and comprises part or all of the sequence from SerpinB9 that binds directly to Granzyme B, *i.e.*, GTEAAASSCFVVAECCMESG (SEQ. ID NO:7). This sequence contains the "reactive center" or "reactive center loop" of SerpinB9. In another embodiment, the Granzyme B inhibitor, *e.g.*, a SerpinB9 peptide comprises the amino acid sequence selected from the group consisting of VEVNEEGTEAAAASSCFVVAECCMESGPRFCADHPFL (SEQ ID NO:8); VEVNEEGTEAAAASSCFVVAADCCMESGPRFCADHPFL (SEQ ID NO:9); VEVNEEGTEAAAASSCFVVAACCMESGPRFCADHPFL (SEQ ID NO: 10); and VEVNEEGREAAAASSCFVVAECCMESGPRFCADHPFL (SEQ ID NO: 11).

In another embodiment, the Granzyme B inhibitor is a Serpina3n peptide, or a GzmB inhibitory fragment thereof. Serpina3n is also known as SerpinA3. The amino acid and nucleotide sequence of SerpinA3 are known and may be found in, for example, Genbank Accession No. GI:73858562, the entire contents of which are incorporated herein by reference, and SEQ ID NOs: 12 and 13.

In another embodiment, the GzmB inhibitor is the cowpox virusprotein, CrmA peptide, or a GzmB inhibitory fragment thereof (see, *e.g.*, Quan, *et al.*, *J. Biol. Chem.* 270:10377-10379, 1995) (the amino acid and nucleotide sequences of CrmA are set forth

in SEQ ID NOs: 14 and 15). In one embodiment, a GzmB inhibitor is a CrmA peptide comprising the amino acid sequence IDVNEEYTEAAAATCALVADCASTVTNEFCADHPFI (SEQ ID NO:16).

Other suitable GzmB inhibitory peptides for use in any of the methods, compositions, or uses of the invention, include, for example, Z-AAD-CH₂Cl (Z-Ala-Ala-Asp-chloromethylketone, also known as Z-AAD-CMK (IUPAC: 5 chloro-4-oxo-2[2-[2-(phenylmethoxycarbonylamino)propanoylamino]propanoylamino] pentanoic acid), Ac-IETD-CHO (Ac-Ile-Glu-Pro-Asp-CHO), also known as Granzyme B Inhibitor IV or Caspase 8 inhibitor III (IUPAC: (4S)-4-[[[(2S)-2-acetamido-4-methylpentanoyl]amino]-5-[2-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbamoyl]pyrrolidin-1-yl]-5-oxopentanoic acid); Ac-IETD-CHO, Caspase-8 Inhibitor I or Granzyme B Inhibitor II, (IUPAC: (4S)-4-[[[(2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[[(2S,3S)-3-hydroxy-1-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid) MF: C₂₁H₃₄N₄O₁₀ CID: 16760475; Ac-AAVALLPAVLLALLAPIETD-CHO, and acetyl-IsoleucinyI-Glutamyl-Threoninyl-Aspartyl-fluoromethylketone (Ac-IETD-FMK).

In yet another embodiment, a GzmB inhibitor for use in the compositions, methods and uses of the invention is an antibody, *e.g.*, an anti-GzmB antibody that binds specifically to and neutralizes the activity of GzmB. In one embodiment, the anti-GzmB antibody is a human antibody. In another embodiment, the anti-GzmB antibody is a humanized antibody. In another embodiment, the anti-GzmB antibody is a camelid antibody. In yet another embodiment, the anti-GzmB antibody is a mouse antibody, a rabbit antibody, or a rat antibody. In a further embodiment, the anti-GzmB antibody is a nanobody.

As used herein, the term "antibody" refers to a composition comprising a protein that binds specifically to a corresponding antigen and has a common, general structure of an immunoglobulin. The term antibody specifically covers polyclonal antibodies, monoclonal antibodies, dimers, multimers, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity. The term "antibody" includes, without limitation, camelid antibodies.

Antibodies can be murine, rabbit, rat, human, humanized, chimeric, or derived from other species. Typically, an antibody will comprise at least two heavy chains and two light chains interconnected by disulfide bonds, which when combined form a binding domain that interacts with an antigen. Each heavy chain is comprised of a heavy chain

variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region is comprised of three domains, C_{H1} , C_{H2} and C_{H3} , and may be of the mu (μ), delta (δ), gamma (γ), alpha (α) or epsilon (ϵ) isotype. Similarly, the light chain is comprised of a light chain variable region (V_L) and a light chain constant region (C_L).

5 The light chain constant region is comprised of one domain, C_L , which may be of the kappa or lambda isotype. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy

10 terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies can mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Cl_q) of the classical complement system.

15 The heavy chain constant region mediates binding of the immunoglobulin to host tissue or host factors, particularly through cellular receptors such as the Fc receptors (*e.g.*, Fc γ RI, Fc γ RII, Fc γ RIII, and the like). As used herein, antibody also includes an antigen binding portion of an immunoglobulin that retains the ability to bind antigen. These include, as examples, F(ab), a monovalent fragment of $V_L C_L$ and $V_H C_H$ antibody

20 domains; and F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. The term antibody also refers to recombinant single chain Fv fragments (scFv) and bispecific molecules such as, *e.g.*, diabodies, triabodies, and tetrabodies (see, *e.g.*, US5,844,094).

Antibodies can be produced and used in many forms, including antibody

25 complexes. As used herein, the term "antibody complex" refers to a complex of one or more antibodies with another antibody or with an antibody fragment or fragments, or a complex of two or more antibody fragments.

As used herein, the term "antigen" is to be construed broadly and refers to any molecule, composition, or particle that can bind specifically to an antibody. An antigen

30 has one or more epitopes that interact with the antibody, although it does not necessarily induce production of that antibody.

As used herein the term "epitope" refers to a determinant capable of specific binding to an antibody. Epitopes are chemical features generally present on surfaces of

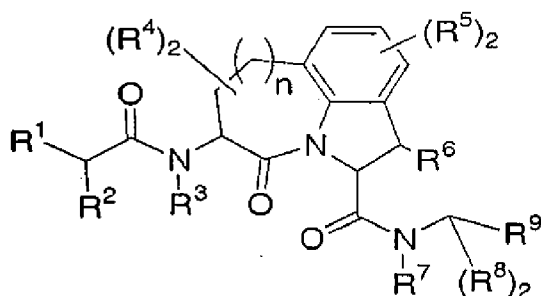
molecules and accessible to interaction with an antibody. Typical chemical features are amino acids and sugar moieties, having three-dimensional structural characteristics as well as chemical properties including charge, hydrophilicity, and lipophilicity. A conformational epitope is distinguished from a non-conformational epitope by a loss of reactivity with an antibody following a change in the spatial elements of the molecule without any change in the underlying chemical structure. The term "epitope" is also understood by those persons skilled in the art as an "antigenic determinant". For example, an antibody that is secreted by a B cell recognizes only a portion of a macromolecule; the recognized portion is an epitope. The foregoing example is provided solely as an example and is not intended not limit the scope of the term "epitope". Epitopes are recognized by numerous cell types including B cells and T cells.

As used herein, the term "humanized antibody" refers to an immunoglobulin molecule containing a minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding nonhuman residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework (FR) regions are those of a human immunoglobulin consensus sequence. A humanized antibody will also encompass immunoglobulins comprising at least a portion of an immunoglobulin constant region (Fc), generally that of a human immunoglobulin (Jones *et al.*, *Nature* 323:635-638, 1986). As used herein the term "antibody fragment" refers to a fragment of an antibody molecule. Antibody fragments can include without limitation: single domains, Fab fragments, and single-chain Fv fragments. As used herein, the term "monoclonal antibody" refers to monospecific antibodies that are the same because they are made by clones of a unique parent cell. As detailed above, the term "antibody" includes without limitation a "monoclonal antibody".

In one embodiment, a GzmB inhibitor is a small molecule.

As used herein, the term "small molecule" refers to a low molecular weight organic compound that binds to a biopolymer such as a protein, a nucleic acid, or a polysaccharide. The foregoing examples of binding partners of a small molecule are non-limiting.

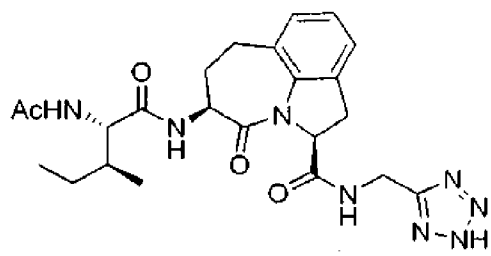
- 5 Optionally, the GzmB inhibitor used herein can be selected from one of the examples detailed herein, which includes but is not limited to azepine compounds of the following formula:



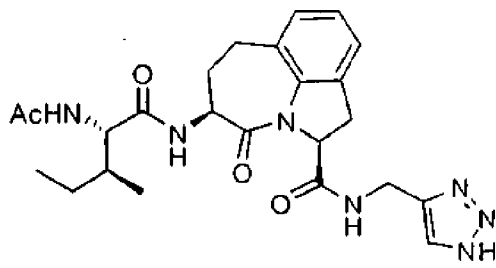
- 10 or a pharmaceutically acceptable salt or hydrate thereof, wherein n is 0, 1, or 2; R¹ and R² are each independently selected from the group consisting of: hydrogen, C₁₋₆alkyl, C₁₋₆alkoxy, C₃₋₆cycloalkyl, aryl, HET and -N(R¹⁰)₂, wherein: (a) said C₁₋₆alkyl, C₁₋₆alkoxy and C₃₋₆cycloalkyl are optionally substituted with 1-3 substituents independently selected from the group consisting of halo and hydroxy; and (b) said aryl
- 15 and HET are optionally substituted with 1-3 substituents independently selected from the group consisting of: halo, hydroxy and C₁₋₄alkyl, optionally substituted with 1-3 halo groups; or R¹ and R² may be joined together with the carbon atom to which they are attached to form a five or six membered monocyclic ring, optionally containing 1-3
- 20 heteroatoms selected from the group consisting of: S, O and N(R¹⁰), wherein said ring is optionally substituted with 1-3 R¹⁰ groups, with the proviso that R¹ and R² are both not hydrogen; each of R³ and R⁷ is independently selected from the group consisting of: hydrogen and C₁₋₄alkyl, optionally substituted with 1-3 halo groups; each of R⁴, R⁵, R⁶ and R⁸ is independently selected from the group consisting of: hydrogen, halo, hydroxy and C₁₋₄alkyl, optionally substituted with 1-3 halo groups; R⁹ is HET, optionally
- 25 substituted with 1-3 substituents independently selected from the group consisting of: halo, hydroxy and C₁₋₄alkyl, optionally substituted with 1-3 halo groups; R¹⁰ is selected from the group consisting of: hydrogen, C₁₋₄alkyl and

-C(O)C₁₋₄alkyl, said -C(O)C₁₋₄alkyl optionally substituted with N(R¹¹)₂, HET and aryl, said aryl optionally substituted with 1-3 halo groups; R¹¹ is selected from hydrogen and C₁₋₄alkyl, optionally substituted with 1-3 halo groups; HET is a 5- to 10-membered aromatic, partially aromatic or non-aromatic mono- or bicyclic ring, containing 1-4
 5 heteroatoms selected from O, S and N(R¹²), and optionally substituted with 1-2 oxo groups; and R¹² is selected from the group consisting of: hydrogen and C₁₋₄alkyl, optionally substituted with 1-3 halo groups.

