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(54) Title: ACTIVATION OF CELLULAR ASSAULT PROCESSES IN THE TREATMENT OF GLIOBLASTOMA MULTIFORME

(57) Abstract: Polypeptides, such as a multi-valent polypeptide designated SVL4, useful in pharmaceutical compositions for the treatment glioblastoma multiforme. SVL4 demonstrated in vivo activity against a syngeneic mouse model of this condition. SVL4 and other polypeptides described herein are mimetics of a natural glycan ligand that binds to activating receptors on immune cells. These polypeptides bind lectins specific for galactose with high-avidity. SVL4, for example, is biologically active at nanomolar concentrations and has a relatively long lifetime in blood. These properties are believed to result as a consequence of the ability of SVL4 and other polypeptides described herein to cross-link cell-surface receptors.



ACTIVATION OF CELLULAR ASSAULT PROCESSES IN THE TREATMENT OF GLIOBLASTOMA MULTIFORME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional application 61/579,479, filed December 22, 2011, the contents of which are hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

- [01] This application incorporates by reference the contents of a 22.1 kb text file created on December 20, 2012 and named "00490800068sequencelisting.txt," which is the sequence listing for this application.
- [02] The present invention relates to polypeptides having binding activity for C-type lectin cell surface receptors, and the use of such polypeptides in the treatment of glioblastoma multiforme, the most frequent and aggressive brain tumor in humans. Representative polypeptides comprise one or more of the sequences VQATQSNQHTPRGGGSK SEQ ID NO:35, for mimicking sugars that bind to these receptors.

BACKGROUND

- [03] Glioblastoma multiforme (GBM) is the most aggressive of the gliomas, a class of tumors arising from glia or their precursors within the central nervous system. Unfortunately, GBM is also the most common of these tumors in humans. Most patients with GBM die in less than a year and essentially none has long-term survival. Despite the significant attention given to this disease, including increasingly complex therapies over the last half-century, treatment remains unsuccessful. A major reason for the resistance of GBM to therapeutic intervention is the multiforme character of the tumor itself, on both the gross and microscopic scales. The tumor is also multiforme genetically, with various deletions, amplifications, and point mutations leading to both activation of various signal transduction pathways and disruption of cell-cycle arrest pathways.
- [04] The migration of glioma cells results in their spread over long distances and into regions of brain essential for survival of the patient. Therefore, although GBM is visible as a mass lesion using MRI, the neoplastic cells actually extend far beyond the area of enhancement. This topically diffuse nature of gliomas renders surgery an ineffective treatment option, despite the fact that surgical resection has remained part of the standard of care for a

considerable number of years. In patients with GBM, surgery reduces tumor load but does not excise all tumor cells. Those cells that remain are resistant to radiation or chemotherapy. Even under the best of circumstances, in which the enhancing tumor seen on the MRI scan is removed completely by surgery, the mean survival time is not appreciably extended. Repeat surgeries for tumor recurrences ultimately do not prevent the tumor from spreading into vital regions of the brain. Current treatment protocols do not extend life expectancy after diagnosis beyond 1 to 2 years.

- [05] Accordingly, there is a need in the art for methods effective for the treatment of GBM, at least in terms of prolonging patient survival and/or quality of life. Such methods may involve conventional surgery and/or radiation as part of an overall treatment regimen.

SUMMARY OF THE INVENTION

- [06] Activation of the immune system is a promising, alternative approach for the treatment of glioblastoma multiforme (GBM). Cells of the immune system express an extensive array of activating, C-type lectin cell-surface receptors whose ligands contain sugar residues. For example, a modified serum protein, designated GcMAF, contains a single N-acetylgalactosamine (GalNAc) residue that is required for activation of macrophage functions. Macrophages, in turn, express a receptor, MGL, which is found on other cells of the immune system and is specific for GalNAc or galactose (Gal).
- [07] Aspects of the invention are associated with the discovery of polypeptides that mimic sugars that bind to lectin-type receptors of immune system cells. Suitable polypeptides therefore include mimetics of GalNAc or galactose, which act as immunostimulatory compounds. Representative polypeptides may be identified, for example, by screening a phage display library with sugar-specific lectins and/or computer-aided molecular modeling of docking of polypeptides to the sugar-binding site of lectins, selected as analogs of cell-surface receptors. Such identification methods were used to predict that the polypeptides described herein would bind receptors with higher affinity than their specific, native glycan ligand. This binding affinity was then confirmed by direct measurement.
- [08] Polypeptides of interest for use in the GBM treatment methods described herein comprise one or more members of a class of amino acid sequences, found to be instrumental in the overall lectin-type receptor binding of the polypeptide. Without being bound by theory, it is thought that the immunostimulatory functionality of such polypeptides contributes to their

effectiveness against GBM, in terms of prolonging patient survival and/or decreasing tumor size. Particular polypeptides comprise multiple members of such amino acid sequence classes, for example with each member forming all or part of a branch in an overall, multi-valent (*e.g.*, tetravalent) polypeptide structure. Representative polypeptides therefore exhibit binding to lectins in a solid-phase lectin binding assay, as described in greater detail herein. Other biological activities of the polypeptide, believed to play a role in stimulating the immune response and contributing to their effectiveness against GBM, include binding to receptors that are specific for galactose/N-acetylgalactosamine ligands in a solid-phase binding assay. One such receptor is CLEC10a/CD301. Further biological activities include (i) inhibition of the release of one or more inflammatory cytokines selected from the group consisting of IL-1 β , IL-6, and TNF- α , in a cytokine release assay using lipopolysaccharide (LPS) as a cytokine release agent, and/or selective induction of the release of the chemokine IL-8, (ii) induction of a greater release of the cytokine IL-21 in a cytokine release assay, compared to IFN- γ (iii) stimulation of phagocytosis, based on internalization of bacterial cells by microglial cells, (iv) stimulation of the immune system, based on increased density of one or more cellular markers on blood monocytes, with representative markers including CD11b protein or MHCII protein and/or (v) stimulation of the immune system, based on increased density of one or more cellular markers on phagocytic cells in the brain and in the presence of glioma cells.

- [09] Specific aspects of the invention relate to the discovery of methods for treating GBM in a patient, the methods comprising administering to a patient a polypeptide comprising an amino acid sequence that imparts binding affinity of the polypeptide to lectin-type receptors of immune system cells. Representative polypeptides comprise amino acid sequences of formula 1, formula 2, or formula 3, as described herein. The amino acid sequence of formula 1 is B1-[X1-Q-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11]-B2 (SEQ ID NO:40); wherein X1 is selected from the group consisting of V, E, and A, or X1 is absent; X2 is selected from the group consisting of A, N, and G; X3 is any amino acid; X4 is selected from the group consisting of P and Q; X5 is selected from the group consisting of S, R, and C; X6 is selected from the group consisting of N, L, G, and K; X7 is selected from the group consisting of Q, A, S, and H; X8 is selected from the group consisting of H, L, and A; X9 is selected from the group consisting of S and T; X10 is selected from the group consisting of P and A; and X11 is selected from the group consisting of R, G, and P, wherein B1 and B2 are independently 1-

5 amino acids, or are independently absent. The amino acid sequence of formula 2 is B1-[X1-X2-X3-X4-I-N-I-X5-N-R-G-X6]-B2 (SEQ ID NO:41); wherein X1 is selected from the group consisting of C, L, and Q, or is absent; X2 is selected from the group consisting of R, P, and S, or is absent; X3 is selected from the group consisting of A, S, and T, or is absent; and X4, X5, and X6 are independently selected from the group consisting of S and T, or are independently absent, wherein B1 and B2 are independently 1-5 amino acids, or are independently absent. The amino acid sequence of formula 3 is B1-[X1-T-D-E-X2-R-R-Q-X3]-B2 (SEQ ID NO:42); wherein X1 is selected from the group consisting of C and T, or is absent; X2 is a 4 amino acid group; X3 is selected from the group consisting of C and P, or is absent; and B1 and B2 are independently 1-5 amino acids, or are independently absent.

- [10] Particular polypeptides comprise the amino acid sequence of formula 1, wherein X1 is V or is absent; X2 is A or N; X5 is S or R; X6 is N; X7 is Q or A; X8 is H or L; and X11 is R or G (SEQ ID NO:43). Among such polypeptides, a class of polypeptides of particular interest are those in which X2 is A; X4 is Q; X5 is S; X7 is Q; X8 is H; X9 is T; X10 is P; and X11 is R (SEQ ID NO:44). Among this class, a group of polypeptides of further interest are those in which X3 is T (SEQ ID NO:45). Other polypeptides comprise the amino acid sequence of formula 1, wherein B2 is the spacer sequence GGGS (SEQ ID NO:46). Further polypeptides comprise the amino acid sequence of formula 2, wherein X1 is L or is absent; X2 is P or is absent; X3 is T or is absent; X4 and X5 are T; and X6 is S (SEQ ID NO:47). Yet further polypeptides comprise the amino acid sequence of formula 3, wherein X2 is the 4 amino acid group Z1-Z2-Z3-Z4, wherein Z1 is A or P; Z2 is L or F; Z3 is Y or V; and Z4 is T or Y (SEQ ID NO:48).
- [11] Other particular polypeptides comprising the amino acid sequence of formula 1, formula 2, or formula 3 are branched, with exemplary polypeptides having multiple branches with any of these amino acid sequences, including the more particular genres of polypeptides within the scope of these formulas, as described above. Branching beneficially provides polypeptides with a multivalent structure (*e.g.*, having multiple amino acid sequences, as described above, that impart binding avidity of the polypeptide to lectin-type receptors of immune system cells). Each of the multiple branches may extend from residues of the same type or different types of amino acids; for example, each branch (or a subset of the branches) may extend from lysine residues. Among branched polypeptides, a class of polypeptides of particular interest are those with multiple branches having the amino acid sequence of formula 1. For example,

a polypeptide of interest has a multivalent structure (*e.g.*, a tetravalent structure) with multiple branches (*e.g.*, four branches) having the sequence VQATQSNQHTPRGGS (SEQ ID NO: 35) in each branch. In the case of branched polypeptides, active portions of the sequences (*i.e.*, portions of the sequences, as described above, having lectin-type receptor binding activity) are preferably at the amino-terminal ends of the branches. The distances of these active portions of the sequences from the branch junctions may be altered, using various spacer portions of the sequences, to achieve the desired conformation of the polypeptide, particularly with respect to orienting the active polypeptide sequences in such a manner to achieve high affinity/association with ligands of C-type lectin cell-surface receptors. As noted above, a representative spacer sequence, as a portion of a sequence of formula 1, formula 2, or formula 3 above, is GGGS (SEQ ID NO:34).

- [12] These and other embodiments and aspects relating to the present invention are apparent from the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

- [13] FIG. 1A depicts a tetravalent polypeptide, having four "arms" or branches, each terminating with a polypeptide sequence that is active for binding to lectin-type receptors of immune system cells, with the active sequences being spaced apart from a tri-lysine core using spacer sequences.
- [14] FIG. 1B shows a particular tetravalent polypeptide, designated SVL4, of the type depicted in FIG. 1A, with each of the four branches extending from the tri-lysine core and having identical polypeptide sequences.
- [15] FIG. 2 is an HPLC chromatogram of SVL4.
- [16] FIG. 3 is a line graph illustrating the relative avidity of tetra-, bi-, and mono-valent polypeptides to several lectins, with the extent of binding affinity of the tetravalent polypeptide being assigned a value of 100%.
- [17] FIG. 4 is a bar graph illustrating the binding of SVL4 to particular lectins.
- [18] FIG. 5 shows bar graphs illustrating, quantitatively, various cytokines released from human peripheral blood mononuclear cells (PBMCs) during a 24-hour incubation of SVL4 in the presence and absence of 1 ng/ml lipopolysaccharide (LPS).

- [19] FIG. 6 is a bar graph illustrating the stimulation of interleukin-21 (IL-21) release by treatment of PBMCs with 1 nM of interferon-gamma (IFN- γ) or 50 nM of SVL4 for 16 hours.
- [20] FIG. 7 is an image showing internalization of fluorescent bacterial cells by microglial cells in an explant of a GBM tumor incubated with 10 nM of SVL4 for 4 hours.
- [21] FIG. 8 shows bar graphs of the increases, compared to a control sample of monocytes in the blood of healthy mice, in cell surface immune markers, CD1 15, CD1 1b, MHCII, and CD1 1c, following subcutaneous administration of SVL4 on alternate days for two weeks.
- [22] FIG. 9A shows bar graphs illustrating the change in the ratio of microglial cells to macrophages, in the brains of mice with implanted glioma cells, following radiation treatment with SVL4, compared to radiation treatment alone.
- [23] FIG. 9B graphically depicts flow cytometric analysis of macrophages and microglial cells.
- [24] FIG. 9C is a nuclear magnetic resonance (NMR) image of a mouse brain, showing early stage tumor development, following implantation of glioma cells.
- [25] FIG. 9D shows bar graphs of the increases, compared to radiation treatment alone, in immune markers, MHCII and CD1 1c on phagocytic cells and the immune markers CD1 15, CD1 1b, and MCHII on microglial cells, in the brains of mice with implanted glioma cells, following radiation treatment 2x4 Gray (Gy) in combination with SVL4 treatment.
- [26] FIG. 10A is a graph illustrating the increase in survival of mice implanted with glioma cells in the brain, following (i) radiation treatment alone, (ii) SVL4 treatment alone, and (iii) a combination of radiation and SVL4.
- [27] FIG. 10B is a bar graph illustrating the decrease in tumor size of mice implanted with glioma cells in the brain, following (i) radiation treatment alone, (ii) SVL4 treatment alone, and (iii) a combination of radiation and SVL4.
- [28] FIG. 11 shows graphs of the concentration of SVL4 in the blood plasma of rats, using both linear and logarithmic scale Y-axes, following intravenous injection of 0.5 ml of a 5 mM solution of SVL4 in phosphate buffered saline (PBS).
- [29] FIG. 12 is a graph of the concentration of SVL4 in the blood plasma of rats, following subcutaneous injection of 2 nmoles/g body weight of SVL4.

