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[Continued on next page]

(54) Title: THE RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS (RAGE) IS A RECEPTOR FOR LYSOPHOSPHATIDIC ACID (LPA)

Fig. 1A

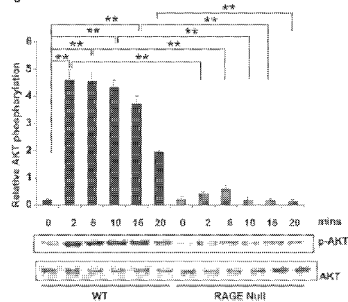


Fig. 1B

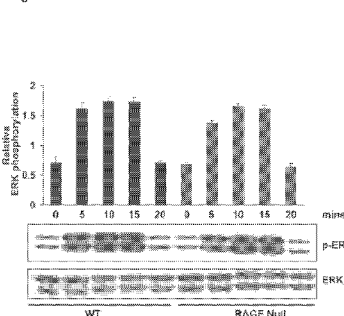


Fig. 1C

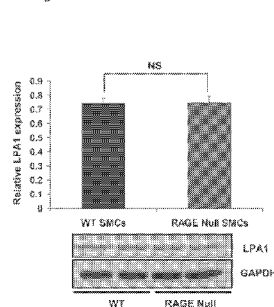
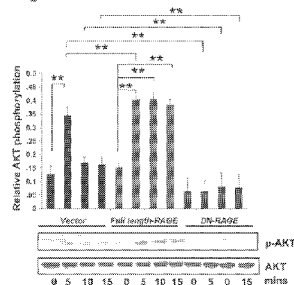


Fig. 1D



(57) Abstract: Provided herein are methods for treating a lysophosphatidic acid (LPA) mediated disorder in a subject comprising administering antagonists of receptor for advanced glycation endproducts (RAGE). Also provided are methods of treating RAGE related disorders comprising administering LPA antagonists. Still further, provided are methods for treating ovarian cancer, inhibiting metastasis of ovarian cancer and treating atherosclerosis comprising administering an agent that inhibits the binding of RAGE with LPA. Also described are methods for inhibiting LPA induced phosphorylation of Akt in a cell comprising administering an antagonist of RAGE. Finally, described herein are methods for determining whether a compound inhibits the interaction of RAGE with LPA.



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THE RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS (RAGE) IS A
RECEPTOR FOR LYSOPHOSPHATIDIC ACID (LPA)

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This application claims priority of U.S. Provisional Application No. 61/442,061, filed February 11, 2011, the entire content of which is hereby incorporated by reference herein.

10

This invention was made with government support under HL60901 awarded by the National Heart, Lung, and Blood Institute and GM62112 awarded by the National Institute of General Medical Science. The government has certain rights in the invention.

15

Throughout this application, various publications are referenced by citation, in parentheses by number, or by superscript by number. Full citations for the numbered references may be found at the end of the specification immediately preceding the claims. The disclosures of all of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

25

Background

Endogenous phospholipids such as lysophosphatidic acid (LPA) regulate cellular signal transduction cascades implicated in diverse homeostatic and pathological conditions.¹⁻³ The production of LPA is tightly regulated through the activity of the enzyme autotaxin, the principal source of LPA in the tissues.⁴ In the vasculature and in tumors, LPA stimulates cellular proliferation, survival, motility, invasion, and production of growth factors.¹⁻³ LPA appears to exert homeostatic effects in development, but in adult organisms, recrudescence of LPA signaling in stressed tissues is met

with pathological responses such as neointimal expansion and tumor growth and metastasis.¹⁻⁶

RAGE is a multi-ligand cell surface receptor of the immunoglobulin superfamily comprised of three extracellular immunoglobulin (Ig) domains, a single transmembrane helix and a short disordered intracellular domain essential for RAGE-mediated signal transduction.¹¹⁻¹³ Activation of RAGE by its ligands triggers a number of signaling cascades through the family of mitogen-activated protein kinases, and phosphatidylinositol 3-kinases.¹²⁻¹⁶ Pharmacological antagonism or genetic modulation of RAGE has been shown to exert protection against disease pathologies characterized by up-regulation and accumulation of RAGE ligands, such as the complications of diabetes, atherosclerosis, inflammation, and tumors.^{12-14, 17-18}

Summary of the Invention

Provided is a method for treating a lysophosphatidic acid (LPA) mediated disorder in a subject comprising administering
5 to the subject an amount of an antagonist of receptor for advanced glycation endproducts (RAGE) effective to treat the LPA mediated disorder in the subject, wherein the RAGE antagonist inhibits the binding of RAGE with LPA.

10 Also provided is a method of treating a receptor for advanced glycation endproducts (RAGE) related disorder in a subject comprising administering to the subject an amount of an lysophosphatidic acid (LPA) antagonist effective to treat the RAGE related disorder in the subject, wherein the LPA
15 antagonist inhibits the binding of LPA with RAGE.

Provided is a method of treating ovarian cancer in a subject comprising administering to the subject an amount of an agent effective treat the ovarian cancer in the subject, wherein
20 the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA).

Also provided is a method of inhibiting metastasis of ovarian
25 cancer in a subject comprising administering to the subject an amount of an agent effective to inhibit metastasis of the ovarian cancer by inhibiting the binding, wherein the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA) .

30 Still further, provided is a method of treating atherosclerosis in a subject comprising administering to the subject an amount of an agent effective to treat the atherosclerosis in the subject, wherein the agent inhibits
35 the binding of receptor for advanced glycation endproducts with lysophosphatidic acid (LPA).

Provided is a method for inhibiting lysophosphatidic acid (LPA) induced phosphorylation of Akt in a cell comprising administering to the cell an antagonist of receptor for advanced glycation endproducts (RAGE), thereby inhibiting the
5 LPA induced phosphorylation of Akt in the cell.

Also provided is a method for determining whether a compound inhibits the interaction of receptor for advanced glycation endproduct (RAGE) with lysophosphatidic acid (LPA)
10 comprising:

- (a) admixing:
 - (i) the compound,
 - (ii) LPA, and
 - (iii) RAGE or a fragment of RAGE which binds to LPA;
- 15 (b) determining the amount of RAGE bound to LPA; and
- (c) comparing the amount of RAGE bound to LPA in step (b) with the amount determined when RAGE or fragment of RAGE is mixed with LPA in the absence of the compound,

thereby determining whether the compound inhibits the
20 interaction of LPA with RAGE, wherein a reduction of the amount of binding in the presence of the compound indicates that the compounds inhibits the interaction.

Brief Description of the Figures**Figures 1A -1D. RAGE is a functional LPA receptor in vascular smooth muscle cells.**

5 Fig. 1A and 1B: Quantified levels of phosphorylated/total AKT and ERK are shown in wild type and RAGE null smooth muscle cells (SMCs), upon 10 μ M LPA stimulation at the indicated times. Representative results from at least three independent experiments are shown. Figure 1C: LPA₁ receptor levels as

10 shown by Western blotting are equivalent in WT and RAGE null SMCs. After probing with the primary anti-LPA₁ antibody, blots were stripped and reprobed with antibody to GAPDH. Figure 1D: Transiently-transfected vector control, RAGE-overexpressing and DN-RAGE-expressing primary murine aortic

15 SMCs were stimulated with 10 μ M LPA for the indicated times. Total lysates were subjected to Western blotting with antibodies against total AKT or p-AKT. Quantified levels of phosphorylated/total Akt in the wild type-transfected SMCs, upon LPA stimulation at different times, are shown. Fold

20 changes are relative to control. **P < 0.05.

Figures 2A - 2G. Direct binding of LPA to RAGE: Surface Plasmon Resonance and NMR.

Figures 2A-2D: SPR sensorgrams showing the interaction between RAGE and LPA. Figure 2A:

25 Binding of LPA (218 nM) to the immobilized RAGE surface on CM5 sensor chip. Figure 2B: Binding of RAGE (4 nM) to the immobilized LPA surface on HPA sensor chip. Figure 2C: Binding of RAGE V domain (1 μ M) to the immobilized LPA surface. Figure 2D: Binding of RAGE C2 domain (1 μ M) to the

30 immobilized LPA surface on HPA sensor chip. Figure 2E: Surface representation of the V domain (PDB 3CJJ) colored by electrostatic field, showing the highly basic (blue) character of the LPA binding surface. Figure 2F: NMR chemical shift perturbations mapped on the structure of the V domain

35 (PDB 3CJJ) for LPA and Ca²⁺-loaded S100B. The significantly perturbed residues are highlighted in red. Figure 2G: 15N-1H HSQC NMR spectrum of 15N-enriched C2 domain in the absence

(black) and presence (red) of LPA.

Figures 3A - 3D. LPA infusion into mouse hearts activates Akt signal transduction via RAGE.

Figure 3A: LPA (200 μ l of a 100 μ M solution) was infused directly into wild-type and RAGE null mice left ventricles. Aortas were retrieved and confocal microscopy was performed on aortic tissue and subjected to immunostaining for detection for p-Akt (15 mins). The **left column** reveals staining with a p-Akt-specific antibody; **middle column** reveals staining with monoclonal mouse smooth muscle actin antibody specific to SMC-actin; **right column** reveals the DAPI staining for nuclei. **Figure 3B:** mice aortas were retrieved at 5 or 15 mins, lysed and LPA induced Akt phosphorylation was determined by immunoblotting. LPA stimulates SMC migration and proliferation via RAGE: **Figures 3C and 3D:** Wild-type and RAGE-deficient SMCs were treated with 10 μ M LPA, S100B (10 μ g/ml) or platelet-derived growth factor (PDGF) 10 ng/ml for 5 or 48 hours, and at the end of that time, migration (Figure 3C) and (Figure 3D) proliferation, respectively, were assessed. Assays were performed in triplicate and results shown are representative of three independent experiments. Error bars represent SD. *P < 0.005.

Figures 4A - 4I. RAGE is a functional LPA receptor on C6 glioma tumor cells, is required for autotoxin/LPA-mediated signaling in vivo, and LPA-induced growth of implanted ID8 tumor cells.

Figure 4A: Quantified levels of phosphorylated/total ERK in the C6, C6 full length-RAGE and C6 DN-RAGE cells upon LPA stimulation (10 μ M) at different times, determined by immunoblotting are shown. Fold changes are relative to control. **Figures 4B-4C:** MMTV-atx mice were bred into the RAGE null background and these mice and littermate RAGE expressing MMTV-atx were studied; at age 6 weeks, mammary tissue from the mice was retrieved and subjected to Western blotting for detection of phosphorylated/total Akt and phosphorylated/total cyclin D1.

N=at least 3 replicates per group; * indicates <0.005.
Figures 4D - Figures 4I: ID8 ovarian cancer cells were
implanted into immunocompetent WT mice receiving PBS (Fig.
4D), WT mice receiving LPA (Fig. 4E), WT mice receiving LPA
5 and sRAGE (Fig. 4F), RAGE null mice receiving PBS (Fig. 4G),
and RAGE null mice receiving LPA (Fig 4H) and tumor
numbers/cm² on day 28 are shown, n = 5 per group (Fig. 4I).
The dose of LPA was 200 µl of a 100 µM solution given per
day. Soluble RAGE (200 µl of a 50 µM solution /day for 28
10 days) was administered to mice in D. Representative
photographs of peritoneal cavity on day 28 of mice bearing
ID8 cells tumors after the indicated treatments are shown.
Error bars represent SD. **P < 0.05, *P < 0.005.

15 Figure 5: Relative expression of transcripts of LPA
receptors. Real time PCR for LPA receptors 1-5 and RAGE gene
products was performed, normalized to 18s transcript levels
and expressed as fold change compared wild type vs. RAGE null
SMCs. The graphs show relative fold of LPA receptor
20 transcripts.

Figure 6: RAGE expression in transiently transfected vector,
full length RAGE and DN-RAGE vascular SMCs. RAGE levels are
shown by Western blotting in transfected SMCs and after
25 probing with the primary anti-RAGE antibody, blots were
stripped and reprobed with antibody to GAPDH.

Figure 7: Immobilization of POPC liposomes and LPA-POPC
liposomes on HPA sensor chip. A monolayer of POPC liposomes
30 was formed on the flow cell 1 (dark blue), and a monolayer of
LPA-POPC liposomes was formed on the flow cell 2 (dark red).

Figure 8: 15N-1H HSQC NMR complete spectrum of 15N-enriched
C2 domain in the absence (black) and presence (red) of LPA .
35

Figure 9: Wound healing assay. Serum starved Wt SMCs and
RAGE null SMCs were scratched and were treated with LPA (10

μM) or S100b (10 μg/ml) for 18hrs. LPA stimulated wound healing in Wt-SMCs but not in RAGE null SMCs. RAGE ligand S100b was used as reference.

- 5 **Figure 10: Expression of autotoxin in RAGE^{+/+}, MMTV-atx, and MMTV-atx/RAGE^{-/-} mice.** Mammary tissue from MMTV-atx/RAGE^{+/+} mice and MMTV-atx/RAGE^{-/-} mice was retrieved at age 6 weeks and subjected to Western blotting for detection of atx. N=3 mice per group. Note that no differences in atx expression
10 were observed between MMTX-atx expressing or devoid of RAGE.

- Figure 11: Ovarian cancer ID8 cells express RAGE.** RAGE expression is shown by Western blotting ID8 cells probing
15 with the primary anti-RAGE antibody. The two left lanes show RAGE expression in lysates prepared from murine SMCs, and the right two lanes show RAGE expression in lysates prepared from murine ID8 cells. In each case, 20 μg protein/lane was loaded.

- 20 **Figure 12: H&E staining of tumors from mice** in (Figure 12A) WT-PBS, (Figure 12B) WT-LPA, (Figure 12C) WT-LPA-sRAGE, (Figure 12D) RAGE null PBS and (E) RAGE null LPA groups. Arrow suggests tumors and each bar represents 100 μM.

25

Detailed Description of the Invention

This inventions provides a method for treating a lysophosphatidic acid (LPA) mediated disorder in a subject comprising administering to the subject an amount of an antagonist of receptor for advanced glycation endproducts (RAGE) effective to treat the LPA mediated disorder in the subject, wherein the RAGE antagonist inhibits the binding of RAGE with LPA.

10 In one embodiment, the antagonist of RAGE is a RAGE antibody. In other embodiments the antagonist of RAGE is a small molecule RAGE antagonist. In further embodiments, the antagonist of RAGE is a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.

15 In one embodiment, the LPA mediated disorder is correlated with elevated levels of LPA.

This invention also provides a method of treating a receptor for advanced glycation endproducts (RAGE) related disorder in a subject comprising administering to the subject an amount of an lysophosphatidic acid (LPA) antagonist effective to treat the RAGE related disorder in the subject, wherein the LPA antagonist inhibits the binding of LPA with RAGE.

25 The one embodiment the disorder is sepsis, atherosclerosis, multiple sclerosis, systemic lupus erythematosus, transplant rejection, asthma, arthritis, tumor growth, cancer, metastasis of cancer, complications due to diabetes, retinopathy, neuropathy, nephropathy, impotence, impaired wound healing, gastroparesis, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, neointimal formation, amyloid angiopathy, inflammation, glomerular injury, seizure-induce neuronal damage, acute skin inflammation, chronic skin inflammation, psoriasis, atopic dermatitis, rheumatoid arthritis, lung inflammation, asthma, chronic obstructive pulmonary disease, diabetes, renal

failure, hyperlipidemic atherosclerosis associated with diabetes, diabetes, diabetic late complication increased vascular permeability, diabetic late complication increase vascular permeability, diabetic late complication nephropathy, diabetic late complication retinopathy, diabetic late complication neuropathy, neuronal cytotoxicity, multiple sclerosis, dementia associated with head trauma, neuronal degeneration, restenosis, amyloidosis, or periodontal disease.

10

In one embodiment the LPA antagonist is an anti-LPA antibody.

In one embodiment of any of the above-described methods, the subject is a human.

15

This invention also provides a method of treating ovarian cancer in a subject comprising administering to the subject an amount of an agent effective to treat the ovarian cancer in the subject, wherein the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA) .

20

Also provided is a method of inhibiting metastasis of ovarian cancer in a subject comprising administering to the subject an amount of an agent effective to inhibit metastasis of the ovarian cancer by inhibiting the binding, wherein the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA) .

25

This invention also provides a method of treating atherosclerosis in a subject comprising administering to the subject an amount of an agent effective to treat the atherosclerosis in the subject, wherein the agent inhibits the binding of receptor for advanced glycation endproducts with lysophosphatidic acid (LPA).

30
35

In one embodiment of any of the above-described methods, the agent is an antagonist of LPA. In one embodiment, the antagonist of LPA is an anti-LPA antibody.

- 5 In another embodiment of any of the above-described methods, the agent is an antagonist of RAGE. In other embodiments, the antagonist of RAGE is a RAGE antibody, a small molecule RAGE antagonist, a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.

10

This invention provides a method for inhibiting lysophosphatidic acid (LPA) induced phosphorylation of Akt in a cell comprising administering to the cell an antagonist of receptor for advanced glycation endproducts (RAGE), thereby
15 inhibiting the LPA induced phosphorylation of Akt in the cell.

In one embodiment of the above-method, the cell is a smooth muscle cell or an aortic smooth muscle cell.

20

In other embodiments of the above-method, the antagonist of RAGE is a RAGE antibody, a small molecule RAGE antagonist, a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.

25

Also provided is a method for determining whether a compound inhibits the interaction of receptor for advanced glycation endproduct (RAGE) with lysophosphatidic acid (LPA) comprising:

30

(a) admixing:

(i) the compound,

(ii) LPA, and

(iii) RAGE or a fragment of RAGE which binds to LPA;

(b) determining the amount of RAGE bound to LPA; and

35

(c) comparing the amount of RAGE bound to LPA in step (b) with the amount determined when RAGE or fragment of RAGE is mixed with LPA in the absence of the compound,

thereby determining whether the compound inhibits the interaction of LPA with RAGE, wherein a reduction of the amount of binding in the presence of the compound indicates that the compounds inhibits the interaction.