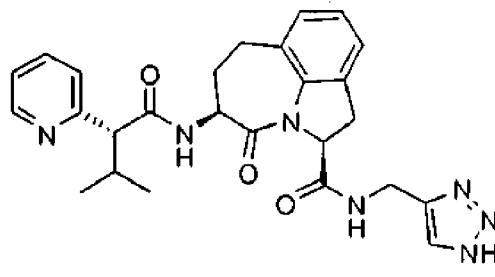
Optionally, the GzmB inhibitor used herein may be selected from one of the examples detailed herein, which includes but is not limited to one or more of the
 10 following:



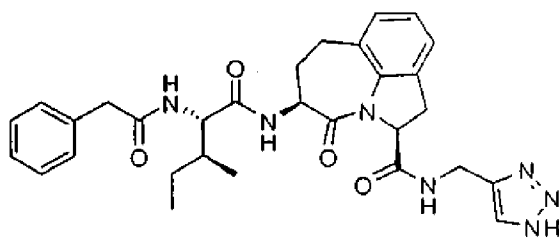
, also referred to herein as (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



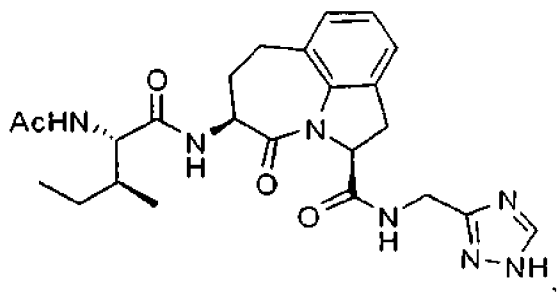
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,
 15



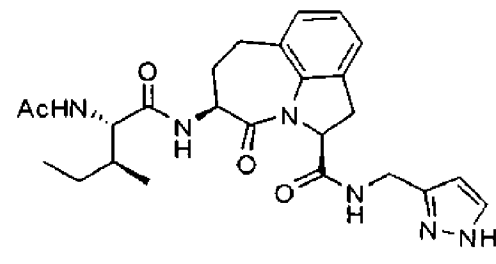
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



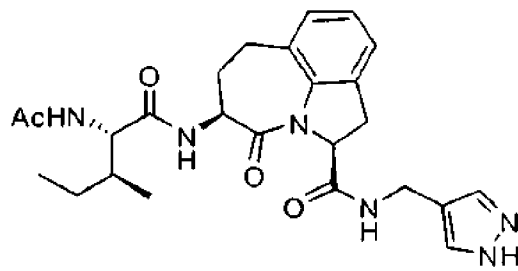
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



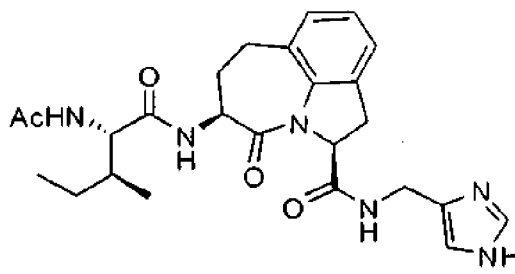
5 , also referred to herein as (2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



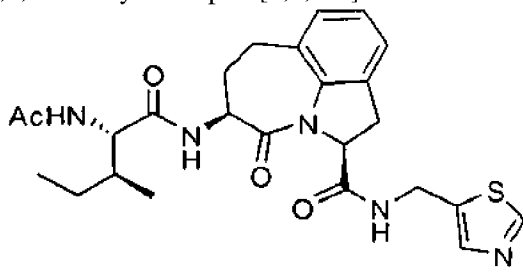
, also referred to herein as (2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



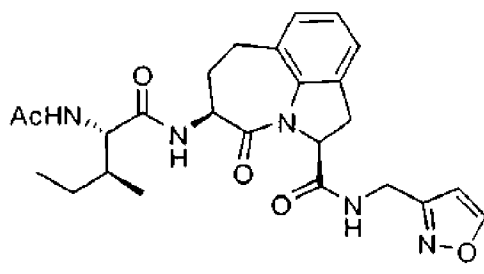
, also referred to herein as (2S,5S)-N-5-((1H-pyrazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



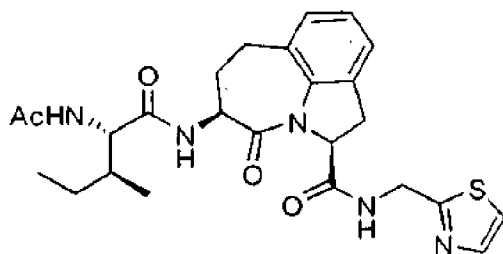
, also referred to herein as (2S,5S)-
N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-
1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



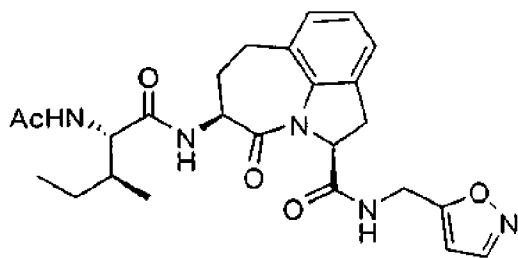
- 5 5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-
hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



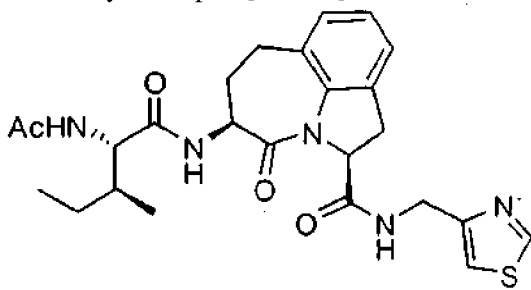
, also referred to herein as (2S,5S)-5-
((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-
hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



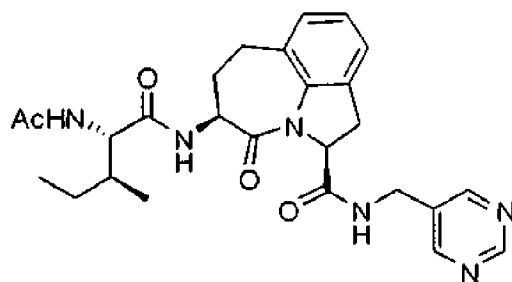
- 10 , also referred herein as (2S,5S)-5-
((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(isoxazol-2-ylmethyl)-1,2,4,5,6,7-
hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



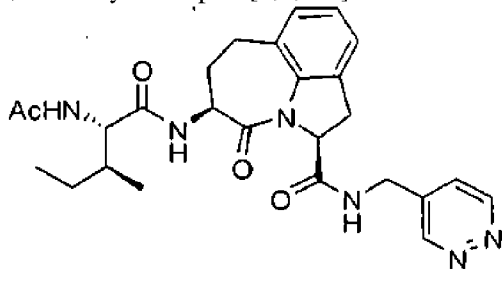
, also referred to herein as (2S,5S)-
5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-
1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



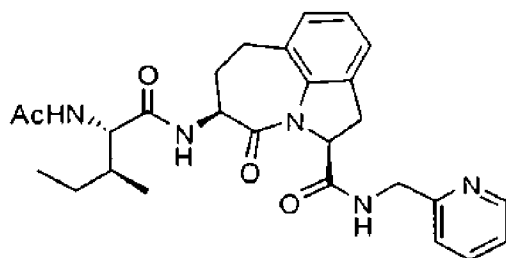
, also referred to herein as (2S,5S)-
5
((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-
hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



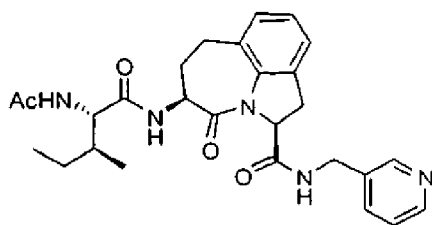
, also referred to herein as (2S,5S)-
5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-
1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



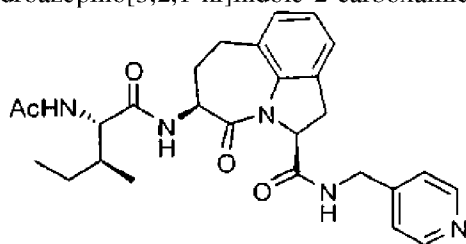
10
, also referred to herein as (2S,5S)-5-
((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-
1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



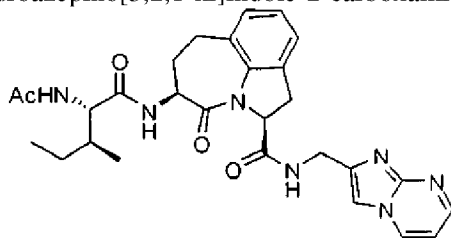
, also referred to herein as (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



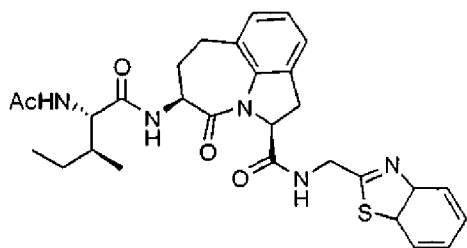
5 ((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



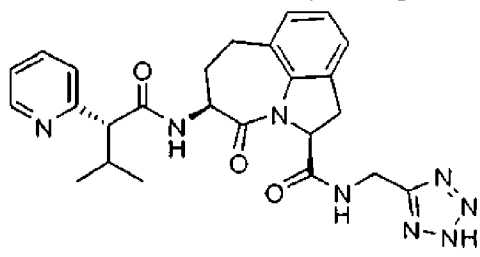
, also referred to herein as (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



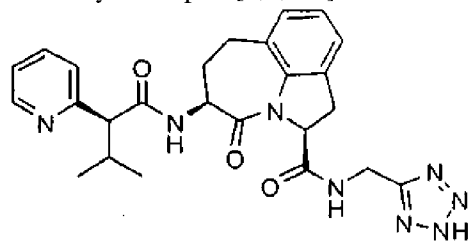
10 , also referred to herein as (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



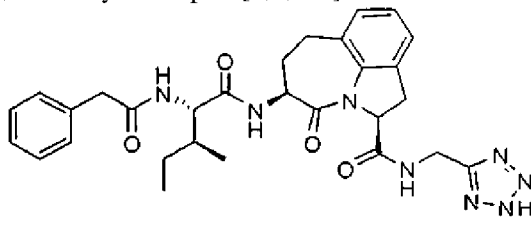
, also referred to herein as (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide,



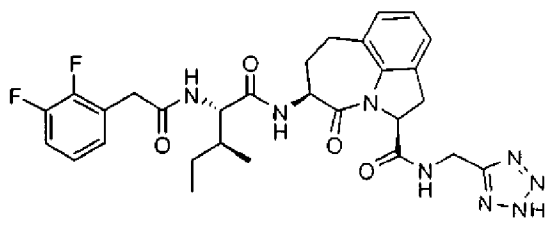
- 5 ((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide,



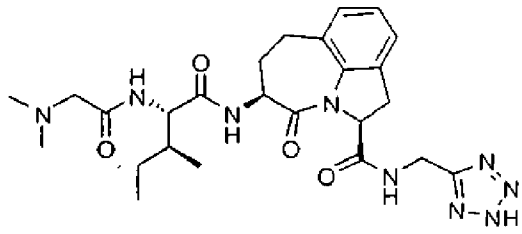
, also referred to herein as (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide,



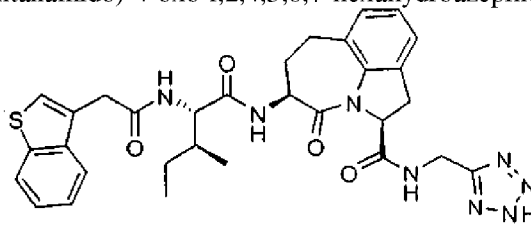
- 10 (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide,



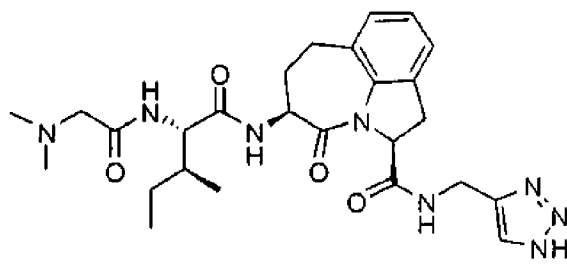
, also referred to herein as (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



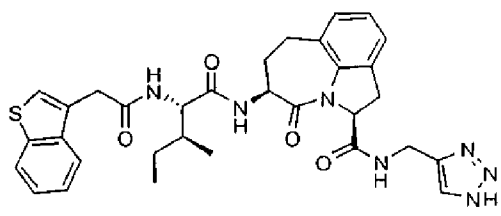
- 5 N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



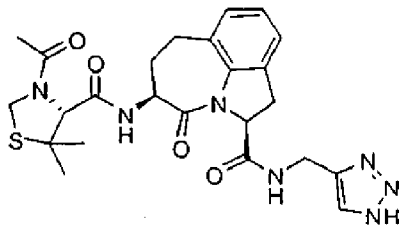
- 10 (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



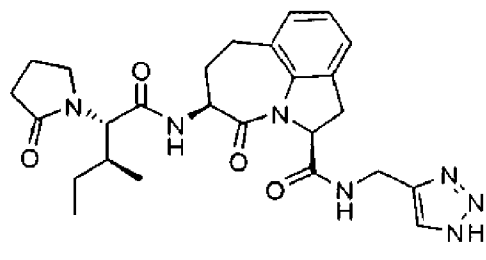
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



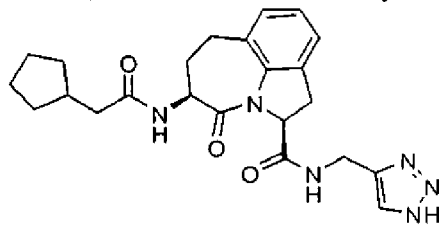
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



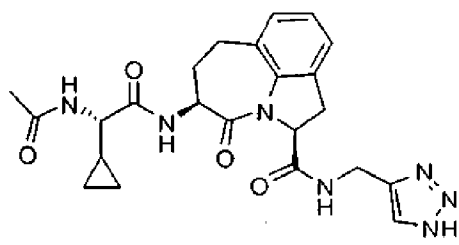
- 5 (R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide,



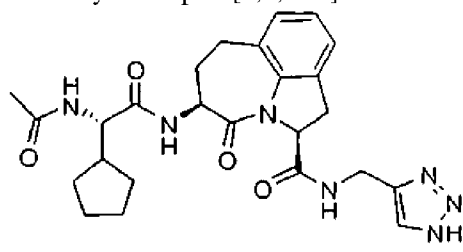
, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,



- 10 (2S,5S)-N-5((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,

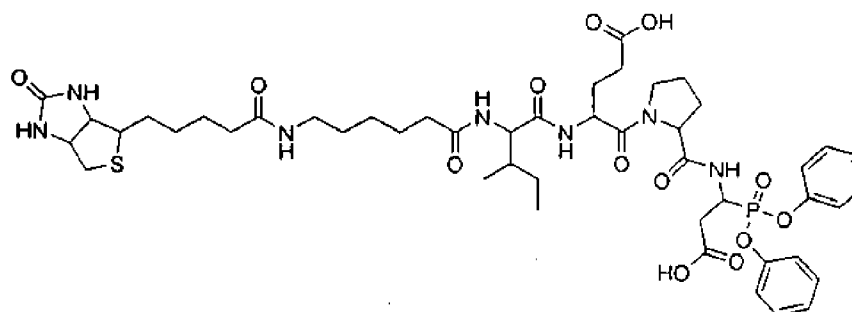


, also referred to herein as (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide,

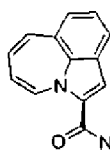


- 5 ((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide, or a salt or a solvate of any of the above.