- [30] FIGS. 1-12 are to be understood to present an illustration of some embodiments of the invention and/or principles involved. These embodiments are illustrated in FIGS. 1-12 by way of example, and not by way of limitation.

DETAILED DESCRIPTION

- [31] As discussed above, aspects of the invention are associated with the use of polypeptide mimetics of GalNAc, as well as other polypeptides that exhibit binding affinity to lectin-type receptors of immune system cells, in the treatment of glioblastoma multiforme (GBM) in patients. Families of such polypeptides, as well as specific examples, are described in US 2009/0041793, incorporated herein by reference with respect to the disclosure of these polypeptides. Representative polypeptides include those having at least one an amino acid sequence (i) with at least 10 contiguous amino acids between XI and XI1 according to the amino acid sequence of formula 1 above, and/or (ii) with at least 8 contiguous amino acids between XI and X6 according to the amino acid sequence of formula 2 above, and/or (iii) with at least 10 contiguous amino acids between XI and X3 according to the amino acid sequence of formula 3 above. The single letter designation for amino acids is used in this disclosure, including in formulas 1-3. According to art-recognized convention, such single letter designations are as follows: A is alanine; C is cysteine; D is aspartic acid; E is glutamic acid; F is phenylalanine; G is glycine; H is histidine; I is isoleucine; K is lysine; L is leucine; M is methionine; N is asparagine; P is proline; Q is glutamine; R is arginine; S is serine; T is threonine; V is valine; W is tryptophan; and Y is tyrosine.
- [32] As discussed in US 2009/0041793, polypeptide sequences of interest were derived from a lectin screen of a phage display library. Such polypeptide sequences, including the consensus sequence (SEQ ID NO: 3), which may be included in polypeptides of the invention that have effectiveness against GBM, include SEQ ID NOS: 1-23, as follows: AQALGLSAISPR (SEQ ID NO:1); CTDEALYTRRQC (SEQ ID NO:2); VQATQSNQHTPR (SEQ ID NO:3); EQATPRNHHSP (SEQ ID NO:4); VQATPRLQHTPR (SEQ ID NO:5); AQGPPSKQHSP (SEQ ID NO:6); LPTTINISNRGS (SEQ ID NO:7); VPFRGYSPQ (SEQ ID NO:8); VQAIQSNQLTPR (SEQ ID NO:9); VQATTVQIQHAP (SEQ ID NO:10); CRASINITNRGS (SEQ ID NO:11); LPSTINITNRGS (SEQ ID NO:12); QSTTINIIRSGS (SEQ ID NO:13); EEAISLISIRRR (SEQ ID NO:14); VQAGQSNHAHTAG (SEQ ID NO:15); TTDEPFVYRRQP (SEQ ID NO:16); VQARQSNQHTPR (SEQ ID NO:17);

VQANQCQSAYAR (SEQ ID NO: 18); VRLQYHRGRG (SEQ ID NO: 19); VQNYQSNQHTPR (SEQ ID NO: 20); FVSTTMKLSDG (SEQ ID NO: 21); FNSYDTEAFGGG (SEQ ID NO: 22), and AETVESCLAK (SEQ ID NO: 23). Other polypeptides of interest have the following sequences with the requisite biological activity as a mimetic of GalNAc: QATQSNQHTPR (SEQ ID NO: 24); QATQSNQHTPRGGG (SEQ ID NO: 25); VQATQSNQHTPRGGG (SEQ ID NO: 26); QATQSNQHTPRK (SEQ ID NO: 27); QATQSNQHTPRKW (SEQ ID NO: 28); QATQSNQHTPRGGGSK (SEQ ID NO: 29); QATQSNQHTPRGGGSKW (SEQ ID NO: 30); VQATQSNQHTPRK (SEQ ID NO: 31); VQATQSNQHTPRKW (SEQ ID NO: 32); and VQATQSNQHTPRGGGSK (SEQ ID NO: 33).

[33] Additional polypeptides include those having one or more of the active mimetic sequences SEQ ID NOS: 1-33, but with an added C-terminal spacer sequence to move these active mimetic sequences away from the core of a branched polypeptide. As discussed above, a representative spacer sequence is GGGG (SEQ ID NO: 34), and therefore such additional polypeptides include those having the added C-terminal spacer sequence GGGG in sequences (*e.g.*, SEQ ID NO: 3) otherwise lacking such a sequence. Therefore, more specifically defined, exemplary polypeptides include those comprising the active mimetic sequence of SEQ ID NO: 3 with the C-terminal spacer sequence of SEQ ID NO: 34, with representative polypeptides comprising the following sequence: VQATQSNQHTPRGGG (SEQ ID NO: 35). Further active sequences may add one or more additional C-terminal amino acids such as lysine (K) of a lysine core, to which any of the above sequences may be joined, and/or tryptophan (W), with specific examples being VQATQSNQHTPRGGGSK (SEQ ID NO: 36); and VQATQSNQHTPRGGGSKW (SEQ ID NO: 37). Further polypeptides include those as described above but having an added N-terminal "V" (valine) residue in sequences (*e.g.*, SEQ ID NO: 4) otherwise lacking such a residue.

[34] Branched polypeptides with any of the above, active mimetic sequences, optionally further including a valine residue and/or a spacer sequence, as described above, bonded to the respective N-terminal and/or C-terminal ends of such sequences, are particularly representative of polypeptides of the invention. Such branched polypeptides may have 2, 3, 4, or more branches, at least one of which, some portion of which, or all of which, comprise(s) an active mimetic sequence as described above, including sequences having additional C-terminal, N-terminal, and/or spacer sequences. A schematic representation of a

polypeptide having four branches, each with an active mimetic sequence 1 and a spacer sequence 2 linking the mimetic sequence 1 to the polypeptide core 3 (*e.g.*, a tri-lysine core), is illustrated in FIG. 1A. The chemical structure of a specific polypeptide with 4 branches, each having the polypeptide sequence of SEQ ID NO. 36, comprising both active mimetic and spacer sequences, is illustrated in FIG. 1B. As shown, this polypeptide has a C-terminal amide group. The branches of this tetravalent polypeptide extend from a tri-lysine core, providing the sequence $[(VQATQSNQHTPRGGGS)_2K]_2K$ (SEQ ID NO:38, see FIG. 1B). The polypeptide consisting of the amino sequence of SEQ ID NO: 38 is designated throughout this disclosure as SLV4. Other polypeptides according to the present invention comprise this sequence.

[35] Further examples of amino acid sequences identified in US 2009/0041793 as GalNAc mimetics, and therefore useful in polypeptides of the present invention, include the amino acid sequences of

[36] LCADYSENTFTEYKKKLAERLKAKLPDATPQATQSNQHTPRGGGSELA KL VNKHSD
FASNCCCINSPPLYCDSEIDAELKNIL (SEQ ID NO: 39) and
MLCADYSENTFTEYKKKLAERLKAKLPDATPQATQSNQHTPRGGGSELA KL VNKHS
DFASNCCSINSPPLYCDSEIDAELKNILHHHHHH (SEQ ID NO: 40) (disclosed as SEQ
ID NOS: 29 and 31 in this publication). According to various embodiments of the invention, therefore, polypeptides useful for the treatment of GBM comprise one or more amino acid sequences selected from the group consisting of SEQ ID NOS: 1-33 and 35-40.

[37] Polypeptides may generally comprise natural or synthetic amino acids, amino acid analogs, or peptidomimetics, which are normally bonded by peptide bonds. Such synthetic amino acids, analogs, or mimetics may replace one or more naturally occurring amino acids in the active amino acid sequences, as described herein, or may replace one or more naturally occurring amino acids elsewhere in the polypeptide. The polypeptides may be synthesized chemically or by recombinant expression. Amino acid sequences as described above may be present in these polypeptides in a single copy or in multiple copies (*e.g.*, 2 or more copies, such as between 2 and 10 copies, or between 2 and 5 copies). In exemplary embodiments, copies of the amino acid sequences are present in separate branches to provide a branched configuration with multiple functionality due to the multiple copies (*i.e.*, as in the case of a multi-valent branched polypeptide). These polypeptides may be prepared by known methods

such as those described herein and also in *Solid Phase Peptide Synthesis: A Practical Approach* (B. Atherton and R. C. Sheppard, eds., 1989. Oxford University Press, New York, N.Y.); *Solid-Phase Synthesis: A Practical Guide* (S. A. Kates and F. Albericio, eds., 2000. Marcel Dekker, Inc., New York, N.Y.); *Fmoc Solid Phase Peptide Synthesis: A Practical Approach* (W. C. Chan and P. D. White, eds., 2000. Oxford University Press, New York, N.Y.). The synthesis of branched polypeptides is described in D. N. Posnett, H. McGrath, and J. P. Tarn (1988); *A novel method for producing anti-peptide antibodies*, JOURNAL OF BIOLOGICAL CHEMISTRY 263: 1719-1 725 .

- [38] Synthetic polypeptides, prepared using known solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and synthetic (unnatural) amino acids. Amino acids used for polypeptide synthesis may be Boc (N-a-amino protected N-a-t-butyloxycarbonyl) amino acid resin with the standard deprotecting, neutralization, coupling and wash protocols of the original solid phase procedure of Merrifield (1963, J. AM. CHEM. SOC. 85: 2149-2154), or the base-labile N-a-amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han (1972, J. ORG. CHEM. 37:3403-3409). Both Fmoc and Boc N-a-amino protected amino acids can be obtained, for example, from Sigma-Aldrich or Cambridge Research Biochemical. In addition, the polypeptides can be synthesized with other known N-a-protecting groups.
- [39] Solid phase polypeptide synthesis may be accomplished by techniques described, for example, in Stewart and Young (1984) SOLID PHASE SYNTHESIS, 2nd Ed., Pierce Chemical Co., Rockford, 111. or Fields and Noble (1990) INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH 35:161-214. Automated synthesizers may also be used. Polypeptides comprising any of the amino acid sequences described herein may comprise the unnatural D-amino acids (resistant to L-amino acid-specific proteases *in vivo*), including combinations of D- and L-amino acids. These D- and L-amino acids may be present in the active amino acid sequences described herein, or may be present elsewhere in the polypeptide. The polypeptides may comprise various "designer" amino acids {e.g., β -methyl amino acids, C-a-methyl amino acids, and N-a-methyl amino acids, *etc*) in the active sequences or elsewhere in the polypeptide to convey special properties. Synthetic amino acids include ornithine for lysine, and norleucine for leucine or isoleucine.

- [40] In addition, polypeptides comprising any of the amino acid sequences described herein can have peptidomimetic bonds, such as ester bonds, to prepare polypeptides with novel properties. For example, a polypeptide may be generated that incorporates a reduced peptide bond, *i.e.*, $R_1-CH_2-NH-R_2$, where R_1 and R_2 are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a peptide bond would be resistant to protease activity, and would possess an extended half-life *in vivo*.
- [41] Polypeptides comprising any of the amino acid sequences described herein may also be present as part of a fusion protein, in which case it may be desirable to synthesize the polypeptide using recombinant DNA technology. Such fusion proteins may include, for example, fusion with one or more amino acid sequences acting as transduction domains to permit movement of a fusion protein with the polypeptide across the cell membrane. These transduction domains can also be linked to other polypeptides to direct movement of the linked polypeptide across cell membranes. In some cases the transducing amino acid sequence(s) do not need to be covalently linked to the rest of the polypeptide. However, peptide bonding is often used to link the transduction domain to the rest of the polypeptide. See, for example, CELL 55: 1179-1 188, 1988; CELL 55: 1189-1 193, 1988; PROC. NATL. ACAD. SCI. USA 91: 664-668, 1994; SCIENCE 285: 1569-1572, 1999; J. BIOL. CHEM. 276: 3254-3261, 2001; and CANCER RES 61: 474-477, 2001).
- [42] According to other embodiments, polypeptides comprising any of the amino acid sequences described herein may be present in a fusion protein with full length vitamin D-binding protein (DBP), or with variations of Domain III of DBP (including but not limited to polypeptides comprising the amino acid sequence of SEQ. ID NOS: 38 and/or 39), as described herein.
- [43] According to further embodiments, the polypeptides comprising any of the amino acid sequences described herein can be fused or otherwise linked to therapeutic agents in order to enhance potential therapeutic effects of both agents. For example, monoclonal antibodies have been generated against a large number of cancers and other pathogenic agents for therapeutic use. Binding of these antibodies to the infectious agent is the first part of the therapy. Phagocytosis of the antibody-bound agent by macrophages and other phagocytic cells must then occur to eliminate the agent from the body. Therefore, a combination of target-directed antibodies with the polypeptides described herein provides an effective

combination therapy. Many other such fusions or linkages to other therapeutic agents will be apparent to those of skill in the art, having regard for the teachings herein.