5

In one embodiment, the RAGE of step (a)(iii) is fragment of RAGE. In other embodiments, the fragment of RAGE is soluble RAGE, a fragment of RAGE which comprises the V-domain of RAGE, a fragment of RAGE which comprises amino acids 1-30 or
10 2-30 of the V-domain of RAGE, or a fragment of RAGE which binds to LPA.

In one embodiment, the compound is an organic molecule. In other embodiments, the compound is a polypeptide, a nucleic
15 acid, or an inorganic chemical.

In one embodiment, the compound is an antibody or a fragment thereof. In other embodiments, the antibody or fragment thereof is an anti-LPA antibody or fragment thereof, such as,
20 for example, any of the anti-LPA antibodies described herein. In one embodiment, the antibody is a polyclonal or monoclonal antibody. In other embodiments, the antibody is humanized, chimeric or primatized.

25 In one embodiment, the LPA is affixed to a solid surface. In other embodiments, the RAGE or the fragment thereof is affixed to a solid surface. In one embodiment, the LPA is detectably labeled. In another embodiment, the RAGE or the fragment thereof is detectably labeled. In other
30 embodiments, the detectable label comprises fluorescence, biotin, or radioactivity.

Receptor for Advanced Glycation Endproducts

The amino acid sequence of the human multi-ligand Receptor
35 for AGE (RAGE):

MAAGTAVGAWVLVLSLWGAVVGAQNITARIGEPLVLKCKGAPKKPPQRLEWKLNTGRTEAW
KVLSPQGGGPWDSVARVLPNGSLFLPAVGIGQDEGIFRCQAMNRNGKETKSNYRVRVYQIPG

KPEIVDSASELTAGVPNKGVCVSEGSYPAGTLSWHLDGKPLVPNEKGVSVKEQTRRHPET
 GLFTLQSELMVfTPARGGDPRTFSCSFSPGLPRHRALRTAPIQPRVWEPVPLEEVQLVVE
 PEGGAVAPGGTVTLTCEVPAQPSPQIHWMKDGVPPLPPSPVLILPEIGPQDQGTYSVCVAT
 HSSHGPQESRAVSISIIIEPGEEGPTAGSVGGSGGLGTLALALGILGGLGTAALLIGVILWQR

5 RQRRGEERKAPENQEEEEERAELNQSEEPEAGESSTGGP (SEQ ID NO:1) The
 amino acid sequence of human RAGE without the signal sequence
 of amino acids 1-22 is set forth in SEQ ID NO:2. The amino
 acid sequence of human RAGE without the signal sequence of
 amino acids 1-23 is set forth in SEQ ID NO:3.

10

The amino acid sequence of human soluble RAGE (sRAGE) is set
 forth in SEQ ID NO:4. The amino acid sequence of human sRAGE
 without the signal sequence of amino acids 1-22 is set forth
 in SEQ ID NO:5. The amino acid sequence of human sRAGE
 15 without the signal sequence of amino acids 1-23 is set forth
 in SEQ ID NO:6.

The amino acid sequence of the V-domain of human RAGE,
 without the signal sequence of amino acids 1-22, is set
 20 forth in SEQ ID NO:7. The amino acid sequence of the V-
 domain of human RAGE, without the signal sequence of amino
 acids 1-33, is set forth in SEQ ID NO:8. N-
 terminal fragments of the V-domain of RAGE are set forth in
 SEQ ID Nos: 9 and 10.

25

Fragments of RAGE without the first 19, 20, 21, 22 or 23
 (leader sequence) amino acids, are set forth, for example, in
 SEQ ID Nos:5, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18, 19, or
 20.

30

A "RAGE" antagonist is a chemical substance, for example a
 small molecule or an antibody, which reverses, reduces or
 blocks the physiological effect induced by RAGE acting as an
 agonist.

35

A "LPA" antagonist is a chemical substance, for example a
 small molecule or an antibody, which reverses, reduces or

blocks the physiological effect induced by LPA acting as an agonist.

A small molecule as used herein is an organic molecule or organic-based molecule having a molecular weight of less than 200 Daltons. In an embodiment, the small molecules of the present invention are those having a molecular weight of less than 160 Daltons. In an embodiment, the small molecule is an organic small molecule.

10

Non-limiting examples of RAGE fusion proteins that can be used as RAGE antagonists in the present invention are described, for example, in the following publications: PCT International Application Publication No. WO/2008/100470; PCT International Application Publication No. WO/2004/016229; PCT International Application Publication No. WO 2006/017647 Al; PCT International Application Publication No. WO 2006/017643 Al; U.S. Patent Application Publication No. US 2006/140933; U.S. Patent Application Publication No. US 2006/078562; U.S. Patent Application No. US 2006/0057679; U.S. Patent Application Publication No. 2006/0030527; PCT International Application Publication No. WO/2007/094926; U.S. Patent Application Publication No. US 2006-0078562 Al; and U.S. Patent No. 7,470,521, all of which are hereby incorporated by reference in their entireties. RAGE fusion proteins which can be used as the RAGE antagonists recited in the present invention include those comprising soluble RAGE (sRAGE) (SEQ ID NO:4) or a derivative thereof, for example a polypeptide being identical to SEQ ID NO:4 except for a glycine as residue no. 1 instead of a methionine, those comprising the V-domain of RAGE (SEQ ID NO:7; SEQ ID NO:8) the RAGE ligand binding site (SEQ ID NO:9; SEQ ID NO:10), and those comprising RAGE (SEQ ID NO:1) or a portion thereof but without the first 19, 20, 21, 22 or 23 (leader sequence) amino acids, for example SEQ ID NOs:5, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, or 18. In addition, fusion proteins comprising fragments of these sequences may be used. The

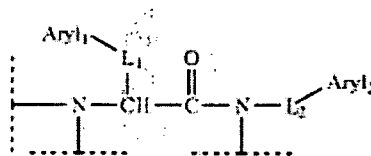
second polypeptide of the RAGE fusion proteins can comprise a non-RAGE polypeptide such as an immunoglobulin derived polypeptide, e.g. a human IgG-derived polypeptide. The second polypeptide of the RAGE fusion proteins can comprise a heavy chain fragment such as an Fc fragment, for example a heavy chain hinge polypeptide. In an embodiment the second polypeptide of the RAGE fusion protein is a C_H2 and/or C_H3 domain of an immunoglobulin (for example see SEQ ID NOs 21 and 22). In an embodiment the second polypeptide of the RAGE fusion protein is a RAGE polypeptide as described above (e.g. sRAGE) linked to a polypeptide comprising a C_H2 domain of an immunoglobulin. In an embodiment the second polypeptide can comprise an interdomain linker derived from RAGE. In an embodiment the C_H2 domain comprises SEQ ID NO:23. RAGE fusion proteins that may be employed as RAGE antagonists in the current invention are also described in PCT International Application Publication No. WO 2006/017643 which is hereby incorporated by reference in its entirety. In an embodiment the RAGE fusion protein is encoded by the sequence set forth in SEQ ID NO:24 or SEQ ID NO:25. In an embodiment the RAGE fusion protein comprises the amino acid sequence set forth in one of SEQ ID NOs:26-31.

Non-limiting examples of other RAGE antagonists are described, for example, in the following publications: U.S. Patent Application Publication No. US 2008/119512; U.S. Patent No. 7,361,678; PCT International Application Publication No. WO/2003/075921; PCT International Application Publication No. WO 2007/089616; PCT International Application Publication No. WO 2007/076200; PCT International Application Publication No. WO 2007/0286858; PCT International Application Publication No. WO/2008/153957; PCT International Application Publication No. WO/2008/123914; PCT International Application Publication No. WO/2007/130302; U.S. Patent No. 7,361,678; U.S. Patent No. 7,423,177; U.S. Patent No. 7,087,632; U.S. Patent No. 7,361,678; U.S. Patent No. 7,067,554; U.S. Patent No. 6,613,801; U.S. Patent No.

5,864,018, all of which are hereby incorporated by reference in their entireties.

Other examples of RAGE antagonists that can be employed in the methods described herein are anti-RAGE antibodies (e.g. see Lutterloh, E., Expert Opinion on Pharmacotherapy, June 2007, Vol. 8, No. 9, Pages 1193-1196 and Flyvbjerg et al. Diabetes January 2004 vol. 53 no. 1 166-172; both of which are hereby incorporated by reference in their entirety). In an embodiment the anti-RAGE antibody is a monoclonal antibody. Examples are those disclosed in Lutterloh et al., Crit. Care 11(6):R122 (2007), ccforum.com/content/11/6/R122), which is hereby incorporated by reference in its entirety.

Other examples of RAGE antagonists are TransTech Pharma TTP448 and TransTech Pharma TTP4000 (TransTech, North Carolina, USA). In an embodiment, the RAGE antagonist is a small molecule. In one embodiment, the small molecule is a compound having the structure:



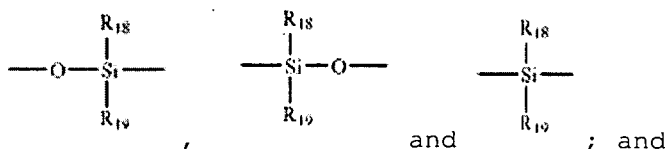
wherein L1 is a C1-C4 alkyl group and L2 is a direct bond, and Aryl₁ and Aryl₂ are aryl, wherein each of Aryl₁ and Aryl₂ are substituted by at least one lipophilic group selected from the group consisting of

- a) --Y-C1-6 alkyl;
- b) --Y-aryl;
- c) --Y--C-1-6 alkylaryl;
- d) --Y--C1-6-alkyl-NR7R8;
- e) --Y--C1-6-alkyl-W-R20;

wherein

Y and W are, independently selected from the group consisting of --CH₂--, --O--, --N(H)--, --S--, SO₂--, --CON(H)--, --

NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂ (H)--, --C(O)--O--,
 --NHSO₂NH-- , --O--CO-- ,



f) halogen, hydroxyl, cyano, carbamoyl, and carboxyl;

5

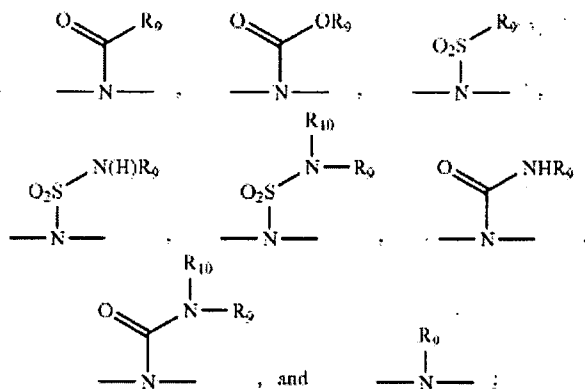
wherein

R₁₈ and R₁₉ are independently selected from the group consisting of aryl, C1-C6 alkyl, C1-C6 alkylaryl, C1-C6 alkoxy, and C1-C6 alkoxyaryl;

10

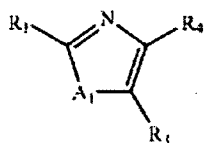
R₂₀ is selected from the group consisting of aryl, C1-C6 alkyl, and C1-C6 alkylaryl;

15 R₇, R₈, R₉ and R₁₀ are independently selected from the group consisting of hydrogen, aryl, C1-C6 alkyl, and C1-C6 alkylaryl; and wherein R₇ and R₈ may be taken together to form a ring having the formula --(CH₂)_m--X--(CH₂)_n-- bonded to the nitrogen atom to which R₇ and R₈ are attached, wherein m and n are, independently, 1, 2, 3, or 4; X is selected from
 20 the group consisting of --CH₂--, --O-- , --S-- , --S(O₂)-- , --C(O)-- , --CON(H)-- , --NHC(O)-- , --NHCON(H)-- , --NHSO₂-- , --SO₂N(H)-- , --C(O)--O-- , --O--C(O)-- , --NHSO₂NH-- ,



or a pharmaceutically acceptable salt thereof, wherein at least one of Aryl₁ and Aryl₂ is substituted with a lipophilic group of the formula --Y--C1-6-alkyl-NR₇R₈.

- 5 In one embodiment, the small molecule is a compound having the structure:



wherein

- 10 R₁ is -hydrogen, -alkyl, -alkenyl, or -alkynyl, A₁ is --N(R₂)-- ;

wherein

R₂ is -phenyl,

15

R₃ is

- a) -hydrogen,
- b) -halogen,
- c) -hydroxyl,
- 20 d) -cyano,
- e) -carbamoyl,
- f) -carboxyl,
- g) -aryl,
- h) -cycloalkyl,
- 25 i) -alkyl,
- j) -alkenyl,
- k) -alkynyl,
- l) -alkylene-aryl,
- m) -alkylene-cycloalkyl,
- 30 n) -fused cycloalkylaryl,
- o) -alkylene-fused cycloalkylaryl,
- p) --C(O)--O-alkyl,
- q) --C(O)--O-alkylene-aryl,

- r) --C(O)--NH-alkyl,
 s) --C(O)--NH-alkylene-aryl,
 t) -SO₂-alkyl,
 u) -SO₂-alkylene-aryl,
 5 v) -SO₂-aryl,
 w) --SO₂--NH-alkyl,
 x) --SO₂NH-- alkylene-aryl,
 y) --C(O)-alkyl,
 z) --C(O)-alkylene-aryl,
 10 aa) -G₄-G₅-G₆-R₇,
 bb) -Y₁-alkyl,
 cc) -Y₁-aryl,
 dd) -Y₁-alkylene-aryl,
 ee) -Y₁-alkylene-NR₉R₁₀, or
 15 ff) -Y₁-alkylene-W₁-R₁₁,

wherein

- G₄ and G₆ are independently selected from the group
 consisting of: alkylene, alkenylene, alkynylene,
 20 cycloalkylene, arylene, -alkylene-aryl, -alkenylene-aryl, -
 alkenylene-heteroaryl, and a direct bond;

- G₅ is --O--, --S--, --N(R₈)--, --S(Ö)--, --S(O)₂--, --C(O)--,
 --O--C(O)--, --C(O)--O--, --C(O)N(R₈)-, --N(R₈)C(O)--,
 25 S(O)₂N(R₈)--, N(R₈)S(O)₂--, --O-alkylene-C(O)--, --(O)C-
 alkylene-O--, --O-alkylene-, -alkylene-O--, alkylene,
 alkenylene, alkynylene, cycloalkylene, arylene, fused
 cycloalkylarylene, or a direct bond, wherein R₈ is -hydrogen,
 -aryl, -alkyl, -alkylene-aryl, or -alkylene-O-aryl;

30

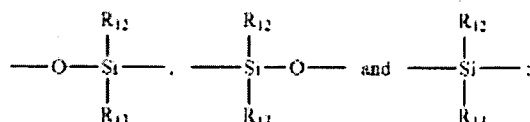
wherein

- R₇ is -hydrogen, -aryl, -cycloalkyl, -alkyl, -alkenyl, -
 alkynyl, -alkylene-aryl, -alkylene-cycloalkyl, -fused
 cycloalkylaryl, or -alkylene-fused cycloalkylaryl;

35

Y₁ and W₁ are independently selected from the group
 consisting of -CH₂--, --O--, --N(H), --S--, SO₂--, --CON(H)--,

--NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--, --C(O)--O--,
 --NHSO₂NH--, --O--CO--,



5 wherein

R₁₂ and R₁₃ are independently selected from the group consisting of: -aryl, -alkyl, -alkylene-aryl, -alkoxy, and -alkylene-O-aryl; and R₉, R₁₀, and R₁₁ are independently selected from the group consisting of: -aryl, -alkyl, and -

10 alkylene-aryl;

R₄ is

a) -phenyl,

b) -phenylene-G5-G6-R7,

15 c) -phenylene-alkylene-G5-G6-R7, or

d) -phenylene-alkenylene-G5-G6-R7,

wherein

20 G6 is alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene, arylene, heteroarylene, -alkylene-aryl, -alkylene-heteroaryl, -alkenylene-aryl, -alkenylene-heteroaryl, or a direct bond;

25 G5 is --O--, --S--, --N(R₈)--, --S(O)--, --S(O)₂--, --C(O)--, --O--C(O)--, --C(O)--O--, --C(O)N(R₈)--, N(R₈)C(O)--, --S(O)₂N(R₈)--, N(R₈)S(O)₂--, --O-alkylene-C(O)--, --(O)C-alkylene-O--, --O-alkylene-, -alkylene-O--, alkylene, alkenylene, alkynylene, cycloalkylene, heterocyclylene,
 30 arylene, heteroarylene, fused cycloalkylarylene, fused cycloalkylheteroarylene, fused heterocyclylarylene, fused heterocyclylheteroarylene, or a direct bond,

wherein

R₈ is -hydrogen, -aryl, -alkyl, -alkylene-aryl, or -alkylene-O-aryl; R₇ is hydrogen, aryl, heteroaryl, cycloalkyl, heterocyclyl, alkyl, alkenyl, alkynyl, -alkylene-aryl, -alkylene-heteroaryl, -alkylene-heterocyclyl, -alkylene-cycloalkyl, fused cycloalkylaryl, fused cycloalkylheteroaryl, fused heterocyclylaryl, fused heterocyclylheteroaryl, alkylene-fused cycloalkylaryl, -alkylene-fused cycloalkylheteroaryl, -alkylene-fused heterocyclylaryl, or -alkylene-fused heterocyclylheteroaryl;

10

wherein

the aryl and/or alkyl group(s) in R₃, R₇, R₈, R₉, R₁₀, R₁₁, R₁₂, and R₁₃, may be optionally substituted 1-4 times with a substituent group, wherein said substituent group(s) are

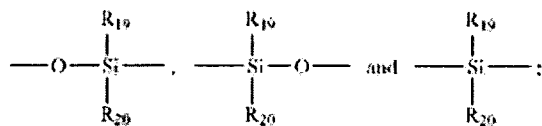
15 independently selected from the group consisting of:

- a) --H,
- b) -halogen,
- c) -hydroxyl,
- d) -cyano,
- 20 e) -carbamoyl,
- f) -carboxyl,
- g) --Y₂-alkyl,
- h) --Y₂-aryl,
- i) --Y₂-alkylene-aryl,
- 25 j) --Y₂-alkylene-W₂--R₁₈,
- k) --Y₃--Y₄--NR₂₃R₂₄,
- l) --Y₃--Y₄--NH--C(=NR₂₅)NR₂₃R₂₄, and
- m) --Y₃--Y₄--C(=NR₂₅)NR₂₃R₂₄,

30 wherein

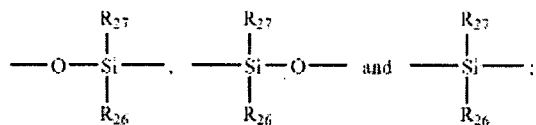
Y₂ and W₂ are independently selected from the group consisting of --CH₂--, --O--, --N(H)--, --S--, SO₂--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--, --C(O)--O--, --NHSO₂NH--, --O--S(O)₂--, --O--CO--,

35



wherein R_{19} and R_{20} are independently selected from the group
 5 consisting of: -hydrogen, -aryl, -alkyl, -alkylene-aryl, -alkoxy, and -alkylene-O-aryl; R_{18} is -aryl, -alkyl, -alkylene-aryl, or -alkylene-O-aryl;

Y3 is selected from the group consisting of a direct bond, --
 10 $\text{CH}_2\text{--}$, --O-- , --N(H)-- , --S-- , $\text{SO}_2\text{--}$, --C(O)-- , --CON(H)-- , --NHC(O)-- , --NHCON(H)-- , $\text{--NHSO}_2\text{--}$, $\text{--SO}_2\text{N(H)--}$, --C(O)--O-- , $\text{--NHSO}_2\text{NH--}$, --O--CO-- ,



15

wherein R_{27} and R_{26} are independently selected from the group
 consisting of: -aryl, -alkyl, -alkylene-aryl, -alkoxy, and -alkyl-O-aryl;

20

Y4 is

- a) -alkylene,
- b) -alkenylene,
- c) -alkynylene,
- 25 d) -arylene,
- e) -cycloalkylene,
- f) -alkylene-arylene,
- g) -alkylene-cycloalkylene,
- h) -arylene-alkylene,
- 30 i) -cycloalkylene-alkylene,

- j) --O--,
- k) --S--,
- l) --S(O)₂--, or
- m) --S(O)--,

5

wherein said alkylene groups may optionally contain one or more O, S, S(O), or SO₂ atoms;

- and R₂₃, R₂₄, and R₂₅ are independently selected from the group consisting of: -hydrogen, -aryl, -alkyl, -alkylene-aryl, and -alkylene-O-aryl,

and

15 wherein

R₂ may be optionally substituted 1-4 times with a substituent group, wherein said substituent group(s) are independently selected from the group consisting of:

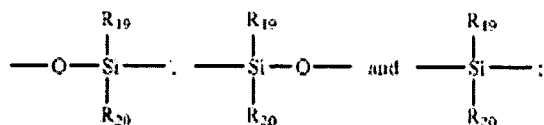
- a) --H,
- 20 b) -halogen,
- c) -hydroxyl,
- d) -cyano,
- e) -carbamoyl,
- f) -carboxyl,
- 25 g) --Y₂-alkyl,
- h) --Y₂-aryl,
- i) --Y₂-heteroaryl,
- j) --Y₂-alkylene-heteroaryl-aryl,
- k) --Y₂-alkylene-aryl,
- 30 l) --Y₂-alkylene-W₂--R₁₈,
- m) --Y₃--Y₄--NR₂₃R₂₄,
- n) --Y₃--Y₄--NH--C(=NR₂₅)NR₂₃R₂₄,
- o) --Y₃--Y₄--C(=NR₂₅)NR₂₃R₂₄, and
- p) -Y₃--Y₄--Y₅-A₂,

35

wherein

Y2 and W2 are independently selected from the group consisting of --CH₂--, --O--, --N(H), --S--, SO₂--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--, --C(O)--O--, --NHSO₂NH--, --O--S(O)₂--, --O--CO--,

5



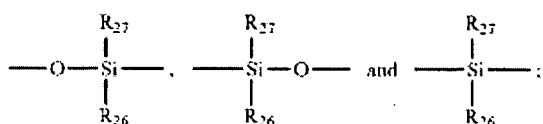
wherein R₁₉ and R₂₀ are independently selected from the group consisting of: -hydrogen, -aryl, -alkyl, -alkylene-aryl, alkoxy, and -alkylene-O-aryl;

10

R₁₈ is -aryl, -alkyl, -alkylene-aryl, -alkylene-heteroaryl, or -alkylene-O-aryl;

Y3 and Y5 are independently selected from the group consisting of a direct bond, --CH₂--, --O--, --N(H), --S--, SO₂--, --C(O)--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--, --C(O)--O--, --NHSO₂NH--, --O--CO--,

15



wherein R₂₇ and R₂₆ are independently selected from the group consisting of: -aryl, -alkyl, -alkylene-aryl, -alkoxy, and -alkyl-O-aryl;

20

Y4 is

a) -alkylene,

b) -alkenylene,

25

- c) -alkynylene,
- d) -arylene,
- e) -heteroarylene,
- f) -cycloalkylene,
- 5 g) -heterocyclylene,
- h) -alkylene-arylene,
- i) -alkylene-heteroarylene,
- j) -alkylene-cycloalkylene,
- k) -alkylene-heterocyclylene,
- 10 l) -arylene-alkylene,
- m) -heteroarylene-alkylene,
- n) -cycloalkylene-alkylene,
- o) -heterocyclylene-alkylene,
- p), --O--,
- 15 q) --S--,
- r) --S(O)₂--, or
- s) --S(O)--,

wherein said alkylene groups may optionally contain one or
 20 more O, S, S(O), or SO₂ atoms;

A₂ is

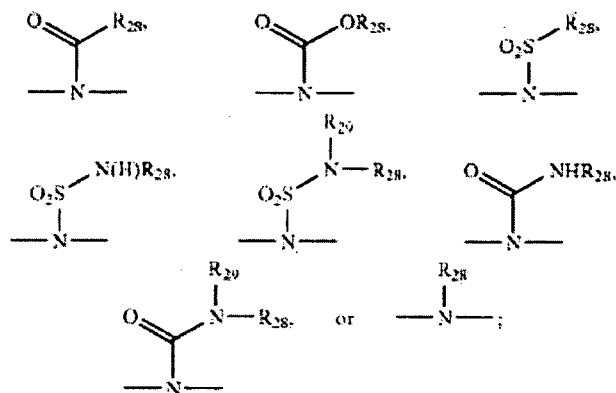
- a) heterocyclyl, fused arylheterocyclyl, or fused
 heteroarylheterocyclyl, containing at least one basic
 25 nitrogen atom, or
- b) -imidazolyl, and

R₂₃, R₂₄, and R₂₅ are independently selected from the group
 consisting of: -hydrogen, -aryl, -heteroaryl, -alkylene-
 30 heteroaryl, -alkyl, -alkylene-aryl, -alkylene-O-aryl, and -
 alkylene-O-heteroaryl; and R₂₃ and R₂₄ may be taken together to
 form a five-membered ring having the formula --(CH₂)_s--X₃--
 (CH₂)_t-- bonded to the nitrogen atom to which R₂₃ and R₂₄ are
 attached

35
 wherein

s and t are, independently, 1, 2, 3, or 4;

X3 is a direct bond, --CH₂--, --O--, --S--, --S(O)₂--, --C(O)--,
 --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--,
 5 --, --C(O)--O--, --O--C(O)--, --NHSO₂NH--,



- 10 wherein R₂₈ and R₂₉ are independently selected from the group consisting of: -hydrogen, -aryl, -heteroaryl, -alkyl, -alkylene-aryl, and -alkylene-heteroaryl;

15 wherein the alkyl and/or aryl groups in the optional substituents

- g) --Y₂-alkyl,
 h) --Y₂-aryl,
 i) --Y₂-heteroaryl,
 j) --Y₂-alkylene-heteroaryl,
 20 k) --Y₂-alkylene-aryl,
 l) --Y₂-alkylene-W₂--R₁₈,
 m) --Y₃--Y₄--NR₂₃R₂₄,
 n) --Y₃--Y₄--NH--C(=NR₂₅)NR₂₃R₂₄,
 o) --Y₃--Y₄--C(=NR₂₅)NR₂₃R₂₄, and
 25 p) --Y₃--Y₄--Y₅-A₂,

of R2 may be optionally substituted 1-4 times with a substituent independently selected from the group consisting of:

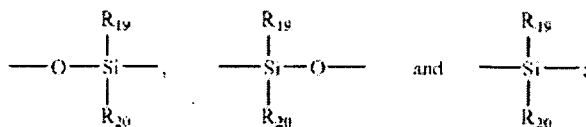
- a) halogen,
- 5 b) perhaloalkyl,
- c) alkyl,
- d) cyano,
- e) alkyloxy,
- f) aryl, and
- 10 g) aryloxy, and

wherein the aryl and/or alkyl group(s) in R4 may be optionally substituted 1-4 times with a substituent group, wherein said substituent group(s) are independently selected from the group consisting of:

- a) --H,
- b) -halogen,
- c) -hydroxyl,
- d) -cyano,
- 20 e) -carbamoyl,
- f) -carboxyl,
- g) --Y2-alkyl,
- h) --Y2-aryl,
- i) --Y2-heteroaryl,
- 25 j) --Y2-alkylene-heteroaryl-aryl,
- k) --Y2-alkylene-aryl,
- l) --Y2-alkylene-W2--R₁₈,
- m) --Y3--Y4--NR₂₃R₂₄,
- n) --Y3--Y4--NH--C(=NR₂₅)NR₂₃R₂₄,
- 30 o) --Y3--Y4--C(=NR₂₅)NR₂₃R₂₄, and
- p) --Y3--Y4--Y5-A2,

wherein

- Y2 and W2 are independently selected from the group consisting of --CH₂--, --O--, --N(H), --S--, SO₂--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)--, --C(O)--O-- , --NHSO₂NH--, --O--S(O)₂--, --O--CO--,

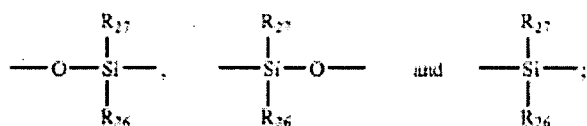


wherein R₁₉ and R₂₀ are independently selected from the group
 5 consisting of: -hydrogen, -aryl, -alkyl, -alkylene-aryl, alkoxy, and -alkylene-O-aryl;

R₁₈ is -aryl, -alkyl, -alkylene-aryl, -alkylene-heteroaryl,
 or -alkylene-O-aryl;

10

Y₃ and Y₅ are independently selected from the group
 consisting of a direct bond, --CH₂--, --O--, --N(H), --S--,
 SO₂--, --C(O)--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --
 NHSO₂--, --SO₂N(H)--, --C(O)--O--, --NHSO₂NH--, --O--CO--,



15

wherein R₂₇ and R₂₆ are independently selected from the group
 consisting of: -aryl, -alkyl, -alkylene-aryl, -alkoxy, and -
 alkyl-O-aryl;

20

Y₄ is

a) -alkylene,

b) -alkenylene,

c) -alkynylene,

25

d) -arylene,

e) -heteroarylene,

f) -cycloalkylene,

g) -heterocyclylene,

- h) -alkylene-arylene,
- i) -alkylene-heteroarylene,
- j) -alkylene-cycloalkylene,
- k) -alkylene-heterocyclylene,
- 5 l) -arylene-alkylene,
- m) -heteroarylene-alkylene,
- n) -cycloalkylene-alkylene,
- o) -heterocyclylene-alkylene,
- p) --O--,
- 10 q) --S--,
- r) --S(O)₂--, or
- s) --S(O)--,

wherein said alkylene groups may optionally contain one or
 15 more O, S, S(O), or SO₂ atoms;

A2 is

- a) heterocyclyl, fused arylheterocyclyl, or fused
 heteroarylheterocyclyl, containing at least one basic
 20 nitrogen atom, or
- b) -imidazolyl, and

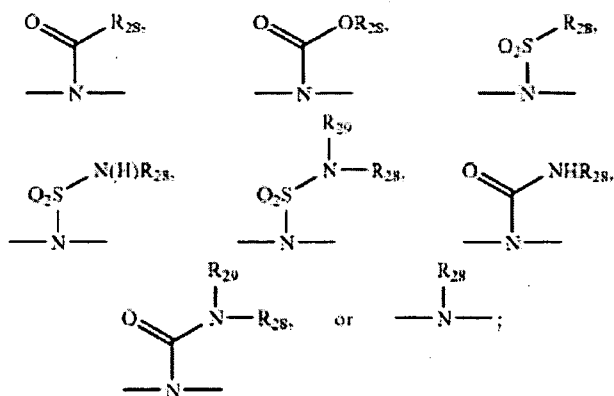
R₂₃, R₂₄, and R₂₅ are independently selected from the group
 consisting of: -hydrogen, -aryl, -heteroaryl, -alkylene-
 25 heteroaryl, -alkyl, -alkylene-aryl, -alkylene-O-aryl, and -
 alkylene-O-heteroaryl; and R₂₃ and R₂₄ may be taken together to
 form a five-membered ring having the formula --(CH₂)_s--X3--
 (CH₂)_t-- bonded to the nitrogen atom to which R₂₃ and R₂₄ are
 attached

30

wherein

s and t are, independently, 1, 2, 3, or 4;

- 35 X3 is a direct bond, --CH₂--, --O--, --S--, --S(O)₂--, --C(O)-
 -, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂--, --SO₂N(H)-
 -, --C(O)--O--, --O--C(O)--, --NHSO₂NH--,



wherein R_{28} and R_{29} are independently selected from the group
 5 consisting of: -hydrogen, -aryl, -heteroaryl, -alkyl, -alkylene-aryl, and -alkylene-heteroaryl;

- wherein the alkyl and/or aryl groups in the optional substituents
- 10 g) --Y2-alkyl,
 h) --Y2-aryl,
 i) --Y2-heteroaryl,
 j) --Y2-alkylene-heteroaryl,
 k) --Y2-alkylene-aryl,
 15 l) --Y2-alkylene-W2-- R_{18} ,
 m) --Y3--Y4--NR₂₃R₂₄,
 n) --Y3--Y4--NH--C(=NR₂₅)NR₂₃R₂₄,
 o) --Y3--Y4--C(=NR₂₅)NR₂₃R₂₄, and
 p) --Y3--Y4--Y5-A2,
 20 of R_2 and R_4 may be optionally substituted 1-4 times with a substituent independently selected from the group consisting of:
 a) halogen,
 b) perhaloalkyl,
 25 c) alkyl,
 d) cyano,
 e) alkyloxy,
 f) aryl, and

g) aryloxy, and

wherein the ring or rings containing a heteroatom in the heteroaryl, heteroarylene, heterocyclyl, heterocyclene, fused
 5 arylheterocyclyl, or fused heteroarylheterocyclyl groups in R₂ or R₄ or in a substituent of R₂ or R₄ is a five membered nitrogen containing ring, and

wherein

10 at least one of R₂ and R₄ is substituted with at least one group of the formula

--Y3--Y4--NR₂₃R₂₄,

--Y3--Y4--NH--C(=NR₂₅)NR₂₃R₂₄,

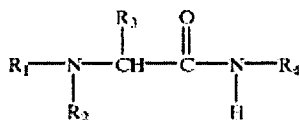
--Y3--Y4--C(=NR₂₅)NR₂₃R₂₄, or

15 --Y3--Y4--Y5-A2,

with the proviso that no more than one of R₂₃, R₂₄, and R₂₅ is aryl or heteroaryl;
 or a pharmaceutically acceptable salt thereof.