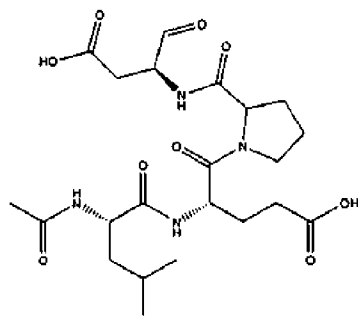
Optionally, the Granzyme B inhibitor used herein can be selected from one of the examples detailed herein, which includes but is not limited to one or more of the following:



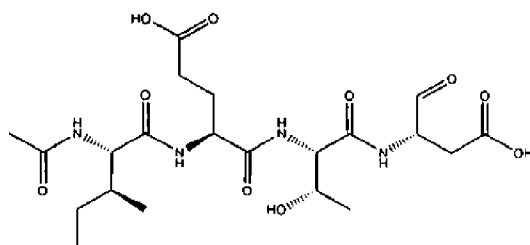
also referred to as Bio-x-IEPD^P-(OPh)₂,



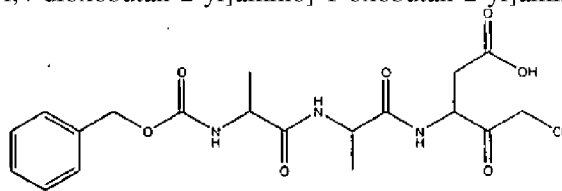
, also referred to as azepino[3,2,1-hi]indole-2-carboxamide,



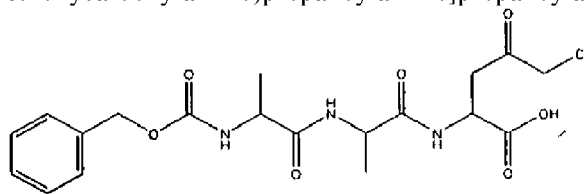
also referred to herein as (4S)-4-[[[(2S)-2-acetamido-4-methylpentanoyl]amino]-5-[2-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbamoyl]pyrrolidin-1-yl]-5-oxopentanoic acid,



- 5 [[(2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[[(2S,3S)-3-hydroxy-1-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid,

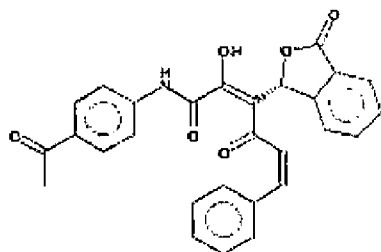


also referred to herein as 5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino)propanoylamino]propanoylamino]pentanoic acid,



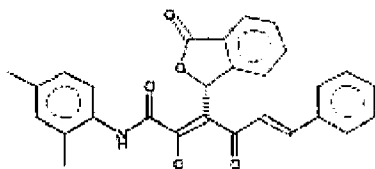
- 10 also referred to herein as 5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino)propanoylamino]propanoylamino]pentanoic acid, or a salt or solvate thereof.

Optionally, the Granzyme B inhibitors used herein is selected from the following:



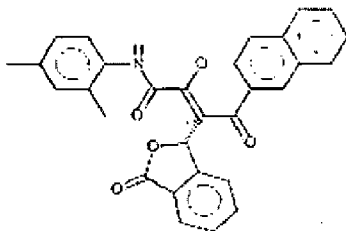
, also referred to herein as ZINC05723764

and NCI 644752,



, also referred to herein as ZINC05723787

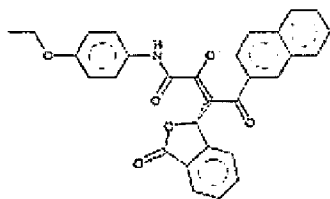
and NCI 644777,



5

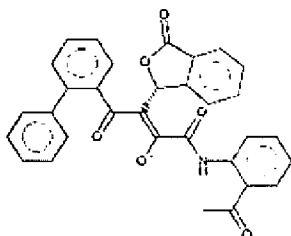
, also referred to herein as ZINC05316154 and

NCI641248,



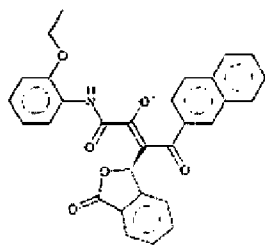
, also referred to herein as ZINC05723499 and

NCI 641235,

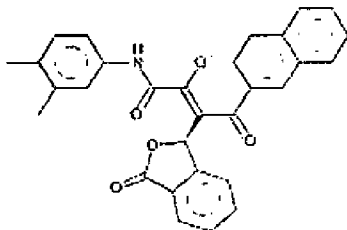


, also referred to herein as ZINC05723646 and NCI

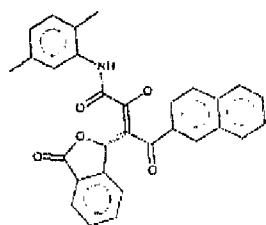
10 642017,



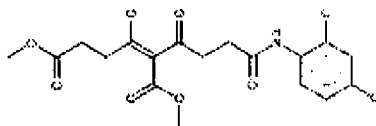
, also referred to herein as ZINC05398428 and NCI
641230,



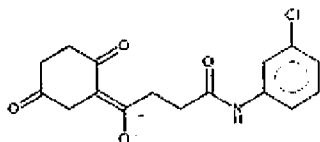
, also referred to herein as ZINC05723503 and
NCI641236,



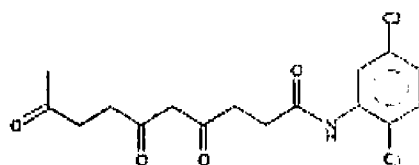
5 , also referred to herein as ZINC05723446 and NCJ
640985,



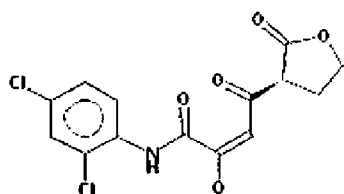
, also referred to herein as ZINC05317216
and NCI 618792,



10 , also referred to herein as ZINC05315460 and
NCI630295,

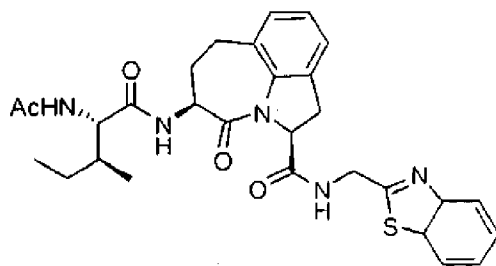


,also referred to herein as ZINC05316859 and NCI 618802, and



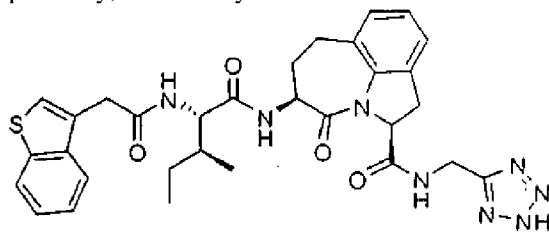
, also referred to herein as ZINC05605947 and NCI 623744, or a salt or solvate thereof.

5 Optionally, the Granzyme B inhibitor used herein is:



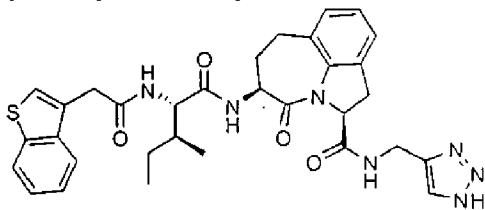
or a salt or solvate thereof.

Optionally, the Granzyme B inhibitor used herein is:



or a salt or solvate thereof.

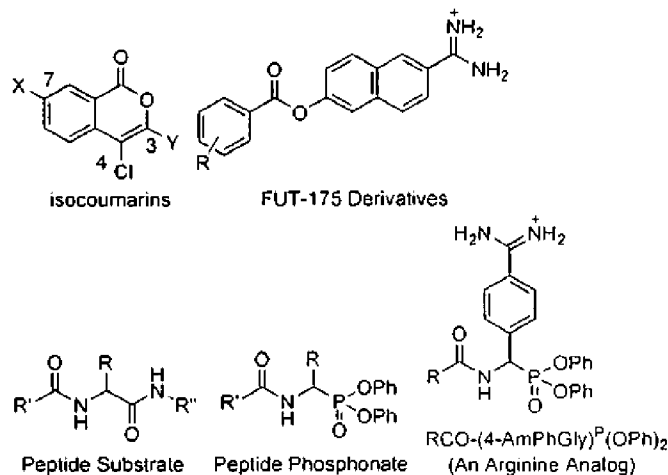
Optionally, the Granzyme B inhibitor used herein is:



10 or a salt or solvate thereof.

A GzmB inhibitor for use in the methods, compositions, and uses of the invention may also be a synthetic inhibitor such as, for example, isocoumarin, a peptide

chloromethyl ketone, or a peptide phosphonate (see, *e.g.*, Kam *et al.*, *Biochim Biophys Acta* 1477:307-323, 2000). As such, the GzmB inhibitor used herein is one or more of:



Isocoumarin derivatives (upper left): 3,4-dichloroisocoumarin, DCI, X = H, Y = Cl; 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin, X = NH₂, Y = O(CH₂)₃-SC(=NH⁺)₂NH₂; 4-chloro-3-ethoxy-7-guanidinoisocoumarin, X = NHC(=NH⁺)₂NH₂, Y = OCH₂CH₃. FUT-175 analogs (upper right). Bottom line: structures of a peptide substrate, a peptide phosphonate and a 4-amidinophenylglycine phosphonate [(4-AmPhGly)^P(OPh)₂] derivative. The latter is an arginine analog.

- 10 In one particular embodiment, a GzmB inhibitor useful in the methods of discloses herein is selected from the group consisting of (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,l-hi]indole-2-carboxamide; (2S,5S)-N-((1H-pyrazol-4-yl)methyl)-

- 5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-

yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; Bio-x-IEPD^P-(OPh)₂; azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-benzo[b]thien-3-ylacetyl)amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-ylmethyl)-, ((2S,5S)- (Compound 20 from Willoughby *et al.*, *Bioorganic Med. Chem. Lett.* 12:2197-2200, 2002); (2S,5S)-5-[(N-acetyl-L-isoleucyl)amino]-4-oxo-N-(1H-tetrazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-[N-acetyl-L-isoleucyl]amino]-4-oxo-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-5-[[2R0-3-methyl-2-pyridin-2-ylbutanoyl]amino]-4-oxo-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (2S,5S)-4-oxo-5-[[n-(phenylacetyl)-L-isoleucyl]amino]-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide; (4S)-4-[[2S)-2-acetamido-4-methylpentanoyl]amino]-5-[2-[[2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbamoyl]pyrrolidin-1-yl]-5-oxopentanoic acid; (4S)-4-[[2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[2S,3S)-3-hydroxy-1-[[2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid; 5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoylamino]

pentanoic acid; 5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoylamino] pentanoic acid, or a salt or solvate thereof; ZINC05723764 (NCI 644752); ZINC05723787 (NCI 644777); ZINC05316154 (NCI 641248); ZINC05723499 (NCI 641235); ZINC05723646 (NCI 642017); ZINC05398428 (NCI 641230);
5 ZINC05723503 (NCI 641236); ZINC05723446 (NCI 640985); ZINC05317216 (NCI 618792); ZINC05315460 (NCI 630295); ZINC05316859 (NCI 618802); ZINC05605947 (NCI 623744); and an isocoumarin.

III. Pharmaceutical Compositions

Many GzmB inhibitors are water-soluble and can be formed as salts. In such
10 cases, compositions of GzmB inhibitors can comprise a physiologically acceptable salt, which are known to a person of skill in the art. Preparations will typically comprise one or more carriers acceptable for the mode of administration of the preparation, be it by topical administration, lavage, epidermal administration, sub-epidermal administration, dermal administration, sub-dermal administration, sub-cutaneous administration,
15 systemic administration, injection, inhalation, oral, or any other mode suitable for the selected treatment. Suitable carriers are those known in the art for use in such modes of administration.