- [44] It will be understood by those of skill in the art that such fusion proteins can result from the addition of a polypeptide having an amino acid sequence as described herein to the carboxy or amino terminal end of another polypeptide, or can comprise the placement of a polypeptide having an amino sequence as described herein within another polypeptide. Those skilled in the art, having regard for the teachings herein, will recognize many such fusion proteins can be made and used.
- [45] Polypeptides comprising amino acid sequences as described herein may be modified by, or bound to, non-polypeptide compounds to produce desirable characteristics, such modifications including but not limited to PEGylation with polyethylene glycol to improve *in vivo* residency time of the polypeptide, alkylation, phosphorylation, acylation, ester formation, amide formation, lipophilic substituent addition, and modification with markers including but not limited to fluorophores, biotin, dansyl derivatives, and radioactive moieties. Such non-polypeptide compounds can be directly linked, or can be linked indirectly, for example via a spacer such as the B1 and/or B2 amino acid group of polypeptides according to formulas 1-3 as described above, β -alanine, gamma-aminobutyric acid (GABA), L/D-glutamic acid, and succinic acid.
- [46] Further embodiments of the invention relate to pharmaceutical compositions useful in the treatment of GBM, comprising one or more polypeptides having amino acid sequences described herein, and a pharmaceutically acceptable carrier. Representative carriers include adjuvants appropriate for the indicated route of administration. For example, the polypeptides may be admixed with alum, lactose, sucrose, starch powder, cellulose esters of alkanolic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidone, polyvinylalcohol, dextran sulfate, heparin-containing gels, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the polypeptides may be dissolved in carriers such as physiological saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, or tragacanth gum, optionally with a physiological buffer system. Other adjuvants and associated modes of administration are

known in the pharmaceutical arts. Representative carriers also include time delay materials, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other known materials. The polypeptides may be covalently or non-covalently bonded to other compounds to promote an increased half-life *in vivo*, such as polyethylene glycol.

- [47] Further embodiments of the invention relate to the administration of a polynucleotide that encodes a polypeptide described herein, for the treatment of GBM in patients. Representative polynucleotides therefore encode polypeptides comprising amino acid sequences of SEQ ID NOS: 1-33 and 35-40, described above. Polypeptides may be administered via direct delivery (for example, by injection), or by gene therapy via administration of an appropriate expression vector that can be expressed in the target tissue of the brain. In embodiments employing gene therapy, it is preferred to use viral expression vectors, including but not limited to adenoviral and retroviral vectors.
- [48] In carrying out representative GBM treatment methods, a pharmaceutical composition comprising the active agent (*e.g.*, a polypeptide as described herein) in a solid form (including granules, powders, transdermal or transmucosal patches or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions) may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional pharmaceutically acceptable carriers, such as adjuvants as described above, stabilizers, wetting agents, emulsifiers, preservatives, cosolvents, suspending agents, viscosity enhancing agents, ionic strength and osmolality adjustors, and/or buffering agents. Suitable water soluble preservatives include sodium bisulfite, sodium thiosulfate, ascorbate, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric borate, parabens, benzyl alcohol, phenylethanol or antioxidants such as Vitamin E and tocopherol and chelators such as EDTA and EGTA. These preservatives and other carriers may be present in pharmaceutical compositions, generally in an amount from about 0.001% to about 5% by weight, and often from about 0.01% to about 2% by weight.
- [49] Whether the active agent is a polypeptide or a polynucleotide, it is administered, according to methods described herein, to a patient in need of treatment for GBM. Preferably the patient is a mammal and more preferably a human. Administration may be by any suitable route, including local delivery, parentally, transdermally, inhalation, and topically, in dosage unit formulations containing conventional pharmaceutically acceptable carriers, as described

above. Parenteral administration includes subcutaneous, intravenous, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, intraperitoneal, and infusion techniques. Preferred administration routes are subcutaneous and intravenous injection, as well as buccal and sublingual administration.

- [50] Methods for the treatment of GBM, by administration of therapeutic agents (*e.g.*, polypeptides described herein, or polynucleotides encoding these polypeptides), can be used in combination with surgery on the patient, including primary surgery for removing one or more tumors, secondary cytoreductive surgery, and secondary palliative surgery. In addition to surgery, or in the absence of surgery, representative methods comprise administering the therapeutic agent and also administering a secondary chemotherapeutic agent and/or radiation therapy. In some embodiments, administering the therapeutic agent can reduce the chemotherapy and/or radiation dosage necessary to inhibit tumor growth and/or metastasis. The therapeutic agent may be administered prior to, at the time of, or shortly after a given round of treatment with a chemotherapy and/or radiation therapy (radiotherapy). Radiation therapy includes external-beam radiation therapy, as well as the use of radiolabeled compounds targeting tumor cells. Any reduction in a chemotherapeutic or a radiotherapeutic dose, as a result of administration of a polypeptide as described herein, benefits the patient by decreasing side effects relative to standard chemotherapy and/or radiation therapy treatment.
- [51] In a preferred embodiment, for example, the polypeptide, as a therapeutic agent, is administered subsequent to a non-curative dose of radiation, with a representative dose being in the range from about 8 gray (Gy) to about 80 Gy. The polypeptide may be administered in multiple doses over a treatment regimen; for example, it may be administered on alternate days, or possibly only one or two times per week, during the treatment regimen. Representative treatment regimens may comprise the multiple doses of polypeptide, in addition to multiple doses of radiation and/or multiple doses of a chemotherapeutic agent. According to preferred embodiments, multiple doses of polypeptide and the multiple doses of radiation are administered alternatingly. For example, according to a specific embodiment involving alternating administration, a first radiation dose is administered prior to all radiation doses of the treatment regimen, and this is then followed by a first polypeptide dose, administered prior to all polypeptide doses of the treatment regimen. A second radiation dose is then administered, followed by a second polypeptide dose, and so on. No intervening

radiation dose occurs between the first radiation dose and the first polypeptide dose, between the second radiation dose and the second polypeptide dose, and so on.

- [52] A specific polypeptide of interest, SVL4, is characterized above as a tetravalent polypeptide, with each of four branches having the sequence VQATQSNQHTPRGGGS (SEQ ID NO: 35) and extending from a tri-lysine core. The overall sequence of this polypeptide is therefore $[(VQATQSNQHTPRGGGS)_2K]_2K$ (SEQ ID NO:38), as illustrated in FIG. 1B, with a molecular weight of 6,828 daltons. This sequence of SVL4, comprising all L-amino acids, was identified based on a screen of a 12-mer phage display library with the lectin from *Helix pomatia*, which is specific for GalNAc or Gal. The polypeptide structure was designed to facilitate cross-linking with cell-surface receptors. According to derivative polypeptides, also useful according to embodiments described herein for the treatment of GBM, the length of the linker, which is GGGS (SEQ ID NO: 34) in the case of SVL4, can be varied to position the active mimetic sequences further away from or closer to the core.
- [53] SVL4 binds to Gal/GalNAc-specific lectins and strongly stimulates cells of the immune system *in vivo*. As discussed in greater detail below, this polypeptide was used in an experimental mouse model for human glioblastoma multiforme (GBM), the most frequent and aggressive brain tumor in humans. Specifically, SVL4 was found to advantageously reduce tumor size and extend the life of mice in which glioma cells were implanted in the brain. SVL4 was particularly effective when a low non-curative dose of radiation was given to the mice prior to administration of the polypeptide. The experimental results demonstrated that SVL4 administration, especially in combination with current standards of care, can provide an effective treatment for patients suffering with GBM.
- [54] Overall, aspects of the invention are directed to methods for the treatment of GBM, including methods for prolonging the survival of patients suffering from this disease. According to representative embodiments, a patient suffering from GBM is administered a pharmaceutical composition comprising a monovalent or multivalent (*e.g.*, tetravalent) polypeptide having the structural and/or functional characteristics described herein, such as the active amino acid sequences described above, exhibiting lectin-binding properties. Administration of the pharmaceutical composition is performed, according to some embodiments, in conjunction with radiotherapy, the administration of other chemotherapeutic agents, and/or surgical procedures. Those having skill in the art, with the knowledge gained from the present

disclosure, will recognize that various changes can be made in these treatment methods without departing from the scope of the present disclosure.

[55] The following examples are set forth as representative of the present invention. These examples are not to be construed as limiting the scope of the invention as other equivalent embodiments will be apparent in view of the present disclosure and appended claims.

EXAMPLE 1

- [56] The tetravalent polypeptide SVL4, as described above, was synthesized, purified, and evaluated in terms of physical and biological properties that are important in the pharmaceutical compositions described herein.

Synthesis and Purity of SVL4

- [57] Tetravalent polypeptides were synthesized by general, standard chemistry methods utilizing Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids. Protecting groups for amino acid sidechains during synthesis were tert-butyl for the hydroxyl group of serine, Boc (tertbutyloxycarbonyl) for the ϵ -amino group of lysine, PBF (2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl) for arginine, or trityl for the imidazole group of histidine. The most difficult blocking groups to remove (and the additional mass provided by a residual blocking group) were tert-butyl (56 Da) and PBF (253 Da). The blocking groups were removed by trifluoroacetic acid (TFA) during cleavage from the resin. Modifications at the C-terminus consisted of (a) an amide group (no tag) or (b) an extension of the polypeptide by addition of C-terminal ϵ -biotinyl-lysine-amide or β -alanine-cysteine-amide. The thiol group on C-terminal cysteine was available for addition of a dansyl group by reaction with 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (Molecular Probes, Eugene, OR).
- [58] For initial biochemical studies, the polypeptides were synthesized on a small scale in batches of less than 1 gram. After the polypeptides were cleaved from the resin and dried, 200 to 300 mg of crude material were dissolved in water, neutralized with Na_2CO_3 , applied to a column (1 × 5 cm) of CM-Sephadex C-50, and washed extensively with water. While the polypeptide was retained on the column, fragments of the resin and most other side products of the synthesis were washed from the column. Polypeptides were then eluted with 0.1 N HCl and purified on a preparative Jupiter Proteo C12 column (21.2 x 250 mm) (Phenomenex, Torrance, CA) using a gradient of acetonitrile in water containing 0.1% TFA. Eluted polypeptides were dried, dissolved in water, neutralized to pH 5, and passed through a DEAE-Sephadex A-25 column (1 x 10 cm) at pH 5 to 6 to remove TFA and endotoxin, diluted with endotoxin-free standard phosphate-buffered saline (PBS), pH 7.4, or 150 mM NaCl, and filter-sterilized. Concentration was determined by the bicinchoninic acid assay (Pierce, Rockland, IL) using the dansylated polypeptide (extinction coefficient, $\epsilon \text{ mM}^{-1}\text{cm}^{-1}=5.7$

cm⁻¹ at 336 nm) as standard. The presence of endotoxin was assayed by gel formation with the *Limulus ameobocyte* lysate (Sigma-Aldrich, St. Louis, MO).

[59] For preliminary toxicity studies, and in preparation for synthesis of larger quantities, polypeptides were synthesized in 5-g batches by CBL Biopharma LLC ("CBL," Patras, Greece). The tri-lysine "core" was synthesized on the solid-phase resin and extended with the GGS sequence. The polypeptide "arms" with C-terminal G were synthesized separately by the standard chemistry method described above and condensed with the core. The polypeptide was purified extensively by HPLC using a gradient of acetonitrile in 0.1% TFA. The data sheet from CBL illustrated the HPLC pattern of the purified polypeptide (99.4% by HPLC) and the ESI mass spectrum. An independent analysis of the polypeptide performed by Blue Stream Laboratories, with the HPLC column (CI 8) at 50°C, is shown in FIG. 2, with 5-40% gradient of acetonitrile in 0.1% TFA. A further analysis conducted by Covance Laboratories with the HPLC column temperature at 35°C (not shown) was similar to that shown in FIG. 2 but did not include small components eluting at longer times. Analyses indicated that the polypeptide could be prepared with a purity of 95% by weight or more. This was representative of the purity level reported by CBL.