20

In one embodiment, the antagonist is a compound having the structure:



25

wherein

R₁ and R₂ are independently selected from

a) --H;

b) --C1-6 alkyl;

30 c) -aryl;

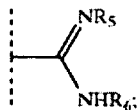
d) --C1-6 alkylaryl;

e) --C(O)--O--C1-6 alkyl;

f) --C(O)--O--C1-6 alkylaryl;

h) --C(O)--NH--C1-6 alkylaryl;

- i) --SO₂--C₁₋₆ alkyl;
- j) --SO₂--C₁₋₆ alkylaryl;
- k) --SO₂-aryl;
- l) -- SO₂--NH-- C₁₋₆ alkyl;
- 5 m) --SO₂--NH--C₁₋₆ alkylaryl;
- n)



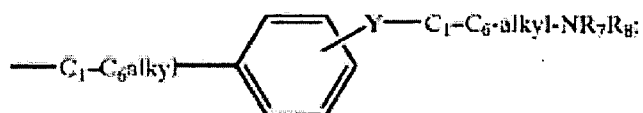
- 10 o) --C(O)--C₁₋₆ alkyl; and
- p) --C(O)--C₁₋₆ alkylaryl;

R₃ is selected from

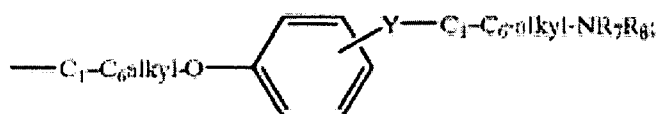
- (a) -aryl; and
 - 15 (b) --C₁₋₃ alkylaryl,
- wherein aryl is substituted by C₁₋₆ alkyl, C₁₋₆ alkoxy, C₁₋₆ alkylaryl, or C₁₋₆ alkoxyaryl;

R₄ is selected from

a)

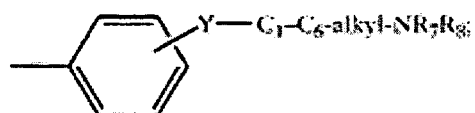


b)



and

c)



R5 and R6 are independently selected from the group consisting of hydrogen, C1-C6 alkyl, C1-C6 alkylaryl, and
 5 aryl; and wherein

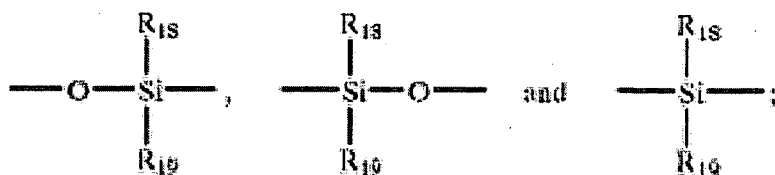
the aryl and/or alkyl group(s) in R1, R2, R4, R5, R6, R7, R8, R9, R10, R18, R19, and R20 may be optionally substituted 1-4
 10 times with a substituent group, wherein said substituent group(s) or the term substituted refers to groups selected from the group consisting of:

- a) $--H$;
- b) $--Y--C_1-6\text{ alkyl}$;
- $--Y\text{-aryl}$;
- 15 $--Y--C_1-6\text{ alkylaryl}$;
- $--Y--C_1-6\text{ -alkyl}-NR_7R_8$; and
- $--Y--C_1-6\text{ -alkyl}-W--R_{20}$; and
- c) halogen, hydroxyl, cyano, carbamoyl, or carboxyl; and

20 wherein

Y and W are independently selected from the group consisting of --CH₂ --, --O--, --N(H)--, --S--, SO₂ --, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂ --, --SO₂ N(H)--, --C(O)--O--, --NHSO₂ NH--, --O--CO--,

5



R₁₈ and R₁₉ are independently selected from the group consisting of aryl, C1-C6 alkyl, C1-C6 alkylaryl, C1-C6 alkoxy, and C1-C6 alkoxyaryl;

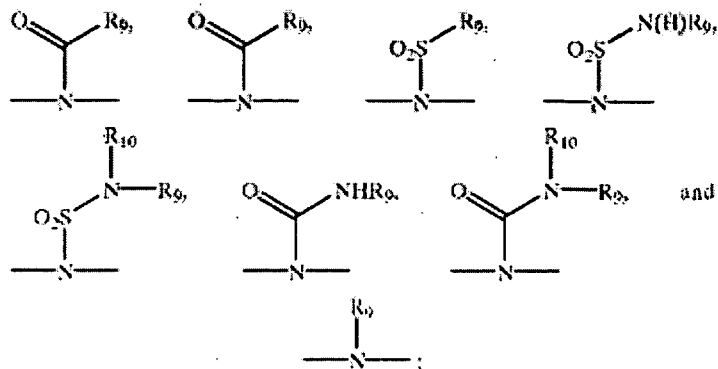
10

R₂₀ is selected from the group consisting of aryl, C1-C6 alkyl, and C1-C6 alkylaryl;

15 R₇, R₈, R₉ and R₁₀ are independently selected from the group consisting of hydrogen, aryl C1-C6 alkyl, and C1-C6 alkylaryl; and wherein

20 R₇ and R₈ may be taken together to form a ring having the formula --(CH₂)_m--X--(CH₂)_n -- bonded to the nitrogen atom to which R₇ and R₈ are attached, and/or R₅ and R₆ may, independently, be taken together to form a ring having the formula --(CH₂)_m--X--(CH₂)_n -- bonded to the nitrogen atoms to which R₅ and R₆ are attached, wherein m and n are,

25 independently, 1, 2, 3, or 4; X is selected from the group consisting of --CH₂ --, --O--, --S--, --S(O₂)--, --C(O)--, --CON(H)--, --NHC(O)--, --NHCON(H)--, --NHSO₂ --, --SO₂N(H)--, --C(O)--O--, --O--C(O)--, --NHSO₂NH--,



or a pharmaceutically acceptable salt thereof. It is understood that the above are all non-limiting examples of
 5 RAGE antagonists that can be employed in the present invention.

Non-limiting examples of anti-LPA antibodies as LPA antagonists include the anti-LPA antibody Lpathmob™ (LT3000)
 10 (LPath Inc., San Diego, CA). Such anti-LPA antibodies are described in PCT International Application Publication NO. WO 2010/121093, published October 21, 2010, the entire contents of which are hereby incorporated by reference.

15 As disclosed herein, LPA indications may be treated using RAGE antagonists. LPA has been associated with a number of diseases and disorders. For review, see Gardell et al., (2006) Trends Mol. Med. 12(2):65-75 and Chun J. and Rosen H., (2006) Curr. Pharma. Design 12:161-171. These diseases and
 20 disorders include, for example, autoimmune disorders such as diabetes, multiple sclerosis and scleroderma; hyperproliferative disorders including cancer; disorders associated with angiogenesis and neovascularization; obesity; neurodegenerative diseases including Alzheimer's disease;
 25 schizophrenia; immune related disorders such as transplant rejection and graft-vs.-host disease. All of the references cited in this paragraph are hereby incorporated by reference in their entirety.

As disclosed herein, RAGE indications may be treated using LPA antagonists. Ishiguro H, (Prostate. 2005 Jun 15;64(1):92-100) describes that the receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development. Hudson BI (Pharm Res. 2004 Jul;21(7):1079-86) describe that RAGE is a novel target for drug intervention in diabetic vascular disease. Flyvbjerg A. describe the long-term renal effects of a neutralizing RAGE antibody in obese type 2 diabetic mice (Diabetes. 2004 Jan; 53(1):166-72). The role of RAGE in cancer is discussed in Riehl A, Cell Commun Signal. 2009 May 8;7:12, "The receptor RAGE: Bridging inflammation and cancer" and Logsdon CD, Curr Mol Med. 2007 Dec;7(8):777-89 "RAGE and RAGE ligands in cancer. The role of RAGE atherosclerosis and diabetes is discussed in Schmidt AM, Curr Atheroscler Rep. 2000 Sep;2(5):430-6, "Atherosclerosis and diabetes: the RAGE connection". Other RAGE indications are discussed in Koyama, H. et al. Arterioscler Thromb Vasc Biol. 2005 Dec; 25(12):2587-93 (Metabolic Syndrome); Katz, J. et al. J Periodontol. 2005 Jul (peridodontal disease, smoking-related); 76(7):1171-4; Cataldegirmen, G. et al. J Exp Med. 2005 Feb 7;201(3):473-84 (hepatocyte regeneration after partial hepatectomy); Sparvero, L.J. et al. J Transl Med. 2009 Mar 17; 7:17; Raman, K.G. et al. Am J Physiol Gastrointest Liver Physiol. 2006 Oct;291(4):G556-65 (intestinal barrier dysfunction); Yan, S.F. et al. Expert Rev Mol Med. 2009 Mar 12; 11:e9 (neuropathy); and Yan, S.F. et al. J Mol Med. 2009 Mar; 87(3):235-47 (nephropathy). All of the references cited in this paragraph are hereby incorporated by reference in their entirety.

"Treating" a disorder/disease shall mean slowing, stopping or reversing the disorder's progression, and/or ameliorating, lessening, or removing symptoms of the disorder. Thus

treating a disorder encompasses reversing the disorder's progression, including up to the point of eliminating the disorder itself.

5 As used herein, an "immunoglobulin domain" is a sequence of amino acids that is structurally homologous, or identical to, a domain of an immunoglobulin. The length of the sequence of amino acids of an immunoglobulin domain may be up to 500 amino acids. In one embodiment, an immunoglobulin domain may
10 be less than 250 amino acids. In an example embodiment, an immunoglobulin domain may be about 80-150 amino acids in length. For example, the variable region, and the CH1, CH2, and CH3 regions of an IgG (including human IgG) are each immunoglobulin domains. In another example, the variable, the
15 CH1, CH2, CH3 and CH4 regions of an IgM are each immunoglobulin domains.

As used herein, a "RAGE immunoglobulin domain" is a sequence of amino acids from RAGE protein that is structurally
20 homologous, or identical to, a domain of an immunoglobulin. For example, a RAGE immunoglobulin domain may comprise the RAGE V-domain, the RAGE Ig-like C2-type 1 domain ("C1 domain"), or the RAGE Ig-like C2-type 2 domain ("C2 domain").

25 Further description of the various diseases recited in this disclosure may be found in The Merck Manual, 17th Edition (1999), Merck Research Laboratories, Whitehouse Station, NJ, U.S.A. which is hereby incorporated by reference for
30 description of the diseases/disorders recited herein.

The administration of RAGE antagonists or LPA antagonists described herein may be by way of compositions containing one of the antagonists and a pharmaceutically acceptable carrier.
35 As used herein, a "pharmaceutical acceptable carrier" is a pharmaceutically acceptable solvent, suspending agent or vehicle, for delivering an active compound to a mammal,

including humans. The carrier may be liquid, aerosol, gel or solid and is selected with the planned manner of administration in mind. In an embodiment, the pharmaceutical carrier is a sterile pharmaceutically acceptable solvent
5 suitable for intravenous administration. In an embodiment, the pharmaceutical carrier is a pharmaceutically acceptable solid suitable for oral administration.

"Administering" the antagonists described herein can be
10 effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be, for example, intravenous, oral, intramuscular, intravascular, intra-arterial, intracoronary, intramyocardial, intraperitoneal, and subcutaneous. Other
15 non-limiting examples include via topical coating of a blood vessel, coating of a device to be placed within the subject, coating of an instrument used during a procedure which, for example, otherwise results in blood vessel injury, or contacting blood of the subject during extracorporeal
20 circulation. In embodiments, administration is effected by injection or via a catheter.

Injectable drug delivery systems that may be employed in the methods described herein include solutions, suspensions,
25 gels. Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic
30 materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc). Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as suspending agents (e.g., gums, xanthans, cellulose and
35 sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine),

preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating agents, and chelating agents (e.g., EDTA).

5 As used herein, the term "effective amount" refers to the quantity of a component that is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner
10 of this invention, i.e. a therapeutically effective amount. The specific effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent
15 therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

Treatment of the diseases recited herein, e.g. of a ovarian cancer, encompasses inducing inhibition, regression, or
20 stasis of the disorder.

As used herein "about" with regard to a stated number encompasses a range of +one percent to -one percent of the stated value. By way of example, "100" therefore includes 99,
25 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9, 100, 100.1, 100.2, 100.3, 100.4, 100.5, 100.6, 100.7, 100.8, 100.9 and 101. Accordingly, "about 100" includes, in an embodiment, 100. Where a range is given in the specification it is understood that the range includes all integers and 0.1 units
30 within that range, and any sub-range thereof. For example, a range of 77 to 90 includes 77, 78, 79, 80, and 81 etc., as well as 77 to 80 and 83-89, etc.

The methods of treatment described herein with the LPA
35 antagonist or RAGE antagonist may be a component of a combination therapy or an adjunct therapy. This combination therapy can be sequential therapy where the patient is

treated first with one drug and then the other, or the two drugs are given simultaneously. These can be administered independently by the same route or by two or more different routes of administration depending on the dosage forms employed.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Summary

Lysophosphatidic acid (LPA) is an endogenous phospholipid produced by stressed vascular cells and in tumors, by transformed cells as well as peri-tumor inflammatory, vascular and stromal cells. LPA regulates fundamental cellular processes such as proliferation, survival, motility and invasion, hence, in order to curtail aberrant vascular expansion and tumor growth, delineation of the precise molecular mechanisms by which LPA exerts its broad effects is essential. Although LPA may interact with G-protein coupled receptors (G-PCRs), abundant evidence implicates other unidentified LPA receptor(s) or possibly non-receptor pathways in the biological actions of this molecule.^{1,6-10} The receptor for advanced glycation endproducts (RAGE) has been implicated in vascular signaling, atherosclerosis and tumorigenesis. Here, it is shown that the receptor for advanced glycation endproducts (RAGE) avidly binds LPA and the LPA binding site is mapped on RAGE. *In vitro*, RAGE was required for LPA-mediated signal transduction in smooth muscle cells and C6 glioma cells, as well as proliferation and migration. *In vivo*, the administration of soluble RAGE and genetic deletion of RAGE mitigated (1) LPA-stimulated vascular Akt signaling, (2) autotoxin/LPA-driven phosphorylation of Akt and cyclin D1 in the mammary tissue of transgenic mice vulnerable to carcinogenesis, and (3) ovarian tumorigenesis. These findings identify novel roles for RAGE as a conduit for LPA signaling and indicate that therapeutic strategies to modify the pathological actions of LPA in vascular disease and tumorigenesis should include targeting the interaction of LPA with RAGE.

To test the hypothesis that LPA binds RAGE and initiates signal transduction through this receptor, we employed gain- and loss-of-function experiments using primary murine aortic smooth muscle cells (SMCs), as the full repertoire of LPA

receptors in SMCs is not fully clarified.^{6,19} SMCs were retrieved from wild type and RAGE null mice.^{14,20} Activation of Akt and ERK was determined in wild type and RAGE null SMCs in response to LPA. Although LPA treatment activated Akt in wild type SMCs, LPA-stimulated activation of Akt was not observed in RAGE null SMCs (Fig. 1A). In contrast, LPA-stimulated ERK phosphorylation and the degree of activation did not differ between wild-type and RAGE null SMCs (Fig. 1B). These data suggest that there are distinct functional receptors for LPA on SMCs, and that RAGE mediates the effects of LPA through Akt, but not ERK signal transduction.

To be certain that RAGE deletion did not impact the major LPA receptors of the G-PCR family in SMCs, we determined the transcript levels for these receptors in both wild type and RAGE null SMCs (Fig. 5), and assessed protein expression of the major LPA₁ receptor and its potential dependence on RAGE (Fig. 1C). These data reveal that known LPA receptors are equivalently expressed in both wild-type and RAGE null SMCs.

To verify that RAGE mediated the effects of LPA signaling, we transiently transfected WT SMCs with full-length RAGE or signaling-deficient dominant negative (DN)-RAGE (Fig. 6). LPA induced robust phosphorylation of Akt in RAGE-overexpressing SMCs compared to vector control, but failed to induce Akt phosphorylation in SMCs expressing DN-RAGE (Fig. 1D).

Together, these data indicated that RAGE was essential for mediating Akt signaling in primary murine aortic SMCs. To support this hypothesis, we performed experiments to test for physical interactions between LPA and RAGE. The extracellular portion of RAGE (soluble RAGE, sRAGE) was immobilized on a carboxymethylated dextran CM5 chip and high affinity LPA binding was observed by surface plasmon resonance (SPR) (Fig. 2A). To confirm the interaction, we reversed the binding assay and examined the binding of sRAGE to immobilized

lysophosphatidic acid (18:1)-1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC-LPA 10:1 w/w) and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) liposomes as a reference on HPA sensor chip (Fig. 7). The SPR binding
5 response in these experiments validated that sRAGE bound LPA (Fig. 2B).

It has been reported that ligand binding to RAGE occurs through the extracellular N-terminal V-type immunoglobulin
10 domain and possibly through the C2 domain.²¹ To understand the mechanism by which RAGE binds LPA, the LPA-modified surface was used to further probe the binding of LPA to sRAGE using the isolated V domain and C2 domains. We observed high affinity binding of LPA to V domain with a dissociation
15 constant of K_d 9×10^{-9} M (Fig. 2C), nearly the same as that to full-length sRAGE. The interaction of the C2 domain to an LPA-modified surface was 1000-fold weaker with the estimated K_d of 9×10^{-6} M (Fig. 2D).

20 To confirm that LPA binds directly to RAGE, heteronuclear NMR experiments were performed using samples of ¹⁵N-enriched V and C2 domains. Titrations of the V domain with LPA resulted in selective broadening of the NMR signals. The residues most perturbed by the addition of LPA include Lys52, Val63, Trp72,
25 Gly9Q, Gln100, Lys110, Tyr113 and Val115, which map to a specific basic surface on the V domain structure (Fig. 2E-F) that is a logical site for interaction of negatively charged LPA. Previous work by our group and others has identified this surface as the site for binding the endogenous RAGE
30 ligands S100B and AGE (Fig. 2F).²²⁻²³ In the titration of the C2 domain with LPA, a limited number of resonances exhibited perturbations in the intermediate to fast exchange regime on the NMR timescale (Fig. 2G, 8). These observations indicate that the interaction of C2 with LPA is significantly weaker
35 than that observed for the V domain and is consistent with the 1000-fold weaker binding reported by SPR. Overall, these studies indicate that LPA binding to RAGE is mediated through

the V domain, whereas the contribution of interaction with the C2 domain to LPA-induced RAGE activation remains uncertain due to LPA's very low binding affinity for C2. Taken together, these *in vitro* experiments indicate that LPA
5 physically interacts with RAGE and that Akt signaling in SMCs is stimulated, at least in part, by LPA activation of RAGE.

To test if LPA serves as a RAGE ligand in vascular cells *in vivo*, LPA was infused into the left ventricles of wild-type
10 and RAGE null mice and activation of signal transduction pathways was assayed. In wild-type mice, LPA induced rapid (15 minutes) activation of Akt *in vivo* in SMCs as revealed by immunostaining of aorta tissue sections and Western blotting for detection of phospho-Akt. In contrast, RAGE null mice
15 displayed significantly less Akt phosphorylation in the aorta after administration of LPA (Fig. 3A-B). Of note, at 5 minutes after infusion of LPA, no activation of Akt was evident in wild-type or RAGE null mice (Fig. 3B).

LPA displays chemoattractant effects in vascular SMCs.⁵⁻⁶ To specifically test if RAGE contributed to LPA-mediated SMC migration and proliferation, we performed studies in SMCs retrieved from RAGE-expressing or RAGE null mouse aortas. Migration and proliferation responses to LPA in RAGE null
20 SMCs were significantly lower than those observed in WT SMCs (Fig. 3C and 3D). Similar patterns of migration and proliferation were noted in SMCs exposed to a distinct RAGE ligand, S100B (Fig. 3C and 3D). However, incubation of SMCs with a non-RAGE ligand, PDGF,¹⁴ mediated comparable degrees of
25 cellular migration in both wild-type and RAGE null SMCs (Fig. 3C). In addition, experiments in SMC wound healing assays confirmed roles for RAGE in LPA signaling (Fig. 9). These data indicate that RAGE is required for the actions of LPA on SMC proliferation and migration.