Suitable compositions can be formulated by means known in the art and their mode of administration and dose determined by a person of skill in the art. For parenteral
20 administration, the compound can be dissolved in sterile water or saline or a pharmaceutically acceptable vehicle used for administration of non-water soluble compounds such as those used for vitamin K. For enteral administration, the compound can be administered in a tablet, capsule, or dissolved in liquid form. The tablet or capsule can be enteric coated, or in a formulation for sustained release. Many suitable
25 formulations are known including, polymeric or protein microparticles encapsulating a compound to be released, ointments, pastes, gels, hydrogels, foams, creams, powders, lotions, oils, semi-solids, soaps, medicated soaps, shampoos, medicated shampoos, sprays, films, or solutions which can be used topically or locally to administer a compound. A sustained release patch or implant may be employed to provide release
30 over a prolonged period of time. Many techniques known to one of skill in the art are described in Remington: the Science & Practice of Pharmacy by Alfonso Gennaro, 20th ed., Williams & Wilkins, (2000). Formulations can, for example, contain excipients, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or

hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers can be used to control the release of a compound. Other potentially useful delivery systems for a modulatory compound include ethylene-vinyl acetate copolymer particles, osmotic
5 pumps, implantable infusion systems, and liposomes. Formulations can contain an excipient, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or can be an oily solution for administration in the form of drops, or as a gel.

Formulations of antisense nucleic acid molecules are also known to a person of skill in the art. Isis Pharmaceuticals is a company that has developed several antisense
10 formulations, including Vitravene™. Such formulations can be used with antisense nucleic acid molecules that are inhibitors of GzmB.

Compounds or pharmaceutical compositions in accordance with this invention or for use in the methods disclosed herein can be administered by means of a medical device
15 or appliance such as an implant, graft, prosthesis, stent, and the like. Also, implants can be devised which are intended to contain and release such compounds or compositions. An example would be an implant made of a polymeric material adapted to release the compound over a period of time.

One skilled in the art will appreciate that suitable methods, *i.e.*, invasive and
20 noninvasive methods, of administering a GzmB inhibitor directly to the eye are available. Although more than one route can be used to administer the GzmB inhibitor, a particular route can provide a more immediate and more effective reaction than another route. The present use is not dependent on the mode of administering the agent to an animal, preferably a human, to achieve the desired effect, and the described routes of
25 administration are merely exemplary and are in no way limiting. As such, any route of administration is appropriate so long as the agent contacts an ocular cell. Thus, the GzmB inhibitor can be appropriately formulated and administered in the form of an injection, eye lotion, ointment, implant, and the like. The GzmB inhibitor can be applied, for example, systemically, topically, intracamerally, subconjunctivally, intraocularly,
30 retrobulbarly, periocularly (*e.g.*, subtenon delivery), subretinally, or suprachoroidally. In certain cases, it can be appropriate to administer multiple applications and employ multiple routes, *e.g.*, subretinal and intravitreal, to ensure sufficient exposure of ocular

cells to the GzmB inhibitor. Multiple applications of the GzmB inhibitor can also be required to achieve the desired effect.

Depending on the particular case, it may be desirable to non-invasively administer the GzmB inhibitor to a patient. For instance, if multiple surgeries have been performed, the patient displays low tolerance to anesthetic, or if other ocular-related disorders exist, topical administration of the GzmB inhibitor may be most appropriate. Topical formulations are well known to those of skill in the art. Such formulations are suitable in the context of the use described herein for application to the skin or to the surface of the eye. The use of patches, corneal shields (see, *e.g.*, US5,185,152), and ophthalmic solutions (see, *e.g.*, US5,710,182) and ointments, *e.g.*, eye drops, is within the skill in the art.

The Granzyme B inhibitor also can be present in or on a device that allows controlled or sustained release, such as an ocular sponge, meshwork, mechanical reservoir, or mechanical implant. Implants (see, *e.g.*, US5,443,505, US4,853,224 and 4,997,652), devices (see, *e.g.*, US5,554,187, US4,863,457, US5,098,443 and US5,725,493), such as an implantable device, *e.g.*, a mechanical reservoir, an intraocular device or an extraocular device with an intraocular conduit, or an implant or a device comprised of a polymeric composition are particularly useful for ocular administration of the expression vector. The Granzyme B inhibitor also can be administered in the form of sustained-release formulations (see, *e.g.*, US5,378,475) comprising, for example, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), or a polylactic-glycolic acid.

Preferably, when used for treating an ocular disease the GzmB inhibitor is administered via an ophthalmologic instrument for delivery to a specific region of an eye. Use of a specialized ophthalmologic instrument ensures precise administration while minimizing damage to adjacent ocular tissue. Delivery of the GzmB inhibitor to a specific region of the eye also limits exposure of unaffected cells to the GzmB inhibitor. A preferred ophthalmologic instrument is a combination of forceps and subretinal needle or sharp bent cannula.

Alternatively, the GzmB inhibitor can be administered using invasive procedures, such as, for instance, intravitreal injection or subretinal injection, optionally preceded by a vitrectomy, or periocular (*e.g.*, subtenon) delivery. The pharmaceutical composition of the invention can be injected into different compartments of the eye, *e.g.*, the vitreal

cavity or anterior chamber. While intraocular injection is preferred, injectable compositions can also be administered intramuscularly, intravenously, intraarterially, and intraperitoneally. Pharmaceutically acceptable carriers for injectable compositions are well-known to those of ordinary skill in the art (see *Pharmaceutics and Pharmacy Practice*, J. B. Lippincott Co., Philadelphia, Pa., Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)).

An "effective amount" of a pharmaceutical composition as described herein includes a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduced release of VEGF, reduced levels of granzyme B activity, improved inflammation state, improved vascular permeability indicated by decrease vascular leakage, improved blood flow, and/or a delay or reduction in the severity of the onset of a vascular leakage as described herein. A therapeutically effective amount of a compound may vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the compound to elicit a desired response in the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as reduced VEGF release from the extracellular matrix, decreased vascular leakage, improved blood flow, normalization of neovessel formation, decreased pro-inflammatory cytokine expression, promotion of stable neovascularization, and the like as described herein. Typically, a prophylactic dose is used in subjects prior to or at an earlier stage of disease, so that a prophylactically effective amount may be less than a therapeutically effective amount.

It is to be noted that dosage values can vary with the severity of the condition to be alleviated. For any particular subject, specific dosage regimens can be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that can be selected by a medical practitioner. The amount of active compound(s) in the composition can vary

according to factors such as the disease state, age, sex, and weight of the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It may be advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

In general, compounds of the invention should be used without causing substantial toxicity. Toxicity of the compounds of the invention can be determined using standard techniques, for example, by testing in cell cultures or experimental animals and determining the therapeutic index, *i.e.*, the ratio between the LD₅₀ (the dose lethal to 50 % of the population) and the LD₁₀₀ (the dose lethal to 100 % of the population). In some circumstances however, such as in severe disease conditions, it may be necessary to administer substantial excesses of the composition.

While a preferred embodiment of the methods, compositions and uses have been illustrated and described herein, it will be appreciated that various changes can be made therein without departing from the scope and spirit of the methods, compositions and uses claimed.

EXAMPLES

The following examples provide a demonstration that Granzyme B (GzmB) increases the release vascular endothelial growth factor (VEGF) from the extracellular matrix (EM) inducing an increase in vascular permeability, and that the inhibition of GzmB from the extracellular matrix decreases the release of VEGF from the extracellular matrix decreases vascular leakage.

Materials and methods:

VEGF release assays:

For Granzyme B-mediated (GzmB-mediated) vascular epidermal growth factor (VEGF) release from fibronectin (FN), 48 well plates were coated with human purified plasma FN (20 µg/ml) in DPBS for 1 hr at 37 °C. Wells were then blocked with 1 % bovine serum albumin (BSA) in DPBS for 30 min at 37 °C. VEGF (50 ng/ml) was added to the FN coated wells and incubated for 2 hrs at 37 °C followed by extensive washing to remove unbound VEGF. Human purified GzmB (50 nM) in Tris buffer (50 mM Tris, pH 7.4) with either vehicle control (1:100 dimethylsulfoxide (DMSO)) or GzmB inhibitor (Compound 20 (50µM); azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-

benzo[b]thien-3-ylacetyl) amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-ylmethyl)-, ((2S,5S)- (Compound 20 from Willoughby *et al.*, *Bioorganic Med. Chem. Lett.* 12:2197-2200, 2002)), were added to the well for additional 2 hrs at 37 °C. Supernatants were analyzed by VEGF ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer instructions. For VEGF release from human umbilical vein endothelial cell (HUVEC) matrix, HUVEC were cultured in a 6 well plate and grown to confluence in complete growth media (EGM2 + 2 % fetal bovine serum (FBS)). Cells were maintained in serum-reduced (0.2 % FBS) media for 9 days with changing media every 2 days. Cell removal was performed as described above (cells were washed 3 times with DPBS and 200 µl/well of 0.25 M ammonium hydroxide was added and incubated for 20 min at room temperature. Wells were washed 3 times with dH₂O and cell removal was confirmed by microscopical examination. Remaining extracellular matrix (ECM) was then blocked with 1 % BSA for 30 min at 37 °C, followed by addition of VEGF (50 ng/ml) in 1 % BSA for 2 hrs at 37 °C. Unbound VEGF was removed by washing the wells with DPBS and GzmB (50 nM) in Tris buffer with either vehicle control or Compound 20 were added to the well for additional 2 hrs at 37 °C. Supernatants were analyzed by VEGF ELISA.

Fibronectin (FN) and VEGF cleavage assay

48 well plates were coated with FN as described above. GzmB (50 nM), plasmin (50 nM), GzmB (50nM) + compound 20 (50 µM), plasmin (50 nM) + aprotinin (125 nM), GzmB (50 nM) + aprotinin, were added to the wells in Tris buffer for 2 hrs at 37°C. Enzyme preparations from the above experiment were used for VEGF cleavage assay. GzmB (50 nM) or plasmin (50 nM) were incubated with 100 ng VEGF in Tris buffer for 2 hrs at 37°C. Supernatants from the FN cleavage assay and the VEGF cleavage assay samples were analysed by Western blot. In brief, samples were resolved by either 10 % (for FN supernatants) or 20 % (for VEGF samples) SDS-PAGE and gels were transferred to PVDF membranes. Membranes were blocked with 2.5 % milk and immunoblotted using anti-human FN antibody (R&D systems, Minneapolis, MN) at 1:1000 dilution in blocking solution or biotinylated anti-human VEGF antibody (R&D systems, Minneapolis, MN) at 1:100 dilution over night at 4 °C. FN signal was detected by incubating membrane with IRDye 800 anti-mouse antibody (LI-COR Biosciences, Lincoln, NE) for 1 hr at room temperature and fluorescent signal was imaged using the Li-COR Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE). VEGF

signal was detected by first incubating membrane with ABC reagent (Vector Laboratories Inc., Burlingame CA) for 30 min at room temperature and adding enzyme substrate (ECL Plus® substrate; GE Healthcare, Buckinghamshire UK). Chemiluminescence signal was captured using Chemigenius Bioimaging System (Syngene, Frederick MD). Mouse FN cleavage assay was performed in the same way as described for human FN, using 48 culture wells coated with mouse FN (20 µg/ml; Sigma St. Louis, MO) treated with either recombinant mouse granzyme B (mGzmb; Sigma St. Louis, MO) or human Gzmb.

VEGFR2 phosphorylation by GzmB-mediated VEGF release from FN

HUVEC were grown to confluence in a 6 well plate in complete growth media. Media was changed to EGM no VEGF + 1 % FBS media for 2 days prior to treating cells with VEGF release supernatants. VEGF release from FN coated wells was performed as described above, however, this time the treatments were prepared in M199 media to allow the use of the supernatants as a treatment media for HUVEC culture. VEGF release supernatants were added to HUVEC culture for 7 min followed by rapid cell lysis with lysis buffer that included a phosphatase inhibitor cocktail (1:100) and a proteinase inhibitor cocktail (1:100). Cell lysates were resolved by 10 % SDS PAGE and immunoblotted using a labeled antibody specific for VEGFR2 when phosphorylated at the tyrosine residue at position 1214, a labeled antibody specific for VEGFR2 when phosphorylated at the tyrosine residue at position 1175, and a labeled antibody specific for total VEGFR2 (pVEGFR2y1214; pVEGFR2y1175; total VEGFR2 (Cell Signaling Technology, Danvers, MA)) (1:700 dilution in 2.5 % milk). β- tubulin immunoblotting was used as a loading control. Densitometry was used to quantify phosphorylated VEGFR2 and values were divided by the expression levels of total VEGFR2 and normalized to loading control (n = 3 with three replicates per treatment).

In vivo permeability assay

8-10 weeks old CD1 female mice were injected with 200 µl Evan's Blue dye (0.5 % in injectable saline)(Sigma St. Louis, MO) via the tail vein. The following treatments were prepared in injectable saline to a final volume of 10 µl (n = 5 for each treatment group): Recombinant mouse granzyme B (mGzmB) (100 ng; Sigma St. Louis, MO), mGzmB (100 ng) + neutralizing anti mouse VEGF antibody (1.5 µg), mGzmB (100 ng) + goat IgG control (1.5 µg). Treatments were injected locally to the ear. After 30 min mice were euthanized and perfused with saline to clear the circulation from any remaining dye. Ear tissues were excised using a 7 mm punch biopsy and dried in 60 °C incubator

overnight. Tissues were weighed and dye was extracted by incubating tissues with 300 µl formamide (Sigma St. Louis, MO) in 60 °C incubator for 24 hrs. Absorbance of the extracted dye was measured at 610 nm and values were normalized to tissue weight. Studies were approved by of the University of British Columbia Animal Care Committee.