[60] The quality of the synthetic product, including correct synthesis and purity, was assessed by matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectroscopy. Possible impurities remaining in the final product were derivatives of the main polypeptide, which may have included smaller products of incomplete synthesis or molecules with incomplete removal of blocking groups. As shown by the mass spectra, these impurities were present in very low amounts in the purified polypeptide.

Physical Properties of the Synthesized Polypeptide

[61] SVL4 in lyophilized form was prepared as a white fluffy powder using trifluoroacetate (TFA) as a counterion, although other physiologically acceptable counterions {e.g., acetate} may also be used. Net polypeptide content with and without the counter ion was approximately 88% and 73%, respectively. The remainder was likely water of hydration of the highly polar polypeptide. Solubility of the polypeptide in water was high, meaning that preparation of solutions is limited largely by the bulk of the powder. In the preparation of aqueous pharmaceutical compositions of the polypeptide with any of the pharmaceutically acceptable carriers described above, concentrations of the polypeptide of 25 mM, and possibly

somewhat higher, can be readily achieved, particularly in aqueous compositions. According to representative embodiments, therefore, the polypeptide is administered to a patient suffering from GBM in an aqueous pharmaceutical composition having a polypeptide concentration generally from about 0.05 mM to about 25 mM, typically from about 0.1 mM to about 20 mM, and often from about 0.1 mM to about 10 mM, and a pharmaceutically acceptable carrier.

Finished Dosage Form

- [62] Aqueous pharmaceutical compositions were adjusted to desired concentrations, in the ranges described above for injection, in pyrogen-free phosphate buffered saline (PBS) at pH 7.4, or in 150 mM NaCl, and sterilized by filtration through a 0.2 micron, low-protein-binding polyvinylidene fluoride (PVDF) or polyethersulfone membrane, for example a 0.2 μm SUPOR[®] membrane (PALL Corporation, Port Washington, NY USA). Assays demonstrated that no measurable loss of polypeptide occurred during filtration. As discussed above, a representative administration route is subcutaneous injection. According to some embodiments, the polypeptide is administered in an amount generally from about 0.01 to about 5 $\mu\text{mole/kg}$ body weight per dose, and typically from about 0.1 to about 3 $\mu\text{mole/kg}$ body weight per dose. In a study with mice, as described below, subcutaneous injection was performed on alternate days with a dose of 1 $\mu\text{mole/kg}$ body weight per dose. In terms of polypeptide weight, representative administration amounts are generally from about 0.05 mg/kg body weight per dose to about 25 mg/kg body weight per dose, typically from about 0.1 mg/kg body weight per dose to about 20 mg/kg body weight per dose, and often from about 0.1 mg/kg body weight per dose to about 10 mg/kg body weight per dose. According to some embodiments, an administration amount from about 0.1 mg/kg body weight per dose to about 1 mg/kg body weight per dose may be optimal. For any of these ranges of dosage amounts, administration may be at least once weekly, at least twice weekly, at least three times weekly, on alternate days, or daily, over a given treatment regimen.

Stability of the Synthesized Polypeptide

- [63] The polypeptides described herein are normally stable indefinitely in dry form. No significant change occurred in the mass spectrum of the polypeptide SVL4 when dissolved in PBS and stored for three years at -20°C with occasional thawing. No significant deterioration in the spectrum was detected after storage at 4°C for 1 year. Table 1 below summarizes the results of a stability study on the polypeptide SVL4 under stress conditions, as performed according to a forced degradation study by an independent laboratory. The polypeptide was initially dissolved in PBS at pH 7.4.

Table 1.

<u>Stress Condition</u>	<u>Percent Polypeptide Remaining</u>
Thermal stress: 60°C/ambient relative humidity, 14 days	~60
High pH: 40°C, 0.1 N NaOH/pH 11, 5 days	0
Low pH: 40°C, 0.1 N HCl/pH 3, 5 days	100
Oxidation: 40°C, 0.5% H ₂ O ₂ , 2 days	100

EXAMPLE 2

- [64] The polypeptide SVL4, synthesized in Example 1 above, was evaluated in terms of a number of biological activities thought to correlate with its effectiveness against GBM.

Lectin Binding

- [65] To study lectin binding avidity as a function of ligand density, several polypeptides, comprising biologically active amino acid sequences as described above, were synthesized as bivalent or monovalent structures. Monovalent polypeptides were synthesized by extension from the spacer sequence GGGS (SEQ ID NO: 34), attached to the α -amino group of an ϵ -biotinyl-lysine-amide residue. Bivalent polypeptides contained the amino acid sequence extended from the α and ϵ amino groups of a lysine residue linked to ϵ -biotinyl-lysine-amide. The tetravalent polypeptide contained the amino acid sequence extended from the four amino groups of a tri-lysine scaffold that was linked to ϵ -biotinyllysine-amide.
- [66] The effect of valency of several polypeptides on binding to lectins was tested in a solid phase assay in which C-terminal biotin-tagged polypeptides were anchored to streptavidin that was

bound in microtiter plate wells. This arrangement allowed maximal flexibility of the synthesized polypeptides for interaction with lectins. The extent of subsequent binding of lectins was detected by peroxidase conjugated to the lectins. To achieve equal numbers of sequences in each study, 25 picomoles of the tetravalent polypeptide, 50 picomoles of the bivalent polypeptide, and 100 picomoles of the monovalent polypeptide were added per well. Using several different lectins, as illustrated by the several different plots shown in FIG. 3, the relative affinity of a series of tetra-, bi-, or mono-valent polypeptides was evaluated. In each case, the extent of binding of the tetravalent polypeptide was set as 100%, as shown in FIG. 3. The results of this binding study indicated that the tetravalent polypeptides bound approximately two times more lectin than the bivalent polypeptides, which bound five times more lectin than the monovalent polypeptides. Polypeptides without the biotin tag were not retained in the assay. FIG. 4 illustrates the binding of the polypeptide SVL4 to specific lectins, with the source of the lectins and their specificities being *Griffonia simplicifolia* (GS: Gal), *Helix pomatia* (HP: GalNAc/Gal), and *wheat germ agglutinin* (WGA: GlcNAc, Neu5Ac). In a solid phase assay, C-terminal biotin-tagged, tetravalent SVL4 was bound to streptavidin in wells of a microtiter plate. Binding of lectins was detected by the activity of conjugated peroxidase after extensive washing. The polypeptide SVL4 was screened from a phage display library with the GalNAc/Gal-specific lectin HP, even though this polypeptide was bound most strongly in this assay to the *G. simplicifolia* lectin, specific for Gal. Representative polypeptides, useful in the methods described herein, are therefore polypeptides exhibiting binding to lectins in a solid-phase lectin binding assay. Particular polypeptides of interest exhibit binding to a receptor that is specific for galactose/N-acetylgalactosamine (GalNAc/Gal) ligands in a solid-phase binding assay. An example of a receptor that may be characterized in this manner is recombinant receptor CLEC10a/CD301.

Cytokine Release

- [67] Biological activity of polypeptides, including the polypeptide SVL4, described above, was tested by treatment of human peripheral blood mononuclear cells (PBMCs) in culture with polypeptide added at concentrations from 3 nM to 90 μ M. After treatment of cultures with the polypeptide for 24 hours, media were removed and assayed for the relative amounts of several cytokines. The response to the polypeptide was assayed when added alone or when added with the prototypic agent commonly used to induce secretion of inflammatory cytokines, namely lipopolysaccharide (LPS). The latter set of samples was an assay to test whether the polypeptide has anti-inflammatory activity. FIG. 5 illustrates the results of the quantitative analysis of cytokines released from human PBMCs during the 24-hour incubation in the presence or absence of 1 ng/ml LPS. As shown in FIG. 5, slight anti-inflammatory responses were detected by a reduction in release of these cytokines, induced by LPS, at high concentrations of the polypeptide. Of particular interest was induction of selective release of IL-8 but not the inflammatory cytokines, IL-1 β , IL-6 or TNF- α by SVL4, at low concentrations, with a maximal response at 3 nM polypeptide. IL-8 is a chemokine released from activated macrophages that attracts neutrophils and plays an important role in host defense by enhancing microbiocidal activity and cytotoxicity. According to representative embodiments of the invention, therefore, polypeptides used in methods described herein inhibit the release of one or more of the inflammatory cytokines IL-1 β , IL-6 or TNF- α , and preferably inhibit the release of all of these cytokines, in a cytokine release assay using LPS as a cytokine release agent.
- [68] In a separate experiment, the effect of the polypeptide SVL4 on the release of IL-21 in PBMC cultures was assayed. IL-21 is a cytokine secreted from activated CD4⁺ T cells and responsible for sustaining maintenance and the cytotoxic function of CD8⁺ T cells, an essential process in control of chronic viral infections. These functions are also of particular importance in regard to the anti-tumor activities of IL-21. As shown in FIG. 6, SVL4 induced a greater release of IL-21 than the known stimulant, IFN- γ , upon treatment of PBMCs with 50 mM SVL4 or 1 nM IFN- γ for 16 h. According to further representative embodiments consistent with these experimental results, therefore, polypeptides used in methods described herein, compared to IFN- γ , induce a greater release of the cytokine IL-21 in a cytokine release assay.

Stimulation of Phagocytosis by Microglial Cells

- [69] To determine whether polypeptides described herein stimulate phagocytosis, dispersed cells of an explant of a human glioblastoma multiforme (GBM) tumor were incubated with 10 nM SVL4 for 4 hours and then the culture was challenged with fluorescently labeled bacterial cells, an assay for phagocytosis. The micrograph in FIG. 7 shows cells containing internalized, fluorescent bacterial cells by microglial cells in the GBM tumor explant. In contrast, fluorescent bacterial cells of control cultures were attached to the surface of a few cells but no internalization was observed. Representative polypeptides for use in methods described herein therefore stimulate phagocytosis, based on internalization of bacterial cells by microglial cells.

EXAMPLE 3

- [70] The polypeptide SVL4, synthesized in Example 1 above, was evaluated in pharmacological studies with mice.

Increased Expression of Activation Markers

- [71] To examine whether SVL4 effectively stimulates the immune system *in vivo*, the polypeptide was injected subcutaneously into healthy mice at a dose of 1 nanomole/gram body weight on alternate days for two weeks. Fluorescently tagged antibodies were used to detect cell surface markers. These markers were namely (i) CD115, receptor for M-CSF (colony stimulating factor 1 receptor, CSF-1R); (ii) CD11b, integrin α_M , a subunit of the heterodimeric integrin molecule, also complement receptor 3; (iii) MHC II, major histocompatibility complex class II molecules, which are involved in antigen presentation; and (iv) CD11c, complement component 3 receptor subunit 4. FIG. 8 illustrates the results from the analysis of these cellular markers on blood monocytes by flow cytometry after the treatment. Several markers, in particular CD11b and MHCII proteins, were significantly increased. According to representative methods, therefore, polypeptides as described herein stimulate the immune system, based on increased density of one or more cellular markers on blood monocytes. Particular cellular markers include CD11b and MHCII protein, with the density increase of one or both of these markers being generally at least about 1.5 times {e.g., from about 1.5 times to about 9 times}, and typically at least about 2 times {e.g., from about 2 times to about 8 times}, compared to a control case in which the polypeptide is not administered.

[72] To further evaluate activity of SVL4 *in vivo*, these markers were quantified on phagocytic cells in the brains of mice in which glioma cells were implanted. At the end of an 11-day treatment period, dispersed brain cells were analyzed by flow cytometry. The analysis revealed that subcutaneous injection effectively activated cells in the brain. The polypeptide also significantly promoted the differentiation of microglial cells. FIGS. 9A-9D illustrate the increase in immune markers on phagocytic cells in the brains of mice with implanted glioma cells. Radiation treatment (4 Gy) was administered on days 7 and 9 after implantation, and SVL4 polypeptide was administered subcutaneously beginning on day 7 at 1 nanomole/gram body weight ($1 \mu\text{moles/kg}$ body weight). The animals were analyzed on day 18. FIG. 9A illustrates the change in the ratio of microglial cells to macrophages, in the brains of mice with implanted glioma cells, after radiation treatment with SVL4, compared to radiation treatment alone. The polypeptide, as is preferred in the case of other representative polypeptides described herein, promotes a microglial/macrophage ratio of greater than 1, when administered in combination with radiation. FIG. 9B illustrates a summary of flow cytometric analysis of macrophages and microglial cells. FIG. 9C shown a nuclear magnetic resonance (NMR) image of a mouse brain, showing early stage tumor development after implantation of glioma cells. FIG. 9D illustrates the increases, compared to radiation treatment alone, in immune markers, MHCII and CD11c on phagocytic cells and the immune markers CD115, CD11b, and MHCII on microglial cells, in the brains of mice with implanted glioma cells, following radiation treatment (4 Gy) in combination with SVL4 treatment. Tissues for examination of cells by flow cytometry were obtained from the brain hemisphere containing the tumor. Fluorescently tagged antibodies were used to detect cell surface markers, as discussed above with respect to the increased expression of activation markers in healthy mice. According to representative methods, therefore, the polypeptide that is administered to a patient suffering from GBM stimulates the immune system, based on increased density of one or more cellular markers (*e.g.*, the cellular markers described above, and preferably all of these cellular markers) on phagocytic cells in the brain and in the presence of glioma cells.