35 In addition to potent effects of LPA on vascular cell signaling, LPA plays key roles in the behavior of transformed

cells. Hence, we tested the potential role of RAGE in this process. ERK mitogen-activated protein (MAP) kinase activity is stimulated by LPA in C6 glioma cells.²⁴ To test whether stimulation of RAGE by LPA leads to ERK phosphorylation in
5 RAGE-expressing C6 glioma cells, we used C6 cells stably expressing vector control, full-length RAGE or DN-RAGE.¹³ C6 glioma cells stably expressing full-length RAGE revealed strong phosphorylation of ERK in response to LPA over 15 minutes stimulation which was greater than that seen in
10 vector alone cells (Fig. 4A). In contrast, LPA stimulation of ERK phosphorylation was not detected in stably expressing DN-RAGE C6 cells (Fig. 4A).

In the above studies, we stimulated cells and animals
15 exogenously with LPA. To be certain that RAGE transduced the signals stimulated by endogenously produced LPA, we employed transgenic mice overexpressing autotoxin (atx) under the control of the MMTV-LTR promoter. In the mammary tissues of MMTV-atx mice, phosphorylation of Akt and hyperplasia were
20 previously shown to be significantly higher versus that observed in control wild-type mice of the FVB background³³. To test the impact of RAGE, we bred MMTV-atx mice into the RAGE null background and compared findings to those observed in littermate MMTV-atx mice expressing RAGE. Mammary glands were
25 retrieved from female animals at age 6 weeks to determine the effects of RAGE deletion on modulation of early signal transduction pathways implicated in tumorigenesis³³. Compared to MMTV-atx mice expressing RAGE, mammary glands retrieved from those mice devoid of RAGE revealed significantly less
30 phosphorylation of Akt (Fig. 4B) and phosphorylation of cyclin D1 (Fig. 4C). Levels of atx in the mammary tissue did not differ between MMTV-atx expressing rage versus MMTV-atx mice devoid of RAGE (Fig. 10).

35 As it is established that LPA strikingly enhances tumorigenesis and metastasis of ID8 cells, a murine epithelial ovarian cancer cell line, in immunocompetent

C57BL/6 mice, we tested the role of RAGE. When ID8 cells are injected by intraperitoneal administration, near complete dependence on LPA treatment to stimulate tumor growth and metastasis has been observed.²⁵⁻²⁶ Daily injections of LPA (100
5 $\mu\text{mol/L} \times 200 \mu\text{L PBS/mouse}$) versus PBS alone for 4 weeks enhanced tumorigenesis of ID8 cells in RAGE-expressing wild-type mice (Fig. 4E vs. 4D, respectively and 4I). RAGE-expressing-LPA-treated groups presented tumors on the peritoneal wall (Fig. 4E and 4I), diaphragm, omentum, and
10 mesentery, and tumors were also found on the surface of the spleen, liver, kidney, and small intestine in these mice.

We established that ID8 cells express RAGE (Fig. 11). LPA-induced tumor numbers were significantly reduced in sRAGE-
15 treated WT and RAGE null groups (Fig. 4 F, H, I). Thus, sRAGE and the RAGE deficient environment significantly affected LPA-induced tumorigenesis and metastasis. The representative hematoxylin and eosin (H&E) staining of the tumors from all groups is shown in Figs. 12A - 12E.

20 Ligand-stimulated cellular migration contributes integrally to multiple facets of RAGE biology, notably in inflammatory processes and tumorigenesis.¹²⁻¹⁴ There is also substantial evidence of specific contributions to diabetes pathogenesis
25 and complications; as blockade of RAGE (i) limited influx of macrophages and lymphocytes into pancreatic islets in NOD/scid mice,²⁷ (ii) suppressed smooth muscle cell migration in injured arteries,^{14,28} and (iii) mitigated macrophage and smooth muscle cell infiltration into atherosclerotic plaques
30 in mice deficient in apolipoprotein E.^{20,29} Surprisingly, little is known about potential fate and actions of LPA in diabetes and its complications; the present results suggest that such investigation may reveal novel mechanisms in this disease for which targeted therapeutic approaches may be
35 readily envisioned.

A particularly revealing finding in our studies is that LPA actions are attenuated upon introduction of RAGE-expressing ID8 cells into a RAGE deficient environment. This suggests discrete roles for LPA both directly on tumor cells and within the tumor milieu. In mice deficient in LPA₂, reduced infiltration of macrophages and expression of monocyte chemoattractant protein-1 and macrophage migration inhibitory factor were noted in parallel with fewer colonic tumor numbers in mice heterozygous for the adenomatous polyposis coli (Apc) allele.³⁰ As RAGE has been implicated in multiple facets of the inflammatory response,¹² it is plausible that RAGE-dependent inflammatory responses triggered by LPA contribute to the significant decrease in ID8 tumor numbers in LPA-treated RAGE null mice. In this context, in tumors and studies using chimeric mice bearing RAGE deficient bone marrow, RAGE-driven infiltration of bone marrow-derived inflammatory cells into dermal tumors was found to be important for early tumor promotion³⁴. Hence, in settings such as vascular disease, inflammation and tumors, the complexity of LPA signaling is likely linked both to the repertoire of its receptors and to the discrete contributions of these receptors both directly in target cells as well as those cells influencing cellular stress in an autocrine or paracrine manner.

25

We thus surmise that the LPA-RAGE axis may be a hitherto unrecognized etiological factor in chronic inflammatory diseases such as diabetes, atherosclerosis and in tumorigenesis. LPA has additional links to RAGE, as mildly oxidized forms of LDL may contain LPA.⁵ We previously showed that AGE-containing epitopes within oxidized LDL contributed to its pro-inflammatory effects.²⁹ From the present work we infer that LPA species within oxidized LDL may signal via RAGE as well. RAGE ligands exhibit different structure, size and symmetry or even no symmetry as in the case of glycated proteins or amyloid β . The finding that LPA is a functional ligand for RAGE expands this repertoire of RAGE ligand

35

families. Although a role for the strongly positive electrostatic potential at the ligand binding surface is clearly evident, the mechanism of activation by the diverse chemical structures of the various RAGE ligands remains
5 enigmatic. We do note however, that although they are chemically distinct, virtually all RAGE ligands are produced in the process of damage and stress within the cellular microenvironment.

10 In human tumors, similar to the findings with LPA, multiple studies reported upregulation of RAGE in tumor versus adjacent non-tumor tissue in malignancies such as those arising in the esophagus, breast, liver, colon and rectum, prostate and brain (glioblastoma multiforme)³⁵⁻⁴⁰, and that in
15 the case of colorectal cancer, even higher levels of RAGE were observed in the more advanced cases with metastasis⁴⁰. Hence, the finding of incomplete suppression of LPA's effects in tumors in some experiments underscores the fact that LPA may exert its pathophysiological effects through multiple
20 receptors. In this context, roles for LPA receptors were suggested by *in vitro* studies in cultured murine and human ovarian epithelial cancer cells in which small interfering RNAs or the LPA receptor₁₋₂₋₃ inhibitor Ki16425 decreased cellular invasion and migration.³¹⁻³² Specific roles for these
25 receptors *in vivo* have yet to be addressed in ovarian cancer models, but roles from LPA₁₋₂₋₃ receptors have recently been demonstrated in mammary tumorigenesis *in vivo*³³.

Taken together, our data identify RAGE as a novel receptor
30 for LPA and illustrate the complex signaling network induced by this biologically-active phospholipid. All previously-reported LPA receptors are members of the G-protein coupled receptor family;¹ thus, our studies identify a unique receptor of the immunoglobulin superfamily active in
35 transducing LPA's biological effects. Whether or not these receptor families function alone or in concert, and their precise mechanism(s) of action, remain important questions

that must be addressed in future studies. We conclude that optimal therapeutic strategies in vascular disease and tumorigenesis should include targeting LPA/RAGE-stimulated signal transduction.

5

Materials and Methods

sRAGE and its domains: expression and purification

Human sRAGE, V domain and C2 domain proteins were expressed
10 and purified as described^{17,22-23}.

LPA and LPA Liposomes. 18:1 Lysophosphatidic Acid (LPA) or
18:1 Lyso PA 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate
(sodium salt), 100 nm size LPA-POPC (1:10, w/w) liposomes and
15 POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine)
liposomes were obtained from Avanti Polar Lipids Inc.

Surface Plasmon Resonance (SPR) Experiments

All experiments were performed at 25 °C using a Biacore X
20 with commercially available Biacore CM5 sensor chips or HPA
sensor chips from GE Healthcare (Piscataway, USA). HBS buffer
(10 mM HEPES at pH 7.4 with 150 mM NaCl) was employed as
running buffer. Proteins were immobilized on the
carboxymethyl dextran surface of flow cell 2 using amine-
25 coupling chemistry with a flow rate of 5 µl/min. Flow cell 1
remained unmodified to serve as a reference cell for the
subtraction of systematic instrument noise and drift. LPA was
immobilized on the HPA sensor chip by solubilization in LPA
liposomes.

30

LPA binding to the sRAGE immobilized surface. The carboxyl
groups on the CM5 sensor chips were activated for 7 min using
0.1 M N-hydroxysuccinimide (NHS) and 0.4 M (N-ethyl-N`-(3-
dimethylaminopropyl) carbodiimide (EDC) mixed at 1:1 (v/v)
35 ratio. In the coupling step, a 30 µl injection of 0.16 mg/mL
sRAGE (in sodium acetate buffer at pH 5.5) was flowed over
the activated surface for 6 min. The remaining activated

sites on the chip surfaces were blocked with a 35 μ l injection of an ethanolamine hydrochloride solution (1 M at pH 8.5), followed by a 60 sec wash with 2 M NaCl to remove any nonspecifically adsorbed materials. About 4000 RU of the
5 immobilized sRAGE proteins were obtained.

Lysophosphatidic acid (LPA) dilutions were prepared in HBS buffer and injected sequentially over two flow cells at a flow rate of 10 μ l/min. Surface regeneration was achieved
10 using a 60 sec injection of 2 M NaCl. The response curve was obtained by subtraction of the signals over the reference surface from the binding response over RAGE immobilized surface.

15 sRAGE and domains binding to LPA immobilized surface. LPA-liposomes were used to generate a stable 'mimic membrane' on the HPA sensor surfaces. The 18:1 Lysophosphatidic Acid (LPA), LPA-POPC (1:10, w/w) liposomes and POPC liposomes were obtained from Avanti Polar Lipids Inc. The chips were cleaned
20 by washing with octyl glucoside for 1 min at 10 μ l/min, and 0.5 mM liposomes as prepared above were passed over the flow cells for 25 min at 4 μ l/min. The chips were washed with a 30 sec injection of 50 mM sodium hydroxide twice to remove the loosely bound liposomes to obtain stable base line. In a
25 typical experiment, a monolayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was deposited in flow cell 1 and referred to as POPC surface. A monolayer of LPA-POPC was deposited in flow cell 2 and referred to as LPA-POPC surface.

30 The dilutions of sRAGE, V domain and C2 domain were prepared in HBS buffer and injected sequentially over two flow cells at a flow rate of 30 μ l/min for 150 s. The response curve was obtained by subtraction of the response signals over POPC surface from the binding response over LPA-POPC surface. The
35 binding curves were analyzed using Biacore software.

NMR Binding Studies

Experiments were performed at 25 °C on a Bruker DRX500 spectrometer equipped with a Z-axis gradient cryoprobe. LPA was titrated into a solution of 50 µM ¹⁵N-labeled V domain in 20 mM phosphate at pH 6.0, and perturbations in NMR signals were monitored by acquiring 2D ¹⁵N-¹H HSQC spectra. The corresponding experiments for C2 were carried out in 20 mM phosphate at pH 6.1. Acquired data were processed using Topspin 2.1 (Bruker) and analyzed using NMRViewJ. The ratios of intensities between spectra with and without LPA (I/I₀) were calculated for all well resolved resonances. Chemical shift perturbations were measured using the equation $\Delta\delta = [(0.17\Delta\delta_N)^2 + (\Delta\delta_{HN})^2]^{1/2}$.

Cell Lines and Materials

Wild-type and RAGE null mice primary aortic vascular smooth muscle cells were isolated and employed through passage 5 to 7. Rat C6 glioma cells were obtained from ATCC. Murine epithelial ovarian cancer ID8 cells were a generous gift from Dr. Katherine Roby (Kansas University Medical Center).

Full-length and DN-RAGE cloning and mammalian expression and transfection

Full-length RAGE cDNA was generated from lung cDNA (Clontech) by PCR as described¹¹ and DN RAGE cDNA was generated as described.¹² Where indicated, Nucleofector kits for primary smooth muscle cells were employed (Lonza).

Real Time PCR

RNA was extracted from cells by using RNAeasy columns (Qiagen). cDNA was prepared (Applied Biosystems) and used as a template for quantitative PCR. Primers and probes for LPA receptors 1-5 and murine RAGE were obtained from Applied Biosystems. Gene expression was normalized to the expression of 18S rRNA. Data were analyzed by the 2^{-ΔΔCT} method.

Western blot analysis

Total cell or aorta lysates were immunoblotted and probed with LPA1, AKT-specific antibody, p-AKT-specific antibody, ERK, pERK (Cell Signaling Technology), HRP-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotechnology) or HRP-conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotechnology) was used to identify sites of binding of the primary antibody. After probing with the primary antibodies, membranes were stripped of bound immunoglobulins and reprobed with GAPDH (Abcam) or for relative total protein. Blots were scanned with an AlfaImager TM 2200 scanner with AlfaEase (AlfaImager) FC 2200 software. Results are reported as a relative absorbance of test antigen to GAPDH or relative total proteins. In all Western blot studies, at least 5 animals or cell lysates per group were used; results of representative experiments are shown.

Immunohistochemistry

Acetone-fixed cryostat aortic sections were preincubated with CAS-BLOCK (Zymed; Invitrogen) for 30 minutes followed by avidin-biotin block for 15 minutes; sections were then subjected to incubation with primary rabbit polyclonal RAGE IgG; Akt and p-Akt (Cell Signaling) overnight at 4°C followed by goat anti-rabbit IgG (Vector Laboratories). Subsequently, Alexa Fluor 555 conjugate (Invitrogen) was incubated for 30 minutes. After washing, mouse monoclonal smooth muscle actin (DakoCytomatin) antibody was incubated for 1 hour followed by anti-rat or anti-mouse IgG for 30 minutes, and then incubated with Alexa Fluor 488 conjugate for 30 minutes, and finally mounted with 4,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories). Rabbit IgG (Zymed; Invitrogen) or omission of the primary antibody was used as a negative control. Slides were mounted with Vectorshield mounting media (Vector) and observed with an oil immersion objective using a Nikon E800 microscope. Images were collected using a Bio-Rad Radiance 2000 Confocal System and the Laserssharp 2000 software (Bio-Rad).

Smooth Muscle Cell Assays

Migration and proliferation assays were performed according to previously published methods.¹⁴ Vascular smooth muscle cells were cultured to 90% confluence. Cells were rinsed with PBS and placed in low serum media (1.5 ml; 0.5% - 0.1% serum in DMEM) overnight. A scratch line was then drawn using a sterile 200 μ l pipet tip. Three separate scratch wounds through the cell monolayer moving perpendicular to the line were drawn in the step above. Cells were rinsed very gently with PBS and replaced with 1.5 ml of media containing additives (10 μ M LPA or 10 μ g/ml S100B final concentration). Photographs were taken after 18 hours using phase contrast and 10X.

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Animal Models

All animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub. No. 85-23, 1996). Four- to six-week-old female C57/BL6 mice were purchased from Jackson Laboratories. Homozygous RAGE null mice backcrossed >12 generations were bred in our laboratory. To test the effects of LPA on vascular signaling, mice were perfused through the left ventricle with 2 ml of sterile phosphate buffered saline (PBS) to remove all blood. The aorta at the level of the bifurcation of the femoral arteries was cross-clamped and 200 μ l of a 100 μ M solution of LPA was injected using a 22-gauge needle. Control animals received equal volumes of PBS. At 5 mins and 15 mins after injection, the aorta from the point distal to exit from the left ventricle to the clamp site was rapidly excised and placed in ice cold buffer. Western blotting for detection of phospho/total Akt and immunohistochemistry to detect phospho-Akt was performed. In the tumor model, mice were injected by intraperitoneal (i.p.) route with 5×10^6 ID8 cells in 1 mL PBS and LPA was

administered daily (100 μ mol/L) in 200 μ L PBS or PBS or LPA(100 μ mol/L) in 200 μ L PBS and sRAGE (50 μ mol/L) in 200 μ L PBS injections for 4 weeks. Tumorigenesis was recorded by counting the numbers and sizes of tumor foci on each organ.

5

In other studies, MMTV-*atx* mice in the FVB genetic background, generously provided by Dr. Gordon Mills (University of Texas M.D. Anderson Cancer Center) were bred into RAGE null background (backcrossed >12 generations into FVB)³³. RAGE-expressing and RAGE null MMTV-*atx* littermate mice were sacrificed at age 6 weeks and all mammary tissue collected and pooled for analysis by Western blotting.

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Data analysis

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The mean $\pm$ standard deviation (SD) is reported. Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA); where indicated, individual comparisons were performed using Students' *t*-test.

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What is claimed is:

1. A method for treating a lysophosphatidic acid (LPA) mediated disorder in a subject comprising administering to the subject an amount of an antagonist of receptor for advanced glycation endproducts (RAGE) effective to treat the LPA mediated disorder in the subject, wherein the RAGE antagonist inhibits the binding of RAGE with LPA.
2. The method of claim 1, wherein the antagonist of RAGE is a RAGE antibody, a small molecule RAGE antagonist, a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.
3. The method of claims 1 or 2, wherein the LPA mediated disorder is correlated with elevated levels of LPA.
4. A method of treating a receptor for advanced glycation endproducts (RAGE) related disorder in a subject comprising administering to the subject an amount of an lysophosphatidic acid (LPA) antagonist effective to treat the RAGE related disorder in the subject, wherein the LPA antagonist inhibits the binding of LPA with RAGE.
5. The method of claim 4, wherein the disorder is sepsis, atherosclerosis, multiple sclerosis, systemic lupus erythematosus, transplant rejection, asthma, arthritis, tumor growth, cancer, metastasis of cancer, complications due to diabetes, retinopathy, neuropathy, nephropathy, impotence, impaired wound healing, gastroparesis, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, neointimal formation, amyloid angiopathy, inflammation, glomerular injury, seizure-induced neuronal damage, acute skin

inflammation, chronic skin inflammation, psoriasis, atopic dermatitis, rheumatoid arthritis, lung inflammation, asthma, chronic obstructive pulmonary disease, diabetes, renal failure, hyperlipidemic atherosclerosis associated with diabetes, diabetes, diabetic late complication increased vascular permeability, diabetic late complication increase vascular permeability, diabetic late complication nephropathy, diabetic late complication retinopathy, diabetic late complication neuropathy, neuronal cytotoxicity, multiple sclerosis, dementia associated with head trauma, neuronal degeneration, restenosis, amyloidosis, or periodontal disease.