5 **VEGF, FN Immunohistochemistry and Immunofluorescence**

Formalin-fixed, paraffin-embedded sections of untreated mouse ear tissue were sectioned at 5 µm thickness. Sections were deparaffinised and rehydrated in xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6) (Invitrogen, Carlsbad, CA) for 15 min followed by a 30 min
10 cooling at room temperature. Endogenous peroxides were quenched by incubating sections in 3 % H₂O₂ for 15 min. Sections were blocked with 10 % rabbit serum for 30 min and incubated over night at 4 °C with 10 µg/ml goat anti-mouse VEGF antibody (R&D Systems, Minneapolis, MN). Sections were incubated with 1:350 dilution of biotinylated rabbit anti-goat antibody for 30 min followed by signal amplification using
15 Vectastain® ABC system (Vector Laboratories Inc., Burlingame, CA), according to manufacturer's instructions. Staining was visualized using DAB (Vector Laboratories Inc., Burlingame, CA). Images were captured using Nikon Eclipse E600 microscope (Nikon). Mouse kidney sections were used as a positive control for VEGF staining. Negative control samples undergo the same treatment without primary antibody
20 incubation.

For double immunofluorescence staining for VEGF and FN, untreated mouse ear tissue sections were blocked with 10 % donkey serum for 1 hr and co-incubated over night at 4°C with goat anti-mouse VEGF antibody (R&D Systems, Minneapolis, MN) and rabbit anti-FN antibody (Abcam, Cambridge, MA) diluted at 1:20 and 1:500,
25 respectively. Sections were then incubated for 1 hr with donkey anti-goat fluorescent labeled antibody (Alexa 594) for VEGF detection, and donkey anti-rabbit fluorescent labeled antibody (Alexa 488) for FN detection (1:350 dilution, Invitrogen, Carlsbad, CA). Fluorescent images were captured using Leica Upright Fluorescent Microscope with Fast Confocal Scanner and CCD camera (Leica Microsystems, Wetzlar, Germany).

30 **In vivo oxazolone-induced delayed type hypersensitivity (DTH) reaction model**

On day 1, 8-11 week old C57/BL6 wild type (WT) and GzmB-deficient knockout mice (GzmB-KO) mice were sensitized by applying 2.5 % oxazolone (Sigma-Aldrich, St. Louis, MO) dissolved in 4:1 acetone:olive oil topically on shaved abdomen (50 µl)

and each paw (25 μ l). On day 7, mice were challenged by applying 1 % oxazolone dissolved in 4:1 acetone:olive oil topically to each ear (15 μ l). Vascular permeability was examined after 24 hrs (WT, n = 8; GzmB-KO, n = 11) and 72 hrs (WT, n = 11; GzmB-KO, n = 7) post-challenge by injecting the mice with 400 μ l Evan's blue dye (1 % in injectable saline) via the inferior vena cava. After 15 min, mice were euthanized and ear tissue collections and analysis were performed as described for the *in vivo* permeability assay.

Statistics

A one way analysis of variance (ANOVA) with Bonferroni's multiple comparison post test was performed to determine statistical differences between multiple groups. A paired t test was performed to determine statistical difference between two matching groups. A value of $P < 0.05$ was considered significant.

Results

GzmB releases VEGF from plasma FN

To examine the ability of GzmB to release VEGF from FN, culture plates were coated with human plasma FN followed by addition of VEGF. VEGF was effectively bound to FN as only a small amount of VEGF spontaneously dissociated to the supernatant in the control group (37.72 pg/ml + 23.95) (Figure 1). However, GzmB treatment elicited significant release of VEGF to the supernatant (350 pg/ml + 61.35; $P < 0.001$). The release of VEGF was dependent on GzmB activity as co-treatment with GzmB inhibitor (Compound 20) significantly reduced VEGF release (147 pg/ml + 8.3; $P < 0.05$). Thus, GzmB promotes the release of VEGF from plasma FN.

GzmB releases VEGF from HUVEC matrix

It has been previously demonstrated that GzmB can cleave both plasma and cell derived FN. Since the endogenous cellular matrix is more complex and may contain other pericellular matrix proteins that can bind VEGF (Yoo *et al.*, *Mediators Inflamm.* 2008:129873, 2008) it was decided to determine whether GzmB could mediate VEGF release from endogenous matrix. VEGF was added to HUVEC-derived matrix and unbound VEGF was removed by washing. Similar to experiments done on plasma FN, GzmB treatment resulted in a significant increase in VEGF release from HUVEC matrix compared to control (control 135 pg/ml + 33.66; GzmB 562 pg/ml + 39.86; $P < 0.01$) (Figure 2). Inhibition of GzmB effectively reduced GzmB-mediated VEGF release

pg/ml + 63.8; $P < 0.05$). Thus, GzmB promotes VEGF release from both plasma FN and from endogenously produced EC matrix.

VEGF is not a proteolytic substrate of GzmB

Several proteases have been demonstrated to release VEGF from the ECM by
5 cleaving VEGF intramolecularly, generating a smaller VEGF fragment with altered biological activity (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992; Lee *et al.*, *J. Cell Biol.* 169:681-691, 2005; Keyt *et al.*, *J. Biol. Chem.* 271:7788-7795, 1996; Kurtagic *et al.*, *Am. J. Physiol. Lung Cell Mol. Physiol.* 296:L534-L546, 2009). As such, the status of GzmB-released VEGF from the matrix was assessed. Plasmin was used as a positive
10 control as it is known to cleave VEGF (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992). To first confirm the activity and specificity of plasmin and GzmB, a FN release assay was performed by adding either GzmB or plasmin to FN coated culture plates and the supernatants were examined for the release of FN fragments by Western blot. Untreated control wells gave rise to a single, faded band corresponding to the full length
15 FN protein that was spontaneously dissociated from the plate during the incubation period. In contrast, GzmB treatment effectively cleaved FN, yielding a number of cleavage fragments that differ in size from those generated by plasmin (Figure 3A). FN cleavage was dependent on the enzymatic activity of both proteases as addition of aprotinin and compound 20 completely prevented FN degradation by plasmin and GzmB,
20 respectively. Interestingly, although aprotinin is a broad range serine protease inhibitor, it did not inhibit GzmB activity. The same enzyme preparations used for the FN release assay were used to examine VEGF proteolysis. As expected, plasmin incubation with VEGF resulted in the generation of a smaller VEGF fragment (Figure 3B, arrow). Conversely, VEGF was not cleaved by GzmB, as no VEGF fragments were observed
25 upon GzmB treatment (Figure 3B). Thus, the release of VEGF from the ECM by GzmB is not due to an intramolecular cleavage of the VEGF molecule.

GzmB-mediated VEGF release from FN activates VEGFR2

VEGFR2 dimerizes and undergoes rapid transphosphorylation in a number of tyrosine residues upon ligation with VEGF (Lee *et al.*, *Cell* 130:691-703, 2007). To
30 examine whether VEGF that is released from FN by GzmB retains its activity, HUVEC were treated with VEGF release supernatants, generated as in Figure 1 and VEGFR2 activation was examined by immunoblot for the phosphorylation of tyrosine residues 1214 (Y1214) and 1175 (Y1175). Even small amounts of VEGF that spontaneously

dissociated from FN activate VEGFR2 as control supernatants showed distinct signal for both Y1214 and Y1175 phosphorylation, while HUVEC treated with media containing no VEGF (-ve control) showed no signal. However, VEGF release supernatants that were generated due to GzmB activity caused a significant increase in VEGFR2 phosphorylation in both Y1214 and Y1175 (Figures 4A and 4B). VEGF released from FN treated with GzmB + inhibitor lead to attenuated VEGFR2 activation. Thus, VEGF that is released from FN by GzmB is active and can effectively lead to VEGFR2 phosphorylation in HUVEC.

GzmB-mediates VEGF dependent vascular permeability *in vivo*

To test the impact of GzmB-mediated VEGF release on vascular leakage the Miles assay was used by injecting mouse GzmB (mGzmB) to the ear and measured Evan's blue extravasation. To minimize the background of Evan's blue dye that was present in the circulation and did not extravasate the animal was perfused with a large volume of saline (10ml) prior to tissue analysis. Vascular leakage at the tip of the ear was due to holding the ear with forceps to stabilize the ear during injections. Only the injection area at the base of the ear was excised with 7 mm punch biopsy for subsequent dye extraction. Absorbance of the extracted dye was normalized to tissue weight. mGzmB injection resulted in increased vascular leakage compared to saline injections (n = 5) (Figure 5A). To further establish that the increase in vascular leakage was dependent on VEGF activity, mGzmB was co-injected with anti-mouse neutralizing VEGF antibody. Vascular leakage was significantly reduced when mGzmB was injected with VEGF neutralizing antibody compared to GzmB + IgG control injections (n = 5) (Figure 5B). Thus, GzmB leads to VEGF dependent increase in vascular leakage *in vivo*.

To confirm the source of VEGF in the above *in vivo* examples, untreated mouse ear sections were assessed immunohistochemically for VEGF expression. As a positive control mouse kidney sections were used as VEGF is reported to be expressed at a higher level by podocytes within the glomerulus and to a lesser extent by the cells of the convoluted tubules; an observation that was confirmed herein. Within the mouse ear, VEGF is expressed in the epidermis and in the deep dermis above the cartilage tissue (Figure 6Ai). This observation is in line with other work indicating the importance of basal VEGF expression in the epidermis and dermis to maintain normal permeability barrier homeostasis (Elias *et al.*, *Am. J. Pathol.* 173:689-699, 2008), while increased VEGF levels promote tissue edema and the development of psoriatic skin phenotype in

the mouse ear (Xia *et al.*, *Blood* 102:161-168, 2003). Immunohistochemical staining for VEGF in the ear revealed intracellular expression within the cells of the of the epidermis and dermis (Figure 6Bi). Additionally, double immunofluorescence for VEGF and FN indicates that VEGF co-localizes with FN in the deep dermis (Figure 6Biii). Thus, VEGF is normally expressed in the mouse ear and serves as a source for the VEGF-dependent vascular leakage observed in the above Miles assay.

It was also determined that murine Granzyme B (mGzmB) can cleave mouse fibronectin (mFN). A FN release assay was performed as for human FN using mFN treated with either mGzmB or human GzmB (hGzmB). mGzmB effectively cleaves mFN as a number of smaller mFN fragments were observed upon mGzmB treatment that are absent in the control sample (Figure 6C). The fragments that were generated by mGzmB proteolysis were similar to the fragments generated by hGzmB. However, hGzmB cleaves mFN more effectively as the resulted fragments were of higher intensity and several lower molecular weight fragments were observed (approximately 90 kDa and approximately 50 kDa; Figure 6C). Nevertheless, mGzmB cleaves mFN, leading to dissociation of fragments to the supernatant.

Vascular permeability is reduced in GzmB-KO mice after DTH induced inflammation

To further confirm the role of GzmB in promoting vascular permeability during inflammation, a DTH reaction was induced on both WT and GzmB-KO mice. In this study, animals were first sensitized to oxazolone by topical administration on the shaved abdomen. 7 days later ears were challenged and vascular permeability was examined after 24 hrs and 72 hrs. Vascular permeability in GzmB-KO mice was significantly lower than WT control, both at the 24 hrs and 72 hrs time points (Figure 7). Thus, GzmB deficiency reduced vascular permeability during a DTH inflammatory reaction.

The ECM exerts a major role in regulating growth factor bioavailability by sequestering and limiting its release (Marci *et al.*, *Adv. Drug Deliv. Rev.* 59:1366-1381, 2007). VEGF is a potent vascular permeability agent that is retained in the ECM by binding to FN (Wijelath *et al.*, *Circ. Res.* 91:25-31, 2002; Wijelath *et al.*, *Circ. Res.* 99:853-860, 2006; Martino and Hubbell; *FASEB* 24:4711-4721, 2010). GzmB has been shown herein to increase VEGF bioavailability through FN proteolysis. GzmB-mediated VEGF release lead to activation of VEGFR2 in HUVEC. GzmB-mediated vascular leakage *in vivo* is, in part, VEGF-dependent as co-treatment of GzmB and an anti-VEGF

neutralizing antibody reduced vascular leakage. It is also demonstrated herein, the role of GzmB-induced vascular leakage in DTH-induced inflammation in mice ears. Thus, GzmB regulates VEGF bioavailability by cleaving FN and promoting vascular leakage *in vivo*.

5 A number of proteases were previously shown to alter VEGF-matrix interaction with significant consequences to vascular morphogenesis. It has been shown previously that VEGF expressing cells treated with plasmin gave rise to a VEGF fragment that dissociates to the supernatant and is capable of inducing vascular leakage in guinea pig skin (Houck *et al.*, *J. Biol. Chem.* 267:26031-26037, 1992). In addition it has been shown
10 that MMP-3 generates an unbound VEGF fragment that promotes the formation of aberrant, dilated and leaky neovessels (Lee *et al.*, *J. Cell Biol.* 169:681-691, 2005). On the other hand, MMP-resistant, matrix bound VEGF promoted smaller vessel diameter and highly branched neovessels (Lee *et al.*, *J. Cell Biol.* 169:681-691, 2005). These studies highlight the fact that VEGF activity is regulated by its interaction with the ECM,
15 while proteolytic processing of VEGF and its dissociation from the matrix promotes vascular permeability and disrupts normal neovessel formation (Ferrara, *Mol. Biol. Cell* 21:687-690, 2010). As opposed to the aforementioned proteases, that directly cleave VEGF, generating a smaller VEGF fragment with altered biological activity, VEGF is not a substrate of GzmB. Since GzmB releases VEGF from the ECM, but does not cleave
20 VEGF directly, it is predicted that GzmB alters VEGF bioavailability through the cleavage of FN in the ECM, leading to the release of the full length VEGF molecule. Indeed, VEGF released from FN by GzmB retain its activity as it leads to VEGFR2 phosphorylation in HUVEC.