Survival of Mice with Brain Tumors

- [73] The survival of mice with implanted glioma cells (murine GL261 cell line) was determined following (i) treatment with radiation (4 Gy on day 7 and 9 following implantation) alone, (ii) administration of polypeptide SVL4 (1 nanomole/gram body weight) alone on alternate days beginning on day 7 following implantation, and (iii) the combination of (i) and (ii), *i.e.*, radiation treatment in combination with polypeptide administration. At day 7 following implantation, the animals were imaged to obtain randomized groups with the same average tumor size. As shown in FIGS. 10A and 10B, the size of the tumor was slightly reduced in polypeptide-treated animals, following treatment regimen (ii), but the life of the animals was not significantly extended. This is also reflected in the results in Table 2 below. However, in conjunction with radiation, the polypeptide dramatically and surprisingly extended life, with the combination therapy resulting in a two-fold increase in life, measured from the start of treatment. Without being bound by theory, it is thought from these results that activated phagocytic cells in the brain cannot effectively attack a solid tumor. In fact, the tumor seemed to expand by invasion of phagocytic cells. However, after radiation, the tumor cells apparently were damaged sufficiently to allow their destruction by phagocytes, as indicated by the marked reduction in tumor size. FIG. 10A illustrates the increase in survival of mice implanted with glioma cells in the brain, following (i) radiation treatment alone, (ii) SVL4 treatment alone, and (iii) a combination of radiation and SVL4. FIG. 10B shows the decrease in tumor size of mice implanted with glioma cells in the brain, following (i) radiation treatment alone, (ii) SVL4 treatment alone, and (iii) a combination of radiation and SVL4. Although the treatment regimen used in this experiment involved administration of SVL4 at a dose of 1 $\mu\text{moles/kg}$ body weight on alternate days, a dose of only 0.1 $\mu\text{moles/kg}$ body weight was similarly effective in causing a reduction in tumor size. It is expected that this dosage amount, administered one or two times per week during a treatment regimen involving combination therapy with radiation, would provide effective treatment in terms of prolonging survival of patients with GBM.

Table 2.

<u>Treatment</u>	<u>Survival after Implantation</u>
Control (sham injection of saline)	21 days
(i) Radiation (2 treatments of 4 Gy each)	22 days
(ii) SVL4 (1 nmole/g body weight, admin. on alternate days)	30 days
Combination of (i) and (ii)	38 days

Antigenicity

- [74] The tri-lysine core of tetravalent polypeptides is immunologically silent. Examination of the amino acid sequence of SVL4 using MHC binding prediction databases indicated that they are not likely to be presented by MHC class I or MHC class II molecules in humans. In a direct test of antibody generation against SVL4, a large dose (5 nanomole/g body weight) of the polypeptide was injected into mice. Negligible antibodies were detected 3 weeks after injection, whether the polypeptide was given with or without alum as an adjuvant. In contrast, alum strongly increased antibody production against ovalbumin as a positive control. A test of SVL4 as an adjuvant with ovalbumin was negative.

Toxicity in vivo

- [75] Toxicity of SVL4 was evaluated after two bolus intravenous injections into rats with a dose of 12.5 $\mu\text{moles/kg}$ body weight (85 mg/kg body weight), one week apart. Sterile solutions of polypeptide were prepared by Susavion, with concentration of polypeptide determined by the bicinchoninic acid assay (Pierce, Rockland, IL), and delivered to an independent laboratory to perform a preliminary toxicity study designed to demonstrate a margin of safety. Because of the lower bioavailability of polypeptide administered subcutaneously, this study should have provided a margin of safety of at least 1000-fold over a proposed standard therapeutic dose. Results of the study indicated that the polypeptide was well tolerated and that no changes were noted in behavior, weight, or food intake. A complete hematology and clinical chemistry panel on all animals showed no changes from control animals. No macroscopic lesions were evident at necropsy one day after the second injection. Each absolute mean organ weight was similar to the control value.

Toxicokinetic and pharmacokinetic curves

- [76] Blood samples were taken during the toxicity study for bioanalytical assay of the polypeptide in plasma. The polypeptide in blood samples was stabilized by acidification with formic acid, a method that extensive preliminary analyses indicated as being the most favorable to preserve polypeptides. Results are summarized in FIG. 11, in which the area under the curve (AUC) value for the polypeptide SVL4 was 43.6 nmole*h/mL, indicating a relatively long lifetime in blood. Data for this curve were obtained following intravenous injection of 0.5 ml of a 5 mM solution of SVL4 in PBS. Curves in FIG. 11 are shown with both linear and logarithmic concentration axes. A "0 point" of about 180 μ M in plasma was achieved.
- [77] Subcutaneous injection of SVL4, tagged with a fluorescent dansyl group, into mice at a dose of 2 μ moles/kg body weight indicated a peak concentration in the serum at about 1 hour after injection and no detectable amount after 4 hours. The plasma concentration versus time curve, illustrating the persistence of the polypeptide SVL4 in the blood, confirmed by mass spectroscopy of full length polypeptides at 30 to 180 min time points, is illustrated in FIG. 12.
- [78] Overall, pharmaceutical compositions of the polypeptide SVL4 exhibit no cytotoxicity at effective concentrations *in vivo*. The amino acids of the polypeptide are normal constituents of the body and of the diet, and representative polypeptides such as SVL4 include sites for proteolytic cleavage for degradation. SVL4 itself is not predicted to be antigenic, as indicated by RANKPEP software, and other polypeptides of this design have been shown to be non-antigenic in animals. Release of anti-viral and anti-tumor cytokines in PBMC cultures is stimulated but that of inflammatory cytokines is below detection.

Conclusions

- [79] The polypeptide SVL4 was found to exhibit a number of biological activities that are believed to correlate with its demonstrated effectiveness in prolonging the survival of mice implanted with glioma cells. For example, the *in vitro* studies with this polypeptide showed that it (i) activated macrophages, with the selective induction of the release of IL-8, (ii) activated T cells, with the induction of the release of IL-21, which activates CD8⁺ T cells, and (iii) activated phagocytosis. The *in vivo* studies with this polypeptide showed that it (i) greatly increased markers of activation on blood monocytes, (ii) increased markers of activation on brain macrophages, (iii) stimulated differentiation of microglial cells in the

brain, and (iv) stimulated differentiation of dendritic cells and antigen-presenting cells in the brain.

- [80] In the mouse model of GBM, the studies demonstrate that SVL4 activates T cells, CD8⁺ T cells and phagocytic cells such as macrophages, microglia and dendritic cells. Activation of these cells is expected to lead to presentation of antigens and generation of antibodies against tumor cells. The synthetic polypeptide SVL4 therefore activates a multicellular assault against the tumor. In other studies, the biological processes exhibited by SVL4 were found to be critical in mice immunized with radiation-killed glioma cells, which effectively prevented growth of tumors after implantation of glioma cells. For example, in such studies, IFN- γ was shown to induce expression of MHC I and II proteins, stimulate phagocytosis, and promote a CD8⁺ cytotoxic cell response, which are the same activities that have now been demonstrated with respect to SVL4. It is also noteworthy that, in the experiments described herein, treatment was initiated after a tumor had developed. This information in its entirety strongly supports the effectiveness of the polypeptide SVL4 in treating GBM or other tumors and provides a reasonable basis to conclude that structurally analogous and/or polypeptides exhibiting similar biological activities would also be effective, particularly in conjunction with low-dose radiation following resection of the tumor.
- [81] Overall, therefore, the observed *in vitro* and *in vivo* biological effects of the polypeptide SVL4, and particularly its immunostimulant effects through activation of phagocytic (macrophages, microglial, etc.) cells, T cells and cytotoxic (natural killer and CD8⁺) cells, are thought to contribute to the functioning of the polypeptide SVL4 to reduce tumor size and extend the life of patients afflicted with GBM. These biological effects also reasonably correlate to the structure of SVL4 and other polypeptides bearing monovalent or multivalent amino acid sequences as described herein, and particularly such sequences exhibiting lectin binding affinity.

CLAIMS:

1. A method for treating glioblastoma multiforme in a patient, the method comprising administering to the patient a polypeptide comprising an amino acid sequence of formula 1, formula 2, or formula 3;

wherein the amino acid sequence of formula 1 is

B1-[X1-Q-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11]-B2;

wherein

X1 is selected from the group consisting of V, E, and A, or X1 is absent;

X2 is selected from the group consisting of A, N, and G;

X3 is any amino acid;

X4 is selected from the group consisting of P and Q;

X5 is selected from the group consisting of S, R, and C;

X6 is selected from the group consisting of N, L, G, and K;

X7 is selected from the group consisting of Q, A, S, and H;

X8 is selected from the group consisting of H, L, and A;

X9 is selected from the group consisting of S and T;

X10 is selected from the group consisting of P and A;

X11 is selected from the group consisting of R, G, and P; and

wherein B1 and B2 are independently 1-5 amino acids, or are absent;

wherein the amino acid sequence of formula 2 is

B1-[X1-X2-X3-X4-1-N-I-X5-N-R-G-X6]-B2;

wherein

X1 is selected from the group consisting of C, L, and Q, or is absent;

X2 is selected from the group consisting of R, P, and S or is absent;

X3 is selected from the group consisting of A, S, and T, or is absent;

X4 is selected from the group consisting of S and T, or is absent;

X5 is selected from the group consisting of S and T;

X6 is selected from the group consisting of S and T; and

wherein B1 and B2 are independently 1-5 amino acids, or are absent; and

wherein the amino acid sequence of formula 3 is

B1-[X1-T-D-E-X2-R-R-Q-X3]-B2;

wherein

X1 is selected from the group consisting of C and T, or is absent;

X2 is a 4 amino acid group;

X3 is selected from the group consisting of C and P, or is absent; and

wherein B1 and B2 are independently 1-5 amino acids, or are absent.

2. The method of claim 1, wherein the polypeptide is administered subsequent to a non-curative dose of radiation.
3. The method of claim 2, wherein the non-curative dose of radiation is from about 8 Gy to about 80 Gy.
4. The method of claim 1, wherein the polypeptide is administered in an aqueous pharmaceutical composition having a polypeptide concentration from about 0.1 to about 10 mM and a pharmaceutically acceptable carrier.
5. The method of claim 4, wherein the pharmaceutical composition is administered by subcutaneous injection.
6. The method of claim 1, wherein the polypeptide is administered in amount from about 0.1 mg/kg body weight to about 10 mg/kg body weight per dose.
7. The method of claim 6, wherein the polypeptide is administered in multiple doses over a treatment regimen.

8. The method of claim 7, wherein the polypeptide is administered on alternate days during the treatment regimen.
9. The method of claim 7, wherein the polypeptide is administered one or two times per week during the treatment regimen.
10. The method of claim 7, wherein the treatment regimen comprises the multiple doses of polypeptide, in addition to multiple doses of radiation.
11. The method of claim 10, wherein the multiple doses of polypeptide and the multiple doses of radiation are administered alternatingly.
12. The method of claim 11, wherein a first radiation dose, administered prior to all radiation doses of the treatment regimen, is followed by a first polypeptide dose, administered prior to all polypeptide doses of the treatment regimen, without any intervening radiation dose.
13. The method of claim 1, wherein the polypeptide exhibits binding to lectins in a solid-phase lectin binding assay.
14. The method of claim 1, wherein the polypeptide exhibits binding to a receptor that is specific for galactose/N-acetylgalactosamine ligands in a solid-phase binding assay.
15. The method of claim 14, wherein the receptor is recombinant receptor CLEC10a/CD301.

16. The method of claim 1, wherein the polypeptide inhibits the release of one or more inflammatory cytokines selected from the group consisting of IL-1 β , IL-6, and TNF- α in a cytokine release assay using lipopolysaccharide (LPS) as a cytokine release agent.
17. The method of claim 1, wherein the polypeptide selectively induces the release of the chemokine IL-8 in a cytokine release assay using lipopolysaccharide (LPS) as a cytokine release agent.
18. The method of claim 1, wherein the polypeptide, compared to IFN- γ , induces a greater release of the cytokine IL-21 in a cytokine release assay.
19. The method of claim 1, wherein the polypeptide stimulates phagocytosis, based on internalization of bacterial cells by microglial cells.
20. The method of claim 1, wherein the polypeptide stimulates the immune system, based on increased density of one or more cellular markers on blood monocytes.
21. The method of claim 20, wherein the one or more cellular markers includes CD11b protein or MHCII protein.
22. The method of claim 1, wherein the polypeptide stimulates the immune system, based on increased density of one or more cellular markers on blood monocytes.