6. The method of claim 4 or 5, wherein the LPA antagonist is an anti-LPA antibody.
7. The method of any of claims 4, 5, or 6, wherein the subject is a human.
8. A method of treating ovarian cancer in a subject comprising administering to the subject an amount of an agent effective to treat the ovarian cancer in the subject, wherein the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA) .
9. A method of inhibiting metastasis of ovarian cancer in a subject comprising administering to the subject an amount of an agent effective to inhibit metastasis of the ovarian cancer by inhibiting the binding, wherein the agent inhibits the binding of receptor for advanced glycation endproducts (RAGE) with lysophosphatidic acid (LPA) .
10. A method of treating atherosclerosis in a subject comprising administering to the subject an amount of an

agent effective to treat the atherosclerosis in the subject, wherein the agent inhibits the binding of receptor for advanced glycation endproducts with lysophosphatidic acid (LPA).

11. The method of any one of claims 8-10, wherein the agent is an antagonist of LPA.
12. The method of claim 11, wherein the antagonist of LPA is an anti-LPA antibody.
13. The method of any one of claims 8-10, wherein the agent is an antagonist of RAGE.
14. The method of claim 13, wherein the antagonist of RAGE is a RAGE antibody, a small molecule RAGE antagonist, a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.
15. A method for inhibiting lysophosphatidic acid (LPA) induced phosphorylation of Akt in a cell comprising administering to the cell an antagonist of receptor for advanced glycation endproducts (RAGE), thereby inhibiting the LPA induced phosphorylation of Akt in the cell.
16. The method of claim 15, wherein the cell is a smooth muscle cell or an aortic smooth muscle cell.
17. The method of claim 15 or 16, wherein the antagonist of RAGE is a RAGE antibody, a small molecule RAGE antagonist, a fusion protein RAGE antagonist or a polypeptide RAGE antagonist.
18. A method for determining whether a compound inhibits the interaction of receptor for advanced glycation

endproduct (RAGE) with lysophosphatidic acid (LPA) comprising:

- (a) admixing:
 - (i) the compound,
 - (ii) LPA, and
 - (iii) RAGE or a fragment of RAGE which binds to LPA;
- (b) determining the amount of RAGE bound to LPA; and
- (c) comparing the amount of RAGE bound to LPA in step (b) with the amount determined when RAGE or fragment of RAGE is mixed with LPA in the absence of the compound,

thereby determining whether the compound inhibits the interaction of LPA with RAGE, wherein a reduction of the amount of binding in the presence of the compound indicates that the compounds inhibits the interaction.

- 19. The method of claim 18, wherein the RAGE is a fragment of RAGE.
- 20. The method of claim 19, wherein the fragment of RAGE is soluble RAGE.
- 21. The method of claim 19, wherein the fragment of RAGE comprises the V-domain of RAGE.
- 22. The method of claim 19, wherein the fragment comprising amino acids 1-30 or 2-30 of the V-domain of RAGE.
- 23. The method of claim 18, wherein the compound is an organic molecule.
- 24. The method of claim 18, wherein the compound is a polypeptide, a nucleic acid, or an inorganic chemical.
- 25. The method of claim 18, wherein the compound is an antibody or a fragment thereof.

26. The method of claim 25, wherein the antibody or fragment thereof is an anti-LPA antibody or fragment thereof.
27. The method of claim 25 or 26, wherein the antibody is a polyclonal or monoclonal antibody.
28. The method of claim 25 or 26, wherein the antibody is humanized, chimeric or primatized.
29. The method of claim 18, wherein the LPA is affixed to a solid surface.
30. The method of claim 18, wherein the RAGE or the fragment thereof is affixed to a solid surface.
31. The method of claim 18, wherein the LPA is detectably labeled.
32. The method of claim 18, wherein the RAGE or the fragment thereof is detectably labeled.
33. The method of claim 31 or 32, wherein the detectable label comprises fluorescence, biotin, or radioactivity.

Fig. 1B

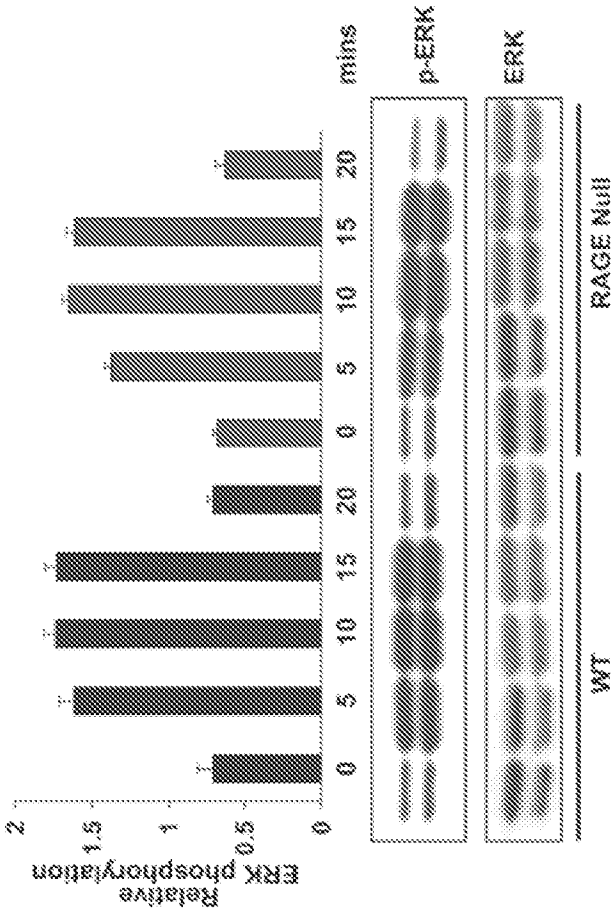
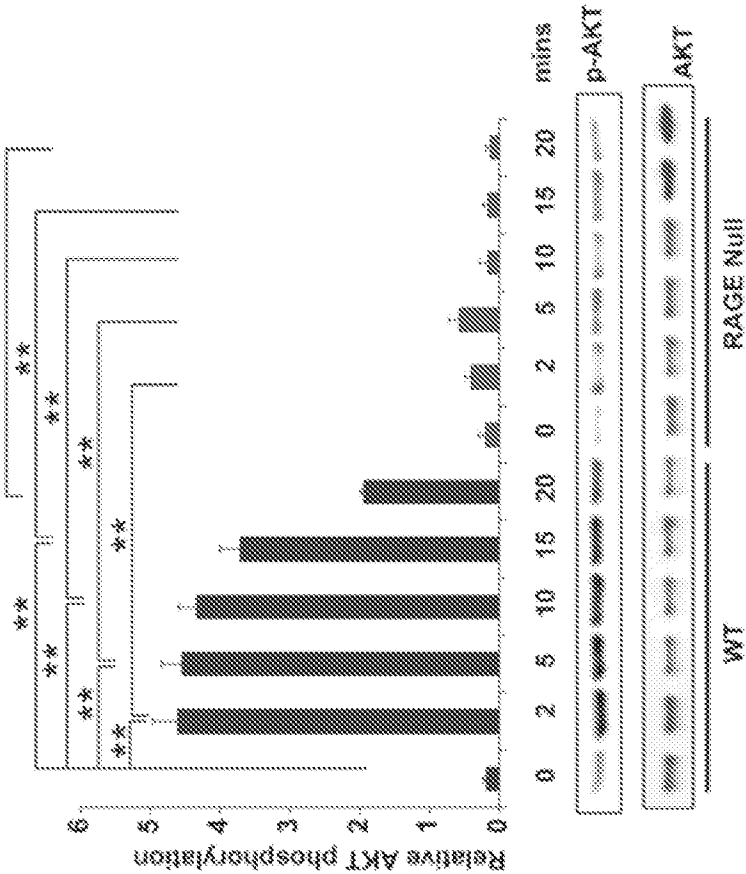
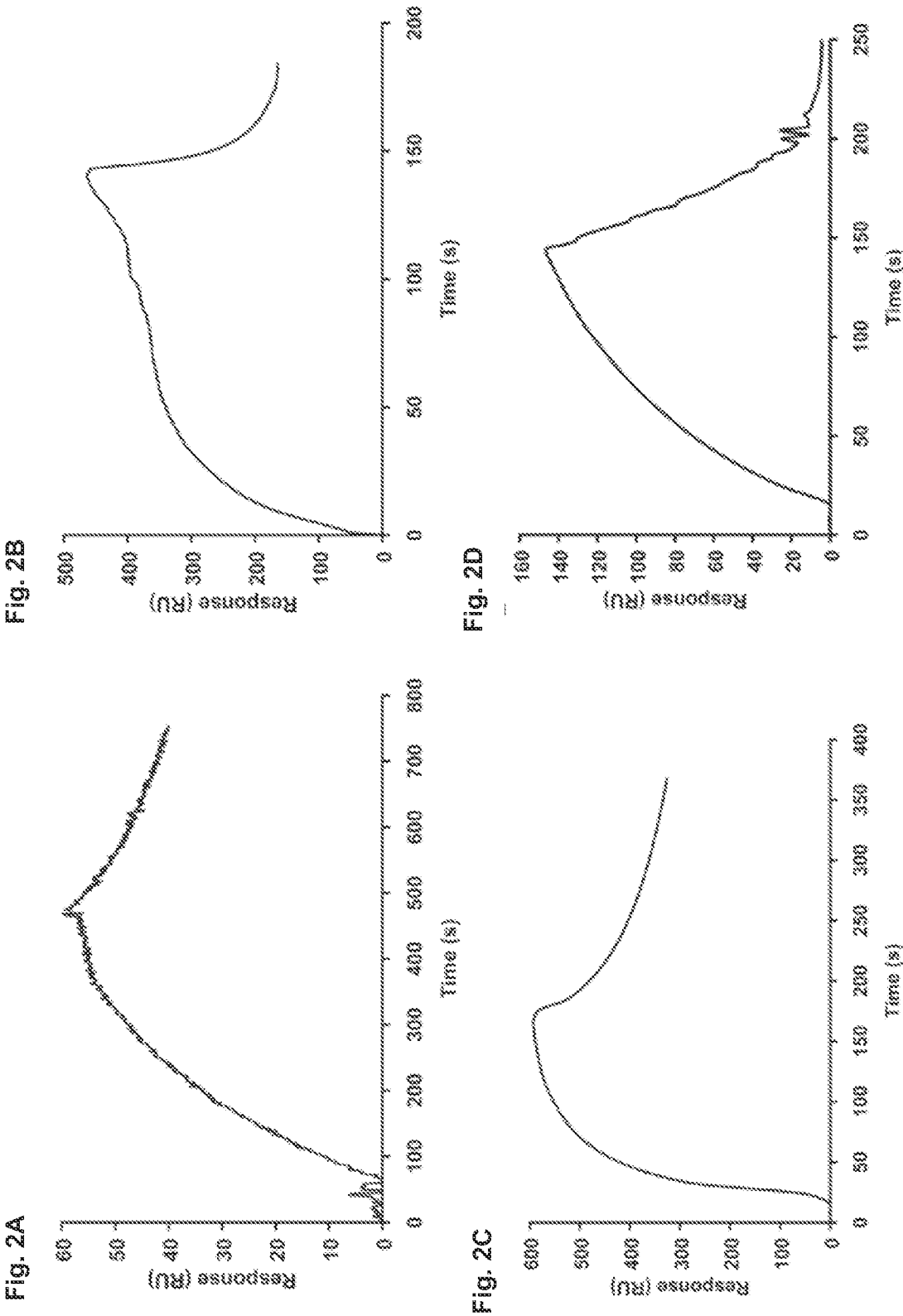


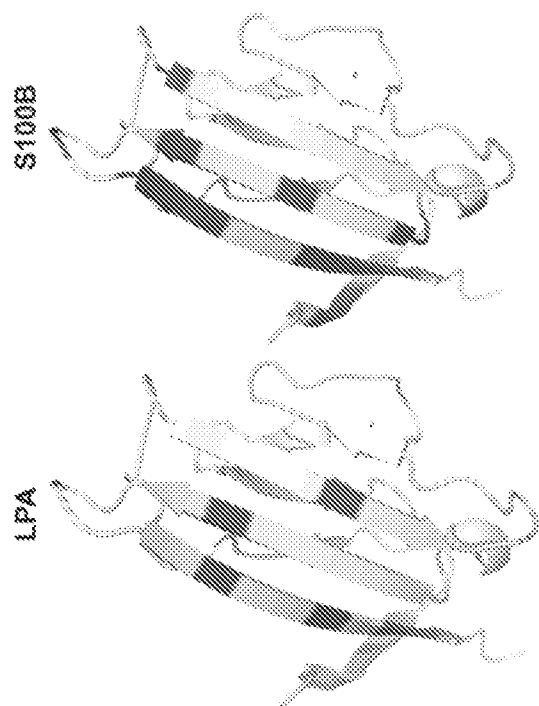
Fig. 1A



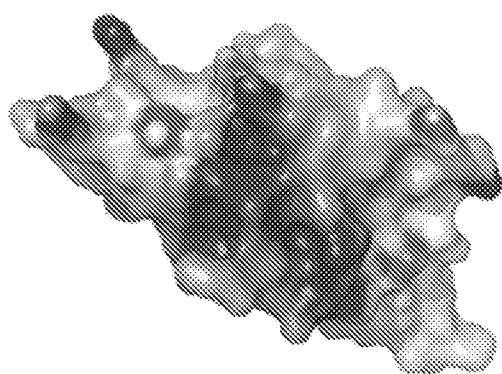
Figures 1A-1B



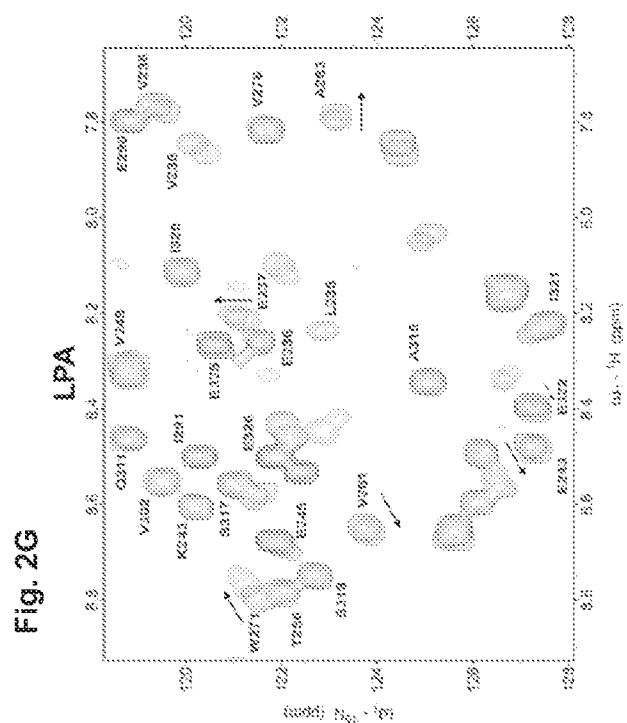
Figures 2A-2D



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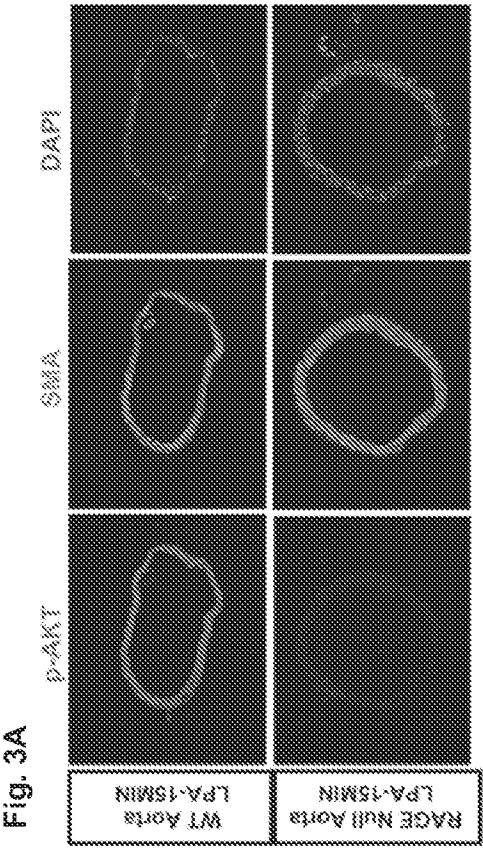
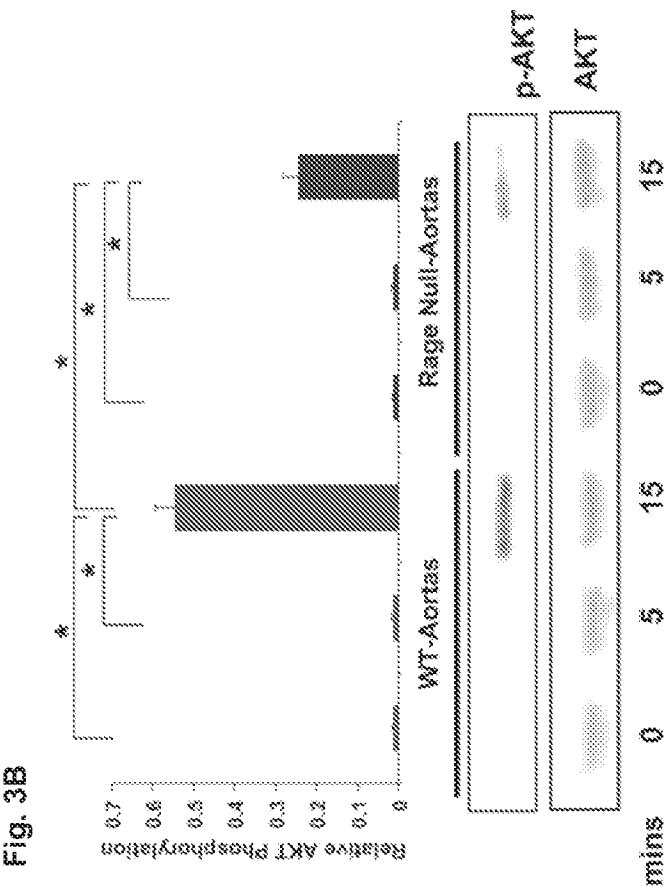