Cumulative evidence supports the role of extracellular GzmB in altering tissue
25 integrity and promoting inflammation through a number of mechanisms. The evidence herein further corroborates the role of extracellular GzmB in altering growth factor and cytokine release and bioavailability during inflammation. Using the DTH mouse model a significant reduction in vascular permeability has been observed in GzmMB-KO mice compared to WT control. The DTH inflammatory reaction is mediated by a number of
30 immune cells including T cells, macrophages and mast cells. Release of cytokines and pro-inflammatory agents promote the development of local inflammation in response to a hapten irritant that is applied 7 days post initial sensitization. Importantly, many inflammatory cells that take part in this reaction can express and release GzmB including

CD8⁺ T cells, mast cells and macrophages. Considering the reduction in vascular permeability due to co-injection of GzmB and anti-VEGF Ab, and the observed reduction in vascular leakage in GzmB deficient mice during local induced inflammation, the extracellular release of GzmB by inflammatory cells promotes vascular permeability mediated by VEGF release from the matrix. Thus, accumulation of extracellular GzmB during chronic inflammation, as observed in a number of chronic diseases, can further exacerbate inflammation and delay resolution by promoting vascular permeability. The reduction in vascular leakage in mice that were treated with GzmB + anti-VEGF neutralizing antibody indicates that GzmB likely mediates vascular permeability predominantly through altering VEGF bioavailability.

In summary, extracellular GzmB induces VEGF release from the ECM by cleaving FN and promoting vascular permeability. This process can serve as a novel mechanism that promotes chronic inflammation in diseases where GzmB is evident while strategies aiming to inhibit GzmB activity can attenuate vascular leakage and reduce the inflammatory response.

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for inhibiting Vascular Endothelium Growth Factor (VEGF) activity in a subject, the method comprising administering a Granzyme B inhibitor to the subject for a time and in an amount effective to inhibit VEGF activity.
2. The method according to claim 1, wherein cleavage of an extracellular matrix protein is inhibited.
3. The method according to claim 2, wherein the extracellular matrix protein is fibronectin.
4. The method according to claim 2, wherein the release of VEGF bound to the extracellular matrix is inhibited.
5. The method according to claim 4, wherein the extracellular matrix protein is fibronectin.
6. A method for inhibiting vascular leakage in subject, the method comprising administering a Granzyme B inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release.
7. The method according to claim 6, wherein cleavage of an extracellular matrix protein is inhibited.
8. The method according to claim 7, wherein the extracellular matrix protein is fibronectin.
9. The method according to claim 7, wherein the release of VEGF bound to the extracellular matrix is inhibited.
10. The method according to claim 9, wherein the extracellular protein is fibronectin.

11. A method for inhibiting VEGF release from the extracellular matrix in a subject, the method comprising administering a Granzyme B inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release.

12. The method according to claim 11, wherein cleavage of an extracellular matrix protein is inhibited.

13. The method according to claim 12, wherein the extracellular matrix protein is fibronectin.

14. The method according to claim 12, wherein the release of VEGF bound to the extracellular matrix is inhibited.

15. The method according to claim 14, wherein the extracellular protein is fibronectin.

16. A method for inhibiting VEGF release from fibronectin in a subject, the method comprising administering a Granzyme B inhibitor to the subject for a time and in an amount sufficient to inhibit VEGF release.

17. The method of any one of claims 1-16, wherein the Granzyme B inhibitor is selected from the group consisting of a nucleic acid molecule, a polypeptide, an antibody, and a small molecule.

18. The method according to claim 17 wherein the antibody is a monoclonal antibody, a humanized antibody, an antigen binding fragment of an antibody, a single chain antibody, a diabody, or a chimeric antibody.

19. The method according to claim 17, wherein the Granzyme B inhibitor is selected from one or more of the following:

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentan amido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentan amido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl) butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi] indole-2-carboxamide;

(2S,5S)-N-((1H-pyrazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi] indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacet
amido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

Bio-x-IEPO^P-(OPh)₂;

azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-benzo[b]thien-3-ylacetyl)
amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-
ylmethyl)-, ((2S,5S)- ;

(4S)-4-[[[(2S)-2-acetamido-4-methylpentanoyl]amino]-5-[2-[[[(2S)-4-hydroxy-1,4-
dioxobutan-2-yl]carbonyl]pyrrolidin-1-yl]-5-oxopentanoic acid;

(4S)-4-[[[(2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[[(2S,3S)-3-hydroxy-1-
[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid,
(Ac-IEPD-CHO);

5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino)propanoylamino] propanoyl
amino]pentanoic acid;

5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino)propanoylamino] propanoyl
amino]pentanoic acid;

(2S,5S)-4-oxo-5-{[N-(phenylacetyl)-L-isoleucyl]amino}-N-(1H-1,2,3-triazol-4-
ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboximide;

or a salt or solvate of any of the above;

ZINC05723764 (NCI 644752);

ZINC05723787 (NCI 644777);

ZINC05316154 (NCI 641248);

ZINC05723499 (NCI 641235);

ZINC05723646 (NCI 642017);

ZINC05398428 (NCI 641230);

ZINC05723503 (NCI 641236);

ZINC05723446 (NCI 640985);

ZINC05317216 (NCI 618792);

ZINC05315460 (NCI 630295);

ZINC05316859 (NCI 618802);

ZINC05605947 (NCI 623744);

an isocoumarin

a peptide chloromethyl ketone;

a peptide phosphonate;
a Granzyme B inhibitory nucleic acid molecule;
an anti-Granzyme B antibody, or an antigen specific binding fragment or derivative thereof;
an inhibitory Granzyme B polypeptide;
a SerpB9 polypeptide, or a Granzyme B inhibitory fragment thereof;
a Serp2 polypeptide, or a Granzyme B inhibitory fragment thereof;
a CrmA polypeptide, or a Granzyme B inhibitory fragment thereof; or
a Serpin A3 polypeptide, or a Granzyme B inhibitory fragment thereof.

20. The method according to any one of claims 1-19 wherein the Granzyme B inhibitor is formulated for topical administration, lavage, epidermal administration, sub-epidermal administration, dermal administration, sub-dermal administration, sub-cutaneous administration, systemic administration, injection, inhalation, or oral administration.

21. The method according to any one of claims 1-20, wherein the subject is a mammal.

22. The method according to claim 21, wherein the subject is a human.

23. Use of a Granzyme B inhibitor in the preparation of a medicament for use in inhibiting VEGF activity in a subject.

24. The use of a Granzyme B inhibitor in the preparation of a medicament for use in inhibiting vascular leakage in a subject.

25. The use of a Granzyme B inhibitor in the preparation of a medicament for use in inhibiting VEGF release from extracellular matrix, or for inhibiting the release of VEGF from fibronectin.

26. The use of a Granzyme B to inhibit the release of VEGF from extracellular matrix, or the release from fibronectin.

27. The use of any of claims 23-26, wherein the Granzyme B inhibitor is selected from the group consisting of a nucleic acid molecule, a polypeptide, an antibody, and a small molecule.

28. The use of claim 27, wherein the Granzyme B inhibitor is selected from one or more of the following:

- (2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamidopentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-1,2,4-triazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-pyrazol-3-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi] indole-2-carboxamide;
- (2S,5S)-N-((1H-pyrazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-N-((1H-imidazol-4-yl)methyl)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-3-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(isoxazol-5-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(thiazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyrimidin-5-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
- (2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridazin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-2-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-3-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-4-oxo-N-(pyridin-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-(imidazo[1,2-a]pyrimidin-2-ylmethyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-5-((2S,3S)-2-acetamido-3-methylpentanamido)-N-((3a,7a-dihydrobenzo[d]thiazol-2-yl)methyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((R)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((S)-3-methyl-2-(pyridin-2-yl)butanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-3-methyl-2-(2-phenylacetamido)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(2,3-difluorophenyl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetlamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((2H-tetrazol-5-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(dimethylamino)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((2S,3S)-2-(2-(benzo[b]thiophen-3-yl)acetamido)-3-methylpentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(R)-N-((2S,5S)-2-((1H-1,2,3-triazol-4-yl)methylcarbamoyl)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indol-5-yl)-3-acetyl-5,5-dimethylthiazolidine-4-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl) methyl)-5-((2S,3S)-3-methyl-2-(2-oxopyrrolidin-1-yl)pentanamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-(2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopropylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;
(2S,5S)-N-((1H-1,2,3-triazol-4-yl)methyl)-5-((S)-2-acetamido-2-cyclopentylacetamido)-4-oxo-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboxamide;

Bio-x-IEPO^P-(OPh)₂;

azepino[3,2,1-hi]indole-2-carboxamide, 5-[[2S,3S)-2-[(2-benzo[b]thien-3-ylacetyl) amino]-3-methyl-1-oxopentyl]amino]-1,2,4,5,6,7-hexahydro-4-oxo-N-(1H-1,2,3-triazol-5-ylmethyl)-, ((2S,5S)-;

(4S)-4-[[[(2S)-2-acetamido-4-methylpentanoyl] amino]-5-[2-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]carbamoyl]pyrrolidin-1-yl]-5-oxopentanoic acid;

(4S)-4-[[[(2S,3S)-2-acetamido-3-methylpentanoyl]amino]-5-[[[(2S,3S)-3-hydroxy-1-[[[(2S)-4-hydroxy-1,4-dioxobutan-2-yl]amino]-1-oxobutan-2-yl]amino]-5-oxopentanoic acid;

5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoyl amino] pentanoic acid;

5-chloro-4-oxo-2-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoyl amino]pentanoic acid;

(2S,5S)-4-oxo-5-{[N-(phenylacetyl)-L-isoleucyl]amino}-N-(1H-1,2,3-triazol-4-ylmethyl)-1,2,4,5,6,7-hexahydroazepino[3,2,1-hi]indole-2-carboximide;

or a salt or solvate of any of the above;

ZINC05723764 (NCI 644752);

ZINC05723787 (NCI 644777);

ZINC05316154 (NCI 641248);

ZINC05723499 (NCI 641235);

ZINC05723646 (NCI 642017);

ZINC05398428 (NCI 641230);

ZINC05723503 (NCI 641236);

ZINC05723446 (NCI 640985);

ZINC05317216 (NCI 618792);

ZINC05315460 (NCI 630295);
ZINC05316859 (NCI 618802);
ZINC05605947 (NCI 623744);
an isocoumarin
a peptide chloromethyl ketone;
a peptide phosphonate;
a Granzyme B inhibitory nucleic acid molecule;
an inhibitory Granzyme B polypeptide;
a SerpB9 polypeptide, or a Granzyme B inhibitory fragment thereof;
a Serp2 polypeptide, or a Granzyme B inhibitory fragment thereof;
a CemA polypeptide of a Granzyme B inhibitory fragment thereof; or
a Serpin A3 polypeptide, or a Granzyme B inhibitory fragment thereof.

29. The use according to any one of claims 23-28 wherein the Granzyme B inhibitor is formulated for topical administration, lavage, epidermal administration, sub-epidermal administration, dermal administration, sub-dermal administration, sub-cutaneous administration, systemic administration, injection, inhalation, or oral administration.