23. The method of claim 1, wherein the polypeptide stimulates the immune system, based on increased density of one or more cellular markers on phagocytic cells in the brain and in the presence of glioma cells.
24. The method of claim 1, wherein the polypeptide comprises the amino acid sequence of formula 1, wherein
- XI is V or is absent;
 - X2 is selected from the group consisting of A and N
 - X5 is selected from the group consisting of S and R;
 - X6 is N;
 - X7 is selected from the group consisting of Q and A;
 - X8 is selected from the group consisting of H and L; and
 - XII is selected from the group consisting of R and G.
25. The method of claim 24, wherein, in the amino acid sequence of formula 1,
- X2 is A;
 - X4 is Q;
 - X5 is S;
 - X7 is Q;
 - X8 is H;
 - X9 is T;
 - XIO is P; and
 - XI1 is R.
26. The method of claim 25, wherein, in the amino acid sequence of formula 1, X3 is T.
27. The method of claim 1, wherein the polypeptide comprises the amino acid sequence of formula 1, wherein B2 is the spacer sequence GGGS.

28. The method of claim 1, wherein the polypeptide is branched.
29. The method of claim 28, wherein the polypeptide has a multivalent structure with multiple branches having the amino acid sequence of formula 1.
30. The method of claim 29, wherein the polypeptide has a multivalent structure with multiple branches having the sequence VQATQSNQHTPRGGGS.
31. The method of claim 28, wherein the polypeptide has a multivalent structure with multiple branches extending from lysine residues.
32. The method of claim 1, wherein the polypeptide comprises the amino acid sequence of formula 2, wherein
- XI is L or is absent;
X2 is P or is absent;
X3 is T or is absent;
X4 and X5 are T; and
X6 is S.
33. The method of claim 1, wherein the polypeptide comprises the amino acid sequence of formula 3, wherein
- X2 consists of an amino acid sequence according to general formula 4:
Z1-Z2-Z3-Z4
wherein
Z1 is selected from the group consisting of A and P;
Z2 is selected from the group consisting of L and F;

Z3 is selected from the group consisting of Y and V; and
Z4 is selected from the group consisting of T and Y.

34. A method for treating glioblastoma multiforme in a patient, the method comprising administering to the patient a polypeptide having one or more biological activities selected from the group consisting of (i) binding to lectins in a solid-phase lectin binding assay, (ii) binding to a receptor that is specific for galactose/N-acetylgalactosamine ligands in a solid-phase binding assay, (iii) inhibition of the release of one or more inflammatory cytokines selected from the group consisting of IL-1 β , IL-6, and TNF- α in a cytokine release assay using lipopolysaccharide (LPS) as a cytokine release agent, (iv) selective induction of the release of the chemokine IL-8 in a cytokine release assay using lipopolysaccharide (LPS) as a cytokine release agent, (v) induction, compared to IFN- γ , of a greater release of the cytokine IL-21 in a cytokine release assay, (vi) stimulation of phagocytosis, based on internalization of bacterial cells by microglial cells, (vii) stimulation of the immune system, based on increased density of one or more cellular markers on blood monocytes, (viii) stimulation of the immune system, based on increased density of one or more cellular markers on blood monocytes, and (ix) stimulation of the immune system, based on increased density of one or more cellular markers on phagocytic cells in the brain and in the presence of glioma cells.

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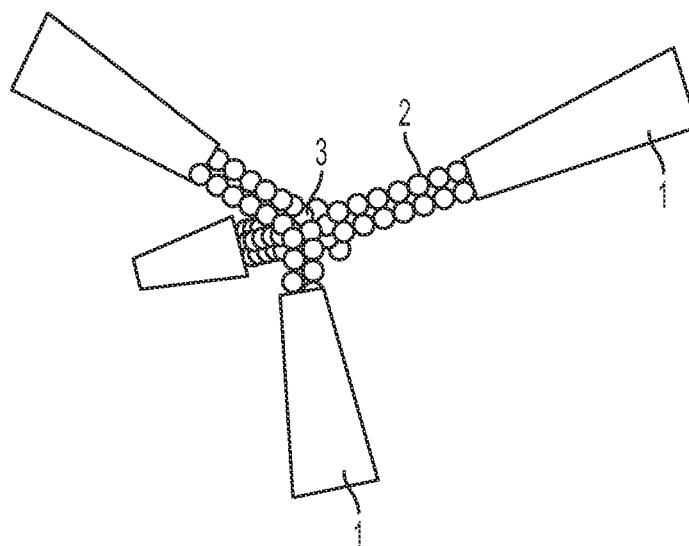


FIG. 1A

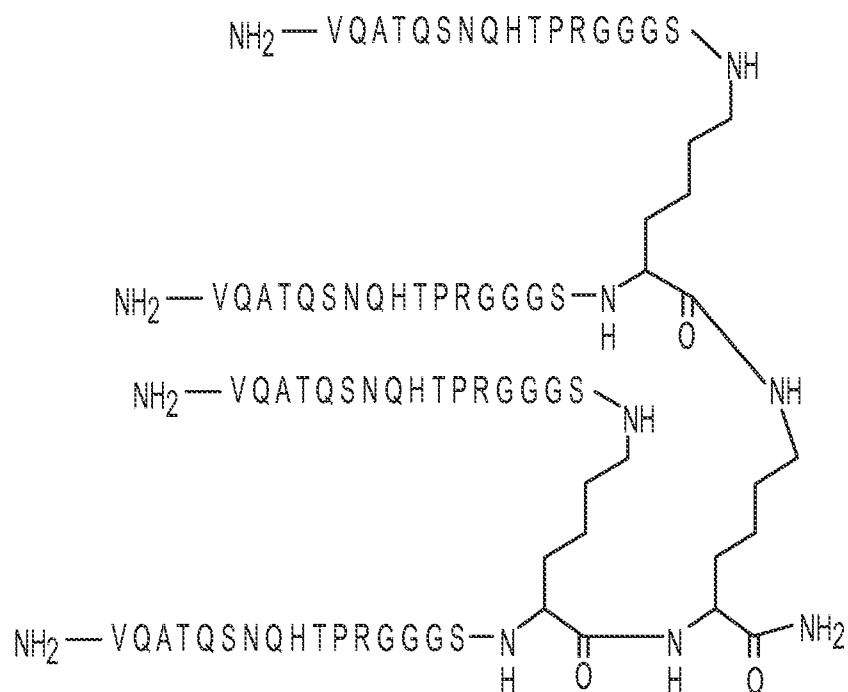


FIG. 1B

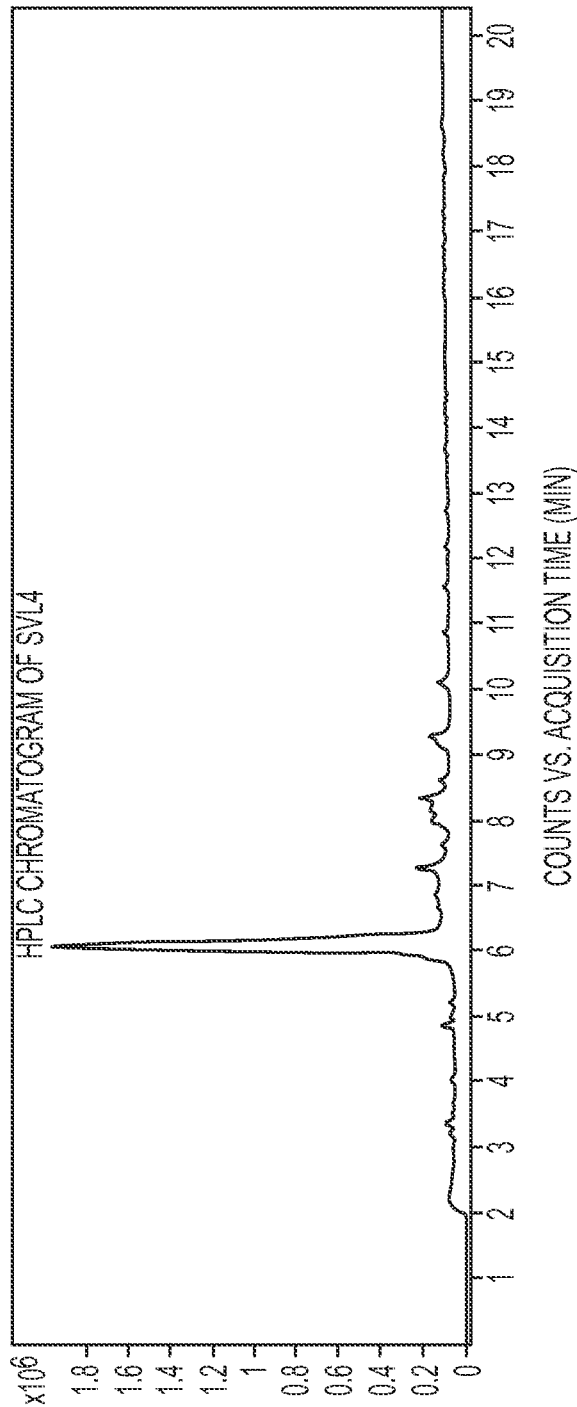


FIG. 2

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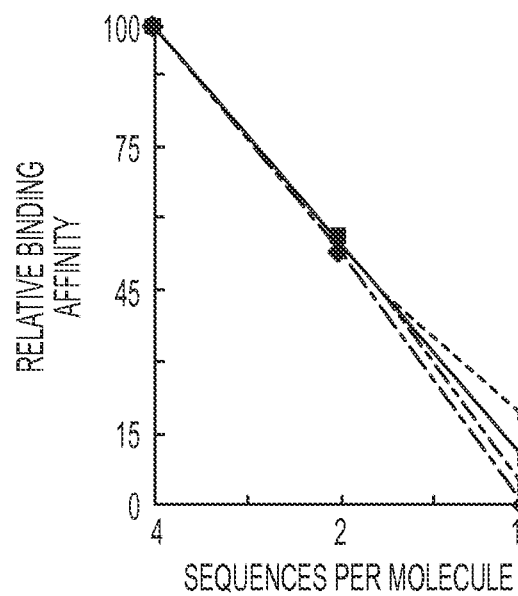