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Figures 2E, 2G



Figures 3A-3B

Fig. 3C

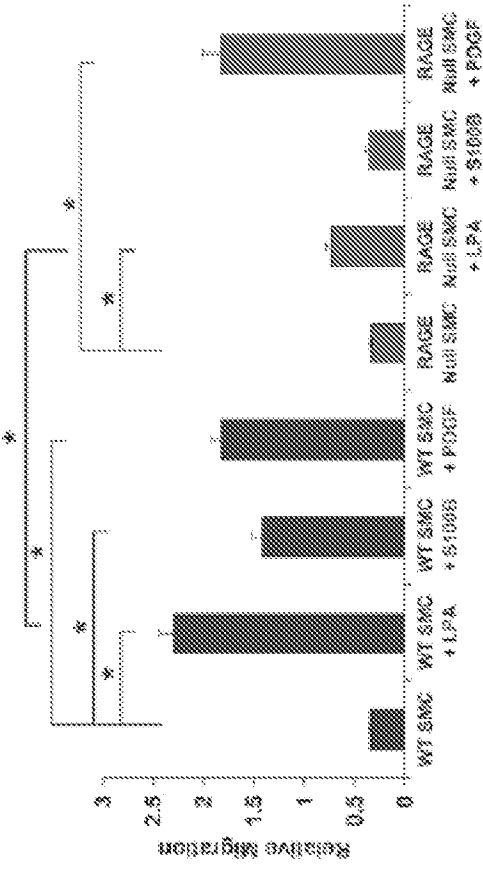
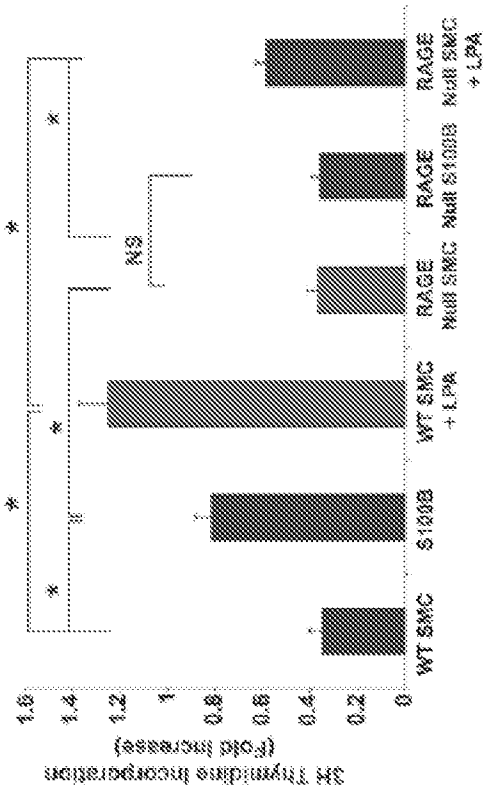
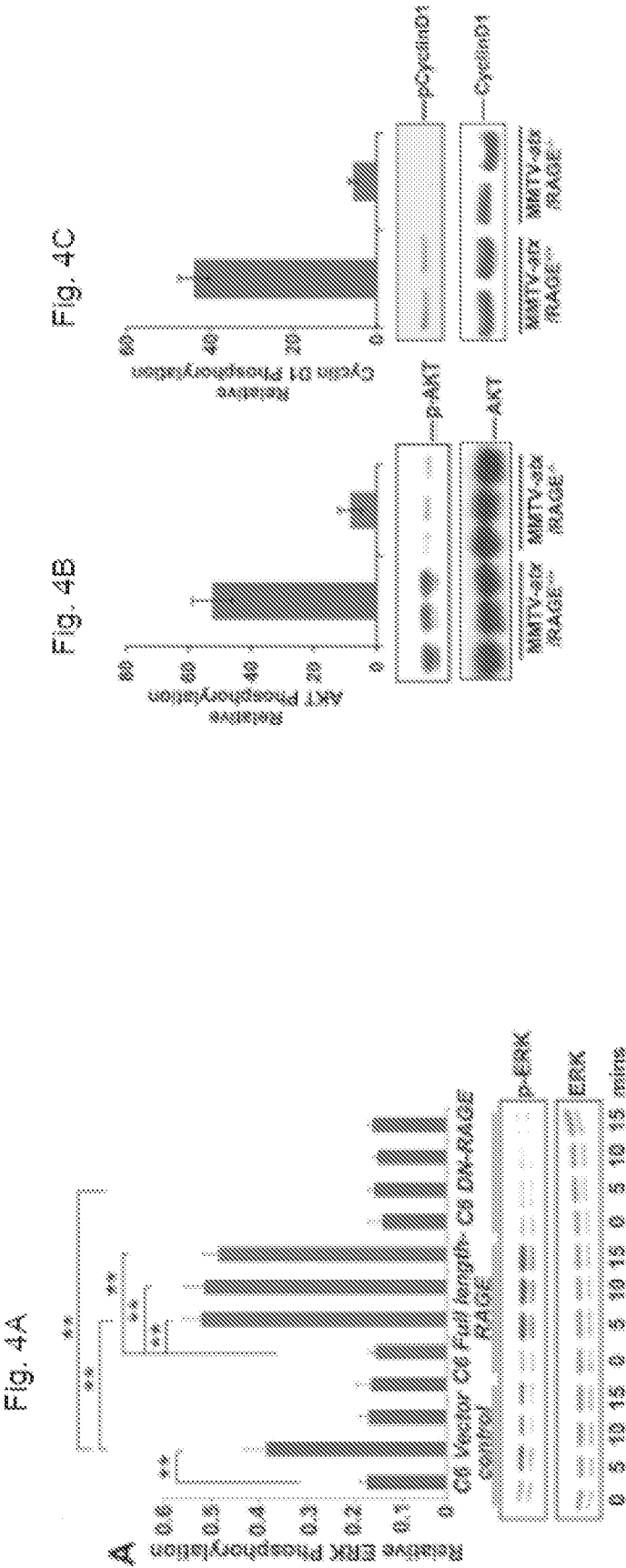


Fig. 3D



Figures 3C-2D



Figures 4A-4C

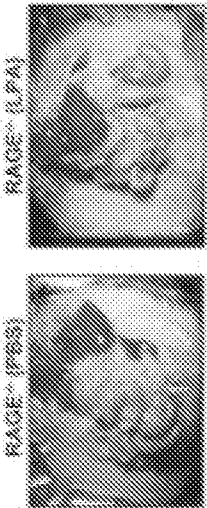
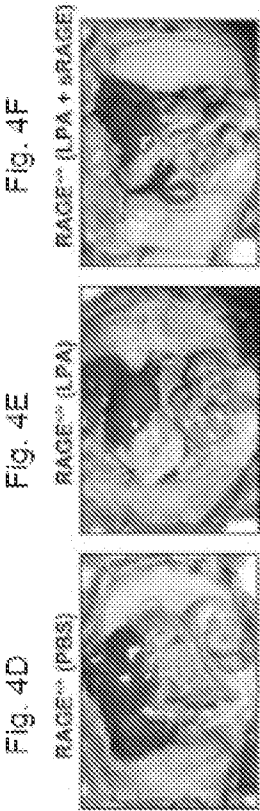
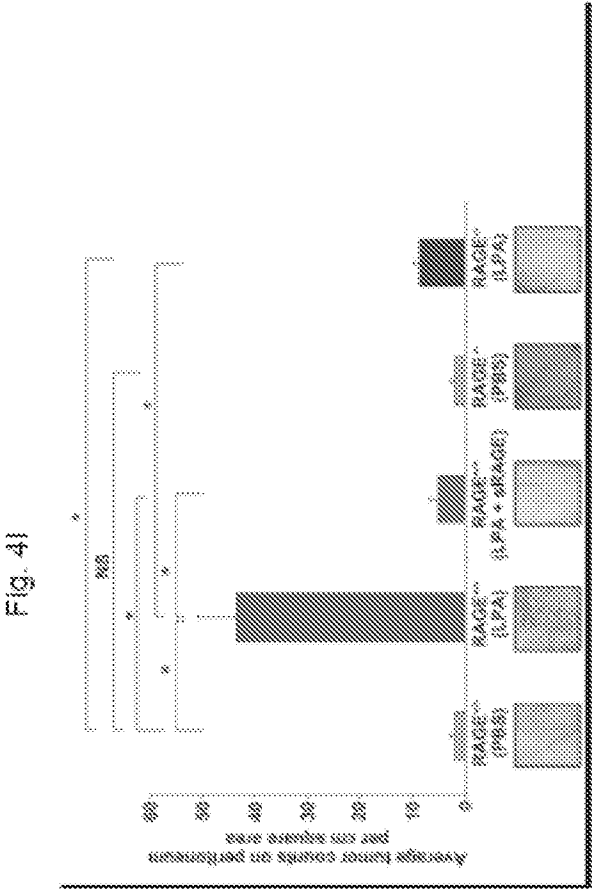