30. The use according to any one of claims 23-28, wherein the subject is a mammal.

31. The use according to claim 30, wherein the subject is a human.

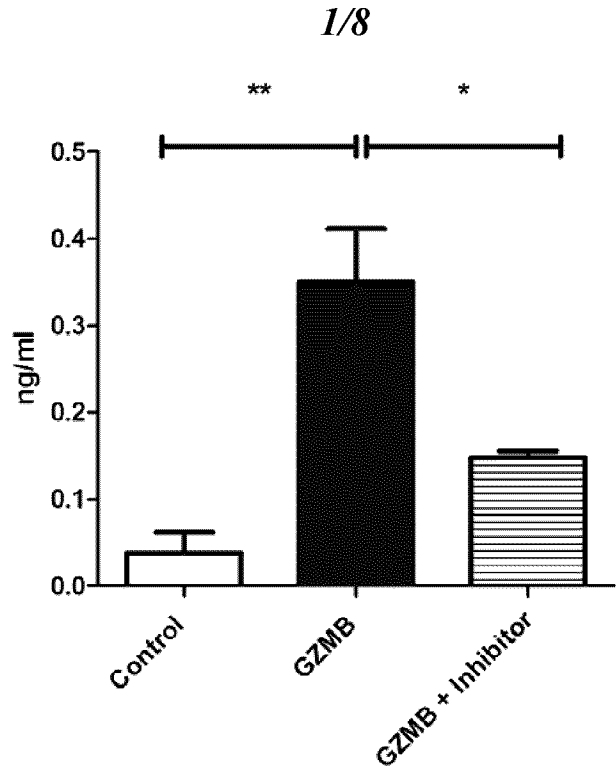


FIG. 1

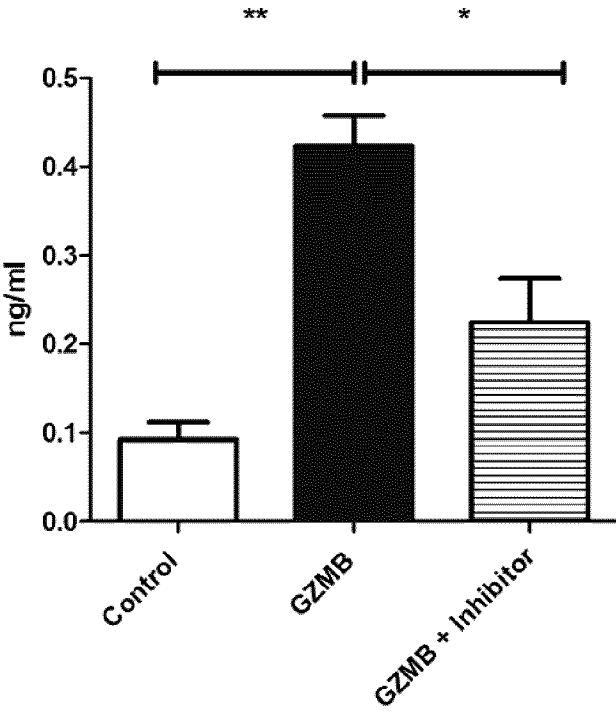


FIG. 2

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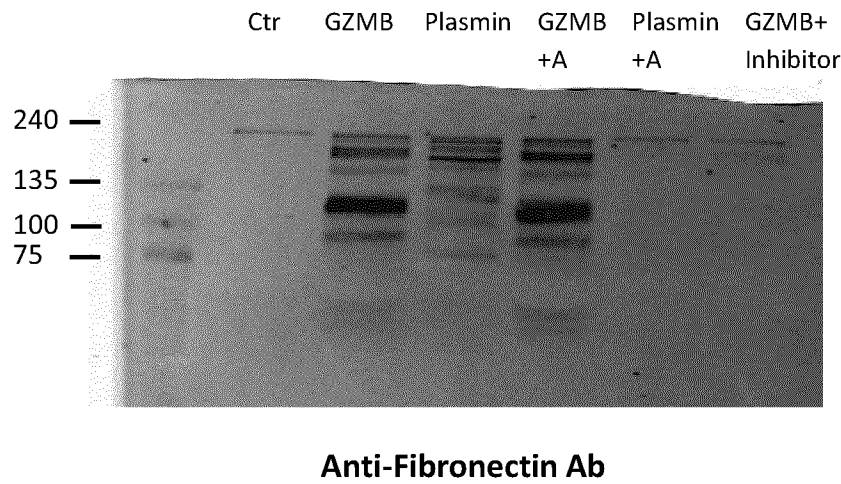


FIG. 3A

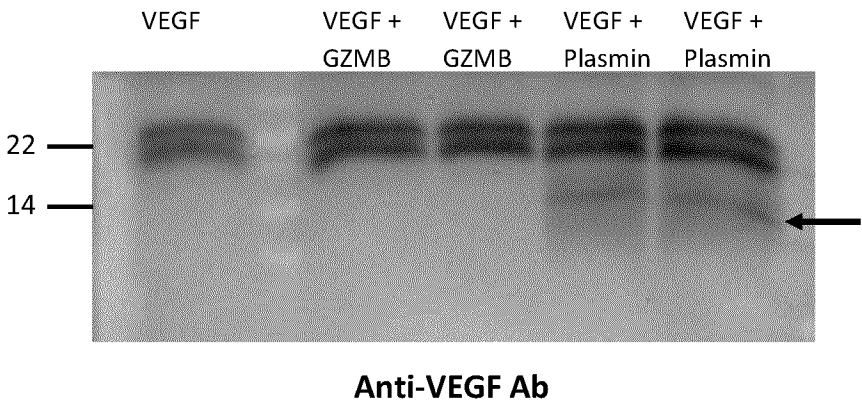


FIG. 3B

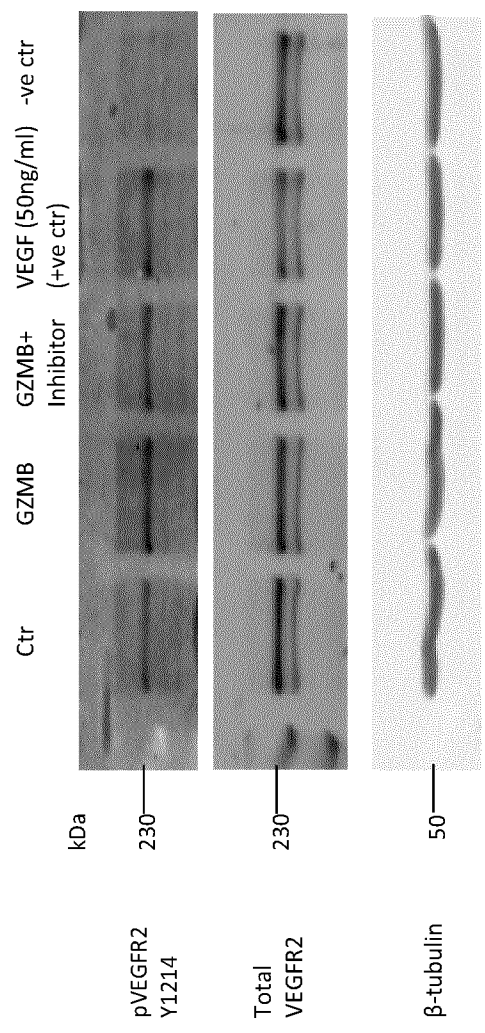
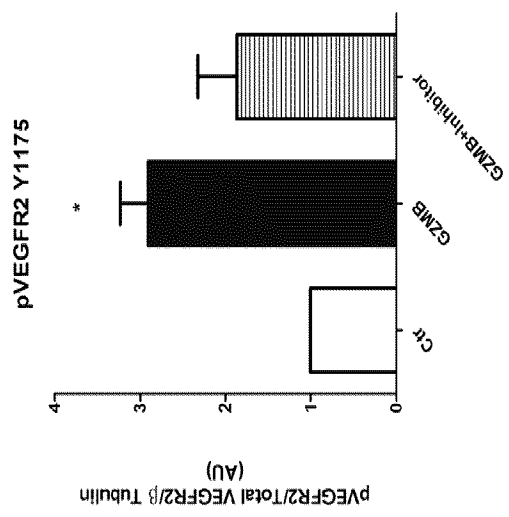
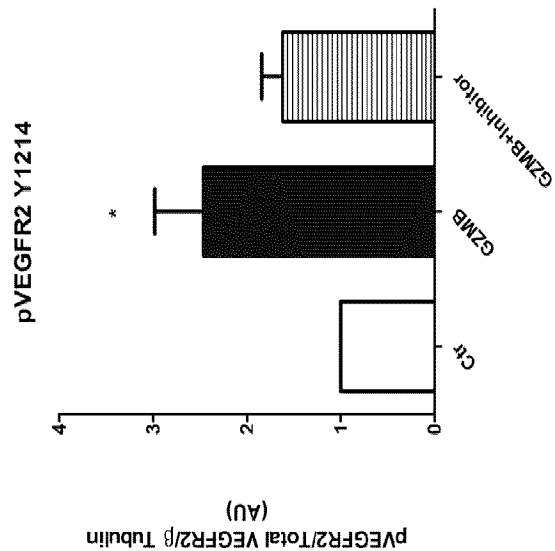


FIG. 4A

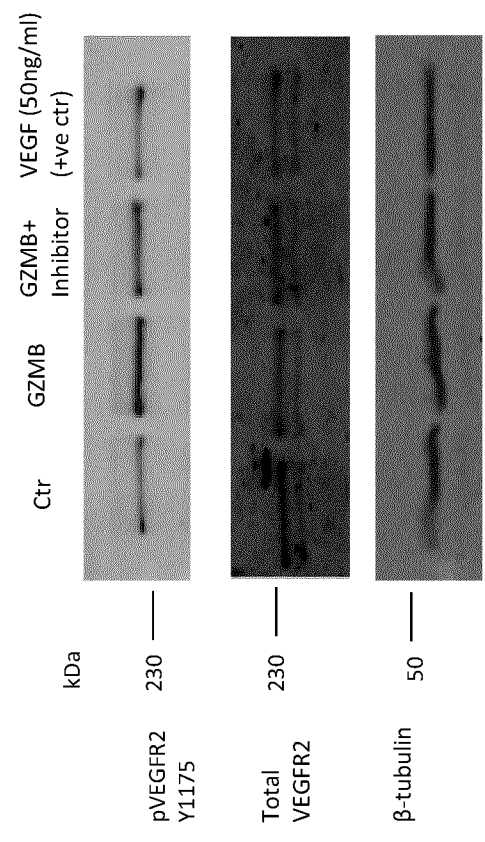


FIG. 4B

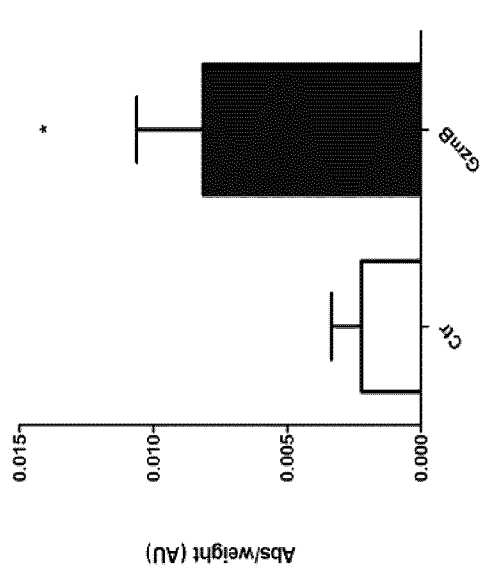


FIG. 5A

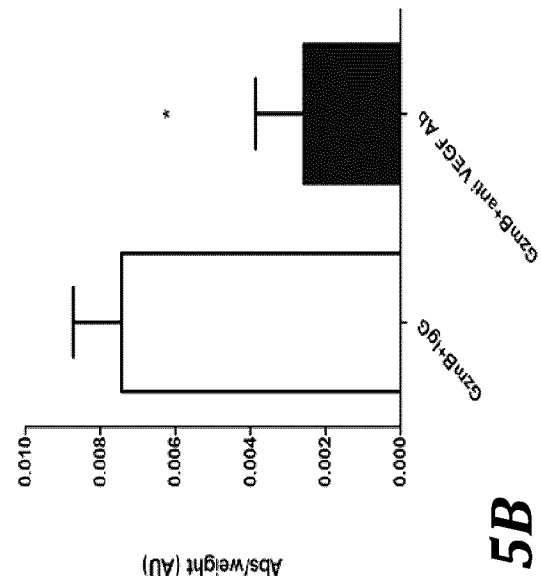
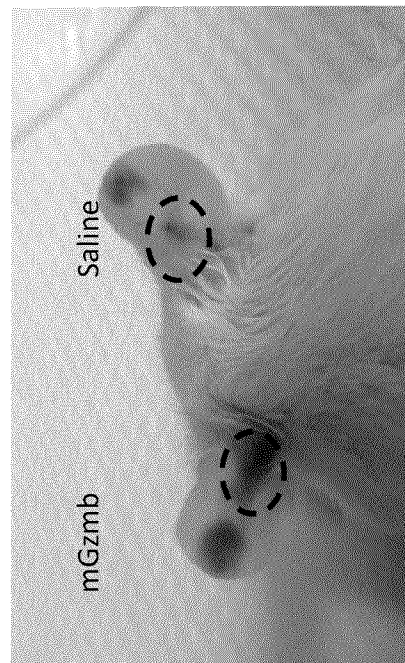
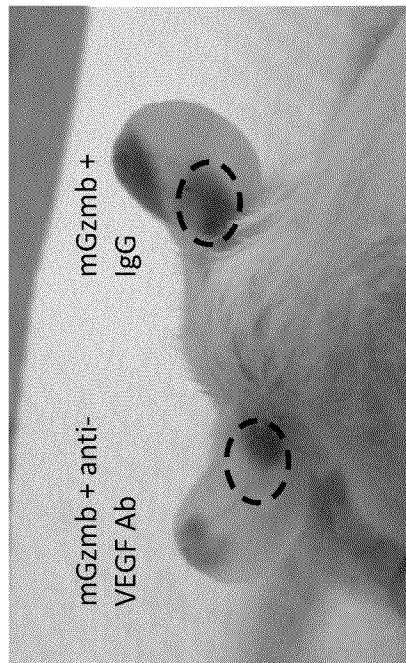