FIG. 3

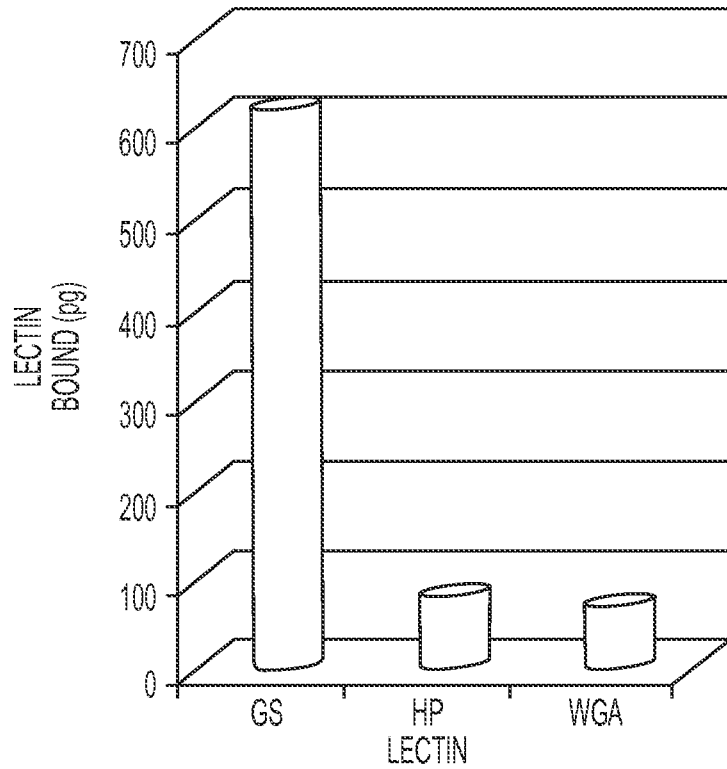


FIG. 4

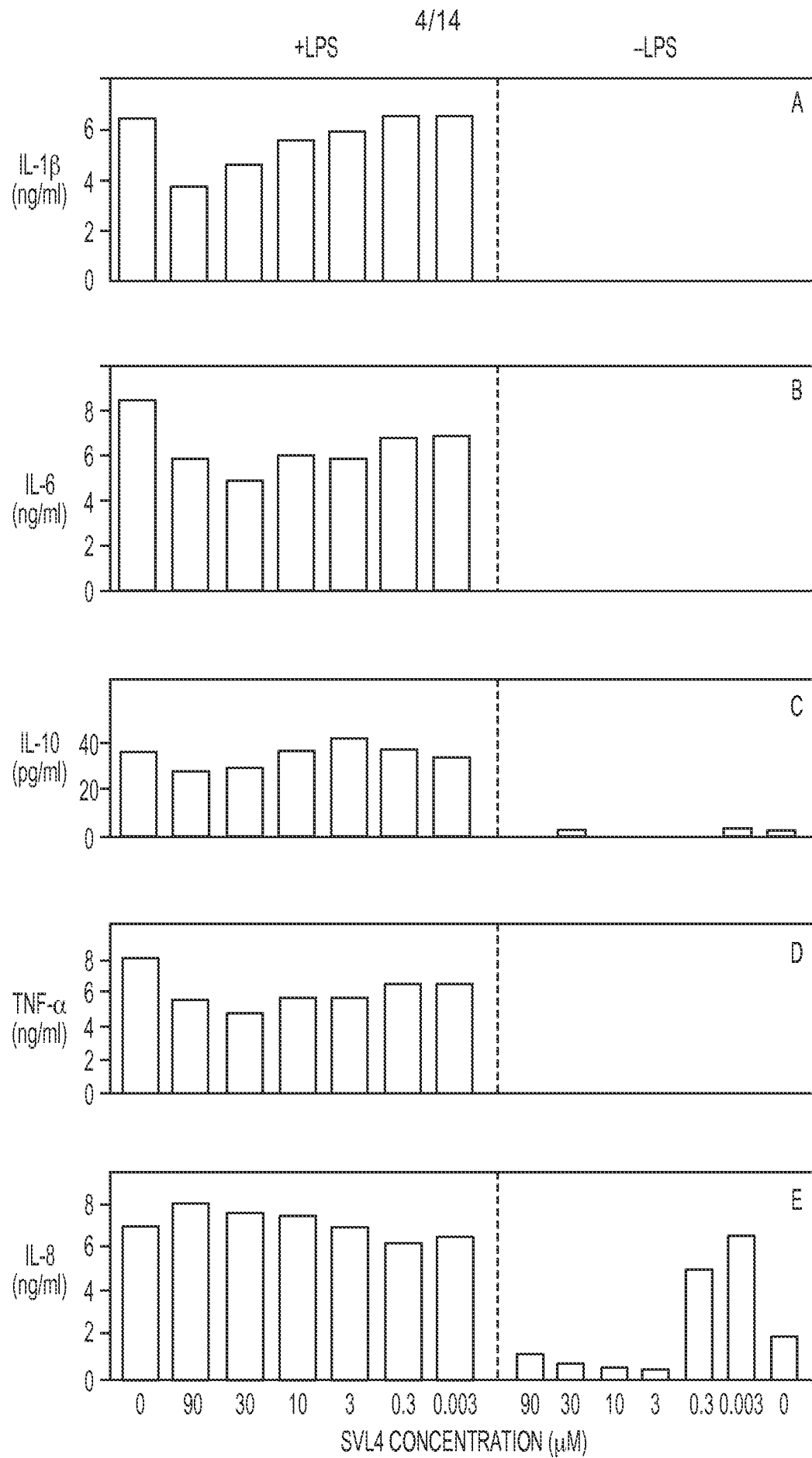


FIG. 5

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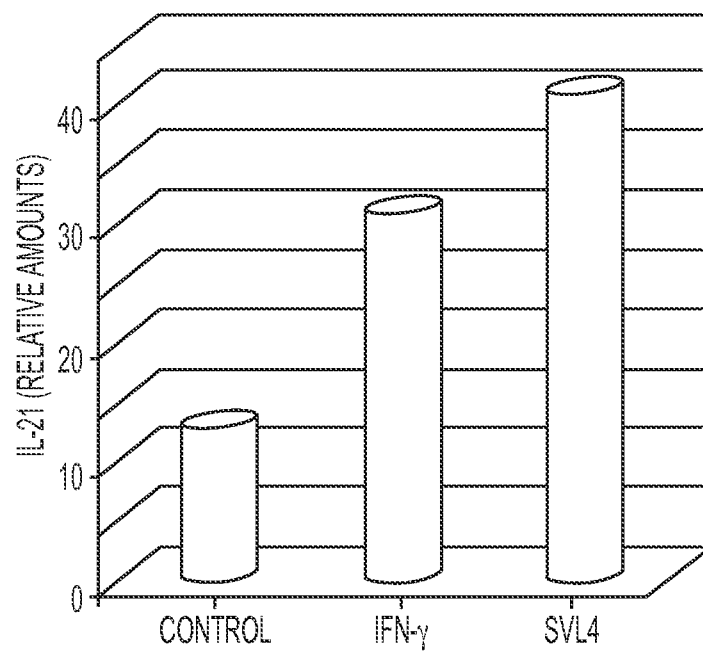


FIG. 6



FIG. 7

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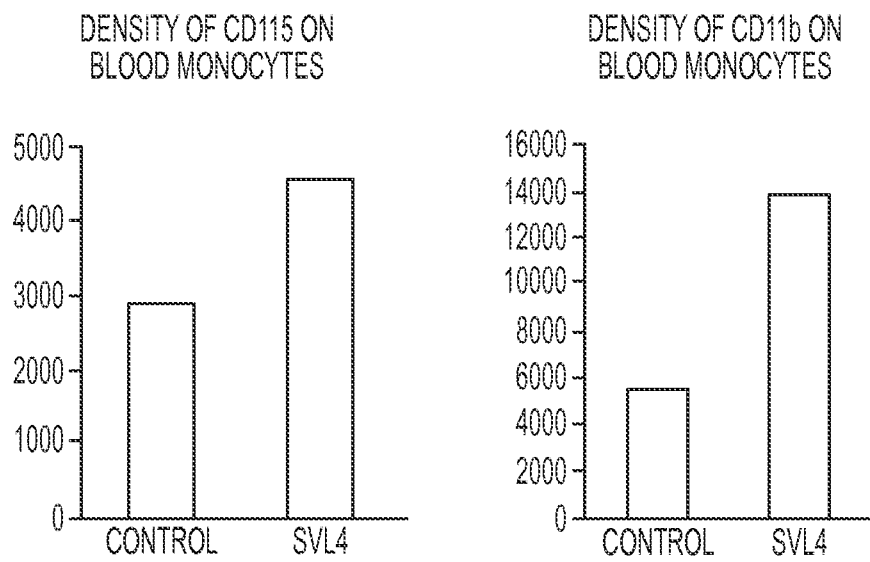


FIG. 8A

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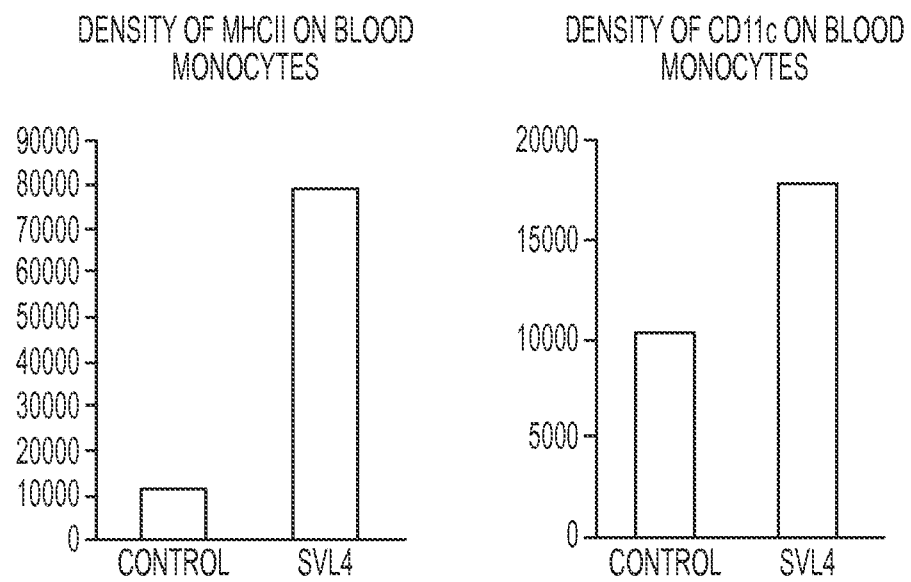


FIG. 8B

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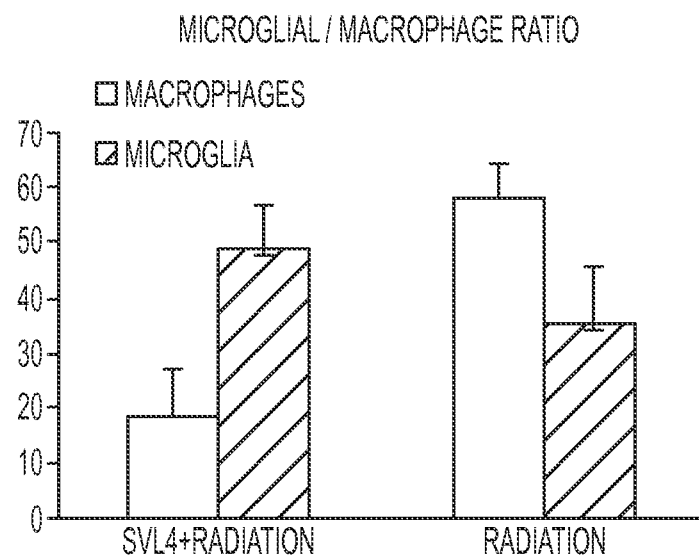


FIG. 9A

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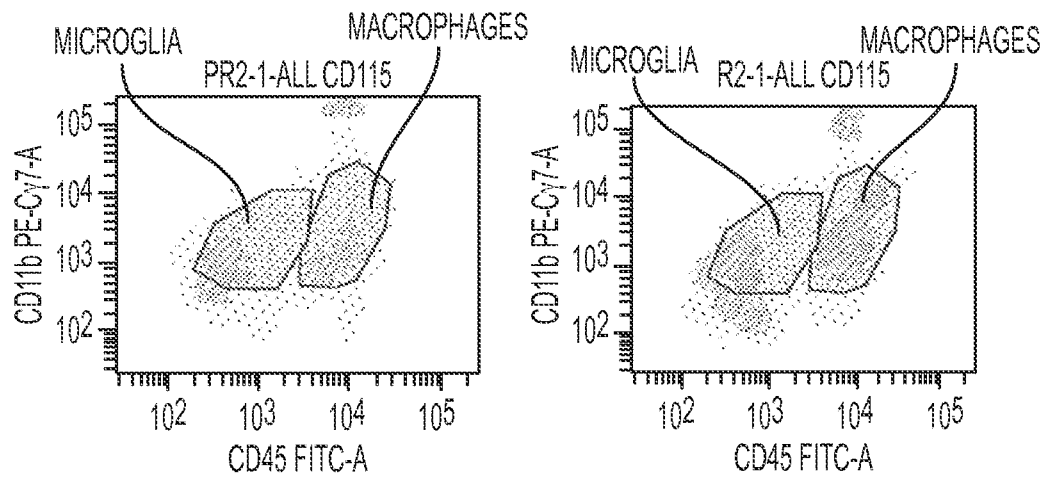


FIG. 9B

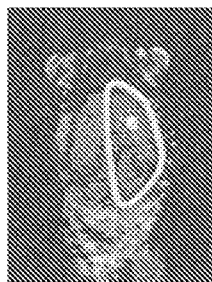


FIG. 9C

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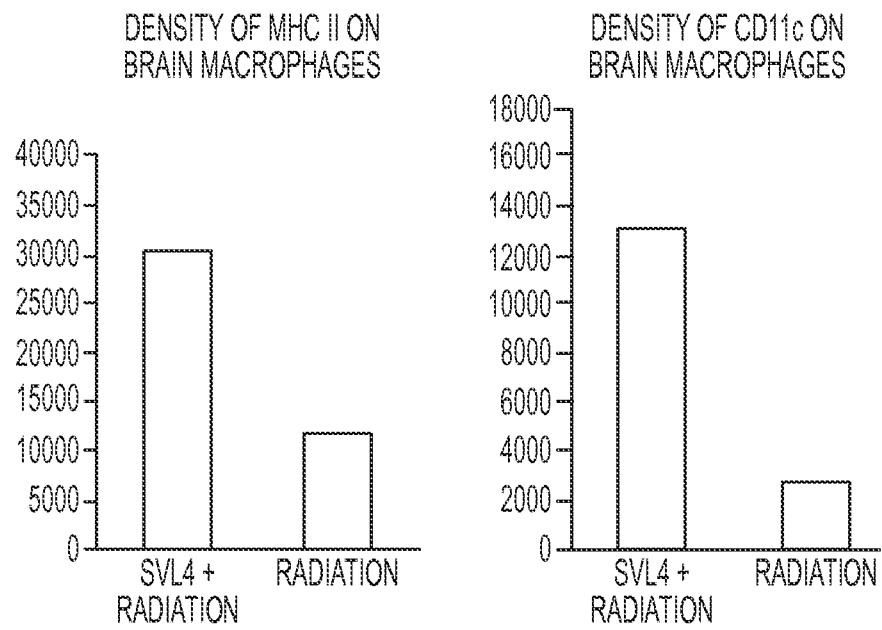