Fig. 4G

Fig. 4H

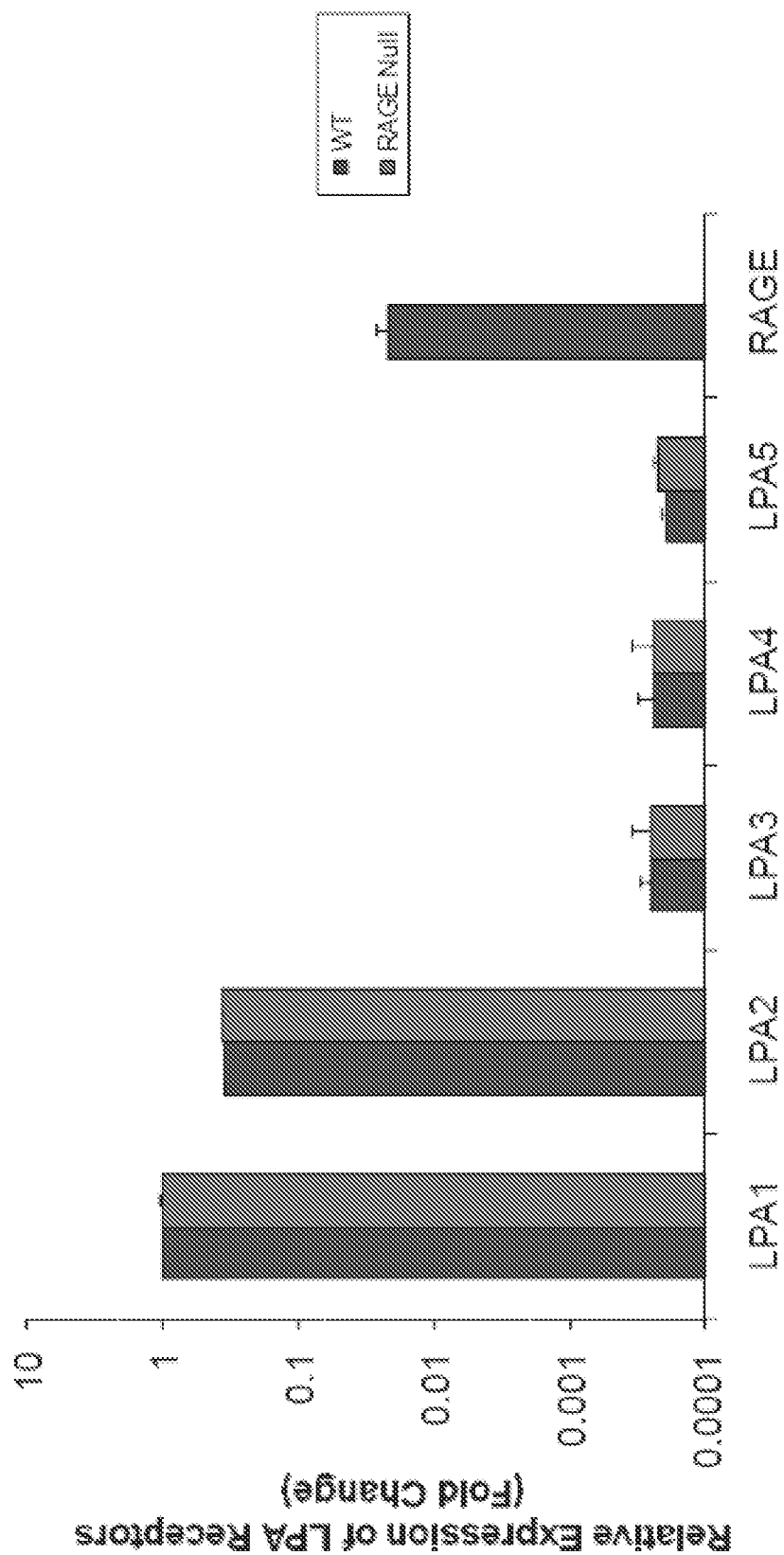


Figure 5

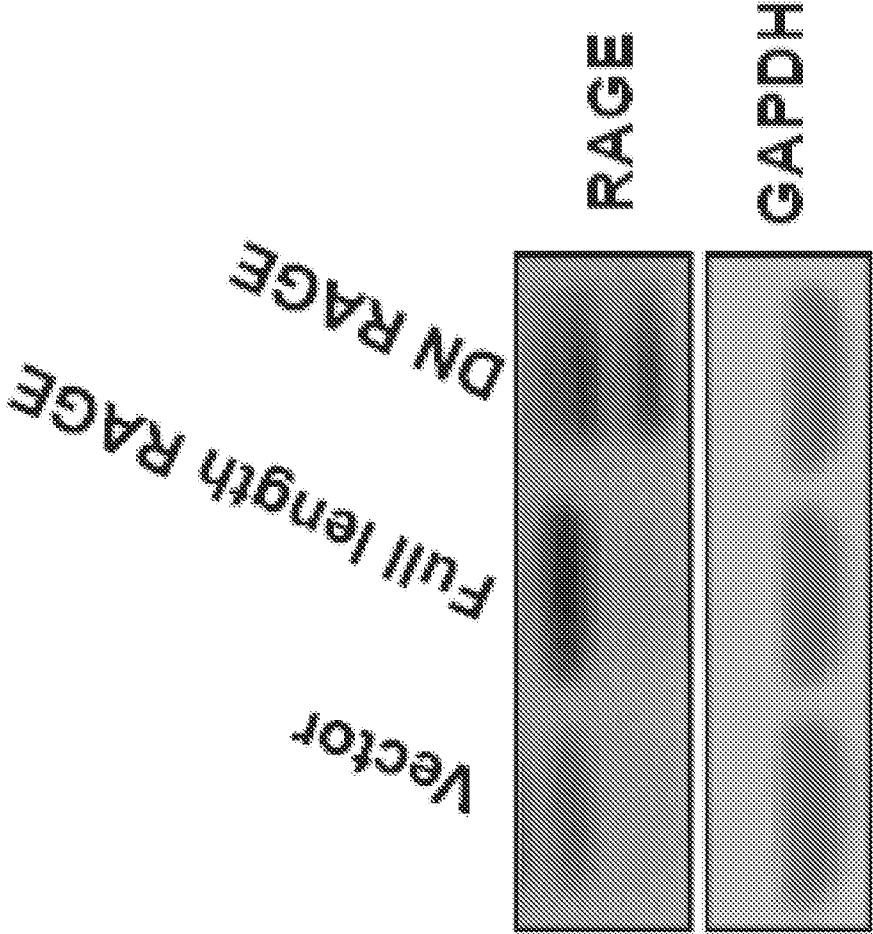


Figure 6

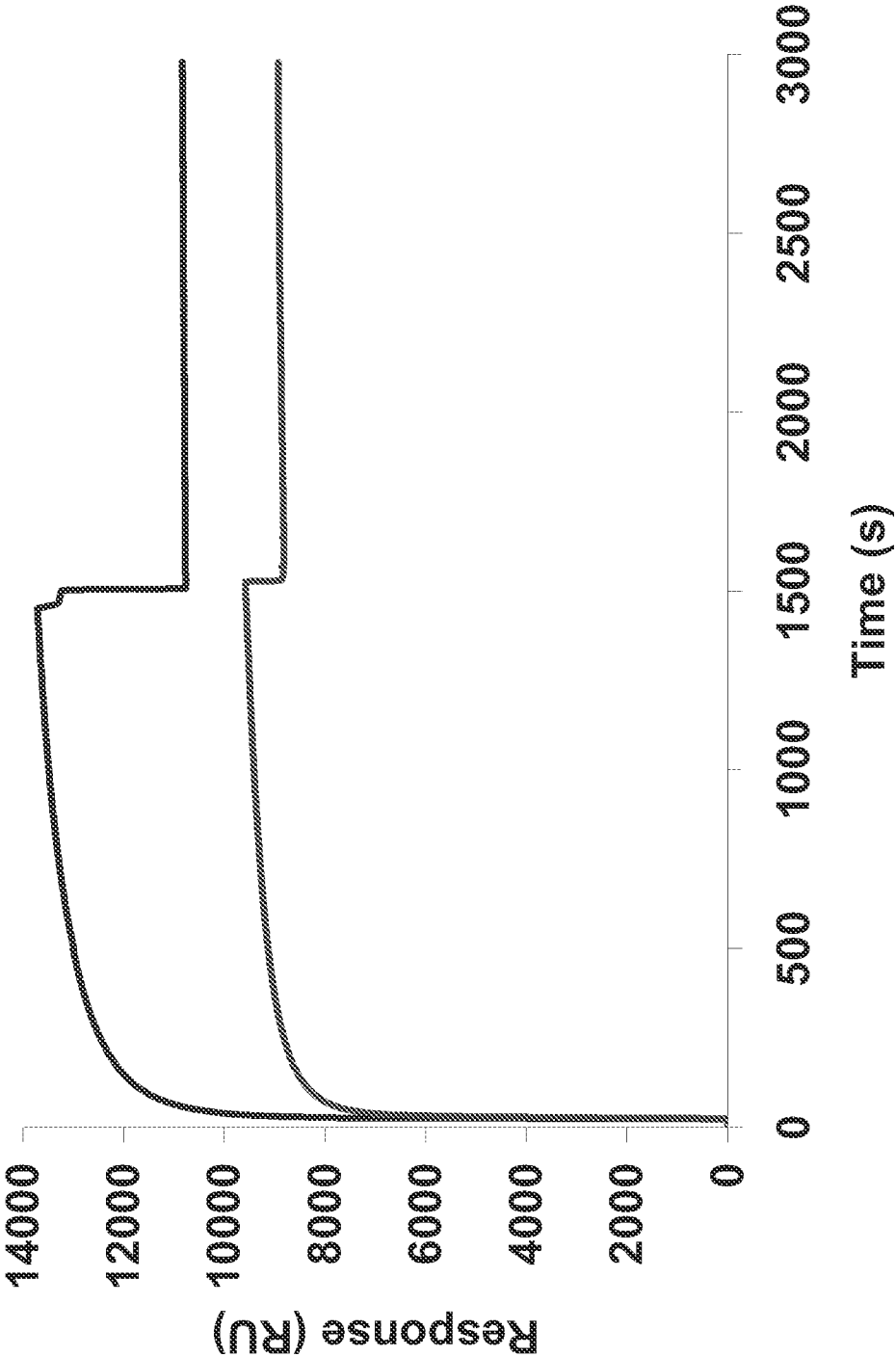
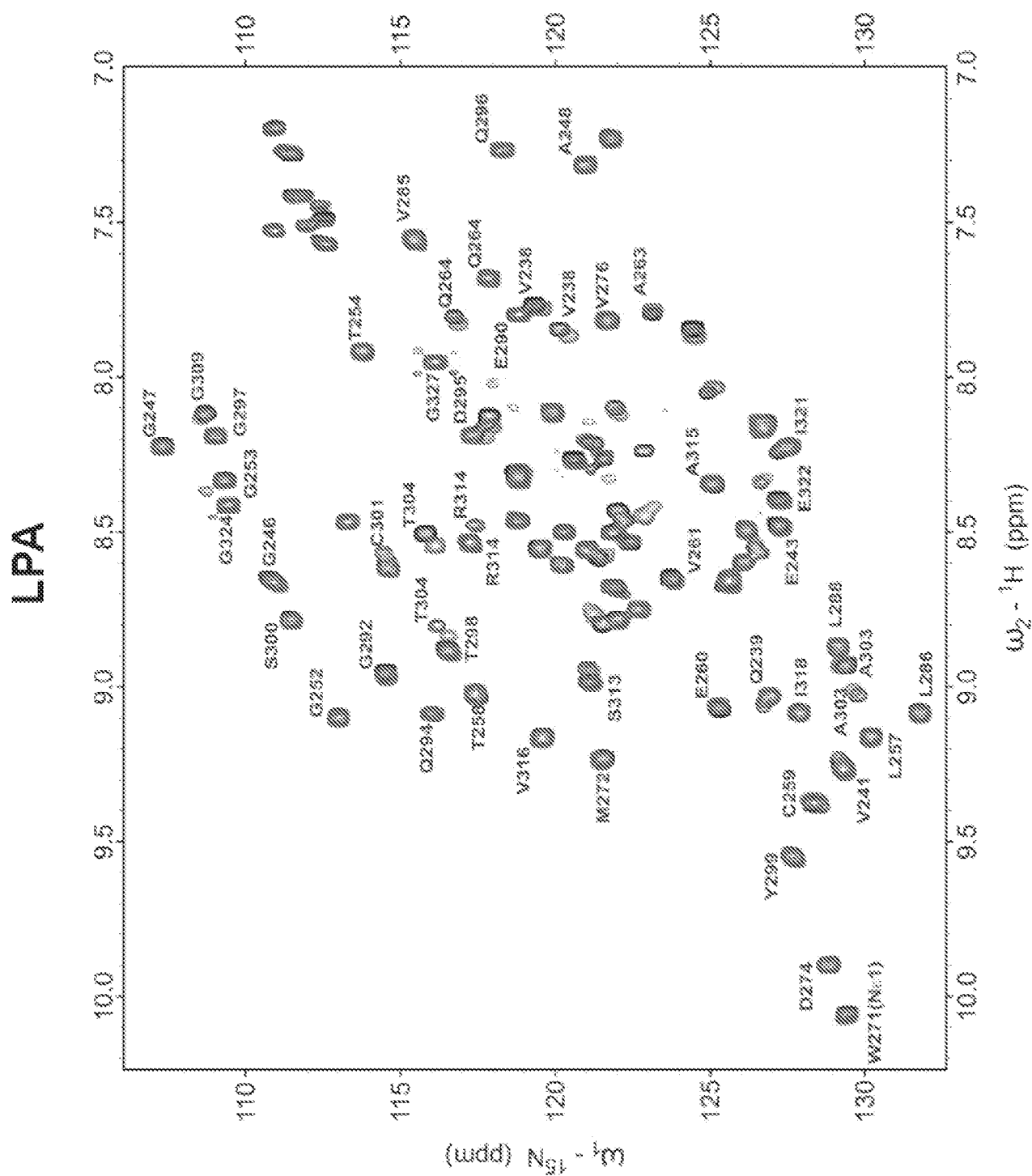


Figure 7



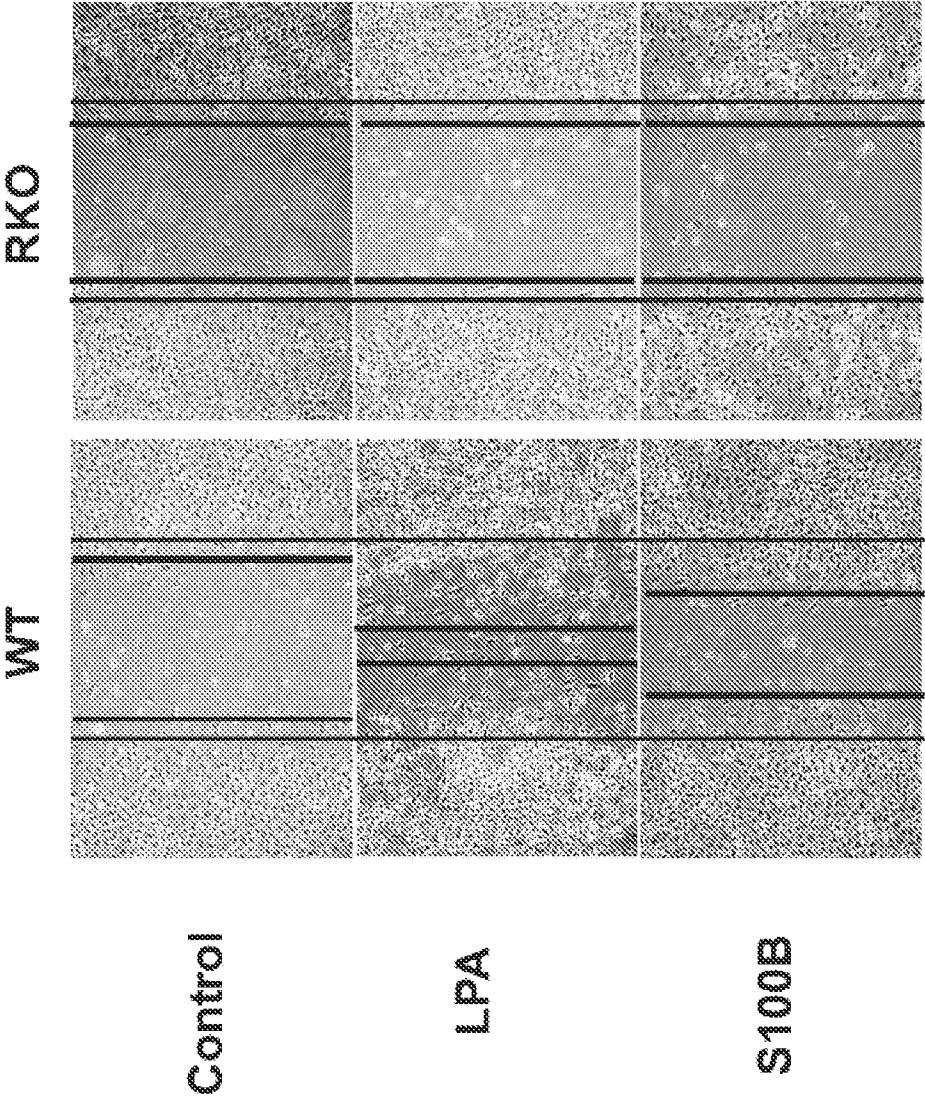


Figure 9

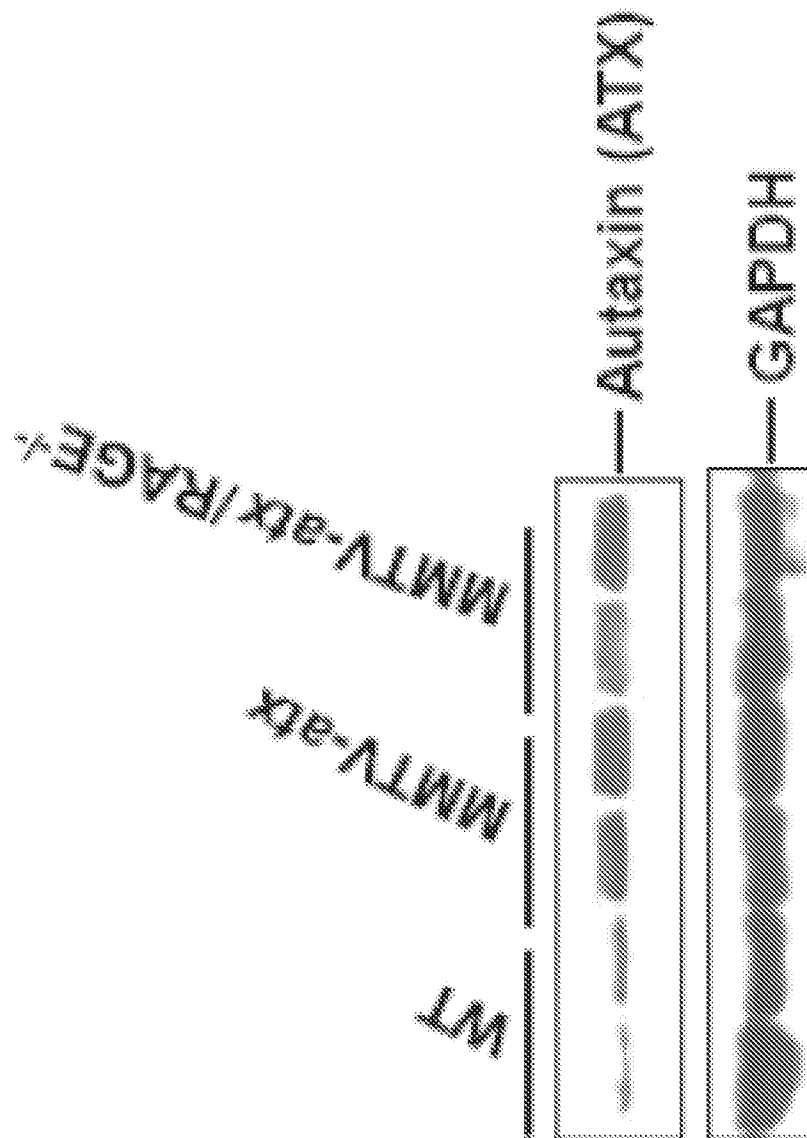


Figure 10

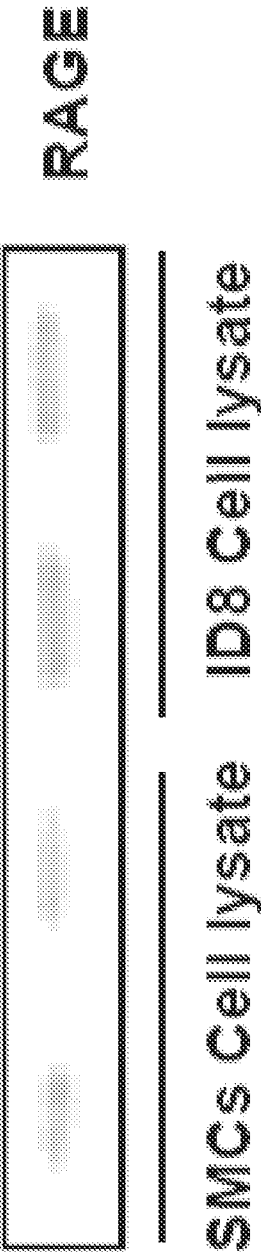


Figure 11

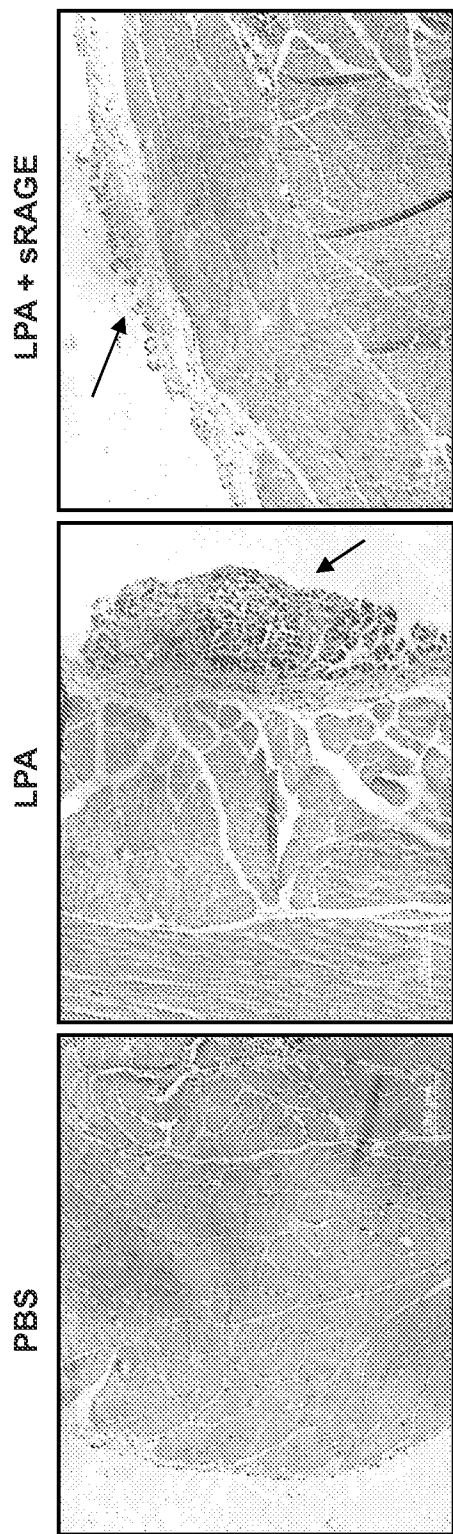


Fig. 12A

Fig. 12B

Fig. 12C

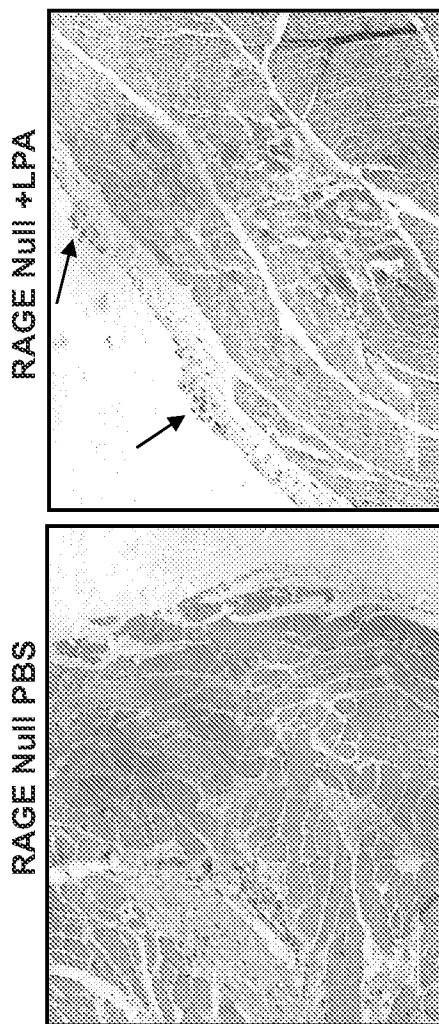


Fig. 12D

Fig. 12E

Figures 12A – 12E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/024701

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/28 (2012.01)

USPC - 424/130.1

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 39/00, 39/395; C07K 16/00, 16/28 (2012.01)

USPC - 424/130.1, 133.1, 134.1, 141.1, 142.1, 143.1; 514/8.1, 12.2, 824, 825, 826

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

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

MicroPatent, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 7,258,857 B2 (STERN et al) 21 August 2007 (21.08.2007) entire document	1-6, 8-33
Y	US 2008/0145360 A1 (SABBADINI et al) 19 June 2008 (19.06.2008) entire document	1-6, 8-33

☐ Further documents are listed in the continuation of Box C.


* 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

02 May 2012

Date of mailing of the international search report

21 MAY 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/024701

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims 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. ☒ Claims Nos.: 7
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 claims 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 claims 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.