FIG. 5B



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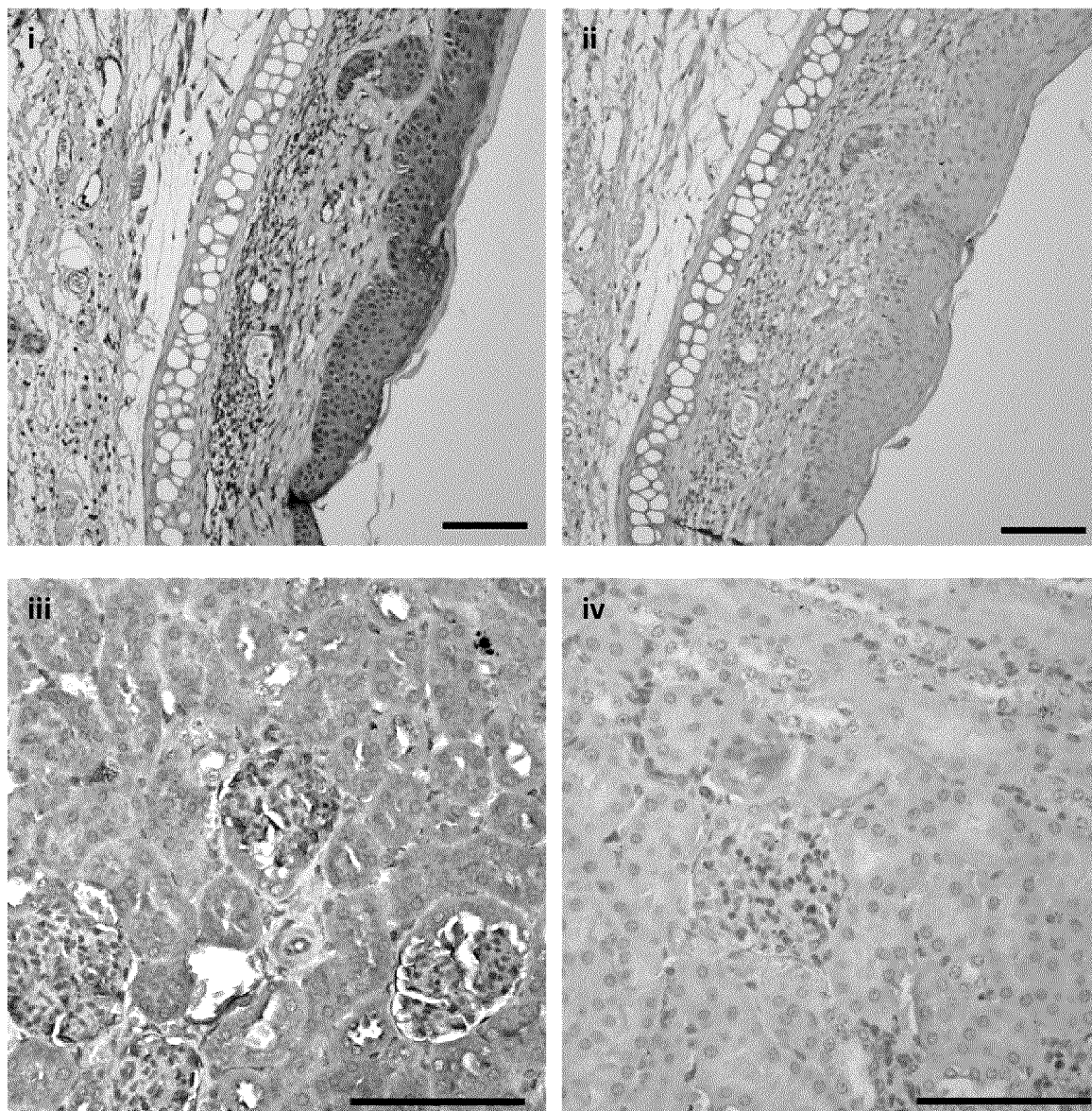


FIG. 6A

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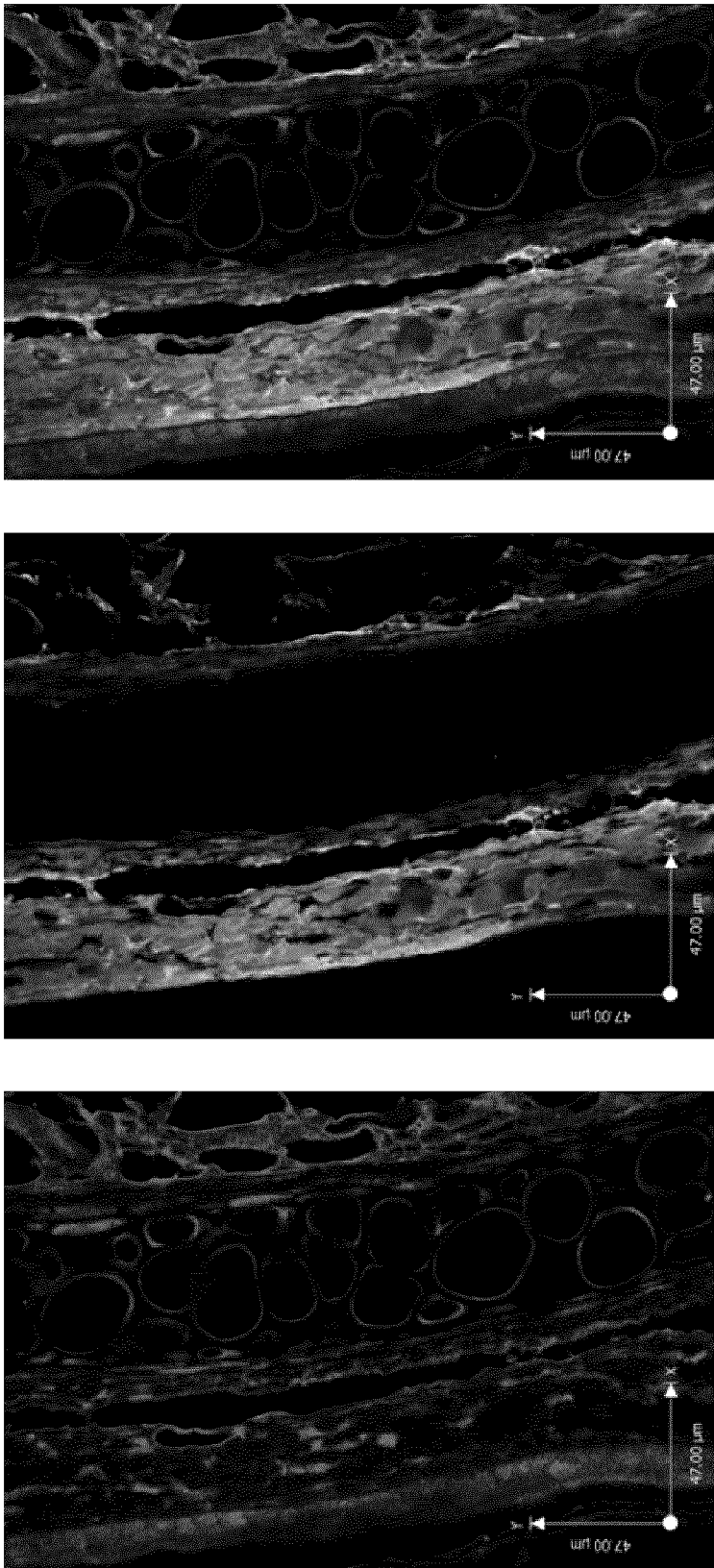


FIG. 6B

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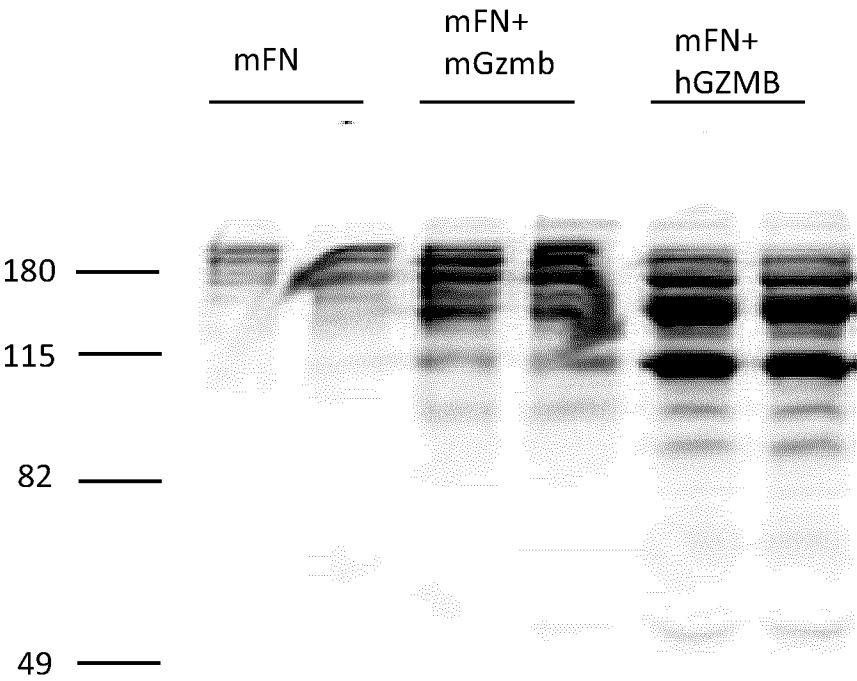


FIG. 6C

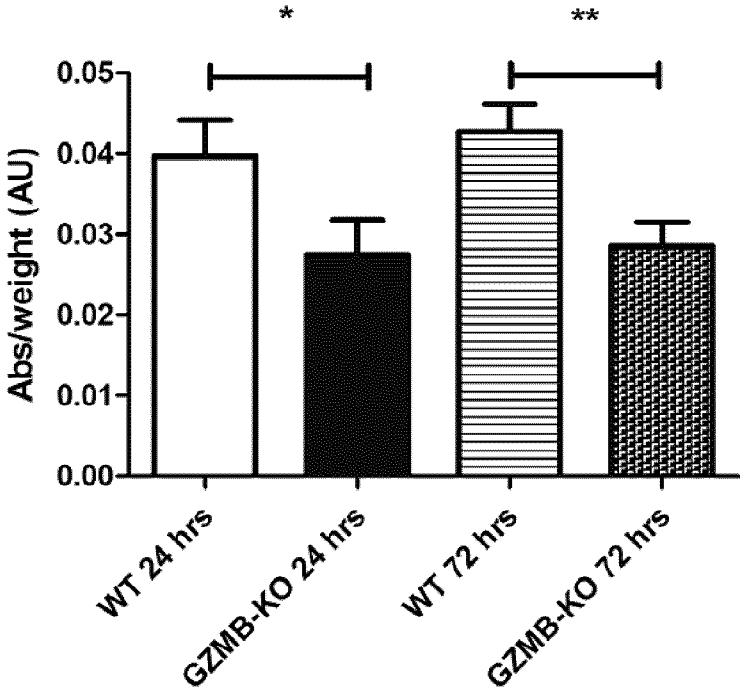


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050261

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61K 31/55 (2006.01), A61K 31/167 (2006.01), A61K 31/225 (2006.01), A61K 31/365 (2006.01), A61K 31/40 (2006.01), A61K 31/662 (2006.01) (more IPCs on the last page)		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 31/* (2006.01)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Databases: Canadian Patent database (Intellect), TotalPatent, PubMed, Google, Google Scholar Keywords: VEGF, vascular, vascular permeability/permeation/leakage, Granzyme B/GzmB, GrB, Fibronectin		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Hendel, et al. "Granzyme B Releases Vascular Endothelial Growth Factor from Extracellular Matrix Stores and Induces Vascular Permeability <i>in vivo</i> ", Atherosclerosis, Thrombosis, and Vascular Biology, May 2013, Vol 33(5 Supp), A326.	23-31
Y	Pardo, et al. "Granzyme B is expressed in mouse mast cells <i>in vivo</i> and <i>in vitro</i> and causes delayed cell death independent of perforin", Cell Death and Differentiation, June 2007, Vol 14, pages 1768-1779. *(see abstract)	24, 27-31
Y	WO 2003/065987 A2 (Willoughby, et al.) 14 August 2003 (14-08-2003). *(entire document)	24, 27-31
A	Boivin et al., "Intracellular <i>versus</i> granzyme B in immunity and disease: challenging the dogma", Laboratory Investigation, November 2009, Vol 89, pages 1195-1220.	23-31
Further documents are listed in the continuation of Box C.		
See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 28 May 2014 (28-05-2014)		Date of mailing of the international search report 18 June 2014 (18-06-2014)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Laura Stuart (819) 934-0421

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050261**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 1-22
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-22 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 1-22.
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2014/050261

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO03065987A2	14 August 2003 (14-08-2003)	WO03065987A2	14 August 2003 (14-08-2003)
		WO03065987A3	19 February 2004 (19-02-2004)
		AU2003210770A1	02 September 2003 (02-09-2003)
		CA2474917A1	14 August 2003 (14-08-2003)
		EP1474093A2	10 November 2004 (10-11-2004)
		EP1474093A4	16 February 2005 (16-02-2005)
		JP2005522430A	28 July 2005 (28-07-2005)
		US2006019945A1	26 January 2006 (26-01-2006)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050261

A61K 31/675 (2006.01), *A61K 38/06* (2006.01), *A61K 38/07* (2006.01), *A61P 9/00* (2006.01),
A61K 31/7088 (2006.01), *A61K 38/17* (2006.01), *A61K 39/395* (2006.01)