FIG. 9DA

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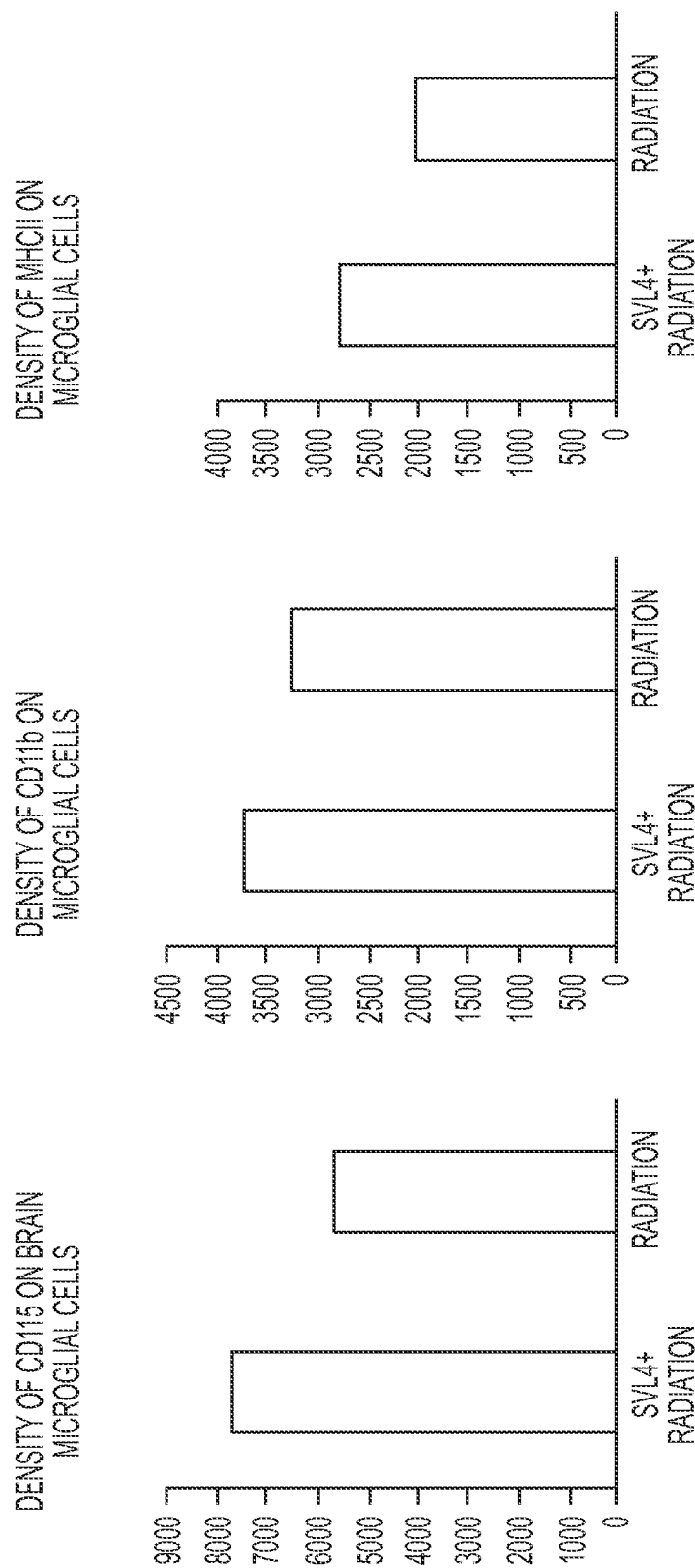


FIG. 9DB

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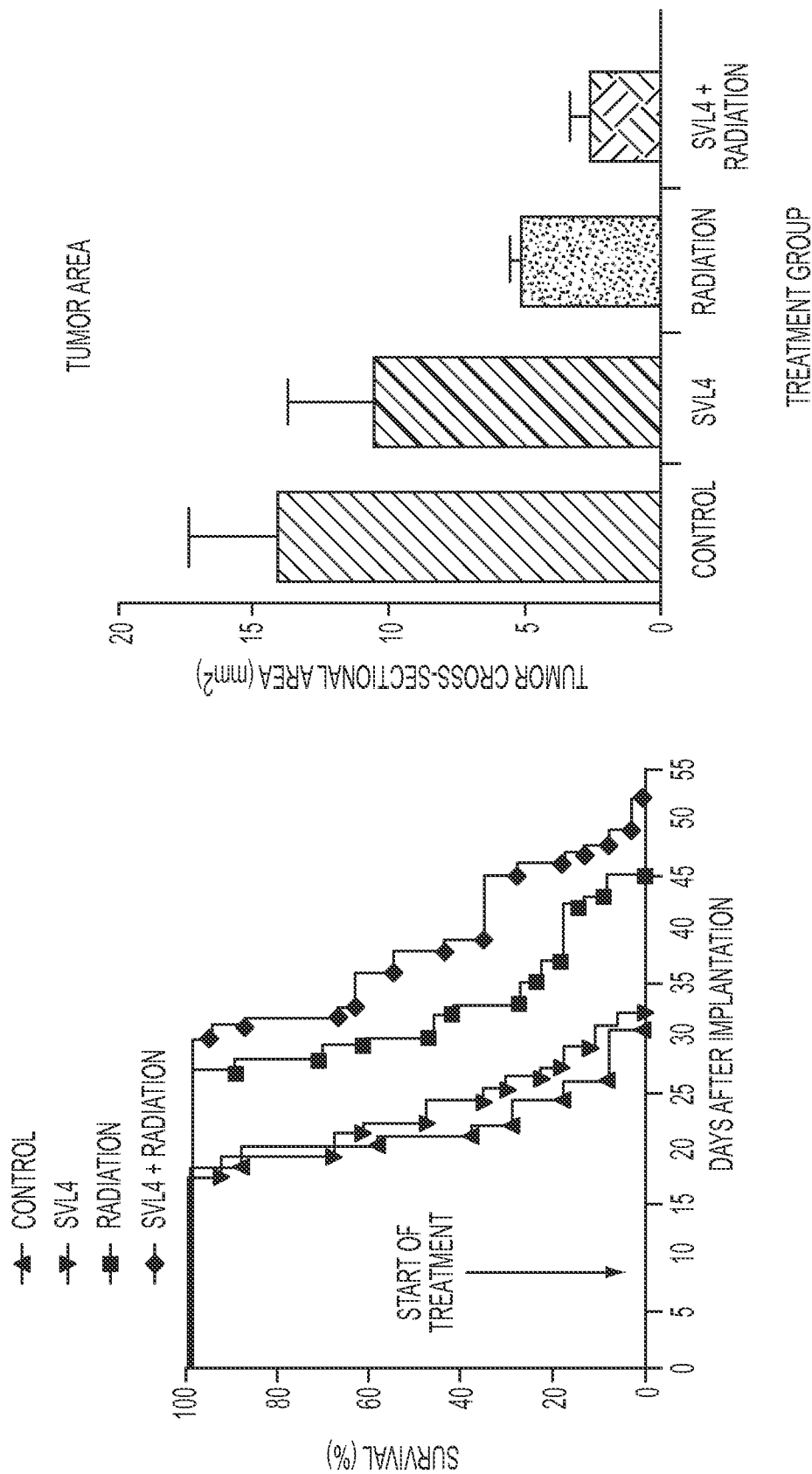


FIG. 10A

FIG. 10B

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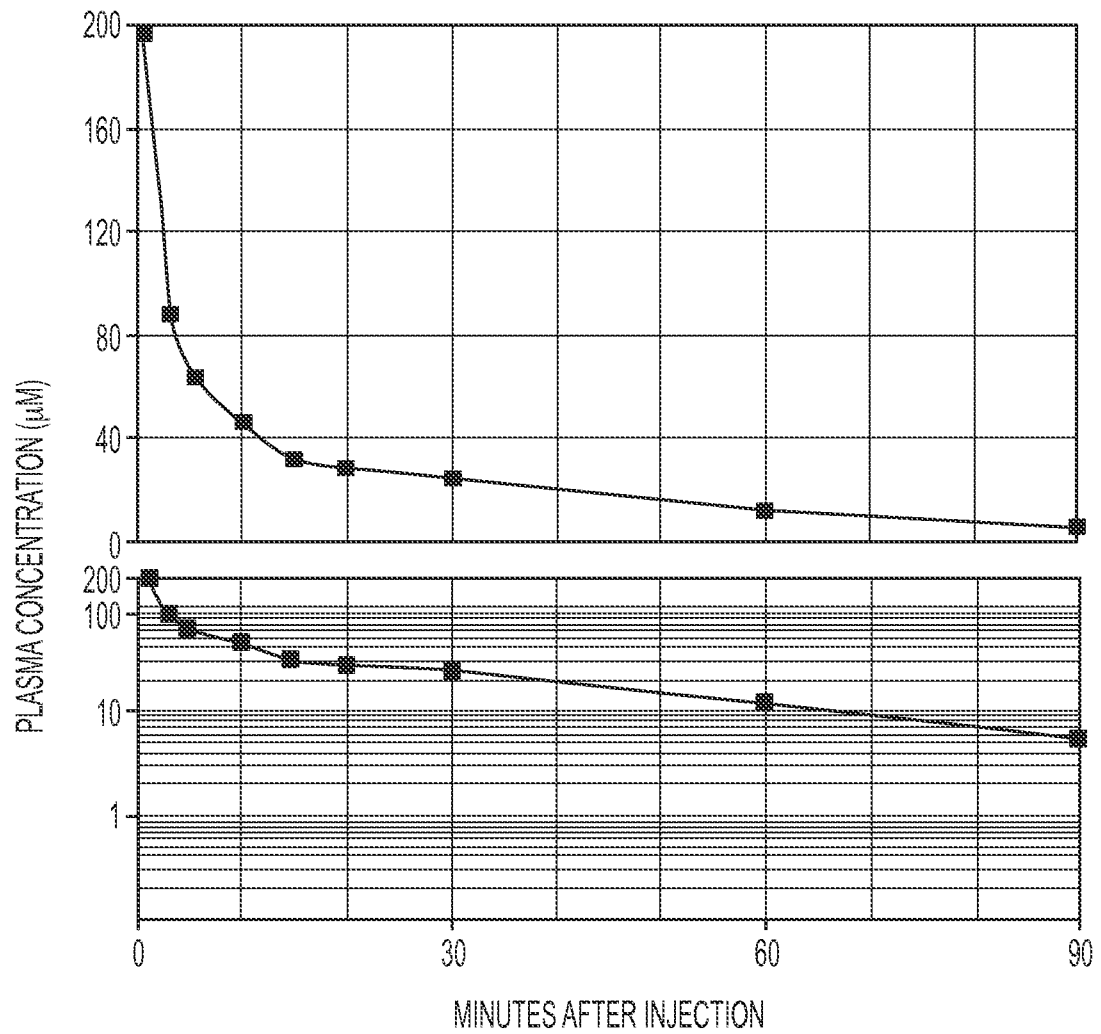


FIG. 11

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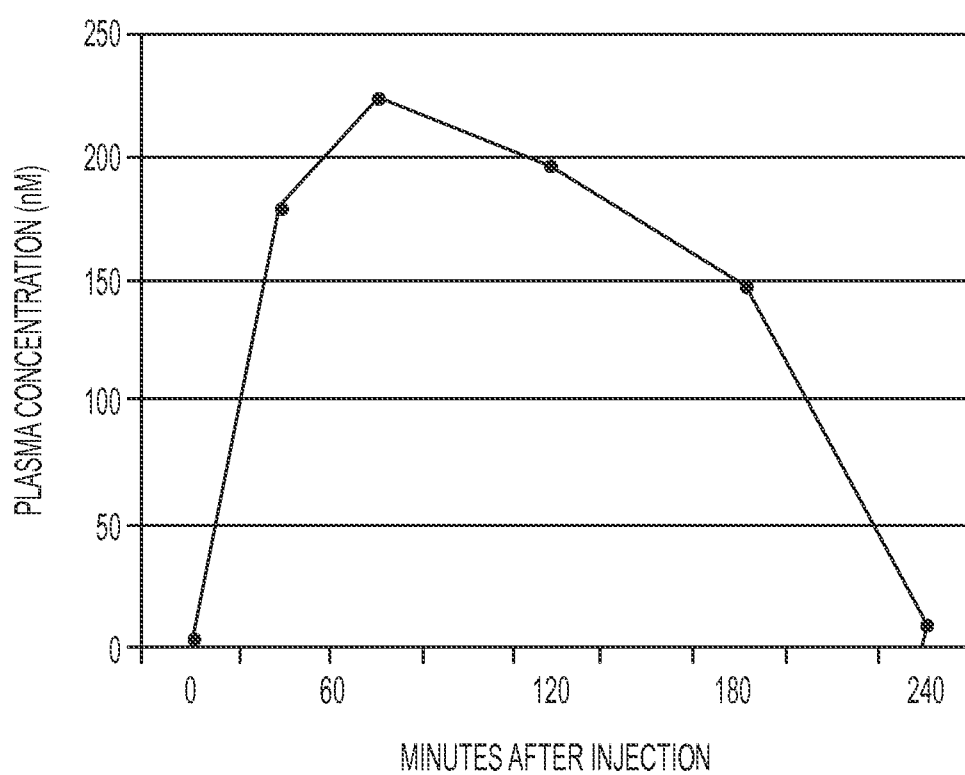


FIG. 12